EVALUATING THE RESISTANCE OF KUTSAGA BRED TOBACCO VARIETIES TO *Meloidogyne javanica*.

By

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Certification of dissertation

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ABSTRACT

Root-knot nematodes (RKNs) are of economic importance in tobacco production especially the specie *M. javanica* with increased virulence. Over the past decades chemical control has been effective in the management of *M. javanica*, but due to its negative impacts to the environment, among all management measures use of resistant varieties has an increased efficiency. This study sought to evaluate the resistance of Kutsaga Research Board bred varieties which are T 66, T 71, T 64, T 29 and KM 10 as a negative control. Determination was done comparing to a resistant tomato variety cv. Rodade containing Mi-gene that inhibits the penetration of some root-knot nematodes. The objective of the research was to evaluate the resistance of the different RKN resistant tobacco bred varieties to *M. javanica*, measuring parameters such as; the gall scores, root weight mass, foliage weight mass, number of second juveniles, number of third and fourth juveniles within the root and number of egg masses among the different varieties this was done at two weekly interval for ten weeks. The experiment was conducted in a Completely Randomized Design (CRD) with four replications. Seedlings were transplanted at six weeks into 6.5 cm diameter pots and 5000 *M. javanica* eggs were inoculated in each pot. Results showed that there were significant differences in galling rates; resistant varieties had low gall scores below the economic threshold value of 2 representing a very slight and trace galls up to 25 galls per root mass. However T 29 had higher gall score at week ten above 2, representing very trace galls but less than 25. T 64 and T 29 had lower root weights than other resistant varieties. T 66 had the highest foliage weights among the resistant varieties. In the J2s, J3s and J4s there were no significant differences among the resistant varieties but however T 66 had the highest number of J3s and J4s amongst the resistant varieties. In the number of eggs KM 10 had increased number however all other varieties had low numbers. The variety T66 had increased resistance among all the resistant varieties and T 29 had the least tolerance among all the varieties .It can be concluded that, use of certified resistant varieties in tobacco production increases yield and quality in tobacco production.

DEDICATION

I dedicate this project to my lovely sister Josephine Hwarari

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ABBREVIATIONS

ANOVA - analysis of variance
CV - coefficient of variation
GMO – genetically modified organism
J2 - second stage juveniles
J3 -third stage juveniles
J4 – fourth stage juveniles
LSD -least significance difference
PVY – potato virus Y
RKN - Root knot nematode
TIMB – Tobacco Industry Marketing Board
TMV – tobacco mosaic virus

CHAPTER ONE

INTRODUCTION AND JUSTIFICATION

1.1 Background

Nematodes refer to minute, thread-like, multi-cellular animals, in the group Ecdysozoa and in Phylum Nematoda (Cranshaw and Zimmerman 2013). They are an abundant species and constitute 80% of the animal kingdom (Hodda, 2011). Most thrive in water and soil and they parasitize on bacteria, fungi, protozoans and other nematodes (Lambert and Bekal, 2002). Some nematodes are entomopathogenic and yet safe for plants and animals. Soil borne nematodes are found near depths of rooting decreasing in population up to the soil surface (Olsen and Skriver, 2003). Plant parasitic nematodes are the most economically important group causing a reduction in quality, yield and surface area on root tissue hence uptake of nourishment from the soil to the crop and also parasitize on the readily stored carbohydrates (Khan, 2001).

Among the economic important plant parasitic nematodes, is the *Meloidogyne* species commonly known as root-knot nematodes, which cause symptomatic galls on roots and are of economic importance in agriculture (William-Woodman and Davis, 2001) . Nematodes which thrive best in tropical regions are *M. javanica, M .incognita and M.hapla*. The species have a short life cycle and reproduce more than a 1000 juveniles from a single egg (Zhang, 2013). Of particular interest to the research is the *M. javanica* with a wide host range, parasitizing more than 770 plant species which include tomato, most vegetables and tobacco making them economic important pests with a need for effective management (Sikora and Fernàndez, 2005). Other researches have suggested that *M. javanica* has increased virulence comparing to other species (Roberts and

Thomason 1986), hence it is responsible for an estimated 14% losses of all worldwide plant, translated to US$100 billion dollars (Tygat, 2000) It causes damage to tobacco by reducing crop quality and overall yield (Moghaddasi, 2003). Small scale tobacco production in Zimbabwe is reduced by 42% and 68% on commercial-scale due to poor nematode management (Mukhtar *et al* 2014). Tobacco production contributes greatly to the GDP of the country and export revenue, it plays a major role in the country's economy, since it is one of the most profitable enterprises in commercial agriculture and the primary reason many commercial farms exist and also generates considerable rural employment.

Current management practices used to control plant parasitic nematodes have been enhanced (Sadeghi *et.al* 2012), these methods include sanitation, use of resistant varieties, fallowing and crop rotation, soil amendments, biological control and irrigation, nematode suppressive plants, good cultural practices and use of nematicides (Jimmy and Robert 2005). However parasitic nematodes are difficult to control because of their wide host range, short life cycles, higher reproductive and their physiology (Lopez *et al* 2004). Among the different nematode management strategies, chemical control has been the most effective in reducing nematode populations; however the continued use of chemicals has resulted in development of resistance in the nematode species (TRB 2013). Other effects of chemical use have been toxicity to non-target organisms and detrimental effects on the environment as well as human health and residual effect. Scientifically it has been proven that Methyl bromide release to the atmosphere increases the rate of ozone depletion resulting in increased global heating, poor rainfall patterns and acid rain (CORESTA 2010). Environmental concerns and the negative impact of the chemicals on human health have caused their reduction in use as well as banning of most nematicides (TRB, 2013). As a result there is an urgent need to come up with environmentally friendly and effective alternative strategies to manage root-knot nematodes. Crop rotation is the commonly used in the management of root-knot nematodes in tobacco, since different crops have different nutritional demands from the soil and pest problems and root-knot has a wide host range, it makes it impossible to control the nematode (Phillips and Trudgill, 1995). Another practice is the use of trap crops such as sun hemp (*Crotalaria spectabilis*). This practice is not efficient in that not all invading nematodes can be destroyed. Another practice is of use antagonistic plants, these have antagonistic properties such as diffusates for example, *Tagetes erectus*, however with time the nematode may develop some resistance. Other practices include the use of cover crops (Bridge, 1996). Soil treatments and organic amendments are believed to induce toxicity and increase nematode tolerance. Also there are other cultural practices, Hominick 1996 suggested that early planting effectively reduces nematode invasion. According to researches done by Overman and Dama (1964), *M. javanica* has to effectively reduce populations at high anoxic conditions; however control is not feasible and impractical on large areas because of inadequate water supply, inadequate instrumentations and high capital requirements (Falkner 1970).

The use of resistant cultivars is important in that, unlike nematicides it has no effect to the environment, it reduces the cost of control, offers crop self-protection depending on the level of tolerance, increases the rotational value of resistance by suppression of J2 populations and reduces management practices in that most of the varieties also have other pathogen resistance capabilities like fungi (*Phytophtora, Pythium*) and bacteria (*Ralstonia*) (Roberts and Thomason 1995). Additionally there is no or limited need for continuous assessment of the disease rate and incidence in the fields (TRB,K 2014). Research has shown that resistant varieties to root-knot nematode (RKN) contain Mi-gene that reduces the penetration of J2s in the crop plant cells, furthermore findings by Anwar and McKenry (2002) have shown that once the J2s have penetrated the vascular tissues there is no barrier to further developments of the RKN species. This gene can be plant bred from wild. Almost all cultural practices proved not efficient in the control of nematode as compared to the use of resistant tobacco varieties to the *M. javanica*. Studies have shown that *M .javanica* causes considerable damage to flue-cured tobacco, the use of resistant varieties is fast becoming one of the most effective and environmentally sound controls (Barker et al. 1998). Schneider (1991) showed that penetration of *M. javanica* in most resistant varieties is inevitable but establishment of feeding sites is hampered due to plant mechanisms. Ibrahim (1987), revealed that tolerance of resistant tobacco varieties is affected by interaction with other species of the *Meloidogyne* but its effect remains outstanding. Most studies done on the use of resistant varieties against *Meloidogyne spp*. have been mainly focused on temperate root-knot nematode species, *M. hapla, M. fallax* and *M. chitwoodi*, there is therefore a need to also test the effectiveness of resistant varieties on the important tropical species such as *M. javanica*. Also mentioned before, the high reproduction capacity of *M. javanica* on resistant varieties gives reason for testing resistant tobacco varieties with local M .javanica populations prior to production so as to increase RKN management in infested soils.

1.2 Objectives

The main objective of the experiment was to evaluate the resistance of the different Kutsaga bred tobacco varieties to *M. javanica*.

1.21 Specific Objectives

1. To determine galling rate of the varieties at two weeks intervals.

2. To determine root wet mass (g)/ plot at two weeks intervals.

3. To determine the foliage weight of each tobacco plant at two weeks interval.

4. To determine number of infective J2/plot at two weeks interval.

5. To determine the number of J3s and J4s at two weeks interval.

6. To determine number of egg masses at two weeks interval.

1.3 Hypotheses

• There are no significant differences in galling rates among the different varieties.

• There are no significant differences in root wet mass (g) among the different varieties.

•There are no significant differences in foliage weight (g) among the different varieties.

•There are no significant differences in J2s infection among different varieties.

•There are no significant differences in J3s and J4s number among the different varieties.

•There are no significant differences in egg mass number among different varieties.

CHAPTER TWO

LITERATURE REVIEW

2.1 History and Origin of tobacco

Cultivation of tobacco in Zimbabwe began with the local people producing a variety called Nyoka (ZTA, 2011) a strain of *Nicotiana. rustica* and it grows naturally in Zimbabwe. Furthermore the ZTA (2011-2015) recorded that, first successful growing of flue-cured tobacco was in Mutare in 1984. In 1987 the first commercially grown tobacco was exhibited at an Agricultural show in Harare (History of Tobacco, ZTA 2011-2015). Thereafter in early 19th century, the growing of tobacco from seeds was introduced by E.H South in the Lake Chivero banks and built the first barn to cure it, which gave reason to the first auction sales in Zimbabwe in 1910(ZTA, 2011-2015). Challenges faced were due to lack of competition between buyers and over-production then abandoned in 1914. From 1914 onwards the crop was sold through sales by private treaty and co-operative selling, where growers were contracted to sell their crops to the Tobacco Co-operative Society. Then flue-curing was first used by Archie Henderson, grower and managing director of Tobacco Auctions Ltd at his farm now then Henderson Research Station and remains an important resource to Zimbabwe farmers. In 1936, the Tobacco Marketing and Levy Act provided the formation of the Tobacco Marketing Board (now the Tobacco Industry and Marketing Board) and the compulsory selling of tobacco through Auction Floors (TRB Annual Report, 2014).

2.2 Physiology and Biology of tobacco

The word tobacco originated from Portugal, word '*Tabaco',* (Bartolomé de las Casas, 1552). Tobacco is in the genus *Nicotiana*, in the nightshade family (*Solanaceae*). There are more than 70 species of tobacco and the most grown is *N. tabacum* (Detroit *et.al*, 2005). The plant is a long annual which has a fibrous root; the stem is erect, round, hairy and viscid. The tobacco plant has 10-20 leaves and grows up to three meters in height, its central stalk branches are long with a narcotic odor and a nauseous taste (Online Etymology Dictionary, 2015). The leaves are high in nicotine and other constituents are albumen, resin, gum, and inorganic matters. Leaves when dry can be smoked; after leaves are smoked then later nicotine decomposes into pyridine, furfurol, collidine, hydrocyanic acid, carbon-monoxide (Cosner , 2015). The poisonous effects of tobacco smoke are due to these substances of decomposed nicotine. Many plants contain nicotine however tobacco contains a higher concentration of nicotine. Unlike many other *Solanaceae* plants, they do not contain tropane alkaloids, which are often poisonous to humans and other animals (Cosner, 2015).

2.3 Economic importance of Tobacco

Tobacco is the most economic important non-food crop in Zimbabwe (BAT, 2001) and its industry plays a role in country's social and economic status. Most of the cultivated lands of the world are covered by tobacco plantations covering 0.1% of land (BAT, 2013). In Zimbabwe tobacco production has enable small-scale farmers to experience higher returns (FAO, 2006). After the land reform in 2000, political instabilities within Zimbabwe have resulted in economy challenges. Hence increased tobacco production by new farmers entering the industry has increased economic stability. From 2009 to 2012 there was a huge increase in tobacco production from 56.800 tons to 140.800 tons (TIMB, 2012). Furthermore tobacco production at its peak in Zimbabwe was at 236 000 tons in 2000 (TIMB, 2012).

Agriculture is the pillar of Zimbabwean economy contributing 17.3% to the GDP and 15.6% is from tobacco production (ZTA 2001). Tobacco production is a major contributor to the GDP of Zimbabwe. Estimated revenue from tobacco in the year 2010 was at US$348 million and highest in the year 2012 with estimated revenue of US$517 million. Income generation from tobacco production is independent of the size of land, so tobacco farming is beneficial to small-scale growers especially for sustenance farming. However production is labor intensive accounting to 50% of production cost (TIMB, 2010).

Also tobacco production contributes to government revenue by means of a levy system to farmers and buyers to which a fixed percent on the value of crop is paid to the government; however this generates several millions annually for the government. Generated money is then used to finance government linked boards like the TRB and TIMB for developing new cultivars and other technological production techniques that are related to tobacco production (TRB Annual Report 2013).

Tobacco production is also essential to the country in that it readily provides employment for the nation reducing poverty and crimes. Estimated employment trends in 1998 were about 170 000 workers who were engaged in the tobacco production. Estimated statics were that; 100 000 in hiring and 80 000 in tobacco related industry, amounting to 50% of Zimbabwean total labor (FAO, 2003).

2.4 Constraints in tobacco production

Production of tobacco in Zimbabwe has many constraints that are; financial shortages, poor infrastructure, erotic rainfall patterns, hailstorm damage, pest and diseases damage (TRB, 2015). Yield and quality of tobacco is greatly reduced by pathogens, these are either foliar or soil borne resulting up to 30% loss (TRB, 2014). These may be bacteria, fungi and viruses (Session 2003). These pathogens are responsible for diseases such as pythium rot, tobacco mosaic virus (TMV), potato virus Y (PVY), alternaria leaf spot; wildfire, white mold, granville wilt and fusarium wilt (Masuka *et.al*, 1998). Also pests reduce crop quality and yield on foliage of plant leaf and root; furthermore some act as vectors of diseases. Pests of economic importance are; tobacco aphids (*Myzuspersicae spp*), tobacco moth (*Ephestiaellutella spp*) and root knot nematodes (*Meloidogyne spp)* which are of high economic value, they include *M. hapla*, *M .incognita*, *M. arenaria*, and *M .javanica* only to mention a few .Problematic pest of particular interest to the research is the root knot nematode (*M .javanica*), this parasitize mainly on the root nourishing stored nutrients by the tobacco plant (Rich and Thomas, 2010). The major control strategies in Zimbabwe include good agronomic Practices.

2.5 Root Knot Nematode (*M.javanica*) Biology and Physiology

RKNs are simple animals which are multi-cellular of 1000 cells or less. The *M. javanica* is vermiform in almost all its life cycle; however it becomes swollen and rounded in later life stages J3 and J4, often described as a tube within a tube (Brusca and Brusca, 1990). Their outer skin or cuticle is secreted from the inner hypodermis, muscles are longitudinally attached to hypodermis, thus it allows only for movement in the dorsal ventral direction (snake-like movement). Its alimentary canal from head to tail, between the alimentary canal and the hypodermis is a fluid to maintain body shape and allow movement. Its mouth is hollow (like a hypodermic needle) called a stylet. It uses this to puncture plant cells, withdraw food and secrete protein and metabolites thus it helps nematode on parasitizing the plant. There is one directional linkage from the stylet, the pharynx and the intestines; this then ends in the rectum in the female and cloaca in the male nematodes. There are three - five salivary glands connected to the pharynx to saliva secreted by the stylet also it aids the RKN in plant invasion and parasitism. The pharynx is muscular, it also contains specialized areas that contract and expand the esophageal lining. This allows the nematode to swallow nourishment into intestine and also secrete saliva (Mohamed, 2010). *M. javanica* has its reproductive system in the middle to posterior and specie has both male and female system, however they do not reproduce asexually by parthenogenesis. The *M. javanica* ranges from 250 µm to 300 µm(J2s) when fully grown mm in length, averaging about 15-35 um in width, they often look segmented due to numerous annulations present on their cuticle this allows allow the nematode to bend but they are un-segmented (Putten et al., 2006). The *M. javanica* possess bilateral symmetry like higher animals although they have a superimposed trilateral and hexalateral symmetry. During their development the nematode is triploblastic (ectoderm, mesoderm and endoderm) in the embryo within the plant root (Kumar and Khanna 2006).

2.6 Life Cycle and Reproduction

The *M. javanica* life-cycle consists of six stages, egg, first-stage juvenile, second-stage juvenile, third-stage juvenile, fourth-stage juvenile, and adult (Shivaji *et al* 2013). In the second-stage juvenile (J2), after the egg is hatched it has increased locomotion with the soil, here it parasitize on a host plant. It penetrates the root vascular cells. The plant cells are triggered by the nematode saliva from the glands to enlarge forming root galls where the nematode feeds. As the juvenile feeds, it matures into J3 and further J4, however J3 and J4 stages are difficult to distinguish and appear as swollen. Finally it reaches the adult, the length a single cycle (from egg to egg) varies from a few days to more than a year and cycle largely depends on the environmental conditions, plant crop response to invasion and other factors. However Viglierchio (1991) explains that after penetration no plant physiological properties can reduce nematode development and invasion. Nematodes lay hundreds to thousands off and this depends on the weather patterns. During summer months, Westerdahl et al., (1998) found that the *M. javanica* life cycle can be long as four weeks in soil temperatures of 28-30^{o}c.

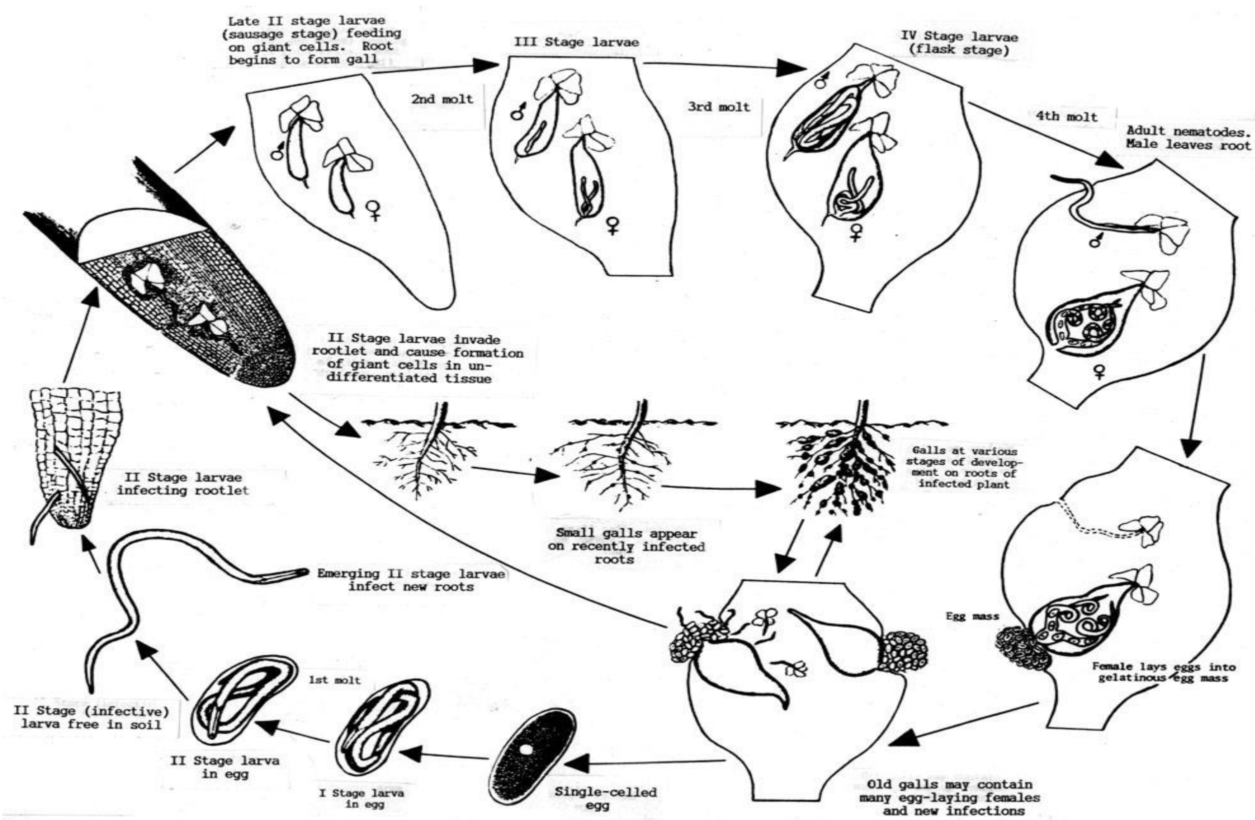

Figure 1 *Generalized life cycle of M. javanica (RKN)* (*Dr.* Colin Johnstone,.1998. University of Pennsylvania)

2.6.1 DAMAGE

Root knot nematodes (*M. javanica*), cause distinctive swellings (galls) on the roots (Cobb 1914). Infested plants are stunted and frequently chlorotic and tend to wilt greatly during the day and recover at night. Infected perennial plants show lack of vigor or poor stunted growth. Hence they reduced yields by up to 90% or more, depending on the degree of infestation, crop plant properties and environmental factors. Findings by the NC State University (2015) has shown that esophageal gland cells of the RKN secrete a protein that is responsible for invading root tissues and form root galls (Huang et.al 2003). Theses root galls are very visible to the naked eye (Perry *et.al* 2009). Hussey RS, 2006 reports that 16D10, a protein in the esophageal gland of the J2s also affects root growth (Dong , 2006). Further findings of 16D10 peptide alluded that, it is also responsible for signaling RKN host interactions. The nematodes thrive and live within the galls; size of the galls can grow up to 3 cm depending on the tobacco variety. The formation of galls disturbs the water and nutrient supply (Mitkowski 2010). Sometimes the galls can crack or split open mostly in tobacco and vegetables, this is detrimental to the plant for it allows entry of soil-borne pathogens. The upper plant symptoms exhibited are as a result of root malfunction due to reduced root cover and disturbed water and nutrient supply. These include poor growth and or stunting of the plant, yellowing or wilting of the foliage, and reduced root systems which can include root necrosis (McKenry *et.al* 2006).

2.6.2 Nematode Feeding and Host-Parasite Relationships

RKNs (*M. javanica*) do not particularly kill plants, they stress plants and in conjunction with factors such; poor water supply, inadequate nutrient supply and environmental conditions, act to reduce growth and yields. Most *M. javanica* infestations occur in areas with poor crop vigor and reduced yields that the plant are weak, however nematode penetrations and movement within the plant tissues results in plant mechanical injury and cell death or leaf necrosis. This injury interrupts the flow of water and nutrients from source to sink. Additionally penetrations create openings through which microorganisms can enter the plant tissues, thus sometimes the M. javanica acts as a primary infector. Some species also act as vectors (Westerdahl, Caswell-Chen and Bugg, 1998). Furthermore the nematodes thrive best in sandy soils and these conditions are conducive to nematode activity and to plant drought stress, (Fribourg, et al, 2009).

2.6.3 Control

Root-knot nematodes (*M. javanica)* are extremely important to the economy of Zimbabwe because of their large reduction to quality and overall yield in tobacco plant production (FAO 2015). Some of the most common useful methods for controlling nematodes include crop rotation practices (Onkendi *et.al* 2014), inspections, chemical control, solarization, use of target crops, flooding and electrocution or heating (though most not feasible) and plant breeding for nematode resistance(Duncan *et.al* 2015). Crop rotation in tobacco production may in include rotations with winter cover crops, such as wheat and rye (Tobacco Growers Guide 2013). Also rotations with summer crops though efficient crops are difficult to find, such as hybrids of sorghum and Sudan grass are effective in managing RKN, particularly cv. Jumbo (Yamamoto et.al 2001), are useful against most populations of the nematode. Research by Matthew Vann in 2013 at (NC State University 2015) has also suggested that rotating with soya beans fixes Nitrogen and reduces nematode populations. Rotations with weed fallows have proven to be conducive and satisfactory in increasing yield and reduce RKN populations (Duncan 2015); however crop rotation will not eliminate infestations because root-knot nematodes can remain in the soil as eggs for at least a year between host crops and most species can feed on a wide range of weeds. Nonetheless, rotation can significantly reduce losses when a field is planted again to a susceptible crop (NC State University 2015). Also longer seasons of fallowing have increased costs and increase soil erosion incidence. Other cultural practices may include cultivation, sanitation this may expose dominant nematode eggs but however it is underutilizing of fields. Biological control is also effective in the control of nematodes, in this method species like fungus, bacteria, insects and virus are widely used because of their readily predative effect to the nematode such as; *Gliocladium roseum* (Nematode Fossils 2013). Recent experiments at TRB 2014 with *Armilliria galleria* have been proven efficient in the control of RKN though this have not been put to test field trials. This method is expensive to establish because of the requirement to culture, maintain and possible ecological imbalances in the field ecosystem. Another method widely used in developing countries is heating, it is an effective method before planting the crop however this method destroys even non-targeted microorganisms in the soil and can only implemented before planting. Furthermore this method reduces the soil fertility of the soil by increasing processes like; nitrogen cycle in the soil and also has an effect the environment by air pollution (Hodda, 2011).

Over the years the use of nematicides has proved to be efficient but however the use of fumigant nematicides, namely EDB, 1, 3-D and Methyl-bromide for use in the lands and seedbed have been prohibited due to their detrimental effects to the environment (TRB 2015). Efforts have been made to replace the nematicides last year the Tobacco Research Board recommended Metham sodium and Oxamyl because of their performance, however, variable efficacy was noted in the performance of Metham sodium, associated with application time. Also there are promising alternatives which are Velum (Fluopyram), Sesamin and Metham potassium. Also supplementary nematicides such as Fenamiphos, Ethoprophos and Solvigo were incomparable.

The last and method of concern to this experiment is the use of resistant cultivars, it is important in that it reduces the effect of nematicides to the environment. Scientifically it has been proven that Methyl bromide release to the atmosphere increases the rate of ozone depletion resulting in increased global heating, poor rainfall patterns and acid rain. The use of resistant varieties reduces the cost of control and management practices, in place of other chemicals to control pathogens like fungi (*Phytophtora, Pythium*) and bacteria (*Ralstonia*) when the plant has a genetic control. Additionally there is no or limited need for continuous assessment of the disease rate in the fields. All these cultural practices proved not be more efficient in the control of nematode as compared to the use of resistant tobacco varieties to the *M. javanica*. Studies have shown that *M .javanica* causes considerable damage to flue-cured tobacco, the use of resistant varieties is fast becoming one of the most effective and environmentally sound controls (Barker et al., 1989). Schneider (1991) showed that penetration of *M .javanica* in most resistant varieties is inevitable but establishment of feeding sites is hampered due to plant mechanisms .Also Ibrahim (1987), revealed that tolerance of resistant tobacco varieties is affected by interaction with other species of the Meloidogyne but its effect remains outstanding. Most studies done on the use of resistant varieties against Meloidogyne spp. have been mainly focused on temperate root-knot nematode species, *M. hapla*, *M. fallax* and *M. chitwoodi*; there is therefore a need to also test the effectiveness of resistant varieties on the important tropical species such as *M. javanica*

2.7 Tobacco breeding for root-knot nematode

Tobacco plant breeding for resistance has proved efficient over the years (Nyoka, 2005), because of its reduced effects to the environment. Plant breeding for resistance has enhanced the characteristics of tobacco plants and their tolerance to root-knot nematodes, resulting in new and better varieties which have desirable qualities (Edward Willbanca, 2008). Crop species of known characteristics; such as resistance to of known plant diseases and pests. These are then inter or cross bred to produce varieties that are fit for cultivation and a higher quality yield (Nyoka, 2005). Interbreeding of desirable qualities produce first generation and propagated (Willbanca, 2008). A plant line with acceptable pest resistance against one pest, such as root-knot nematode can still lack resistance against other pests, so there is a need to breed for multi characteristic resistant crops (Kosack et al., 1997). Tobacco breeding in Zimbabwe is done by Tobacco Research Board, a parastatal solely responsible for research on tobacco, basing on varieties and hybrids. It is limited to commercial seed production of male-sterile hybrids (TRB. Burley Tobacco Handbook 2014). Then the Zimbabwe Tobacco Seed Association multiplies and distribute varieties from TRB, until recently the ZTSA also ventured in seed production. Biotechnology integration in the national tobacco research programs has successful made tobacco breeding successive in Zimbabwe. This effort has nurture the institution to higher levels of tobacco breeding because of manipulation of processes like; tissue culture, anther culture. Furthermore releasing doubled haploid cultivar of tobacco which is still grown today. The Board has currently made attempts to overcome interspecific incompatibility barriers.

2.7.1 Mechanism of Pest resistance

Some plant species are resistant to RKN, however recent researches by the National Academy of Science in the U.S.A have identified resistance loci for root-knot nematode in some crop species (Williamson, 2006). The function of the resistance genes are to recognize RKN biotypes and select for virulent field populations. However recombinant DNA technology has provided alternatives for detrimental effects of chemicals to RKN management (Fassuliotis, 1985) and in some plants the Mi-gene has been located and transferred to other susceptible plants; which is the base of the resistant varieties used in this experiment through plant breeding. The Mi-gene in the tobacco varieties is believed to have been obtained from tomato (*L. esculentum*). Literature outlines that this gene was also transferred from *L. peruvianum* using the embryo rescue technique, thus resistance in modern tomato varieties is from a hybrid by Smith (1944) between the nematode and the plant genotype. Mechanism or pest resistance is little known, but researchers have found that a susceptible plant cells responds to the invading nematode head by enlarging forming galls to serve as feeding sites and for developing, but in a resistant cultivar with the Mi-gene the adjacent plant cells fail to form galls due to necrosis of those cells at the end of the pest and this happens within a day (Dropkin et.al, 1969). This defense mechanism is triggered by the Mi-gene (Williamson, 1991).

Chapter Three

Materials and Methods

3.0 Study site

The trial was carried out at Kutsaga Tobacco Research Board situated in Harare, in natural region IIb of Zimbabwe. The soils are deep well drained, sandy loam. At an altitude of 1479 meters above sea level and latitude 170 55’ S, longitude 310 08’ E and average annual, maximum and minimum temperatures of 24.7 ^{o} C and 12.^{o} C (Vincent and Thomas, 1961). The mean annual rainfall for Kutsaga Research Station is approximately 820mm, but this subject to wide fluctuations and in the last 30 years has varied between 430mm and 1320mm (AREX, 2005). Slightly more than two thirds of the total rainfall normally falls during the months of December, January and February.

3.1 Variety description

Table 1 Tobacco varieties bred at Kutsaga Research Board and a single tomato variety which was used as a control. Varieties in include four RKN resistant varieties and a susceptible variety KM 10 as a negative control and tomato (Rodade) as negative controls since source of the Mi-gene is legally protected.

Tobacco variety	**Origin**
T 66(tobacco)	Kutsaga TRB
T 71(tobacco)	Kutsaga TRB
T 64(tobacco)	Kutsaga TRB
T 29(tobacco)	Kutsaga TRB
KM 10(tobacco)	Kutsaga TRB
Rodade (tomato)	Amber Valley Farm

3.2 Experimental Design

The experiment was laid in a Complete Randomized Design, in a greenhouse with four replication, six treatments and two negative controls KM 10 and tomato (Rodade). Each replication had 120 plants

3.3 Methodology

Nematode Inoculum: *M. javanica* eggs were extracted from cultures tomato cv. Money maker (*L. esculentum*) roots in a greenhouse at 25-30^{o}c. Invaded roots were first washed off excess soil then blended, the blended roots were then immersed in 0.5 % NaOCl in plastic jars 200ml (Hussey and Barker 1973; Boneti and Ferrazi 1981) and vigorously agitated for 5 minutes. Agitation was followed by rinsing of the roots by tap water over a Fleihmeir (1972) sieve method of 74µm and 25µm sieves. Pulp in sieve number 1 with 25µm was then rinsed into a 500ml cylindrical beaker. Then 1ml was drawn using a dropper which was then transferred to a counting grid under then fixed under a light microscope. Counts recorded were 122 egg/ml, counting was done using a grid-slide manual counter and volume of pulp to be inoculated per pot was calculated as follows;

$$\text{Volume of inoculum / pot} = \frac{\text{Number of eggs to be inoculated}}{\text{Number of eggs per / ml}}$$

$$\text{Thus volume} = \frac{5000}{122} = 42\text{ml per pot}$$

Fumigating soil with Methyl Bromide: Sandy-loamy soil was heaped; moisture placed on level ground covering an area of 4.5m^2, and then covered using a black polythene plastic. Canister was used in application of methyl bromide at an application rate of 30g/m^2 or 10ml/m^2. Then the fumigated soil was air tight covered for 14 days. On the 14^{th} day the soil was cultivated to allow escape of trapped gases.

Tobacco and Tomato seedlings: Tobacco cv. T 66, T 71, T 64, T29, KM 10 and Rodade seedlings were first sown in 6.5 cm diameter pots which were Bromide fumigated to ensure zero nematode populations. Same procedure was done to the tomato cv. Rodade in 6.5 cm diameter pots.

Transplanting: Variety seeds sown were then transplanted into 12.5cm diameter pots after 6 weeks, the pots were first filled with fumigated soil to ensure zero nematode population at the beginning of the experiment. A depression was first made in the soil filled 12.5cm diameter pots using an empty 6 cm pots, 120 pots were successive transplanted containing 100 tobacco seedlings and 20 tomato seedling cv. Rodade.

3.4 Data Collection

Root weight Assessment: Roots were collected at two weeks interval and a destructive sampling was done. At first the roots were washed of excess soil using tap water then dried using paper towel. Then they were weighed using an electronic balance then recording were taken.

Foliage weight Recordings: The tobacco plant was cut using a scissor at the mid rib then the upper foliage was rinsed to remove dust and pests, then dried using paper towel. The foliage was then put over an electric balance and recordings were weighed using an electrical balance.

Root gall scoring: Roots weighed were not discarded, were then taken under a light into a white tray then the invade roots were scored using the Dauton (1961) gall index, shown in table below, then a 5g sample of each variety assessed was further boiled in acid Fuschin that gives a red stain for a few seconds, then the stained roots were preserved in a glass bottle in lactophenol for further root assessments.

Table 2 Root gall index (Dauton, 1961) showing infection class of crop plant invaded by the RKN and its description determined by the number of galls present

Infection class	**Index Value as a %**	**Description of degree of galling on roots of indicator plants**
0	0	Free from galls
1	1	Trace infection, less than 5 galls
2	5	Very slight, trace to 25 galls
3	10	Slight, 26 to 100 galls
4	25	Moderate, numerous galls, mostly discreet
5	50	Moderately heavy, numerous galls, many coalesced
6	75	Heavy, very numerous galls, mostly coalesced, root growth slightly retarded
7	90	Very heavy, mass invasion, slight root growth
8	100	Extremely heavy, mass invasion, no root development

J2s, J3s and J4 counting: Samples collected per each variety were then mount on slides using Glycerol then later fixed on a Zeiss Carl Photo microscope for assessment of RKN development and invasion. Images of particular interest in the development of the nematode were captured for further analysis

3.4.1Data Analysis

Analysis of variance of resistance within cultivars was done using statistical package GENSTAT 14th ed. and SSPS version. 21. Separation of means was done using the Least Significant Difference (LSD) and a significance test of 5% was used.

CHAPTER FOUR

RESULTS

4.1 Galling rates for different varieties according to the nematology scale

There were significant differences ($p < 0.01$) in galling rates among the different varieties as shown in Figure 2. At week two; Rodade, KM 10 and T 29 had the highest gall score, with an infection class of 2 with slight trace galls up to 25 per root. On the fourth week T 71 and T66 had increased invasion, however they had the least number of gall score of 1 representing a trace infection of less than 5 galls per root. At week six T 64 and T 71 had the least number of gall score while Rodade had the greatest gall score of 5. In the eighth week, Rodade and KM 10 had the highest gall score of 4 representing a moderate, numerous galls which was discreet and T 71 and T 66 had the least gall score of 1. In the final week Rodade had the greatest invasion with a gall score of 6 described as heavy, very numerous galls, mostly coalesced and root growth is slightly retarded and T 64 had the least gall index of 1.

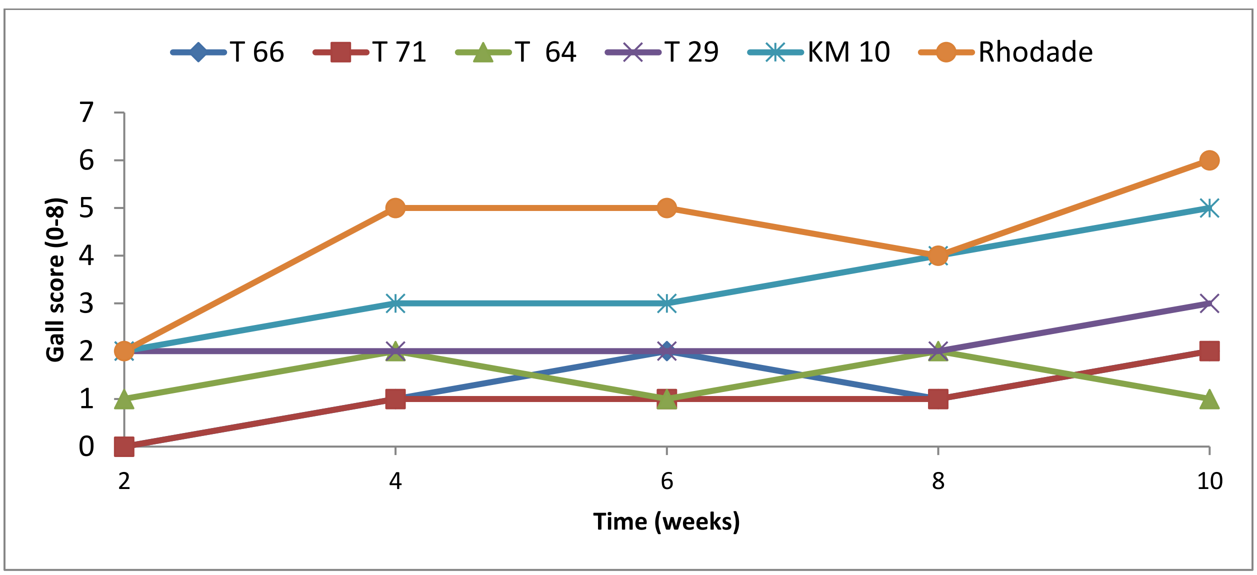

Figure 2 Gall scores of different varieties at a two weekly interval, 2, 4, 6 and 8 weeks after M. javanica eggs inoculation. The lines show gall score trend (Dauton 1967) representing number of gall on the variety roots.

4.2 Root weight for different varieties.

There were significant differences in root weight of the different varieties (p = 0.028) as shown in figure 3; however there were no significant differences at two weeks among the varieties and T 66 had the highest root weight. On the fourth week T 71 was significantly different to T 64, also T 64 was significantly different to KM 10 and KM 10 had the highest weight above 4g. On the sixth week Rodade was significantly different to all other varieties and had the highest root weight above 12g and T 66 was significantly different other varieties. In the eighth week T 29 was significantly different to T 64, T 71 and T 66 but not to other varieties and also it had the highest root weight. In the final week KM 10 and T 29 were significantly different to T 64, T 71 and T 66 and KM 10 had the highest root weight of 14g, as shown in Fig 3

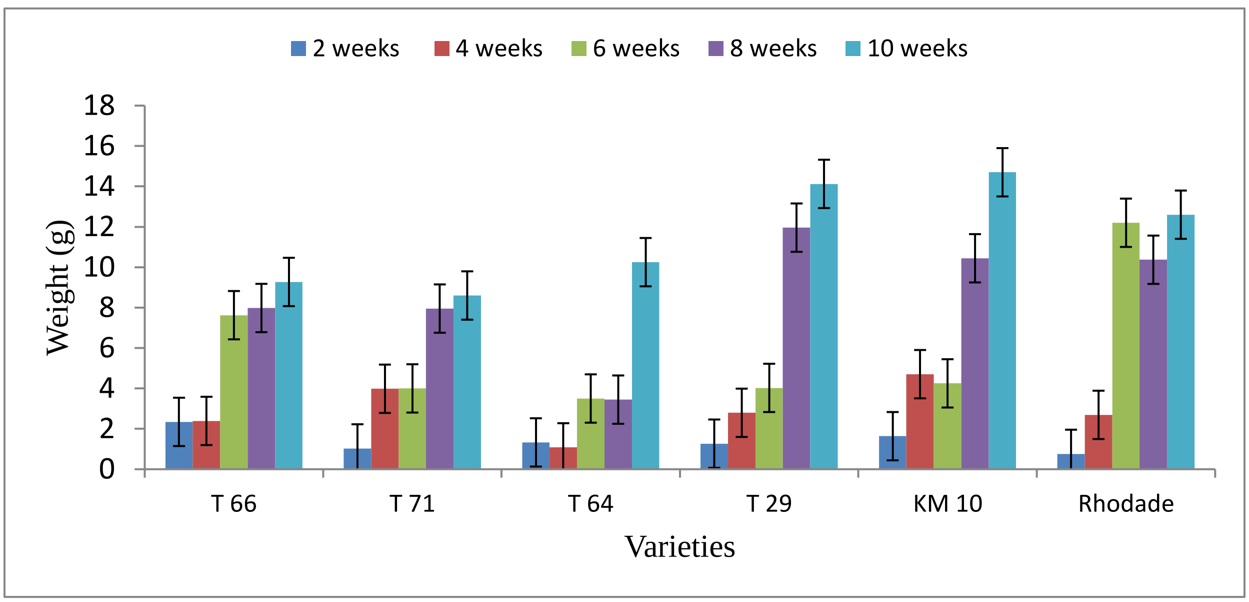

Figure 3 Root weights for different varieties at two weekly intervals at 2, 4, 6 and 8 weeks after M. javanica eggs inoculation. The bars show mean root weight of the different variety roots ±SE of the root weights observed. Different letters indicate significant differences among the treatments (LSD) test (P<0.05).

4.3 Foliage weight for different treatments

There were significant differences ($p < 0.01$) in foliage weight among the varieties as shown in figure 4, at week two, Rodade was significantly different to T 64 and T 66, and however T 64 had the highest foliage weight. In the fourth week Rodade was significantly different to all other varieties and KM 10 had the highest foliage weight. In the sixth week Rodade was significantly different to KM 10, T 29 and T 66, however T 66 had the highest foliage weight. In the eighth week Rodade was significantly different to all other varieties except T 64 and T 29 had the highest foliage weight. In the final week, Rodade was significantly different to KM 10, T 29 and T 66; however T 29 had the highest foliage weight among all the varieties of above 45g.

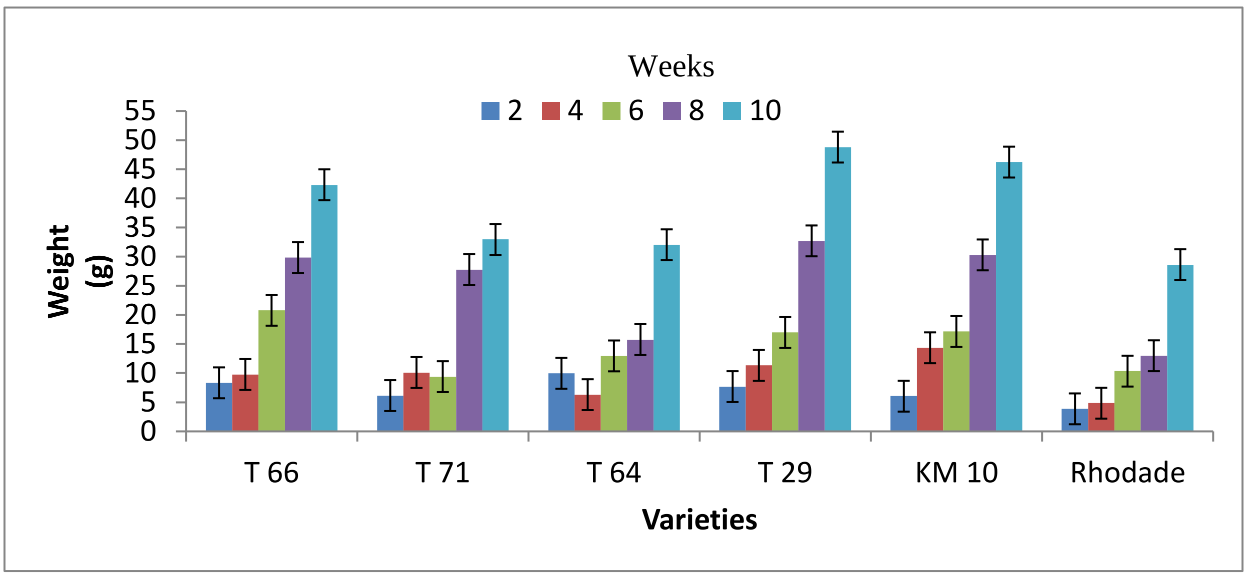

Figure 4 Foliage weights for different varieties at two weekly intervals of 2, 4, 6 and 8 weeks after M. javanica eggs inoculation. The bars show mean foliage weights recorded from an electric scale of the different variety roots ±SE of the root weights observed. Different letters indicate significant differences among the treatments (LSD) test (P<0.05).

4.4 Number of J2s in different varieties

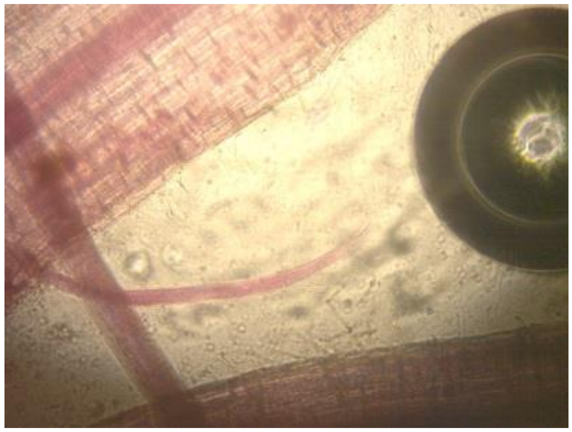

Figure 5: Images showing J2 as taken by the Zeiss Photo microscope at week 10.

There were significant differences in the number of second stage juveniles (J2)($p = 0.02$) among the varieties as shown in figure 6, At two weeks T 29 had the highest number of J2s over 1000 and KM 10 had the least population. In week four KM 10 had increased number and had the highest number of J2s while T 71 had the least number of J2s. In the sixth week all the varieties had reduced number of J2s and KM 10 below 250 and T 71 had no J2s. Similar results were obtained in the eighth week. In final week KM 10 had increased numbers but still below 250 and all other varieties had low numbers of J2s, However slight increase was observed in the final week.

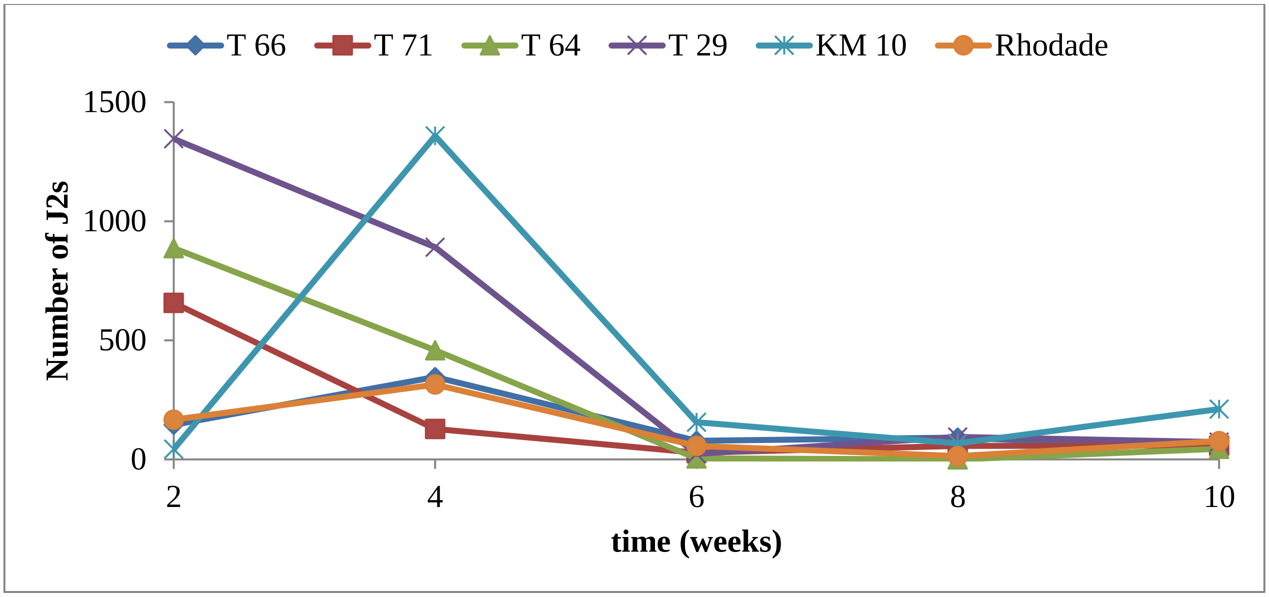

Figure 6 *Number of J2s for different varieties at two weekly intervals of 2, 4, 6 and 8 weeks after 5000 M. javanica eggs inoculation. Different lines show the number of J2s counted using a compound microscope.*

4.5 Number of J3 s and J4s of different varieties

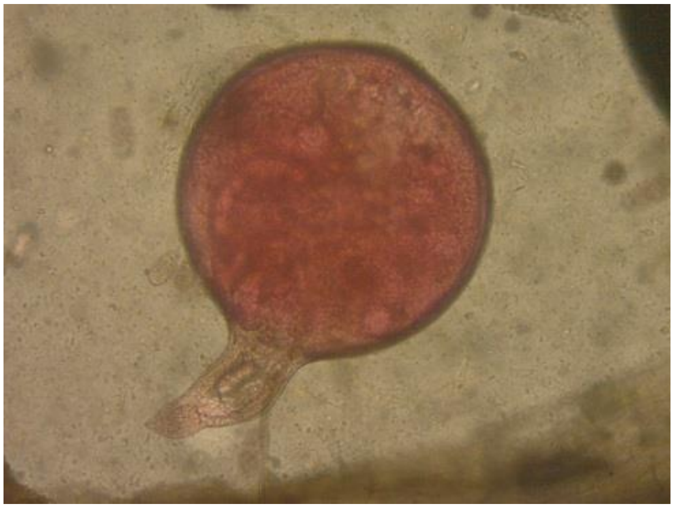

Figure 7: Images showing J3 or J4 at week ten. Since they are indistinguishable and physiologically similar it is not certain of which is J3 or J4

There were significant differences in the number of J3s and J4s ($p < 0.01$) among the different varieties as shown in figure 8, At week two all the varieties had no J3s and J4s present. In the fourth week varieties had increased numbers and KM 10 had the greatest number of J3s and J4s above 50, while T 64 had the least. In the sixth week KM 10 had the highest number of J3s and J4s above 100 while T 64 had the least number below 20. In the eighth week T 66 had increased numbers and had highest number of J3s and J 4s above 70 while T 71 had the least number of J3s and J4s. In the final week T 71 had the least number of J3s and J4s below 20 and T 64 had the highest number of J3s and J4s above 90.

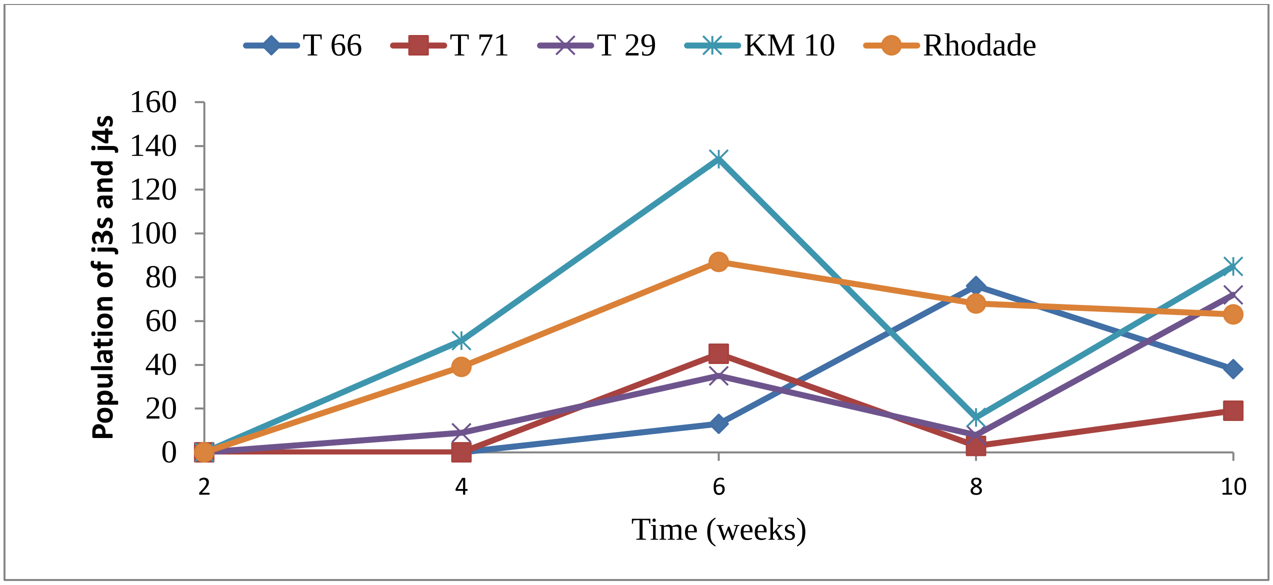

Figure 8 Number of J3s and J4s for different varieties at two weekly intervals of 2, 4, 6 and 8 weeks after 5000 M. javanica eggs inoculation. Different lines show the number of J3s and J4s counted using a Zeiss Carl Photo microscope.

4.6 Egg masses of different

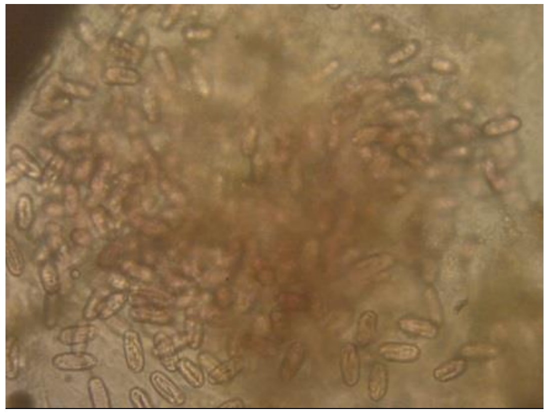

Figure 9: *Images showing egg masses containing a thousand M. javanica eggs at week ten as captured by the Zeiss photo-microscope*

There was a significant difference in the number of egg masses ($p < 0.01$) among the varieties as shown in figure 10, in the week two there were no eggs masses present in all the varieties. In the fourth week KM 10 and Rodade had increased numbers while KM 10 had the highest number of egg masses and other varieties had no egg masses present. In the sixth week all varieties had increased numbers of egg masses but KM 10 had the highest number of egg masses above 200 while T 64 had the least number of egg masses present. On the week eight T 66 had the highest number of egg masses present while T 71 had the least. In the final week Rodade had the highest number of egg masses while T 71, T 64 and T 29 had the least number of egg masses below 20.

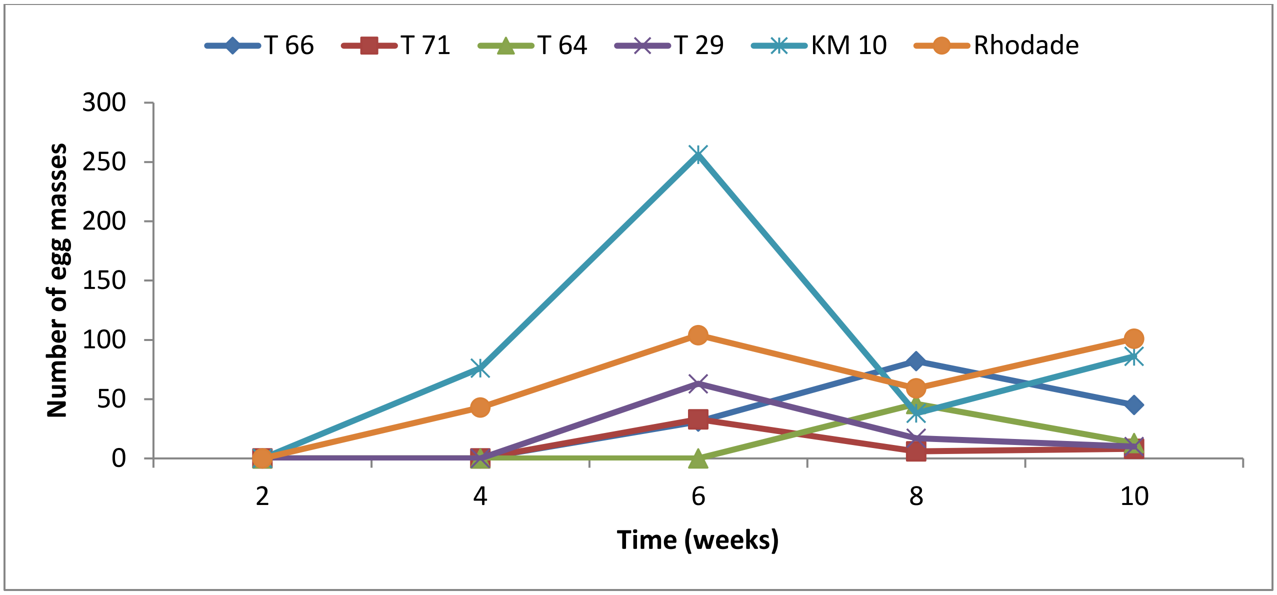

Figure 10: Number of egg masses in varieties at two weekly intervals of 2, 4, 6 and 8 weeks after 5000 M. javanica eggs inoculation. The different lines show the number of J2s counted using a compound microscope

Chapter Five

Discussion

5.1 Gall scores for different varieties

KM 10 and Rodade recorded the highest gall scores among all the varieties. The first invasion was noticed in KM 10 which is a susceptible variety at week two, this follows the findings by Colin (2000) of RKN life cycle that *M. javanica* J2s hatch after seven days but the effects can be noticed thereafter, also Sayre (1958) found out that susceptible varieties to RKN have higher gall scores because of involvements of oral secretions, anal excreta, and accessory gland secretions that initiate growth of galls. Additionally tobacco varieties release phenolic compounds that attract J2s (Schoonhoven et al., 2005; Gullan and Cranston, 2010). However there was reduced penetration in the resistant varieties this is suspected to be due to the presence of the Mi-gene in the resistant varieties; that confers resistance by inhibiting penetration of the RKN juveniles. Hence the resistant varieties managed to confer resistance below economic threshold values of 3, however T 29 had increased gall score on week ten above 2 but below 3 and among the resistant varieties T 29 had reduced resistance. Resistance in resistant varieties may be suspected to follow findings by Bakker et al (2006), which were based on microscopic observation of various incompatible interactions between nematode and plant. First resistance was on the development of galls described earlier due to the presence of Mi-gene and the second is as a result of a compensatory reaction, resulting in necrosis of the galls a few days after infection, as was noticed in Rodade. The reaction is a defense mechanism and is characterized by an oxidative reaction resulting in the production of active kinds of oxygen, such as hydrogen peroxide (H_2O_2), the superoxide radical (O_2) and accumulation of phenylpropanoids which are toxic to feeding J2 inside the gall.

5.2 Root weight for different varieties

Rodade had the greatest mean weight compared to all the varieties but with the greatest coalition and burst of the galls, this is also evident to the modern research by Javed (2008), that invaded tomato varieties increase root growth as a defense mechanism to sustain root-plant tissue exchange of nutrients, thus there is rejuvenation of parasitized plant root tissue. It has been suggested that there is a change in primary metabolites after J2 penetration by Schwachtje and Baldwin, (2008) to increase photosynthate in the roots, therefore this theory may account for the increased root weight. Furthermore Fassuliotis (1985) supported that tomato varieties increase root growth as a defense mechanism. Additionally the root structure of Rodade has a long tap root and has a dense fibrous root growth that may account for the extra weight. However KM 10 had reduced root growth and in some cases only the tap root had only developed much of the fibrous roots was believed to have parasitized by the J2s. Schneider 1991 evidently supported that J2s of *M. javanica* have increased virulence compared to all other species of nematodes due to their increased adaptability and acclimatization in adverse conditions. Van den Burg and Tokken (2009) further found out that penetration, migration, induction of feeding sites may induce plant stress to the plant resulting in hormonal regulatory responses in the growth of plant roots stimulating reduced growth and physiological changes. All the resistant varieties had a normal root growth

5.3 Foliage weight for different varieties

There were significant differences in foliage weight among the varieties, Rodade had the greatest mean foliage weight however T 66 was the first to germinate and Rodade plants had the tallest plants among all the varieties. Among the varieties T 71 and T 29 had the fastest growth among all the varieties. This is evident to the TRB manual and guidelines. Among the resistant varieties T 64 had the largest leaf size at week ten among all the varieties however it was short and it is evident to the TRB tobacco manual (2014). Rodade did not resemble any above ground symptoms of nematode infestation however KM 10 had reduced leaf sizes and necrotic leafs that were yellow. However KM 10 had stunted growth which had chlorotic spots on the leaves and characterized by thin steams. This is evident to findings by Onkendi (2014) of above ground symptoms with rapid yellowing due to reduced root surfaces and disruption of vascular system by the presence of feeding sites on the roots. Furthermore McClure (1977) explains that there is reduced foliage mass in invaded plant due to poor crop growth because of the presence of the parasitizing RKN in the root surfaces.

5.4 J2 populations in different varieties

There were significant differences in J2 population among the varieties, at two weeks there were low populations in KM 10 which doubled at week four and further reduced after week four this follows the life cycle of the root-knot nematode after the findings by Makumbi-kidza et al (2000), that day 14 is the climax of hatching hence there are increased populations, however the low numbers in KM 10 at week two is suspected to be as a result of reduced virulence due to the fact of late root maturity and establishment in KM 10. However resistant varieties conferred resistance and evidently signified the ability to resist penetration of the J2s. This resistance is suspected to be a result in the presence of the Mi-gene in tobacco that triggers the adjacent cells of the plant to the pest head to tighten and prohibit entrance of the nematode (Dropkin et.al, 1969). Viglierchio (1956) explains that after penetration there are physiological characteristics conferred by the plant to provide and reduce further development of the invaded j2s in the crop plant. In some of the resistant varieties like T 64 had higher nematode populations that penetrate the plant this follows findings by Ornat and Verdejo-Lucas (2001) that some *M. javanica* can overcome resistant varieties and penetrate their vascular cells. However in the susceptible varieties population size extracted in the roots was proportional to the size of inoculum this follows the findings of McClure and Viglierchio (1956) that population size in root galls increases with inoculum.

5.5 J3 and J4 population in different varieties

There were significant differences in the number of J3s and J4s among the varieties, however in first populations were evident in KM 10 and Rodade in the fourth week and population increased up to week ten this follows the life cycle of RKN by University of Pennsylvania (2000), that third juveniles are first evident in the invaded plant at twenty-one days and their development proceeds. In the susceptible varieties number of J3s and J4s was almost proportional to the inoculum size as findings by Alan (1993) that once the second juveniles had penetrated the plant root cell development and further invasion has greater probability of success. However in the resistant varieties there were low populations of J3s and J4s, this is believed to because of low penetration rates of the second juveniles and also death of the J2s due to lack of nutrient supply in the feeding sites (Walters 2006). Additionally Walters supports there is 50% chance of hatched J2 survival under ample conditions.

5.6 Number of egg masses in different varieties

There were significant differences among the varieties in the number of egg masses present in the crop plants. This is also evident by the presence of large number of egg masses in susceptible varieties, this follows the life cycle of M. javanica as stated by University of

Pennsylvania (2000). Also in resistant varieties there were reduced numbers of egg masses due to reduced penetration of the j2s.

5.7 Recommendations

1. Farmers to incorporate integrated nematode approach in the management of Root-Knot nematode such as crop rotation, adopting the use of bio-organic amendments, and the use of resistant varieties.
2. Farmers to adopt the use of certified resistant varieties seed in their production
3. Further study and research of source of resistance in tobacco.
4. Experiment need to be repeated to authenticate the results

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APPENDICES

Appendix 1: Galling rates for different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replication stratum	9	0.42527	0.04725	1.68	
replication.*Units* stratum					
treatment	5	1.34629	0.26926	9.55	<.001
Residual	45	1.26852	0.02819		
Total	59	3.04007			

Appendix 2: Root weight for different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replication stratum	9	725.798	80.644	21.53	
replication.*Units* stratum					
treatment	5	52.349	10.470	2.80	0.028
Residual	45	168.532	3.745		
Total	59	946.679			

Appendix 3: Foliage weight for different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicatio stratum	9	7627.24	847.47	42.30	
replicatio.*Units* stratum					
treatment	5	683.92	136.78	6.83	<.001
Residual	45	901.46	20.03		
Total	59	9212.62			

Appendix 4: Number of J2s in different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replication stratum	9	7.42743	0.82527	18.69	
replication.*Units* stratum					
treatment	5	0.64982	0.12996	2.94	0.022
Residual	45	1.98725	0.04416		
Total	59	10.06450			

Appendix 5: Number of J3 s and J4s for different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replication stratum	9	36425.3	4047.3	5.48	
replication.*Units* stratum					
treatment	5	19237.9	3847.6	5.21	<.001
Residual	45	33218.7	738.2		
Total	59	88881			

Appendix 6: Number of egg masses for different varieties

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replication stratum	9	447.199	49.689	7.66	
replication.*Units* stratum					
treatment	5	329.779	65.956	10.16	<.001
Residual	45	292.056	6.490		
Total	59	1069.034			