Using Neem to Control Charcoal Rot Of Chickpea

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Abstract

Charcoal rot is an important soilborne disease that infects a wide range of plant species including chickpea, caused by *Macrophomina phaseolina* resulting in serious yield losses. This study was undertaken to control this disease by a natural fungicide agent, the neem tree which has a well-known fungicidal effect in most of its parts. In vitro experiments examined neem seed oil and neem leaf extract to determine their inhibitory effect on the growth of two strains of *M. phaseolina*. Neem oil extract promoted the mycelial growth of both strains, while neem leaf extract reduced the growth of both strains. Glasshouse experiments examined the effect of neem leaf powder on the development of charcoal rot of two cultivars of chickpeas: desi and kabuli. Neem powder reduced the symptoms of infected plants and promoted their growth so that it was the same as in uninfected controls. However, there was a slight phytotoxic effect when neem was applied in the absence of the pathogen. A glasshouse experiment showed that neem powder could be safely used on the beneficial microbes *Rhizobium* and mycorrhiza. In order to optimise rate and application time, neem leaves were prepared in different formulations, pellets, capsules, aqueous extract and powder. These were used in rates 0.5%, 1%, and 1.5% w/w in pot experiments, applied either pre- or post-emergence of chickpea seeds. Neem pellets at 0.5% applied pre-emergence had the optimum effect on charcoal rot symptoms with less phytotoxicity to the plant. The effect of neem pellets and neem powder on control of charcoal rot of chickpea was examined under field conditions. Neem powder reduced disease symptoms and increased shoot dry weight of the plant. Neem pellets controlled the disease and also had a large growth promotion effect. Neem pellets increased the number and the dry weight of rhizobium nodules. Neem reduced the population of soil fungi but not of bacteria. Tests of growth inhibition against a suite of typical soil fungi showed a range of responses from high to no inhibition. Neem extracts did not inhibit growth of the important chickpea pathogen *Phytophthora medicaginis* and neem pellets did not control Phytophthora root rot of chickpea in glasshouse experiments. Neem shows promise as a control method for charcoal rot and some other soilborne diseases, which does not have a negative effect on beneficial microbes.

Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is currently not being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been fully acknowledged in this thesis.

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Ali Salman

Dedication

I dedicate my thesis to

My sister Nidhal, my wife, and my adopted daughter Hiba

And to all my friends

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Contents

Chapter 1: General Introduction

The history of legumes started with human civilizations and their evolution throughout many different regions of the world. The ancient Egyptians had a high regard for lentils, and the Romans appreciated them. During the reign of Caligula they transported 840 t of lentils to Rome. During this period the use of beans as a foodstuff was negligible in Egypt, although peas had been a staple food in Rome for some time. Their use became popular in the green form in the 17th century, when it was a fashionable dish of the rich (Harlan & de Wet, 1971). The word *halluru* which symbolizes chickpea was found in ancient manuscripts from the earliest time before 3000 BC in Mesopotamia. Chickpea (*Cicer arietinum* L.) was considered one of the first grain legumes to be domesticated in the Old World, and was also one of the crops domesticated with wheat, barley, rye, peas, lentil, flax and vetch (Redden & Berger, 2007) Chickpea is named *gram* in the Anglo-Indian language *(Bengal)* which was derived from the Portuguese *gràо (grain)* (Van der Maesen, 1987). Chickpeas are also known as garbanzo beans. The ancient Egypt and Levant are thought to be the centre of origin of chickpea. However, the world's leading producer of chickpeas is India (Wallace, Murray, & Zelman, 2016). According to Huntrods (2013) there are two main types of chickpea grown in the world: Kabuli which has large rounded, cream coloured seeds while Desi has small angular dark coloured seeds.

Nutrition Value of Chickpea

According to Wallace et al. (2016) pulses contain higher proportions of protein (17%–30% by dry weight) in comparison to other plant foods. Chickpea is a good source of carbohydrates and high quality protein compared with other pulses. Also, chickpea has good amounts of essential amino acids, except for those containing sulphur.

Chickpea proteins are mainly globulins and albumins. A smaller amounts of prolamines and glutelins are also present (Saharan & Khetarpaul, 1994). The major storage carbohydrate in chickpeas according to Jukanti, Gaur, Gowda, and Chibbar (2012) is starch followed by dietary fibre, oligosaccharides and simple sugars such

as glucose and sucrose. Although there is a low proportion of lipids, chickpea is rich in oleic and linoleic acids which are the nutritionally important unsaturated fatty acids. Chickpea is a good source of important vitamins such as riboflavin, thiamine, folate, niacin, and vitamin A. Chickpea oil contains important sterols such as β-sitosterol, campesterol and stigmasterol. As well, chickpea seeds have Ca, P, Mg, and K. Overall, chickpea is an important pulse crop that has several potential health benefits which could have an effect on some human diseases (Jukanti et al., 2012).

In Iraq, Chickpea is considered an important part of the diet for many people. However, most chickpeas in the Iraqi market are imported from other countries such as Iran, and Syria. There are no clear data about the annual production of chickpea in Iraq, but there are a few parts in the northern area of the country where chickpea is grown such as Duhok and Zakho (Emenky & Khalaf, 2010). Growing chickpea in Iraq faces many challenges. One of these challenges is root disease, especially those that are favoured by high temperature and drought conditions such as charcoal root rot caused by *Macrophomina phaseolina.* Finding a solution to such problems is important for allowing greater production of chickpea in Iraq.

Charcoal Root Rot (*Macrophomina phaseolina*)

Charcoal root rot is caused by the soil and seed borne fungus *Macrophomina phaseolina* (Dhingani, Solanky, & Kansara, 2013). This is a widespread pathogen and a causal agent of root disease to more than 500 plant species (Kunwar, Singh, Machado, & Sinclair, 1986). *M. phaseolina* is a facultative saprophyte which can survive in soil by forming microsclerotia. The microsclerotia are pseudoparenchymal tissue masses which are resistant to adverse environmental conditions (Shaner, 1999). Infection occurs when the root exudates of a susceptible plant come into contact with microsclerotia that remain in the soil, promoting their germination and then penetration of the plant (Kendig, Rupe, & Scott, 2000; Smith Jr, Hodges, & Cordell, 1989). The symptoms in the root system can be observed as blackening of the main root and lateral roots with swelling underneath the blackened area. The swelling can increase the diameter to three times the normal. At the time when the infection begins to appear on the root, there are no symptoms to be observed on the

aerial parts of the plant. However, in the progressive stages of the infection most of the root system will be destroyed which causes plants to present growth reduction, wilting, chlorosis, or total death of the plant in advanced stages (Shaner, 1999; Smith Jr et al., 1989). After plant death, the microsclerotia are able to survive in soil or within the plant residue for 2–15 years to become the inoculum source for future infection (Baird, Watson, & Scruggs, 2003; Kendig et al., 2000; Smith Jr et al., 1989).

Due to its ability to survive in the soil for a long time as microsclerotia even in the absence of the host plant there are no effective methods known to control *M. phaseolina*. Charcoal root rot has become a serious threat to chickpea crop production. In addition, no chickpea cultivar resistant to charcoal root rot has been discovered yet. Also, the fungus has broad range of alternative hosts which enable the fungus to survive if crop rotation is applied (Srivastava, Singh, Srivastava, Saxena, & Arora, 2008). Therefore, it is so necessary to find other ways and methods to manage and control the disease.

Using neem extract to control soil borne fungi

Neem (*Azadirachta indica* A. Juss.) is a tree belonging to the family Meliaceae which is also known as "Indian Lilac". Neem produces toxins that have a great potential to control a broad range of phyto-pathogenic fungi (Tewari, 1992). Thus, neem has attracted the interest of scientists for many years, and the expectation was that neem will be effective as a fungicide. Nowadays, there are several neem based commercial products in the markets. Also, neem cake and neem leaves have achieved good results when they were used as soil treatments against various soil borne fungi such as *Rhizoctonia solani* and *Pythium aphanidermatum* (M. W. Khan, Khan, & Saxena, 1974). The study of Govindachari, Suresh, and Masilamani (1999) revealed antifungal activity of hexane extract of neem leaves and its chromatographic fractions against *Fusarium oxysporum* and *Colletotrichum lindemuthianum*. The extract completely inhibited the growth of both fungi. Ashraf and Javaid (2007) found that concentrations of 5-20% of aqueous leaf extracts of *Azadirachta indica* reduced the biomass of *M. phaseolina* by 34-85%.

The results of previous studies showed the antifungal activity of neem extract against a broad spectrum of fungi in general and soil borne fungi in particular. The present study considered whether neem could be useful to control *M. phaseolina* the causal agent of rot root disease of chickpea.

The aims of this study

The overall aim of this study was to determine whether neem could be used to control charcoal root rot of chickpea. The experiments had the following objectives:

1- To examine the relative effect of neem seed oil and leaf powders and extracts on growth of *M. phaseolina.*
2- To determine the best formulation for use to manage the disease.
3- To find out the optimum rate and the optimum time of application of neem products.
4- To test the effects on disease in glasshouse experiments, and to confirm the results in experiments under field conditions.

Thesis Outline

Chapter 2 is a literature review. The general background of the chickpea plant, interactions with beneficial microbes, and its common root diseases, in particular charcoal root rot, are discussed. Attempts to control charcoal root rot with non-chemical methods, in particular using plant extracts, are described. An intensive review about the neem tree and neem compounds and their uses has been presented in this chapter.

Chapter 3 describes experiments on the sensitivity of two strains of *M. phaseolina* to neem products and preliminary experiments on the effect of neem on disease.

In Chapter 4 neem powder has been formed into different formulas, then applied to the soil at different times and rates in pot trials.

In Chapter 5, a field trial has been conducted to confirm the glasshouse results and to examine the findings under field conditions.

Chapter 6 is the General Discussion, which discusses the results compared with what other researchers did, and to evaluate the finding of my study with my strong evidence.

Chapter 2: Literature review

Chickpea (*Cicer arietinum L.*)

Eight annual and 34 perennial species of chickpea are known (Van der Maesen, 1987). The general belief is that the wild progenitor of chickpea was *C. reticulatum* Lad. That belief was based on karyotype (F. Ahmad, Slinkard, & Scoles, 1987), interspecific hybridization studies (Ladizinsky & Adler, 1976), and seed storage protein profiles (Ladizinsky & Adler, 1975). Chickpea plants grow to 20–50 cm high and have small, feathery leaves on either side of the stem. At the maturity stage the plant has white flowers with blue, violet, or pink veins. The seedpod contains two or three seeds. Huntrods (2013) reported that there are two main types of chickpea grown in the world: kabuli (large ram-shaped, cream coloured seeds) and desi (small angular and dark coloured seeds). The kabuli types are usually grown in the Mediterranean region, while desi types are mainly grown in the Indian subcontinent

The chickpea crop

Abbo, Berger, and Turner (2003) quoted FAO 2002 data that ranked chickpea as third among the world's food legumes. The total production reached 7.8 million metric tonnes from an area of ~ 9.9 million ha. Approximately 75% of global production of chickpea was from the Indian sub-continent, while the rest was produced in the Mediterranean basin, East Africa, North and South America, West Asia, Australia, and Southern Europe. The recent report of M. Sharma, Ghosh, and Pande (2015) was based on statistic data of FAO 2012. The annual production had increased to 11.30 million tonnes from 12.14 million hectares worldwide. The 68% of chickpea production was from South Asia which was the largest producer at that time. India itself produced 7.70 million tonnes from 8.32 million hectares.

Mutualistic symbionts of chickpea

Rhizobia

The term 'rhizobia' includes a wide range of bacterial genera such as *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. Under conditions of nitrogen limitation these bacteria elicit on their leguminous hosts the formation of specialized organs, nodules. In these root structures (nodules), the bacteria are able to convert atmospheric nitrogen into ammonia, which is used by the plant as a nitrogen source (van Rhijn & Vanderleyden, 1995). The infection and nodulation mechanism is a multistep process starting with pre-infection events in the rhizosphere. Rhizobia respond by positive chemotaxis to plant root exudates and move toward localized sites on the legume roots (Gulash, Ames, Larosiliere, & Bergman, 1984). Both *Rhizobium* spp and *Bradyrhizobium.* are attracted by dicarboxylic acids, and amino acids present in the exudates, and very low concentrations of excreted components, such as flavonoids, that may not have high nutritional value (Kape, Parniske, & Werner, 1991; Peters & Verma, 1990).

Inoculation of legumes with selected strains of rhizobia chosen for their ability for high fixing of nitrogen and nodulation of the host can promote crop growth especially in soils with low populations of rhizobia. However, many new strains of rhizobia might fail to compete and adapt with the new population of microbes. Therefore, many studies have been done to improve nodulation by trying to know the factors which affected the interaction between rhizobia and other soil microbes.

According to Sprent, Ardley, and James (2013) legume nodules can be classified as determinate or indeterminate in growth. Determinate nodules have a transient meristem while indeterminate nodules maintain meristematic tissue. Nodule type is dependent on host plant, and legume species that can produce both determinate and indeterminate nodules are rare (Fernández-López et al., 1998; Liu et al., 2014). Chickpea has indeterminate nodules, and large nodule clusters may form that are composed of 6-20 small nodules (Lee & Copeland, 1994). The recent review conducted by Andrews and Andrews (2017) revealed that only *Mesorhizobium* species have been reported to nodulate chickpea. Although the chickpea rhizobia are

commonly referred to as *M. ciceri*, several species of *Mesorhizobium* have been reported from chickpea in different parts of the world (Andrews & Andrews, 2017).

Mycorrhizas

The word mycorrhiza refers to a symbiotic relationship of fungi with roots of many kinds of plants. This relationship is found from at least 350 million years ago (Remy, Taylor, Hass, & Kerp, 1994). Mycorrhiza are divided into two groups *(ectomycorrhizas* and *endomycorrhizas)* depending on the position of the hyphae. The hyphae of endomycorrhizal fungi penetrate the cell wall of cortical cells of the plant roots. However, ectomycorrhizal fungi hyphae are located between the root cells and do not penetrate the cell walls (Allen, 1991). Most legumes form a type of endomycorrhiza known as arbuscular mycorrhiza (AM). Arbuscular mycorrhizal fungi (AMF) are obligate endosymbionts that rely on carbohydrates from the root system as their sole carbon source (Azaizeh, Marschner, Römheld, & Wittenmayer, 1995). The presence of AMF is widespread in most agricultural soils, and they can represent 10% or more of the soil microbial biomass, establishing a mutualistic symbiosis with the majority (approx. 80%) of land plant species and agricultural crops (S. E. Smith, Read, D.J, 2008). During the long development of this symbiotic relationship numerous interactions have been involved in this co-evolution such as physiological, ecological, and molecular levels (Trappe, 1987). Mycorrhiza improve plants absorption by both chemical and physical ways of many poorly mobile elements such as phosphorus and iron. The size of mycorrhizal mycelia are smaller than the root hairs of any plant, thus increasing the absorption surface area at low cost. Mycorrhizal fungi can also produce chemical compounds to increase solubility of hard to absorb elements such as phosphorus and iron (Harley & Smith, 1983). It is well known that arbuscular mycorrhizal fungi modify the functions of the root system (i.e. root exudation) (Marschner, Crowley, & Higashi, 1997). They change the carbohydrate metabolism of the host plant (Shachar-Hill et al., 1995), and impact the microbial populations of the rhizosphere (Hobbie, 1992). On the other hand, microorganisms which live in the hyphosphere of arbuscular mycorrhizal fungi

might have an influence on the functions of mycorrhiza such as the uptake of water and nutrients (M. S. Khan, Zaidi, & Wani, 2007).

Arbuscular mycorrhiza fungi (AMF) of chickpea

As with many legume plants, chickpea plants normally have arbuscular mycorrhizas in their root. There is much evidence that AMF increase the nutrient uptake in chickpea (Akhtar & Siddiqui, 2007; Alloush, Zeto, & Clark, 2000; Zaidi, Khan, & Amil, 2003). The study of Farzaneh, Wichmann, Vierheilig, and Kaul (2009) showed that colonization by AMF increased the growth of chickpea up to 43%. According to Farzaneh, Vierheilig, Lössl, and Kaul (2011) a moderate level of AMF colonization (18–55% of root length with fungal colonization) increased the uptake of the nutrients P, Mn, Cu, Fe, and K by chickpea plants. During their study Pellegrino and Bedini (2014) found a great potential role of AMF as a biofertilization and biofortification of chickpea. AMF improved the nutritional value of grain by protein, Fe and Zn biofortification.

Root exudates of chickpea

According toVeneklaas et al. (2003) chickpea roots are able to exude large amounts of carboxylates. These are organic anions that have low-molecular weight that can enhance the availability of soil phosphorus to the plant (Hocking, Keerthisinghe, Smith, & Randall, 1997). The report of Jones (1998) showed that organic anions can either displace phosphate from the soil matrix or complex metal cations, which bind phosphates (Al^{3+}, Fe^{3+}, Ca^{2+}). Carboxylate exudation is associated with proton extrusion, therefore, the lower pH may itself contribute to greater P availability, if the soil pH is relatively high. On the other hand, the protons cotransport is not associated with organic anion release in all species and in all circumstances (Roelofs, Rengel, Cawthray, Dixon, & Lambers, 2001; Ryan, Delhaize, & Jones, 2001).

Root exudates and rhizosphere

The term rhizosphere can be defined according to Hartmann, Rothballer, and Schmid (2008) as the zone which includes the soil influenced by the root along with the root tissues colonized by microorganisms. The number of species of microbes in the soil may vary from thousands to millions (Nihorimbere, Ongena, Smargiassi, & Thonart, 2011). So, the interactions between roots and soil microbes are usually specialized based on co-evolutionary pressures (Morgan, Bending, & White, 2005; Morrissey, Dow, Mark, & O'Gara, 2004). The interactions of plants and microbes in the rhizosphere play important roles in many bio- ecosystem processes, such as nutrient cycling and carbon sequestration (B. K. Singh, Millard, Whiteley, & Murrell, 2004). There are many beneficial plant–microbe interactions including plant-microbe symbioses such as mycorrhizal fungi and plant-growth promoting rhizobacteria (PGPR). These interactions can have positive effects on plants like increased immunity to biotic and abiotic stresses (Badri, Chaparro, Zhang, Shen, & Vivanco, 2013; Selvakumar, Panneerselvam, & Ganeshamurthy, 2012; Zamioudis & Pieterse, 2012; Zolla, Badri, Bakker, Manter, & Vivanco, 2013). As well , they may suppress plant diseases (Haas & Défago, 2005; Weller, Raaijmakers, Gardener, & Thomashow, 2002) and increase nutrient availability and uptake (Lugtenberg, Chin-A-Woeng, & Bloemberg, 2002; Morrissey et al., 2004). All that beneficial impact will lead to increases in plant productivity (Berg, 2009).

Impacts of root exudates on soil microbial communities

A large number of studies have shown that plants secrete specific root exudate compounds which can drive and shape the selection of the rhizosphere microbial community (Bakker, Manter, Sheflin, Weir, & Vivanco, 2012; Berendsen, Pieterse, & Bakker, 2012; Chaparro, Sheflin, Manter, & Vivanco, 2012). According to Fang et al. (2013); Michalet et al. (2013) there are important roles of phenolic compounds in plant root exudates in shaping the rhizosphere microbial community. As well, the study of Badri et al. (2013) reported that in the absence of the plant (*Arabidopsis. thaliana*), a natural blend of phytochemicals present in the root exudates can change and modulate the soil microbiome. Root exudates have an effect to attract most soil

microorganisms (Shengjing, 2009). Exudates of organic compounds are considered a good energy supplier to many parasites, which activate spore germination to enhance infection of the host (Z. El-Gali, 2003). These compounds consist of, amino acids, sugars, phenolics, organic anions (OAs) and many other secondary metabolites. These may inhibit or stimulate the growth and the development of the pathogens and that will lead to decrease or increase in the incidence of the disease (Bais, Weir, Perry, Gilroy, & Vivanco, 2006; Mohamed, 1990). Z. I. El-Gali (2015) studied the effect of root exudates and extracts, and seed exudates and extracts of the more susceptible and less susceptible bean cultivars Libyan and Giza-6, on the mycelial growth of *Macrophomina phaseolina, Rhizoctonia solani,* and *Botrytis cinerea*. The study revealed that root and seed exudates and extracts of cv. Libyan gave the greater stimulating effect on the mycelia growth of all tested fungi than root and seed exudates and extracts of Giza-6.

Chickpea diseases

Chickpea suffers from diseases that seriously affect the plant in all growth stages. There are many pathogens that affect chickpea such as fungi, oomycetes, viruses, bacteria, mycoplasma, and nematodes which causes an economic loss to the crop yield all over the world. Fungal infections are the most common diseases that threaten chickpea. Fungi could infect the aerial and the root system of the plant (Nene et al., 2012). According to Nene and Sheila (1996) about 172 pathogens have been reported from different parts of the world that infect chickpea plant, however, few of them cause major losses to the crop. Soil borne diseases of chickpea are very critical in realizing the yield potential of improved cultivars in several agricultural crops. The diseases caused by pathogens which survive in the soil matrix and in the plant residues on the soil are defined as soil borne pathogens. Soil borne pathogens can survive in the soil as soil inhabitants (survive in soil for a long time), soil invaders, or soil transients (survive in soil for short time) (Divya Rani & Sudini, 2013). There are many reasons which make the control of soil borne pathogens difficult such as the pathogens often have a wide host range including weeds, the pathogens can survive for long periods of time in the absence of the normal crop

host, chemical control does not work well, and it is difficult to develop resistant varieties of plants (Divya Rani & Sudini, 2013).

Ascochyta blight

Ascochyta blight is the most important and common disease that affects chickpea plants. Disease occurs on the plant in all growth stages, causing serious loss to the yield. The disease is caused by *Ascochyta rabiei*, and the more serious infection happens in the kabuli type which is higher in nutritional value than the desi type (Kahraman & Ozkan, 2015). The main symptoms of the disease are leaf lesions, chlorosis on leaf margins, pod lesions causing seed disease, and stem lesions causing breaking. The lesions on the stem and pods are considered the most important damages which result in stem breakage and pod diseases. Lesions appear in curved or elongated brown-red line shapes on leaflets, with the appearance of pycnidia on green pods as nested concentric curves which then turn to black lines, and 3-4 cm long brown lesions with black spots (Abbo et al., 2003).

Botrytis grey mould (BGM)

Botrytis grey mould (BGM) is an economically important disease of chickpea especially in areas where there is continuous optimum conditions such as cool temperatures and high humidity. BGM is caused by *Botrytis cinerea.* This pathogen mainly survives between seasons on infected crop debris and seeds. BGM may cause complete crop loss of chickpea yield (Pande et al., 2006). The appearance of BGM symptoms on aerial parts of the plant which are the most susceptible is firstly as water-soaked lesions on the stem near ground level, which then extend along the stem and spread the infection to the other stems (Knights EJ, 2002). The lesions can reach 10-30mm long and surround the stem of the plant completely, causing breaking at the rotting point (Bakr, 2002). The fungus can form grey or brown to light brown lesions on leaflets, branches, and pods, covered with conidiophores and masses of single celled, hyaline spores (Haware MP, 1992).

Collar rot of chickpea

The main cause of this fungal disease is *Sclerotium rolfsii*. Disease can occur in the presence of favorable conditions, high temperatures of 30°C and more at the sowing time, and moist soil. The common symptoms of the disease are collar rot of the seedling of the plants after week 6 of plant age, with leaves not dropping from the plant. However, young seedlings may collapse, but older seedlings may dry without collapsing. When uprooted, the seedlings show rotting at the collar region and downwards. A white mycelium then will cover the rotting parts (Nene et al., 2012).

Fusarium wilt

Fusarium wilt is caused by *Fusarium oxysporum* f. sp. *ciceri*. The disease can affect the crop at any stage, and wilt incidence is generally higher when chickpea is grown in drier climates with temperature more than 25°C and when crop rotations are not applied. The disease appears on seedling 3 weeks after sowing, causing total death of seedlings which appear then as yellow patches in the field. The symptoms on adult plant starts with drooping of the petioles, rachis and leaflets. The drooping starts from the upper parts of the plant then extends to the entire plant. When uprooting the plant there is no external rotting, drying, or root discoloration, but when the stem of the plant is cut vertically, an internal discoloration can be seen in the centre of the stem that is a clear discolored dark brown or black. The discoloration can extend several centimeters above the collar region into the main stem and branches (Nene et al., 2012).

Verticillium wilt

According to Nene et al. (2012) the main cause of Verticillium wilt is *Verticillium albo-atrum.* This fungal disease was very important in many countries such as Tunisia, Italy, Pakistan, and Syria. There are similarities between Verticillium wilt and Fusarium wilt in their needs of weather conditions. As well, disease symptoms are similar to Fusarium wilt, however, the vertical cut of infected stems shows that

the brown discoloration of the xylem tissue is lighter than that caused by *F. oxysporum* f. sp. *ciceri* (Erwin, 1958).

Wet root rot (*Rhizoctonia solani*)

Wet root rot of chickpea is caused by *Rhizoctonia solani.* In the presence of the optimum weather conditions, wet soil and temperatures ranging between 11-18°C, the pathogen can spread and infect a wide range of plants. The symptoms of wet root rot in the field are almost the same as collar rot disease, yellow patches which are dying plants scattered throughout the field. Most infection happens after 6 weeks from sowing in soils with relatively high moisture content. However, the infection may occur at later stages of growth. Infected seedlings usually do not collapse, but their leaves turn yellow gradually and then petioles and leaflets start to droop. Dark brown lesions then appear at the top of main stem collar, and in the lower branches in older plants (Nene et al., 2012).

Pythium root and seed rot

This less important disease has been recorded in different countries such as India, Canada, Spain, USA, Iran, and Turkey. The chickpea type desi is consider to be more resistant to infected by the oomycete *Pythium ultimum* than kabuli type, due to its thick seed coats. Disease can appear either as poor emergence or damping off of the seedlings of the plant. Symptoms on infected larger roots are discolored, necrotic, and devoid of rootlets, and the infected plants die before the flowering stage (W. J. Kaiser & Hannan, 1983).

Phytophthora root rot (PRR)

Phytophthora root rot (PRR) in chickpea is one of the important diseases that infects chickpea plants. The cause of this disease is *Phytophthora medicaginis* which is a soil borne oomycete that produces three kinds of asexual spores: zoospores, chlamydospores, and sporangia. Sporangia germinate to produce hyphae or

differentiate to produce 10 - 30 zoospores. Zoospores are considered an important route to infect the roots of the plant, especially under flooded soil conditions (Tyler, 2007). According to Tyler (2002) zoospores can swim chemotactically toward the root exudates which are released from the roots of the host plants. Then zoospores encyst on the surface of the root from where the hyphae penetrate the root directly from the cyst. Sporangia and zoospores can also be spread to the upper plant by splashing (Tyler, 2007). Phytophthora root rot disease can develop in chickpea plants at any stage from emergence of seedlings to near maturity. The disease is known to cause chlorosis and wilting followed by rapid death of plants a week or more after rain. Infected plants turn yellow with drying of leaflets and rot on the base of the stem. Later on, a decay will start on the lower portion of the tap root and lateral roots. Dark-brown to black lesions then appear on the upper portion of the tap root. These lesions may extend to the base of the stem in some cases, and in the development of the disease the margins of these lesions often turn to reddish brown. In the field the appearance of the infection is seen as patches of dead plants (Nene et al., 2012; Vock, Langdon, & Pegg, 1980).

In Australia PRR caused yield losses estimated at 20% for a district and 50% for individual crops (Knights, Southwell, Schwinghamer, & Harden, 2008). One of the main reasons for the severity of PRR is there is no highly resistant chickpea cultivar found yet. The primary ways to control Phytophthora root rot are using chemicals (Nene & Reddy, 1987), biocontrol bacteria (Myatt, Dart, & Hayward, 1993), and crop rotation (Manning, Ackland, Moore, Lucy, & Brinsmead, 2000). However, crop rotation is not considered very effective because the *Phytophthora* can survive on other legume plants as alternative hosts, such as pasture medics and lucerne, as well as surviving in the soil for up to 3 - 4 years. The other difficulty of controlling the disease is the ability of the pathogen to spread from field to field by water and machinery (Manning et al., 2000).

Charcoal rot (*Macrophomina phaseolina*)

This disease is caused by *Macrophomina phaseolina,* which is a soil, seed and stubble borne fungus (Dhingani et al., 2013). Initially, the disease was known as dry

root rot which was first reported by Mirta in 1931. The pathogen is also referred to in the literature as *Rhizoctonia bataticola*. *M. phaseolina* is very widespread across the world and poses a threat to chickpea crop production (Hoes, 1985). There was a widespread increase in incidence of charcoal rot root of chickpea in the central and southern states of India according to surveys during 2010–2013 (Ghosh, Sharma, Telangre, & Pande, 2013). The fungus is considered a causative agent of different diseases such as charcoal rot, wilt, damping-off (Cowan, 1999), leaf blight, dry rot, and ashy stem blight, to more than 500 plant species (Kunwar et al., 1986).

M. phaseolina survives by producing microsclerotia which are tuber or cushion shaped black bodies up to 0.3 mm in diameter (S. Kaiser & Das, 1988). Microsclerotia are responsible for spreading the primary inoculum on the surface of the field by floating in the free water when a field is flooded. Furthermore, microsclerotia become dislodged and are able to rise to the water surface when the seed bed is flooded or with any other factor, such as water waves due to wind that disturbs the surface layer during growing season. In dry soil conditions *M. phaseolina* can survive for more than 10 months (Keim & Webster, 1974; Webster, Bolstad, Wick, & Hall, 1976)

Khan SN (2000) showed that disease severity is correlated positively with the number of microsclerotia of *M. phaseolina* in the seedbed. The density of microsclerotia in the soil represents the amount of inoculum. However, the hyphae of *M. phaseolina* mycelium in the soil is not considered as a source of inoculum (Meyer, Sinclair, & Khare, 1974). A study conducted on a Pakistani soil which had 25 microsclerotia of *M. phaseolina* in 1g soil showed that any single sclerotium of the fungus can cause death of the plant in its susceptible host (M. S. Khan et al., 2007). As well, there is a level of ability of *M. phaseolina* strains to infect seed embryos in pre-emergence mortality (Burney, Ahmad, & Aslam, 1984; Dhingra & Sinclair, 1978; Francl, Wyllie, & Rosenbrock, 1988). The severity of nutritional compatibility with the host tissue will play a role with the pathogenicity of these strains (Khan SN, 2000). I. Ahmad, Burney, and Asad (1991) reported that high plant density, mechanical injury, and insect attacks are considered to be predisposing factors for transmission of the disease.

Symptoms of charcoal root rot of chickpea

Symptoms on seedlings

Seedlings can be infected when the conditions are favourable for the disease with temperatures up to 35ºC with drier soil continuously for 2-3 weeks. These conditions will enable the pathogen to infect chickpea seedlings and the symptoms will appear as brown to dark spots. The margins of the cotyledons turn brown to black colour and are then shed at an early stage. Reddish-brown lesions with a circular to oblong shape can then be visible on the leaflets at the unifoliate leaf stage. After several days with the presence of hot and dry conditions, the lesion turns brown-black in colour and it may extend up the stem which leads to total dryness of chickpea seedling if the conditions persist (Girish K Gupta, Sharma, & Ramteke, 2012).

The most striking belowground symptoms are a black-charcoal discoloration of the roots and a lack of feeder roots (Figure 2-1). Therefore, the disease is often called charcoal rot. The root cortex and the pith of infected plant is filled with microsclerotia of the fungus, which give it a black appearance (M. Sharma et al., 2015).

Figure 2.1. Symptoms of charcoal rot on chickpea seedling (Photo by author)

Symptoms on Adult Chickpea plants

The first symptoms can be noticed on the growing plants between 1- 4 weeks before normal maturity of chickpea plants. The pathogen causes uniform lesions on different parts of the plant such as roots, stems, pods and seeds. A light brown to grey lesion starts to appear on the stem from ground level upwards. Microsclerotia then form in the vascular tissues and in the pith, giving a greyish-black appearance to the sub-epidermal tissues of the stem. Then a discolouration starts to be observed at nodes as small black profuse specks which are randomly distributed (Girish K Gupta et al., 2012). According to Bristow and Wyllie (1986) in glasshouse experiments, the infected plants had a twin stem abnormality and the foliar symptoms started from top to bottom of the plant. The report by Girish Kumar Gupta and Chauhan (2005) stated that the leaves of infected plants remain smaller than normal plant leaves and start to get yellow in colour before total wilting. A reddish-brown discolouration appears on the vascular tissues on the root and the lower parts of the stem. Black bodies (microsclerotia) will cover either totally or as spots the dry pods of the fully mature chickpea plants. The fungus can penetrate the pods of chickpea grains to infest seeds which will also get black spots of microsclerotia, while the seed coat sometimes gets fissures and cracks. After the total death of the chickpea plant, a huge number of microsclerotia appear which will spread out to give a charcoal like appearance to the chickpea plant tissue. In the field, the infected chickpea crops can be seen as a yellow scattered patches (Girish K Gupta et al., 2012). Symptoms also described in the recent study of M. Sharma et al. (2015) mostly appear in chickpea plants at the post flowering stage which include chlorosis, and drooping leaflets and petioles starting with top leaves of the plant. Generally, stems and leaves of infected chickpea plants get a yellow colour, while the lower leaves and stems are brown in some cases. Symptoms on chickpea roots appear firstly as a black colour at the top of the root with clear rotting signs. When plants are uprooted, the tip of the root is easily broken leaving the lower portion of the tap root in the soil. The dead roots are brittle and easily show shredding of the bark. Later on, the microsclerotia can be observe as dark minute bodies on the roots (M. Sharma et al., 2015).

Charcoal rot Management

So far, there are no effective and economical control methods for charcoal rot. The fungus has a wide range of alternative hosts which enable the fungus to survive if crop rotation is applied and no cultivar of chickpea genetically resistant to the disease has been identified (Srivastava et al., 2008). Microsclerotia of this pathogen can survive in soil for 2–15 years, even in the absence of the host plant (Baird et al., 2003; Young, Gilbertson, & Alcorn, 1982). Charcoal rot disease can be controlled by soil fumigation using methyl bromide and chloropicrin (Smith Jr & Krugman, 1967). However, methyl bromide is an ozone-depleting gas and its general use in agriculture has been banned worldwide. As well there are risks of killing beneficial microorganisms (Munnecke & Van Gundy, 1979). Therefore, many attempts have been conducted to find alternative ways to control charcoal rot such as inducing plant resistance by using biotic and abiotic treatments (Hammerschmidt, 1999).

Rathore BS (2000) showed that one of the successful way to charcoal rot disease by organic amendments such as farmyard manure, mustard and neem cake. The study of Lodha S (2002) reported that using soil amendment with pearl millet compost reduced *M. phaseolina*-induced plant mortality to 63–72% at harvest in cluster bean. Ndiaye (2007) found that amending cowpea field with 6 metric tonnes of compost/ha increased the yield and controlled charcoal rot disease.

In addition, many studies tested biological control agents as potential means of disease control, such as using *Trichoderma harzianum* (Srivastava et al., 2008). *Bacillus* spp. have been identified as potent antagonists against *M. phaseolina* (Muhammad & Amusa, 2003; Pal, Tilak, Saxcna, Dey, & Singh, 2001). Khare and Arora (2010) showed that indole-3-acetic acid (IAA) produced by *Pseudomonas aeruginosa* suppressed charcoal rot of chickpea. Saikia et al. (2006) reported that using riboflavin can induce resistance in chickpea against charcoal rot and *Fusarium* wilt diseases.

The natural fungicides

There are many botanical products that are reported as antifungal compounds (Chowdhury, Banerjee, & Walia, 2008). Plant extracts stand as an alternative source for controlling plant diseases. Plant extracts are eco-friendly products which are biodegradable (Duke, Romagni, & Dayan, 2000). Jabeen, Javaid, Ahmad, and Athar (2011); Javaid and Amin (2009) have reported a fungicide effect of both crude extracts and purified isolated compounds from plants in the management of plant diseases, which can be considered as natural fungicides.

Using plant extract to control *Macrophomina phaseolina* growth

Because of the difficulty of controlling charcoal rot, there have been many attempts to find plant extracts that are effective against *M. phaseolina*

An in vitro study by using poisoned food technique was conducted by Hussain et al. (2014) to evaluate the fungicide effect of five plant extracts on the growth of *M. phaseolina* the causal agent for charcoal rot of sunflower. The results showed that aqueous plant extracts at 100% concentration significantly inhibited the mycelium growth of the fungus. The maximum inhibition was by *Allium sativum* followed by *Parthenium hysterophorus* and *Cassia fistula,* while the minimum inhibition was by *Dalbergia sissoo*

The study of Javaid and Saddique (2012) found a highly suppressive effect of methanolic leaf and fruit extracts of *Datura metel* against *Macrophomina phaseolina*, the cause of charcoal rot disease. According to Lakshmeesha, Sateesh, Vedashree, and Sofi (2013) among the 10 medicinal plant extracts, *Datura metel* methanol leaf extract and *Azadirachta indica* leaf extract showed the most promising activity against *Macrophomina phaseolina*.

In order to achieve the main aim of the present study, which is to control and manage charcoal rot root disease of chickpea, it is worth focusing on a natural fungicide plant which is the neem plant.

Neem tree

Neem (*Azadirachta indica*) is the most useful traditional medicinal plant in India. Neem is a member of the family *Meliaceae* (*mahogany* family).

At the middle of the twentieth century a chemical investigation on the products of the neem tree was extensively conducted (Tewari, 1992). Because it has a great potential to control various phytopathogenic fungi, the neem tree has attracted special interest of scientists. During the last five decades the chemistry of the neem compounds has achieved progress in the biological activity and medicinal applications of neem. It is considered as a valuable source for the development of industrial products and for the development of medicines against different kinds of diseases (Biswas, Chattopadhyay, Banerjee, & Bandyopadhyay, 2002). Neem leaves and neem have been used as a soil treatment against various soil borne fungi such as *Colletotrichum atramentarium* (R. Singh, 1968), *Pythium aphanidermatum* and *Rhizoctonia solani* (M. W. Khan et al., 1974), and *Fusarium oxysporum* (Kannaiyan & Prasad, 1981). The fungicidal effect of neem extracts has been reported by many researchers such as Ilyas, Iftikar, Anwar, and Haq (1997); Lokhande, Lanjewar, and Newaskar (1998); B. Sharma and Basandrai (1997). Later, Dubey and Kumar (2003) have found approximately similar effect of neem extract on growth and microsclerotial survival of *M. phaseolina*.

Chemical compounds of neem

Siddiqui (1942) reported there were more than 135 compounds that have been isolated from different parts of neem. Tewari (1992) found that neem contains a variety of chemical constituents such as azadirachtin, azadirachtol, nimolicinol, nimbocinone, isolimolicinolide, nimlinone, nimbocinol, nimocin, etc.

Devakumar (1996) reported that neem compounds have been divided into two main classes: nonisoprenoids and isoprenoids. The nonisoprenoids include carbohydrates (polysaccharides), proteins (amino acids), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, coumarin, dihydrochalcone, and tannins, aliphatic compounds, etc. The isoprenoids include triterpenoids and diterpenoids

containing limonoids, protomeliacins, azadirone and its derivatives, vilasinin type of compounds, gedunin and its derivatives, and C-secomeliacins such as salanin, nimbin, and azadirachtin.

The bioactivity of neem against *M. phaseolina*

In an in vitro study, Dubey, Kumar, and Pandey (2009) compared aqueous extracts from different parts of the neem tree such as leaf, bark, oil cake and neem oil, on the growth, mycelial yield and microsclerotial survival of *M. phaseolina*. Autoclaved extracts from neem bark, leaves or seed cake promoted the radial growth of *M. phaseolina.* However, there was a strong inhibitory effect on growth and mycelial biomass yield of the fungus when using neem extracts that had been sterilized through sintered glass filters. Dubey et al. (2009) concluded that the autoclave sterilization (121 ^{0}C, 15 psi, 15-20 min) will denature the active material of the neem extract, and the optimum way to sterilize the extract is through filtration. They also found that neem oil was more toxic to the fungus than the other extracts.

All other studies on the effect of neem on *M. phaseolina* have been in vitro tests where neem was one of a number of extracts from a range of plants that were being compared. Ashraf and Javaid (2007) studied the antifungal activity of aqueous leaf extracts of *Azadirachta indica* and two other plants in the same family as neem (*Meliaceae*) against *M. phaseolina*. The results showed that neem leaf extract and extract of *Melia azedarach* significantly reduced the biomass of the fungus in liquid culture. However, extracts from *Toona ciliata* promoted the growth of the fungus. The growth of *M. phaseolina* was significantly reduced compared to the control in the study of Tandel, Sabalpara, and Pandya (2010) that examined the effect of extracts from eleven plant species on colony diameter using the poisoned food technique. Neem leaf extract reduced growth by about 40% compared with 98% for onion extract. However, the extracts were autoclaved and so active ingredients may have been denatured. Tests by Javaid and Rehman (2011) on leaf extracts of four medicinal trees showed that *Azadirachta indica* had higher antifungal activity than extracts of *M. azederach*, *Syzyzgium cumini* or *Eucalyptus citriodora* to reduce the biomass of *M. phaseolina* in liquid culture. The ethyl acetate and chloroform extracts

of neem leaves had the greatest inhibitory effect on growth of *M. phaseolina* compared with methanol extracts (Javaid & Rehman, 2011).

Dhingani et al. (2013) evaluated aqueous extracts from various botanicals and oil cakes against *Macrophomina phaseolina* in poisoned food assays in agar culture. Neem leaf extract was among the least effective plant extracts (12% reduction) for inhibiting the growth of *M. phaseolina*. However, the extracts were autoclaved. Extract from neem seed cake was more effective in reducing mycelial growth of *M. phaseolina* than farmyard manure or castor or mustard seed cakes in a second experiment in the same study (Dhingani et al., 2013). Meena, Tripathi, Gotyal, and Satpathy (2014) used identical methods to those of Dhingani et al. (2013) with a different range of plants and found that neem extract was more effective at reducing growth of *M. phaseolina* (64% reduction) and confirmed that extracts from neem cake were more inhibitory than those from mustard cake. Among filter-sterilized aqueous extracts from nine plant species, neem extract was intermediate in inhibition (up to 53%) of the mycelial growth of *M. phaseolina* (Savaliya, Bhaliya, Marviyaand, & Akbari, 2015). Microsclerotial formation was also reduced in cultures grown on neem extracts.

The results of these studies show inconsistent results for the effectiveness of neem products against *M. phaseolina*. However, the most detailed experiments (Dubey et al., 2009) showed strong effects of neem extracts and oil on growth. The common use of autoclaving in other experiments may be one reason why neem was not always effective.

Bioactivity of neem products against other soil borne fungi

An intensive study was conducted by U. Singh, Singh, and Singh (1980) to evaluate the fungicidal effect of the extract of different parts of neem plant against four soil borne fungi, *Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium rolfsii,* and *Fusarium oxysporum*. In one or two concentrations neem oil had the most effect on inhibition and the survival of the four pathogens. This was followed by neem fruit pulp and neem leaf extract and neem bark extract in different concentrations. However, there was a gradual decline in the percentage of germination of chickpea

seeds with increasing concentration of neem oil. In a pot experiment, chickpea seeds were treated with different concentrations of neem oil, then sown in pots of autoclaved sand-clay mix that had single inoculation with each pathogen and in pots of mixed inoculation. Treated seeds were significantly protected against disease compared with control for *R. solani, S. rolfsii, and F. oxysporum* respectively, while *S. sclerotiorum* was the least affected pathogen. The results of the pot experiment also showed that the severity of root rot and wilt diseases was greater in chickpea plants that were grown with mixed inoculum than single inoculum (U. Singh et al., 1980).

Nwachukwu and Umechuruba (2001) studied the effects of various plant leaf extracts on mycelial growth of major seed-borne fungi (*Aspergillus niger, Aspergillus flavus, Botryodiplodia theobromae* and *Fusarium moniliforme*) of African yam bean (*Sphenostylis stenocarpa*). The efficacy of the leaf extracts was tested using the blotter method and soil mix in buckets. The naturally infested seeds were treated with extracts then sown in sterilized soil. The results showed that neem leaf extract (crude and aqueous) gave the highest inhibition to mycelial growth of all fungi. As well, neem leaf extract gave complete protection to seedlings against *A. niger* and *A. flavus*.

Obongoya, Wagai, and Odhiambo (2010) examined the effect of selected crude extracts from whole neem plant material on Fusarium wilt (*Fusarium oxysporum.* f. sp. *phaseoli*) of common bean. The study revealed that neem had the greatest effect of all botanicals to reduce the incidence of Fusarium wilt when it was sprayed onto foliage. Hanaa, Abdou, Salama, Ibrahim, and Sror (2011) reported that treating tomato seedlings with neem aqueous extract 4 days before inoculation induced systemic resistance of the plant and reduced the incidence of Fusarium wilt. Although Obongoya et al. (2010) suggested that neem extract was translocated within bean plants and inhibited growth of *F. oxysporum* in the xylem, it is also possible that their results were due to induced resistance.

The study carried out by Reddy, Bagyaraj, and Kale (2012) evaluated the effect of seed treatment with 10% aqueous extracts of vermicompost prepared from different organic substrates including leaves of *Azadiracta indica* (neem) against *Fusarium*

oxysporum and *Ralstonia solanacearum* wilt in tomato plants. A microplot method was conducted to inoculate the seeds artificially. Results showed there was 100% survival of tomato plants in a treatment of (seeds soaked in aqueous extract of vermicomposted neem leaves +vermicomposted neem leaves added to soil) followed by 96% in the treatment of (seeds soaked in aqueous extract of vermicomposted agricultural waste + vermicomposted neem leaves added to soil) compared with control treatments which had 70% and 58% survived respectively when farm manure was added or not. The yields of tomato fruits per plot from three harvests were higher in the second harvest in all the treatments and it decreased during the third harvest. The duration between each harvest was 10 days. The treatment (seeds soaked in aqueous extract of vermicomposted neem leaves +vermicomposted neem leaves added to soil) had good foliar growth and early flowering and fruiting. As well, this treatment had the highest yield production among all treatments compared with control treatments.

Hassanein, Ali, Youssef, and Mahmoud (2010) reported that using neem leaf aqueous extract in vitro suppressed mycelial growth of *Fusarium oxysporum*, the causal agent of tomato wilt. As well, in a pot trial, the plants that were irrigated with aqueous neem leaf extract had the lowest disease incidence. In their study Islam and Faruq (2012) examined the effect of some plant aqueous extracts (neem leaf, garlic clove, allamonda leaf, ginger rhizome, kalijira seed, bel leaf, turmeric rhizome, katamehedi leaf and onion bulb) against damping-off disease caused by *Fusarium oxysporum* of some winter vegetables (tomato, eggplant and chilli). Seeds were soaked for 30 minutes in the solution of the plant extract before sowing in naturally inoculated soil. Neem leaf extracts showed the best effect on increasing the germination and reducing damping-off as well as increasing the growth characters of vegetable seedling. The highest seed germination of tomato, eggplant and chilli was recorded after seed was treated with neem leaf extract.

There is therefore a reasonable amount of literature suggesting the neem extracts can reduce incidence or severity of diseases caused by *Fusarium oxysporum* in pot and field trials, but very limited work has been done with other soilborne pathogens.

Effect of neem on beneficial microbes

There is a lack of information about the effect of neem products on beneficial microbes, including mycorrhiza and rhizobium, because few studies has been conducted on this aspect yet.

In their report Muthukumar, Udaiyan, and Rajeshkannan (2001) studied the symbiotic relationship between neem trees with both arbuscular mycorrhizal (AM) fungi and phosphate-solubilizing bacteria (PSB). The study was used to understand the relationship between neem seedlings and the two beneficial microbes in two inoculation types, individually or in various combinations. Inoculated seedlings had greater plant height, leaf number, and stem girth, compared to uninoculated controls. The combined inoculation of AM+PSB increased plant growth better than individual inoculation with one of them. Although this is not related to the application of neem products to soil, it does show that mycorrhizal fungi and beneficial bacteria can function in the rhizosphere of neem trees.

Another experiment published later by Gopal, Gupta, Arunachalam, and Magu (2007) studied the effect of the azadirachtin granules (alcoholic extract of neem seed kernel mixed with China clay) on the population of bacteria, actinomycetes, fungi, *Azotobacter* and nitrifying bacteria in soil. The study showed that using azadirachtin increased the number of the free living nitrogen fixing *Azotobacter* with a suppressing effect on the other kinds of microbes. Neem is also an inhibitor of nitrification, as has been well documented by Kiran and Patra (2003) who used it to reduce the loss of nitrogen by preventing the activity of nitrifiers. Gopal et al. (2007) also have concluded that the reason for high phosphatase activity in soil where neem products have been used was due to the biocidal effect of azadirachtin on the soil microorganisms and subsequent decomposition and release of the phosphates from the dead microbial biomass. Sarawaneeyaruk, Krajangsang, and Pringsulaka (2015) found a reduction in the number of rhizobium nodules on mung bean plants treated with azidirachtin and neem leaf extract. However, the level of neem used were phytotoxic so it is not whether this was due to a direct effect on the symbiosis.

Conclusion

To sum up, the results of previous research have shown the fungicidal effect of neem extract against *Macrophomina phaseolina* the causal agent of charcoal root rot in chickpea plants. Because most studies were conducted in vitro, there is a lack of information about the effect of neem extract on the growth of *M. phaseolina* and charcoal root rot disease in contact with plants, such as in glasshouse pot trials or field work. The review of the present study revealed the obvious fact that neem extract has been included in most studies that examine the fungicidal effect of plant extracts against *Macrophomina phaseolina.* However, there are different factors that will determine the efficacy of neem extract on the targeted pathogen (*M. phaseolina*). These include the nature of the neem products (seed oil, neem leaf powder, neem bark, etc.), the solubility of these products in water and breakdown time in the soil, and the minimum effective rate of neem on the pathogen. Some in planta work has been done using neem against other soilborne pathogens, especially *F. oxysporum*, but the data are too limited to indicate how best to use neem against charcoal rot.

The present study focuses on examining the effect of two kinds of neem products (neem seed oil, and leaf extract) individually. Much thinking and effort was spent to find out appropriate formulations which delayed neem extract breakdown and to ensure a lasting effect of neem against the targeted pathogen. In addition, the study examined the best rate, and timing to apply neem extract. The interactions with the pathogen, disease and beneficial microbes was studied in in vitro, glasshouse, and field work.

Chapter 3: Neem leaves protect chickpea from charcoal rot root

Introduction

There are different neem products and they differ in their effect on fungi. Neem oil and neem leaf extract are the most popular products used as a fungicide among the other products of neem. The purpose of this chapter was to compare these products for their toxicity to the fungus, to determine whether the best product could reduce the severity of disease, and to test for any adverse effects on beneficial symbionts of chickpea.

Therefore, in this chapter neem seed oil and neem leaf extract were examined for their fungicidal effect against two strains of *M. phaseolina*. The in vitro tests examined the effect of both kinds of neem on the mycelial growth of *M. phaseolina* in broth culture media. Then a poisoned food method in solid culture media was conducted to examine the effect of neem on the radial growth of the fungus. Then a glasshouse experiment was conducted to evaluate the effect of neem leaf extract on the development of charcoal rot on chickpea plants. Two chickpea cultivars (desi and kabuli) were tested with and without the presence of rhizosphere microbes (sterilized and unsterilized soil). An experiment was conducted in liquid culture to extract root exudates from chickpea plants and examine the effect of these root exudates on the growth of *M. phaseolina.* A pot trial experiment was done to examine the effect of neem leaf extract on the beneficial microbes of chickpea plant (mycorrhiza and rhizobium).

Material and Methods

Preparation of neem extracts

Neem seed oil extract, neem leaf extract, and neem leaf powder were obtained from Neeming Australia (Ashmore, Queensland, Australia). The company certified its product extraction methods and compositions as Neem Seed Oil: cold pressed 100% pure with no additives; Neem Leaf Extract (Ethanolic): water and ethanol extraction method; Neem Leaf Powder: 100% ground neem leaves.

Microbial cultures and seeds

Two strains of *Macrophomina phaseolina* were obtained from Queensland Plant Pathology Herbarium, Brisbane, Australia. They were BRIP39280 from chickpea from Kingaroy, QLD, and BRIP39348 from sunflower from Bowenville, QLD.

Chickpea seeds of desi and kabuli types were obtained from Eden Seeds, Lower Beechmont, QLD, Australia. The supplier was unable to say which cultivar they were.

EasyRhiz inoculum Group N for chickpea was obtained from New Edge Microbial, Albury, NSW, Australia.

In vitro effects of neem extracts on the growth of *Macrophomina phaseolina*

Estimation of mycelial biomass

This experiment examined two neem products; neem seed oil and neem leaf extract to determine the growth inhibition of both isolates of the fungus based on the weight of mycelial biomass. Neem seed oil was used at final concentrations of 0, 0.05, 0.010, 0.025, 0.050, 0.100, 0.250, and 0.5% in liquid medium. The ethanolic extract of neem leaves was used at concentrations of 0, 0.25, 0.50, 0.75, and 1%. Both kinds of neem extract were sterilized by filtration through 0.22 µm filters and added to autoclaved potato dextrose broth (PDB). Flasks contained 50 ml of PDB; the flasks

were inoculated with one agar block 5 mm diameter which was taken from *M. phaseolina* culture on potato dextrose agar (PDA) incubated for 7 days at 30°C (Dubey et al., 2009). Flasks were incubated at 30 C for 10 days on a rotary shaker at 150-170 rpm. After that, cultures were filtered through pre-weighed Whitman filter paper No.1, then the filter papers with the mycelial mats were dried in an oven at 60°C for 24 h. The weight of mycelia was determined by subtracting the weight of the filter paper from the total weight of the filter paper with mycelia. Data of three replicates were subjected to statistical analysis. The experiment was done twice.

Mycelial inhibition by poisoned food technique

Powdered neem leaves were obtained from Neeming Australia. A suspension of 100 g of neem powder and sterilized distilled water (1:1) was firmly blended in a blender for 10 min. The mixture was then poured though double-layered Miracloth (Merck Millipore, Melbourne, Australia) then centrifuged at 4000 g for 10 min. The supernatant was then filtered through Whatman filter paper No.1 (Mohana & Raveesha, 2007). The extract was sterilized by filtration through 0.22 μm filters. The extract was preserved in the freezer as crude 100% concentration extract for further use (Satish, Raveesha, & Janardhana, 1999). Neem aqueous extracts were diluted with sterilized distilled water to achieve the concentrations of 5%, 10%, 15%, and 20% and 1 ml from each concentration was added per Petri-plate. Sterilized distilled water without any aqueous extract served as control. A PDA medium was prepared and sterilized and then cooled down, and 15 ml of this medium added to each plate. The plates were shaken gently to mix the extract with PDA.

Neem oil was diluted with 40% ethanol to achieve the concentrations 0.5%, 1%, 1.5%, 2%, and 3%. 1 ml from amended solution from each concentration was added to each Petri-dish (Coventry & Allan, 2001). Ethanol 40% was diluted with sterilized distilled water to achieve the same ethanol concentrations to serve as control for each concentration. PDA medium was added as above. After complete solidification of the medium, each plate was inoculated with a 5 mm diameter mycelia disc of *Macrophomina phaseolina* taken from culture incubated at 30°C for 7 days. All the plates were incubated at 30°C for three days and then the colony diameter was measured (J. Singh & Tripathi, 1999).

Effect of neem on the development of charcoal root rot disease on chickpea plant

The experiment was conducted in the glasshouses of the University of New England-Australia. Two types of chickpeas: desi and kabuli were planted in two kinds of soil, sterilized and unsterilized soil: sand (1:1). Plants were inoculated or not with *M. phaseolina* and treated or not with neem extract. There were four replicates of each treatment. The effect of disease and neem leaf powder was determining by measuring the dry weight of the shoot and root of each plant.

Preparation of fungus inoculation

In order to obtain an actively growing culture containing microsclerotia the method of Mihail et al. (1992) was followed. Sand-cornmeal medium was prepared by mixing sand, cornmeal, and distilled water 1.1:0.4:0.4 respectively. The mixture was then put in a polyester oven bag before autoclaving at 121°C for 1 hour on two consecutive days. After sterilization, the bag with the 250 g mixture was then inoculated with 10 agar blocks 5mm taken from growing cultures of *M. phaseolina* BRIP 39280 (chickpea strain) on PDA. Then the mixture was incubated for 6 days at 32-34°C. During the incubation the mixture was shaken vigorously once daily to promote uniform fungal colonization. The inoculum was used immediately after incubation.

Soil preparation

A cracking clay soil (Vertosol) was collected from Laureldale Farm on the western side of University of New England, Armidale, New South Wales, Australia. The soil had pH 6.5, Colwell P 57 mg/kg, cation exchange capacity 31.2 cmol/kg. Soil was air dried and sieved (2 mm mesh) to remove stones and large particles. Sand was also air dried and sieved, then sand and field soil was mixed thoroughly in proportion 1:1 in a cement mixer. Half of the mixed soil was sterilized, by using

autoclavable plastic bags at 121°C for 1 hour. This process was repeated for 3 consecutive days. Pots (12 cm diameter) were filled with 500 g soil. Neem powder was added to the pots individually in proportion of 0.5% and thoroughly mixed with soil. To achieve a successful inoculation, soil was pre-moistened with tap water before the inoculum was added in each pot, then 10 g from the inoculum mixture was mixed into a 3 cm deep layer in the pot one day before seed planting. Each pot had one plant. Glasshouse temperature was 25°C day and 16°C night.

Data collecting

Plants were allowed to reach flowering stage (8 weeks) when the plant is sensitive to the disease with a shortage in water and nutrition. After harvest, plants were carefully soaked in water to remove the soil particles from the root system, and then washed gently under tap water. Each plant was then divided into two parts, shoot and root, each part put in pre-weighed paper bags. All the paper bags were put in an oven at 60°C for 48 hours. The dry weight of the root and shoot separately were determined by subtracting the weight of the paper bag from the total weight of the paper bag and the plant part.

Root exudate production

In their method to produce root exudates from tomato plants Shang, Grau, and Peters (2000) used tubes of 150 ml to grow tomato seeds. However, due to the size of chickpea seeds and the size of chickpea plant at 21 days (age of plant when root exudates were collected) this method needed to be modified. Therefore, a new technique and growth chambers were created for the purpose of this study. The bottom part of a compartmented plastic storage box was glued onto the lid of an identical box. Each box had 12 chambers 100 × 50 × 40 mm. A 5 ml micropipette tip with the end cut off was inserted in each chamber in a hole drilled through the base of the top box and the lid of the lower box. The boxes were sterilized with 3% sodium hypochlorite for 10 min then air-dried in a laminar flow cabinet. Seeds from two cultivars of chickpea plants desi and kabuli were rinsed for 3 min with 1% sodium hypochlorite

and washed three times with sterilized distilled water. Seeds were germinated in Petri dishes on 2 layers of filter paper wet with sterilized distilled water. Plates were then incubated in a growth chamber at 25°C for 2 days. Seedlings were gently placed in the microcentrifuge tubes. Sterilized cotton wool and then two layers of sterilized gauze were placed around the shoot of the seedlings to prevent contamination of the root area. All bottom chambers were provided with 40 mL of sterile Hoagland nutrient solution (Figure 3-1). The containers were then moved to a growth chamber at 25°C with 16 h day, and 8 h dark. Root exudates were collected after 21 days. The solution was filtered by using Millipore filters 0.22 µm. For checking microbial contamination, 1 ml of each root exudate was cultured on PDA medium and incubated for five days at 25°C. The solution was stored frozen for the rest of the experiment.

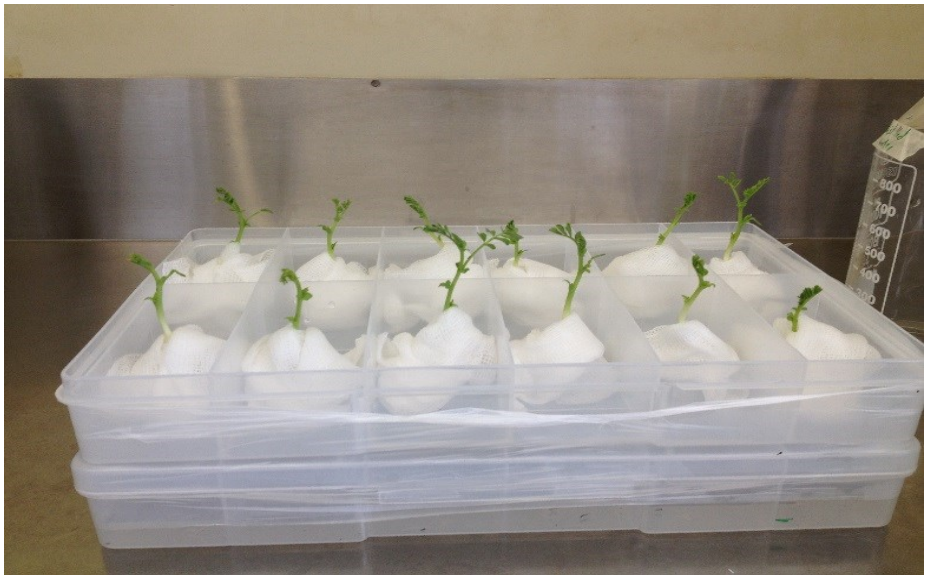

Figure 3-1 Root exudates extraction chambers

Effect of the root exudates of chickpea plant on the growth of *Macrophomina phaseolina*

The growth assay was achieved in sterile tubes (150 ml size) to measure the mycelial biomass of the fungus *M. phaseolina* in the root exudates of desi chickpea and in the root exudates of kabuli chickpea separately. Two types of control were used: Hoagland nutrient 50 ml solution and sterilized distilled water individually50 ml. Tubes were inoculated with 5 mm diameter mycelia disc of the chickpea strain *Macrophomina phaseolina* which was taken from culture incubated for 7 days at 30°C and tubes were then incubated at 30°C for 7 days at 120 rpm on shaker. After incubation, cultures were filtered through pre-weighed Whatman filter paper No 1.Then, filter papers with the mycelial mats were dried in an oven at 60°C for 24 h. The weight of mycelia was determined by subtracting the weight of the filter paper from the total weight of the filter paper with mycelia. Data of three replicates were subjected to statistical analysis.

Effect of neem extract on the beneficial microbes Rhizobium and Mycorrhiza

An experiment was conducted in the glasshouse of the University of New England to examine the effect of neem powder extract on rhizobium and mycorrhiza of two cultivars of chickpea. Plus and minus neem treatments were crossed with plus and minus rhizobium inoculation. Mycorrhizal infection came from natural inoculum in the soil. Non-sterile soil sand mix (1:1) was prepared as in the previous experiment, then 4 kg of soil put in each pot size 20 cm diameter. Neem powder was added to the pots in proportion of 0.5% and mixed thoroughly. Chickpea seeds of each cultivar were soaked for one hour in the suspension of *Rhizobium* Group N bacteria immediately before planting according to the manufacturers recommendations.

Plants were harvested at flowering stage (8 weeks) by removing them totally from the pots, and dividing into shoots and roots. Shoots were put in pre-weighed paper bags to dry them at 60°C for 48 hours. Shoot dry weights were calculated as in the previous experiment.

Pots were soaked in water to take the roots out carefully, then roots were washed gently in tap water, then soaked in water for 8 hours to remove adhering soil and sand grains from nodules which may affect the accurate weight of the nodules. For the later evaluation of mycorrhizal colonization a subsample of the roots was weighed and stored in an alcoholic solution (50% ethanol). Roots were air dried then forceps were used to take the rhizobium nodules off the roots and transfer them to empty Petri- plates to count them. Nodules for each root were put in small paper bags to dry them in the oven at 60^{o}C for 8 hours. To estimate the colonization of mycorrhiza, root samples were cleared with 10% KOH solution and stained with a 5% ink solution (Shaeffer jet black + acetic acid) according to Vierheilig, Coughlan, Wyss, and Piché (1998). Evaluation of AMF colonization was done under a microscope by a grid line method (Giovannetti & Mosse, 1980).

Statistical analysis

Data were analysed by ANOVA in SPSS version 22. The criterion for significance was $P < 0.05$. Homogeneity of variance was assessed by Levene's test and plotting residuals. Log transformation was used when necessary. The effect of concentration of oil or extract on fungal growth was analysed by linear regression.

Results

Effect of neem extracts on the growth of *Macrophomina*

Mycelial biomass

Neem seed oil promoted the mycelial yield of both isolates of *M. phaseolina* (chickpea and sunflower) compared with control. The mycelial biomass yield for both strains was increased significantly ($P < 0.01$) with increasing neem oil concentration (Figure 3-2).

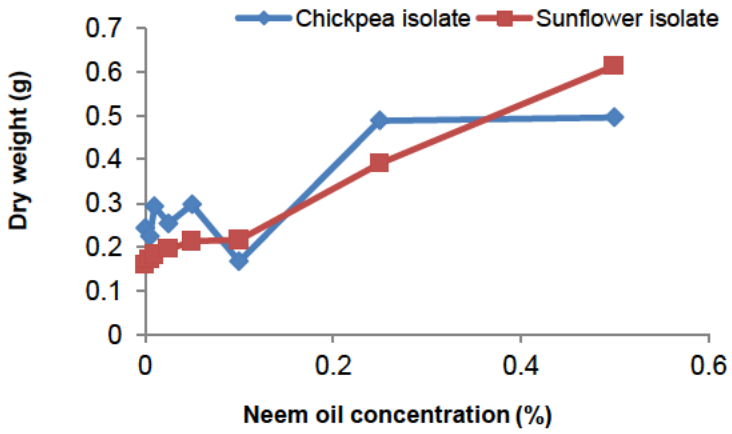

Figure 3-2. Biomass of two isolates of *M. phaseolina* on medium amended with neem oil.

The experiment was repeated with similar results (Figure 3-3).

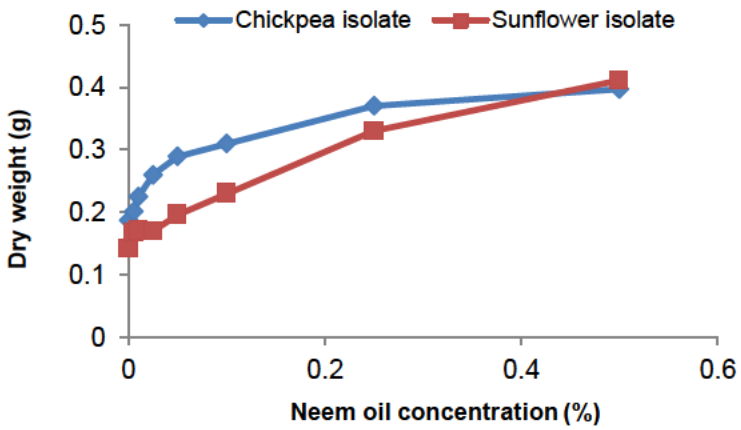

Figure 3-3. Biomass of two isolates of *M. phaseolina* on medium amended with neem oil.

Leaf extract showed inhibition of the growth of the fungus by reducing weight of biomass. For both strains of *M. phaseolina* (Chickpea and Sunflower strain) the dry weight of biomass decreased significantly ($P < 0.05$) when the concentration of the leaf extract increased. The experiment was repeated to confirm the result (Figure 3-4).

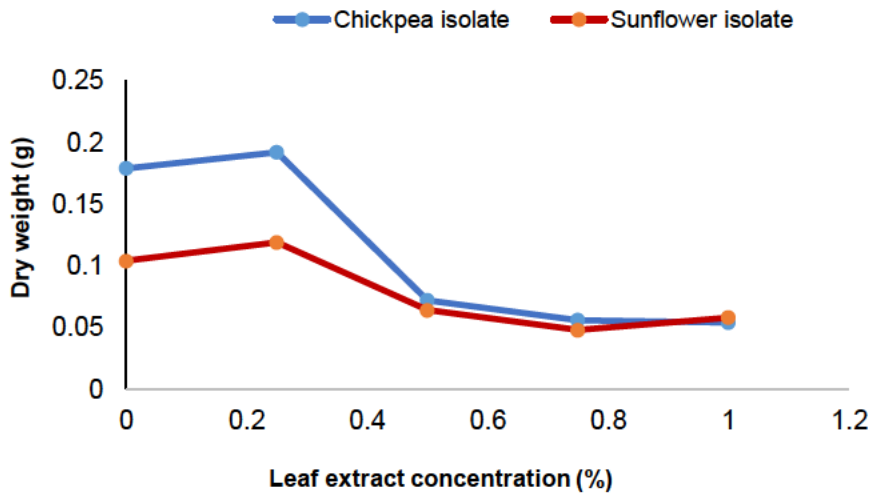

Figure 3-4. Biomass growth of two isolates of *M. phaseolina* on medium amended with neem leaf extract.

The experiment was repeated, with similar results (Figure3-5).

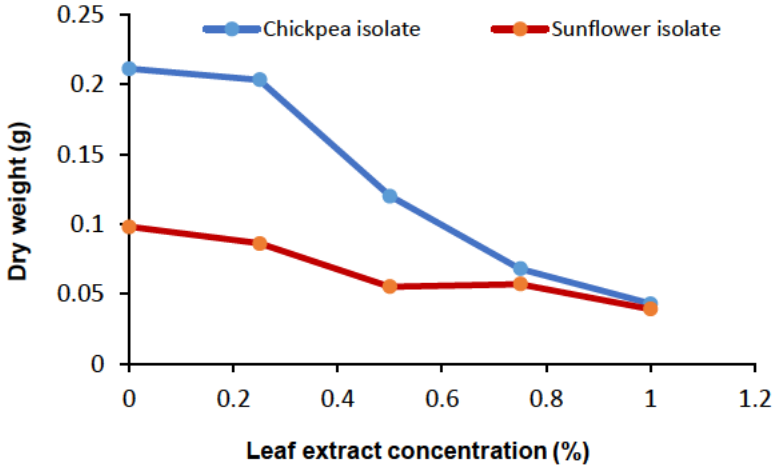

Figure 3-5. Biomass of two isolates of *M. phaseolina* on medium amended with neem leaf extract.

Determination of mycelial inhibition by poisoned food technique

Neem oil showed slight, but highly significant ($P < 0.01$) inhibition to the radial growth of the fungus. The diameter of the colony decreased when concentration of the neem oil increased (Figure 3-6).

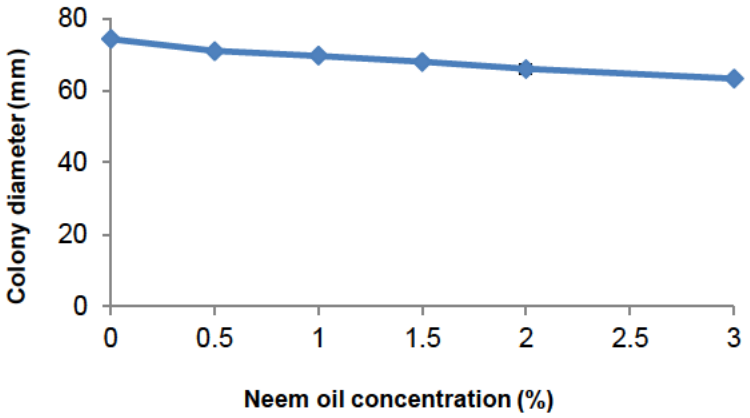

Figure 3-6. Radial growth of *M. phaseolina* on medium amended with neem oil.

The aqueous extract of neem leaves showed clear reduction to the fungus radial growth. The inhibition of growth increased highly significantly ($P < 0.01$) when the concentration of the extract increased (Figure 3-7).

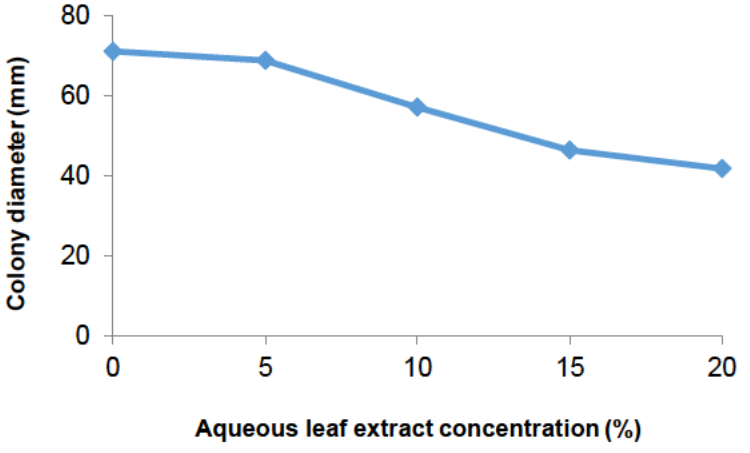

Figure 3-7. Radial growth of *M. phaseolina* on medium amended with aqueous extract of neem leaf.

Effect of root exudates of on the growth of *M. phaseolina*

Root exudates of two varieties desi and kabuli significantly increased the growth of *M. phaseolina* compared with the two controls distilled water, and Hoagland solution. There was no difference in effect of exudates from the two varieties (Figure 3-8).

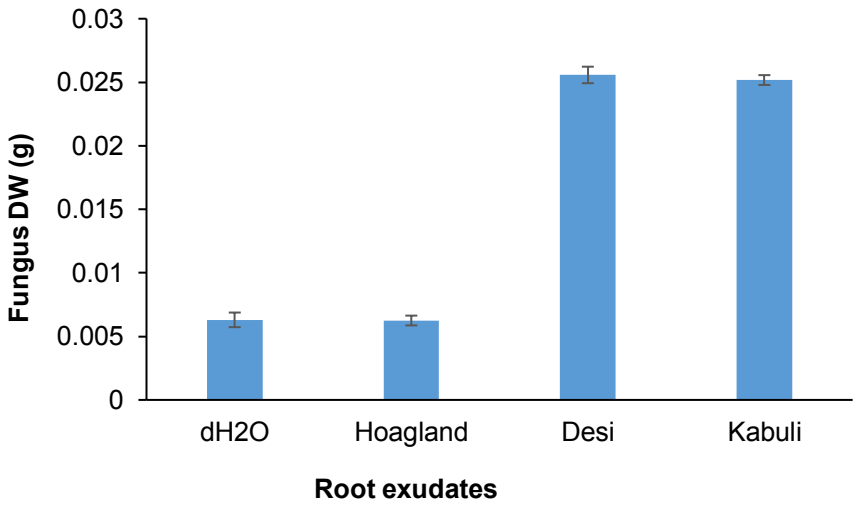

Figure 3-8. Effect of root exudates of Desi and Kabuli on the growth of *M. phaseolina* in liquid culture. Error bars show standard errors.

Effect of neem extract on interactions with beneficial microbes

Growth

There were significant effects of chickpea variety and neem on shoot dry weight. Shoot dry weight was greater in kabuli (6.2 g) than desi (4.6 g), and was lower in the neem treatment (4.9 g) than without neem (5.9 g). There was a significant interaction between neem and rhizobium inoculation, with inoculation increasing dry weight from 5.4 g to 6.3 g in the absence of neem but having no effect on shoot dry weight in the presence of neem. There were no other significant interactions between factors in their effects on shoot dry weight.

Rhizobium nodulation

In both varieties desi and kabuli, there was no significant effect of neem extract on the number of rhizobium nodules (Figure 3-9). Inoculation with rhizobium increased nodule number by 51% in desi and 135% in kabuli. There was no significant interaction between neem treatment and inoculation.

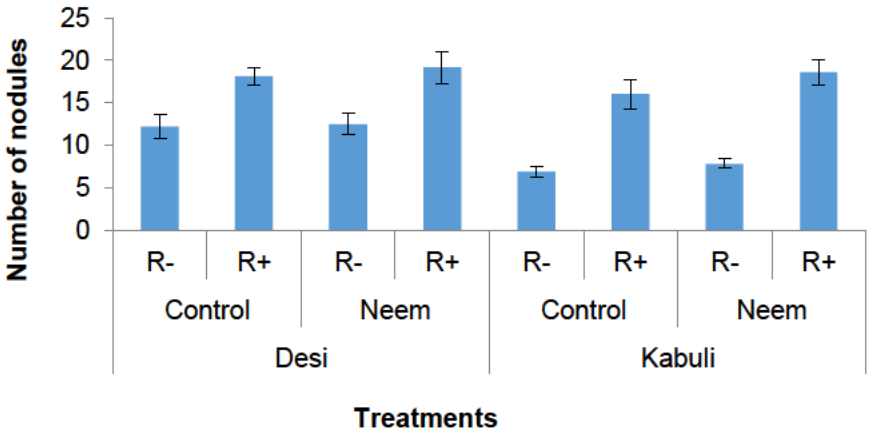

Figure 3-9. Effect of neem leaf powder on the number of nodules of two chickpea cultivars (R+: Inoculation with *Rhizobium* N). Error bars show standard errors.

There was a significant positive effect of inoculation with *Rhizobium* group N on the nodule dry weight for both varieties (Figure 3-10). There was a significant interaction between neem treatment and inoculation. Neem extract increased dry weight of nodules in the inoculated treatment for both varieties (Figure 3-10).

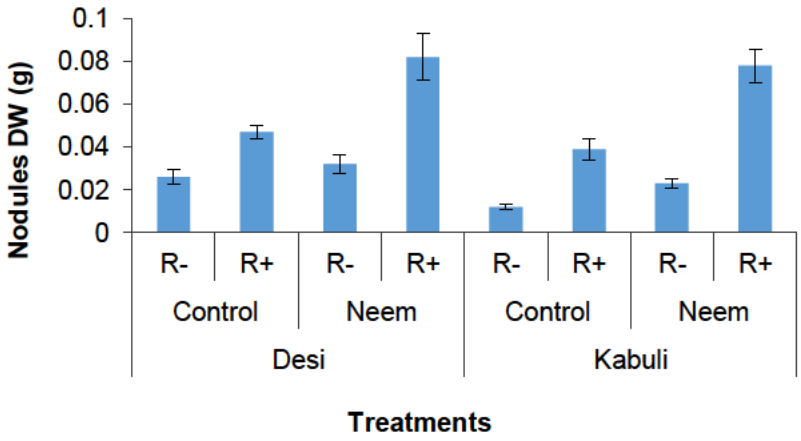

Figure 3-10. Effect of neem leaf powder on the dry weight of nodules of two chickpea cultivars (R+: Inoculation with *Rhizobium* N). Error bars show standard errors.

Mycorrhiza

Mycorrhizal colonization was 31% in the desi variety and 39% in the kabuli variety. There was no significant effect of neem powder in either variety on the percentage of mycorrhiza colonization.

Effect of neem on the development of charcoal root rot disease

Symptoms of the disease were seen in the control treatment inoculated with *M. phaseolina* and untreated with neem. The symptoms started with leaf discoloration then yellowing and blackening of the stem base. There were no obvious symptoms in plants treated with neem.

There were significant effects of variety, sterilization, inoculation with *M. phaseolina*, and addition of neem leaf powder on shoot dry weight. The kabuli variety grew larger than the desi variety (Figure 3-11), and shoot weight was 26% higher in the sterilized soil. Soil sterilization did not interact significantly with pathogen inoculation or neem treatment, so data for sterilized and non-sterilized soils have been combined for presentation (Figure 3-11).

There was a very highly significant interaction between inoculation with the pathogen and addition of neem extract (Figure 3-11). In the absence of the pathogen, there was a slight reduction in growth with the addition of the neem extract. Inoculation with the pathogen in the absence of neem reduced shoot dry weight by 80% in desi and 71% in kabuli. Addition of neem extract to inoculated plants increased shoot dry weight 3.5-fold in desi and 2.4-fold in kabuli (Figure 3-11).

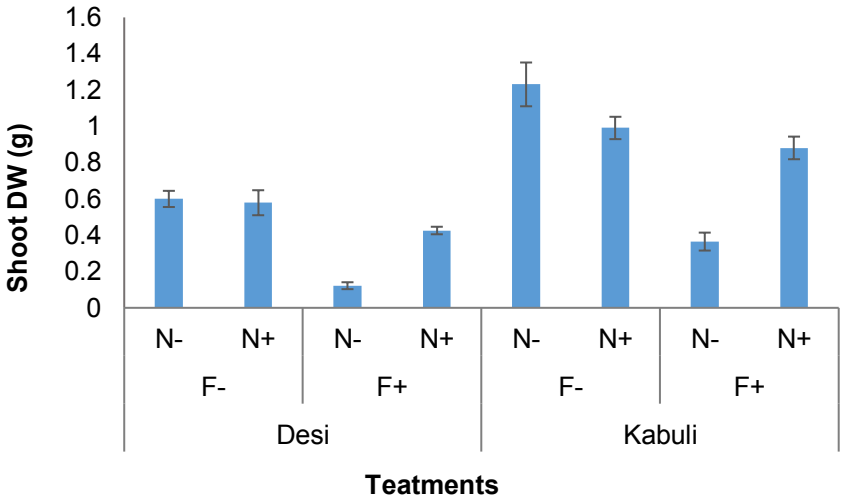

Figure 3-11. Effect of inoculation of *M. phaseolina* on the shoot dry weight of two chickpea cultivars (N+: neem) (F+: fungus inoculation). Error bars show standard errors.

The effects on root dry weight of inoculation with the pathogen and application of neem extract showed the same patterns as for the shoot dry weight (Figure 3-12).

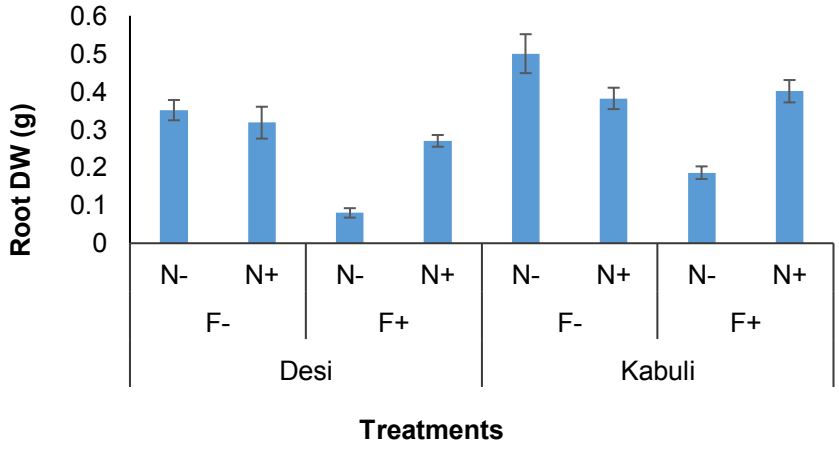

Figure 3-12. Effect of inoculation of *M. phaseolina* on the root dry weight of two chickpea cultivars (N+: neem) (F+: fungus inoculation). Error bars show standard errors.

Discussion

The work described in this chapter revealed that there were differences in fungicidal effect between the neem products (seed oil and leaf extract). The biomass yield of two strains of *M. phaseolina* was promoted by neem seed oil. That promotion increased with increase of the concentration of neem oil. The results of this study are totally different from what Dubey et al. (2009) found. They reported that neem oil had the most toxic effect against *M. phaseolina* among the other neem products that were examined. The result was also different from the earlier study of Dwivedi and Dubey (1986) as they found that neem oil decreased the germination of microsclerotia of *M. phaseolina.*

However, neem leaf aqueous extract significantly inhibited the mycelium yield (dry weight) of *M. phaseolina.* This agreed with results of Ashraf and Javaid (2007) and (Dubey et al., 2009) who showed the inhibitory effect of neem aqueous extract on the mycelial biomass growth of *M. phaseolina.*

In the poisoned food method, neem leaf extract inhibited the mycelial radial growth of *M. phaseolina.* This supported the results of Tandel et al. (2010) who also showed that neem leaf aqueous extract reduced mycelial radial growth of *M. phaseolina.* Also, Lakshmeesha et al. (2013) found that the antifungal activity of neem aqueous extract against *M. phaseolina* was the second highest among ten medicinal plants that were used in disc diffusion method assays.

There was a slight reduction of the mycelial radial growth of *M. phaseolina* by neem seed oil. This contrasted with its effects on dry weight, and may be because it affected the growth form on agar medium. The effect on biomass in liquid culture is likely to give a better indication of the true toxicity.

Root exudates of two varieties desi and kabuli were extracted in modified growth containers. The results gave a clearer understanding of the relationship between root exudates of chickpea and *M. phaseolina.* Root exudates promoted the growth of the fungus, which explains one of the most important factors that promote microsclerotia of the fungus to germinate, leading to infection. W. H. Smith (1969) showed that root exudates from pine stimulated germination of microsclerotia, but there have

been no other studies focused on the effects of root exudates on *M. phaseolina*. This preliminary experiment was done to test whether the role of exudates should be examined in later experiments. There was no difference between root exudates of the two chickpea varieties in their effect on the fungus growth, so no further experiments were done.

There was no reduction in number of rhizobium nodules and the degree of mycorrhizal colonization of chickpea plants treated with neem powder. This is the first time that the effect of neem on these beneficial symbioses has been tested explicitly in any plant. It shows that neem can be used without negatively affecting the beneficial microbes. For both chickpea cultivars, the inoculation with Rhizobium N increased the number and the dry weight of nodules. In addition, neem extract increased dry weight of nodules in the inoculated treatment for both varieties. That will give an obvious understanding of the relationship between neem extract and beneficial organisms. Besides the role of neem extract as a fungicide agent, neem extract could promote the health of the plant by increasing the effectiveness of rhizobium infection which has the main role of nitrogen fixation for the plant. That will lead to healthy and disease resistant plants. The increase in nodule weight was not accompanied by an increase in growth in this experiment. This could be because the neem treatment had a phytotoxic effect or because there was sufficient nitrogen in the soil for plant growth.

The pot trial experiments of the present study which were conducted on two cultivars of chickpea revealed that neem leaf aqueous extract reduced the severity of charcoal rot disease. In plants not treated with neem extract, *M. phaseolina* caused severe stunting of the plants. Treatment with neem extract increased growth so that it was like non-diseased plants. This growth promotion must have been because of the effect of neem on the disease because it was not seen in uninoculated plants. This is the first time that disease control of charcoal rot by neem has been shown, and one of the very few reports of effects of neem on any soil-borne plant disease. In all previous studies with *M. phaseolina*, neem extract has been tested against growth or microsclerotial germination only in vitro. This is the first time neem extract has been shown to control charcoal rot of chickpea in planta.

Although Symptoms were seen in diseased plants, these were difficult to quantify. Because inoculation with *M. phaseolina* gave a clear reduction in growth, this was chosen as a measure of disease severity. Growth rate will be directly related to yield. Dry weight can also be measured accurately, so was used in later experiments.

To sum up, neem seed oil promoted the growth of two strains of *M. phaseolina*. However, neem leaf extract reduced the growth of both strains of the fungus. As well, neem leaf powder reduced the symptoms of root rot caused by *M. phaseolina* in the glasshouse and there was no reduction in the activity of beneficial symbiotic microbes. Neem leaf extract is therefore a promising control method for charcoal rot in chickpea. Further work is needed on optimising method of application, and testing in the field.

Chapter 4: Neem formulation and time of application

Introduction

The results of the previous chapter revealed that neem oil promoted the growth of *M phaseolina,* and on the other hand, neem leaf extract reduced the growth of the fungus. Based on these results neem leaf extract was chosen as the antifungal agent to use against *M phaseolina*. The work described in this chapter aimed to achieve the optimum way of using neem leaf extract. The method of using neem has to be effective against the pathogen at low concentration (rate) and with long lasting effect. Most previous work on control of soilborne diseases by botanicals (reviewed in chapter 2) has used liquid extracts. it is not known whether this is the best method of application, or whether solid or slow-release formulations may work better.

In this chapter, neem leaf material was formulated into different forms: neem powder, neem capsules, neem aqueous extract, and neem pellets. Different rates of these formulas; 0.5%, 1%, and 1.5% were used at two application times: pre-emergence and post-emergence of chickpea seeds. Then, the effect of neem pellets were evaluated at low rates 0.001%, 0.0025%, and 0.005%. After that, a seed coating method was tested.

Material and Methods

Neem leaf powder was obtained from Neeming Australia and was used as crude 100% powder. In order to achieve the long-lasting activity of the neem products to protect chickpea plant from root rot disease, pellets and capsules were chosen as ways to release neem extract over a longer period. These were raw powder, aqueous extract, capsules, and pellets (Figure 4-1). Three concentrations 0.5%, 1.0%, and 1.5% from each formula were used at two different times pre- and post-emergence of seedlings.

Preparation of formulation

Neem powder: The crude 100% neem powder was used as one of the formulations.

Neem capsules: Empty clear gelatin capsules size 00 were obtained from the Capsule Guy, Adelaide, Australia. Each capsule was manually filled with 500 mg neem powder and stored in a dry place for future use.

Neem aqueous extract: A water extract was prepared as described in the previous chapter.

Neem pellets: A new technique was used to create pellets (granules) from neem powder. Polyvinyl acetate (PVA) was used as an adhesive. PVA Craft Glue was obtained from Mont Marte, Salisbury, Queensland, Australia. Several attempts were made with many mixtures to achieve optimum proportions of neem powder, PVA glue, and distilled water. The best mixture was 250 g of glue thoroughly mixed with 300 ml distilled water then mixed with 1kg neem powder. Small pellets, approximately 10 mm diameter and 5 mm thick, were made by extruding drops of the paste through a 7 mm diameter nozzle on a cake icing pump. The pellets were air dried for four hours. After solidification, pellets were stored in a dry place at room temperature for future use.

Figure 4-1. Formulation types of neem.

Seeds of kabuli chickpea variety Genesis 090 were obtained from Australian Agricultural Crop Technologies, Wee Waa, NSW, Australia. Seeds were washed with detergent and rinsed several times in water to remove any fungicide treatment.

Soil: sand mix was prepared as described in the previous chapter and 4 kg soil was weighed for each 20 cm diameter pot. The pre-emergence treatments were applied five days before seeds were planted and pots were irrigated regularly. A sand-cornmeal medium was prepared as described in the previous chapter for inoculation of *M phaseolina*. 10 g of fungus inoculum was added to each pot (premoistened) one day before seed planting. Post-emergent treatments were applied five days after seedlings emerged. All neem treatments were applied at 0.5, 1.0 or 1.5% w/w (solid products) or v/w (aqueous extract) onto the surface of the pots. All neem treatments were inoculated with *M. phaseolina*.

There were two control treatments that did not receive any neem products, one inoculated with *M. phaseolina* and one uninoculated. There were 3 replicates of every treatment. Because inoculation with rhizobium did not affect plant growth in chapter 3, this was not used in this experiment.

Data collection

At 10 days age of the plants data were collected on the height, number of leaves, and number of leaflets for each plant. The height of each plant was measured at ages 40, 50, and 60 days. Plants were harvested at age 110 days. Shoot dry weight, number of seeds, and the seed dry weight for each plant were measured.

Effect of lower rates of neem pellets

A pot experiment was conducted to examine the effect of different rates of neem pellets 0.5%, 0.25%, and 0.1% on the development of *M. phaseolina* root rot in chickpea plants. Inoculated and uninoculated plants that were not treated with neem served as control. Soil was prepared as described in the previous chapter and 500 g soil was weighed for each 12 cm diameter pot. The pre-emergence neem treatments rates were applied five days before seeds of chickpea cv. Genesis 090 were planted and pots were irrigated regularly. A 10 g of sand-cornmeal medium as a *M. phaseolina* inoculum was added one day before seed planting. Plants were harvested at eight weeks, and plants checked for the symptoms of charcoal root disease. The height and the shoot dry weight of the plants were measured to determine the disease severity.

Seed coating

A pot trial was conducted to evaluate seed coating as a method of applying neem. Chickpea cv. Genesis 090 seeds were coated by neem powder in a sticker solution. According to Bardin and Huang (2003) 10% polyvinyl alcohol in water showed efficiency to combine organic material to the seeds of beetroot. Polyvinyl alcohol (M.W. approximately 125000, BDH Chemicals Ltd, Poole England) at 10% was dissolved in boiling water, then left to cool down to room temperature. Seeds were soaked in the sticker solution for 15 min, then scooped out and mixed with the neem powder (0.45 g/10 seeds) in a plastic bag. To ensure good seed coating the inflated bag was vigorously shaken for 2 min. The seeds were dried overnight (16 h) at room temperature. Coated seed and uncoated seed were planted in 20 cm diameter pots (10 seeds/pot, 10 pots per treatment). The pots were placed in a glasshouse. The seed germination rate was counted every day for the first 5 days. Seedling growth was observed by measuring the length of the shoot and the root of each seedling.

Statistical analysis

Data were analysed by ANOVA in SPSS version 22. For experiments with only two treatments, t-tests were done in Microsoft Excel. The criterion for significance was $P < 0.05$. Log transformation was used when necessary to correct for homogeneity of variance.

Results

Formulation experiment

Plant height and health was monitored every 10 days for the first 60 days. Symptoms of disease (leaf yellowing and blackening of stem base) first appeared in the inoculated control at 30 days, and the height of inoculated control plants increased more slowly than uninoculated controls or any of the neem treatments from 40-60 days (Figure 4-2). Averaged across rates and application times, the tallest plants were in the treatments with neem pellets (Figure 4-2).

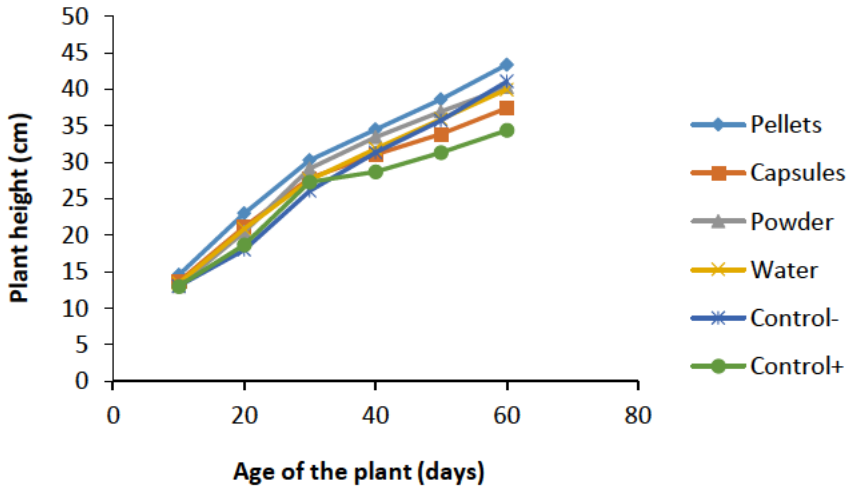

Figure 4-2. Effect of different formulation of neem on the height of chickpea plants in a pot experiment.

The number of leaves and leaflets were counted every 10 days from 10 to 30 days of plant age. The number of leaves and leaflets was similar for all neem treatments, but was much lower in the inoculated controls at 30 days (Figure 4-3 and Figure 4-4).

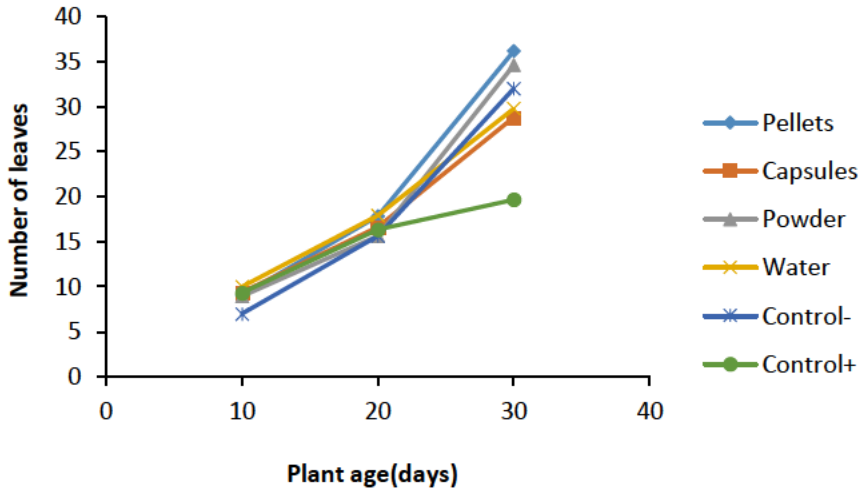

Figure 4-3. Effect of different formulation of neem on the number of leaves of chickpea plants.

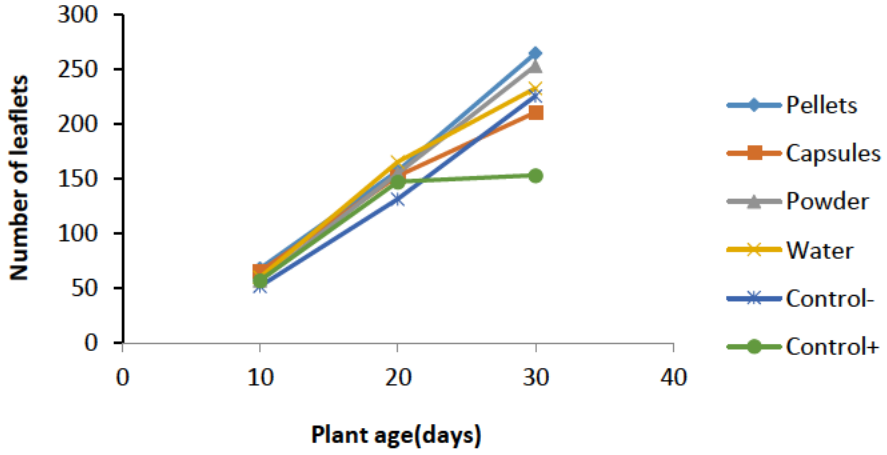

Figure 4-4. Effect of different formulation of neem on the number of leaflets of chickpea plants.

Plant growth at 30 days

An analysis of growth parameters (height, number of leaves, number of leaflets) was done at 30 days, when disease symptoms first occurred in the inoculated controls. There were significant effects of formulation, rate, and time of application on the height of plants, but no significant interactions between these factors. Plants treated with pellets were significantly taller than those treated with water extracts or the controls (Figure 4-5). Plants treated with the highest rate of neem (1.5%) were slightly but significantly taller than those treated with 0.5% or 1% neem (Figure 4-6). Plants treated with neem before sowing were significantly taller than those treated after sowing (Figure 4-7).

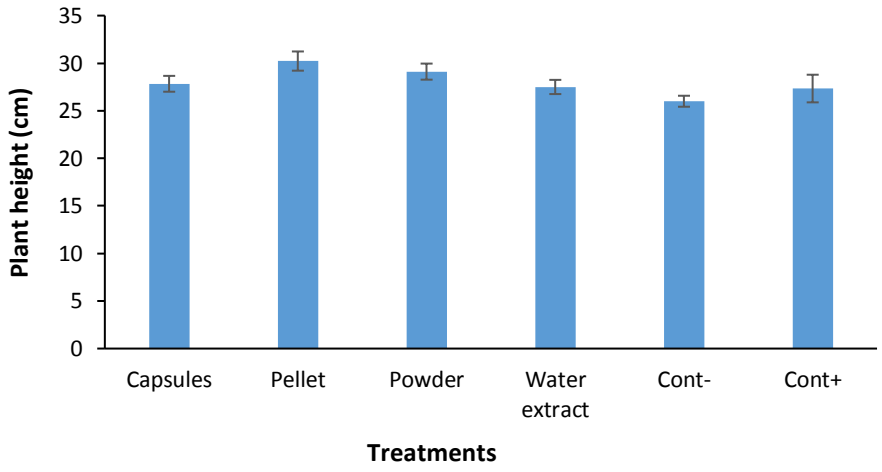

Figure 4-5. Effect of neem formulation on the height of chickpea plants at 30 days. Error bars show standard errors.

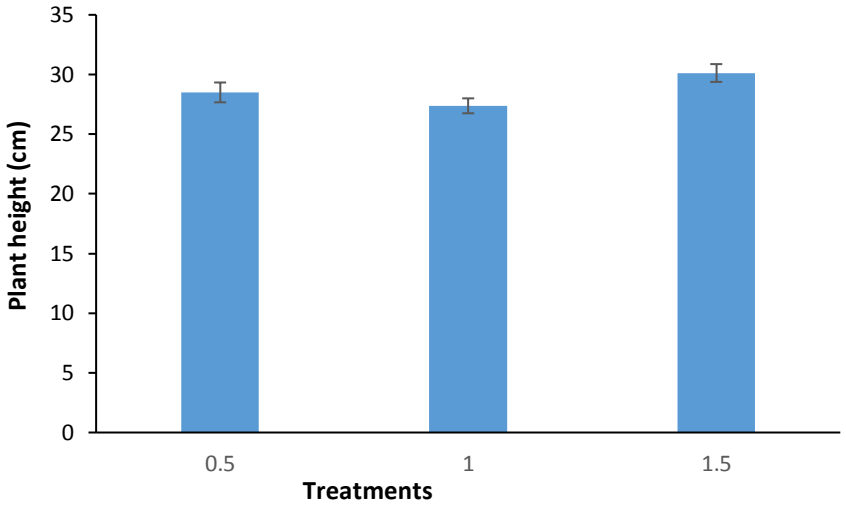

Figure 4-6. Effect of neem rates on the height of chickpea plants at 30 days. Error bars show standard errors.

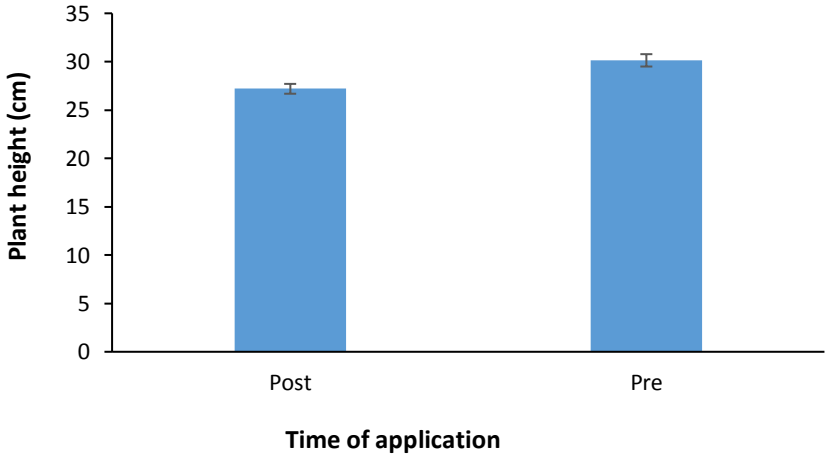

Figure 4-7. Effect of application time (Pre/Post-emergence) on the height of chickpea plants at 30 days. Error bars show standard errors.

There were significant effects of formulation and time of application, and a highly significant (P = 0.001) effect of the interaction between formulation and application time on the number of leaves per plant. There was a 50% increase in the number of leaves of plants treated with pellets when the pellets were applied before sowing compared with application after sowing (Figure 4-8). There was no significant effect of rate on the number of leaves.

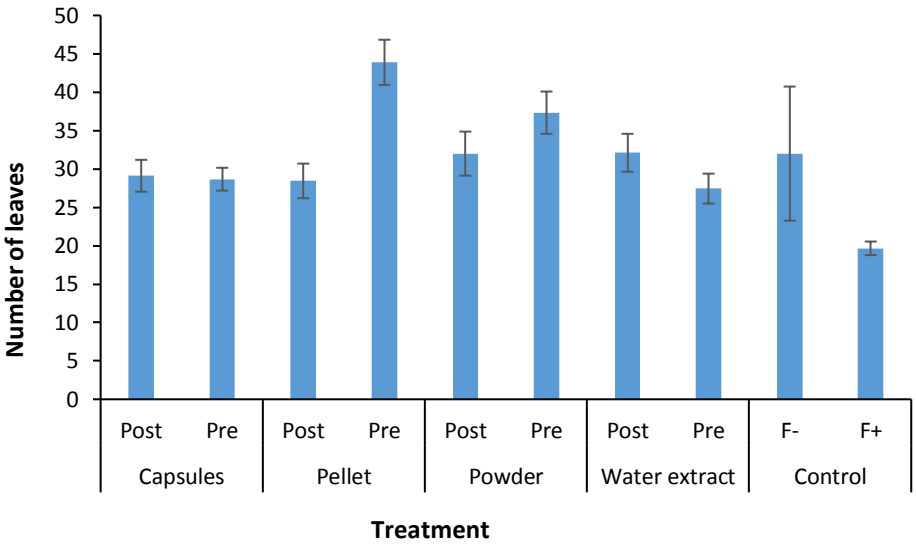

Figure 4-8. Effect of the interaction between formulation and application time on the number of leaves per chickpea plant at 30 days. Error bars show standard errors.

There was also a highly significant interaction between formulation and application time on the number of leaflets per plant, with more leaflets when pellets were applied before sowing than after sowing (Figure 4-9). There was a significant effect of rate of neem on the number of leaflets, with more leaflets on plants treated with 0.5% neem than with 1% or 1.5% (Figure 4-10).

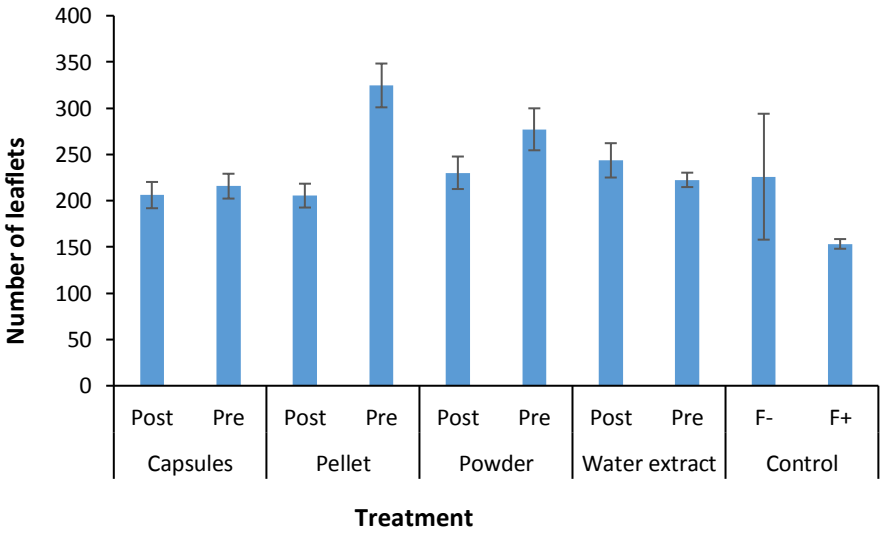

Figure 4-9. Effect of the interaction between formulation and application time on the number of leaflets per chickpea plant at 30 days. Error bars show standard errors.

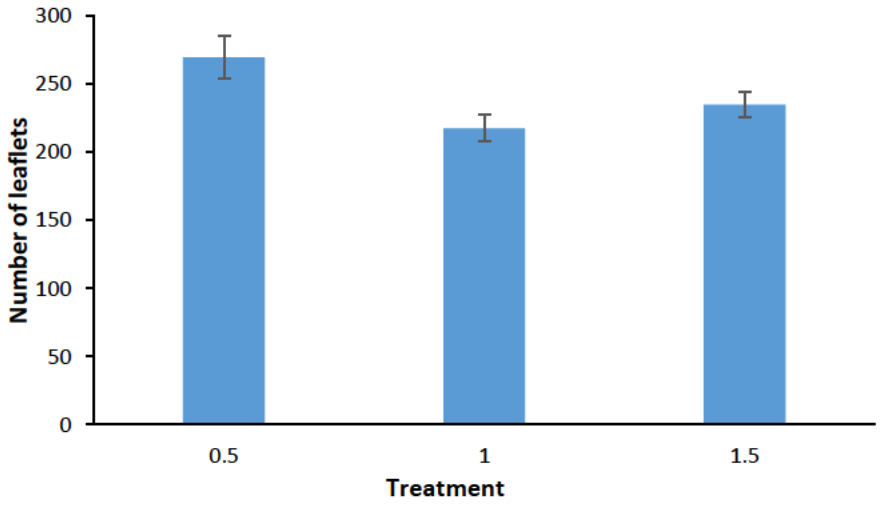

Figure 4-10. Effect of rate of neem on the number of leaflets per chickpea plant at 30 days. Error bars show standard errors.

Plant growth at 110 days

At harvest (110 days) formulation type and time of application had significant effects on shoot dry weight of the plants. The neem pellets had the highest shoot dry weight of the plant (Figure 4-11). There was no significant effect of neem rates. The pre-emergence application time had significantly higher shoot dry weight of the plant than post-emergence application (Figure 4-12)

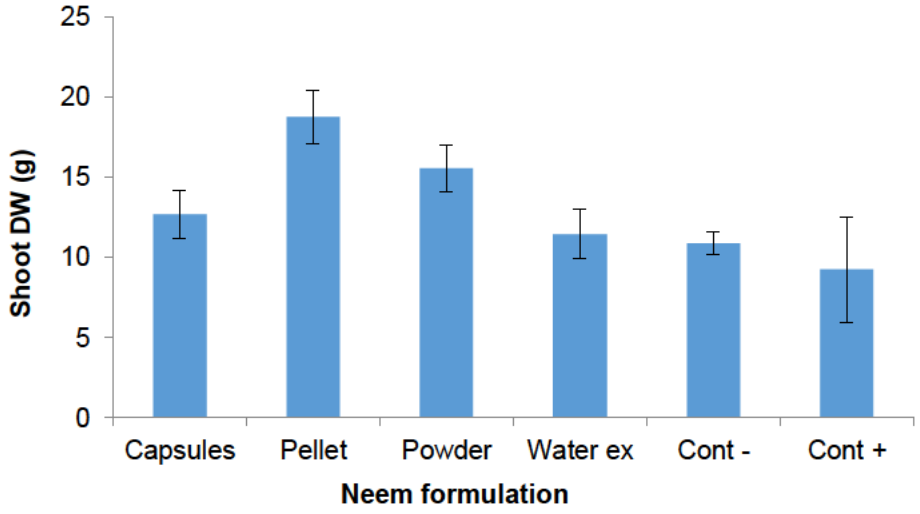

Figure 4-11. Effect of different formulation of neem on shoot dry weight of chickpea plants at 110 days. Error bars show standard errors.

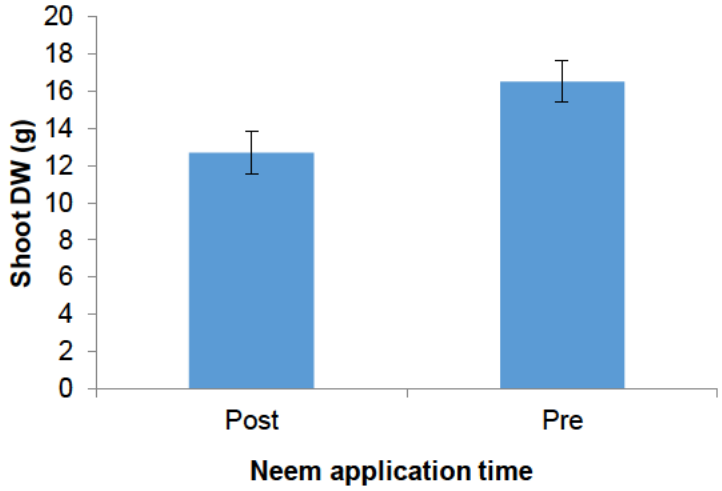

Figure 4-12. Effect of neem application time (Pre/post-emergence) on shoot dry weight of chickpea plants at 110 days. Error bars show standard errors.

The number of seeds was significantly affected by neem formulation, with fewer seeds in the capsule treatment than the other neem formulations or the uninoculated control (Figure 4-13). The number of seeds per plant in the controls was reduced by 70% by inoculation with *M. phaseolina*.

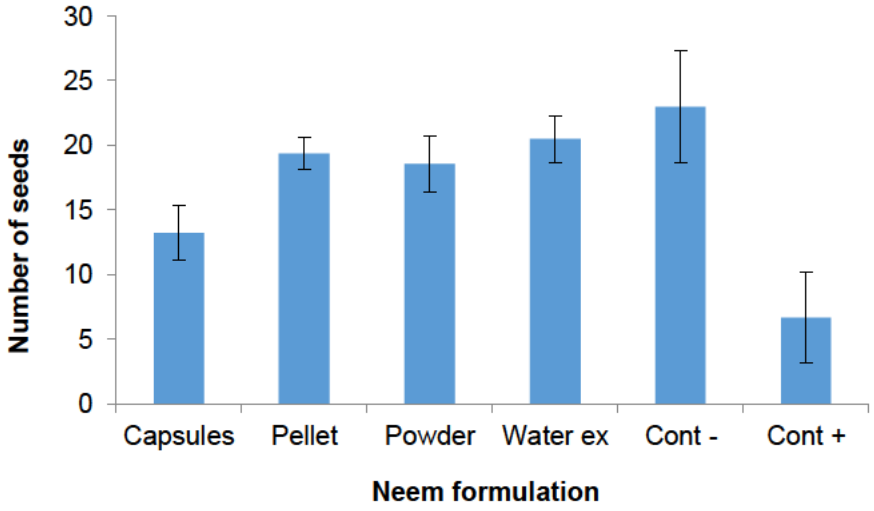

Figure 4-13. Effect of different formulation of neem on the number of seeds of chickpea plants at 110 days. Error bars show standard errors.

Grain yield (seed dry weight per plant) was not significantly affected by the main effects of formulation, rate or time. However, there was a significant interaction of application time and rate, with the seed dry weight being reduced by 47% when 0.5 % treatments were applied post-emergence compared with pre-emergence (Figure 4-14). Inoculation with *M. phaseolina* significantly reduced the seed dry weight per plant in the controls from 4.4 g to 1.4 g.

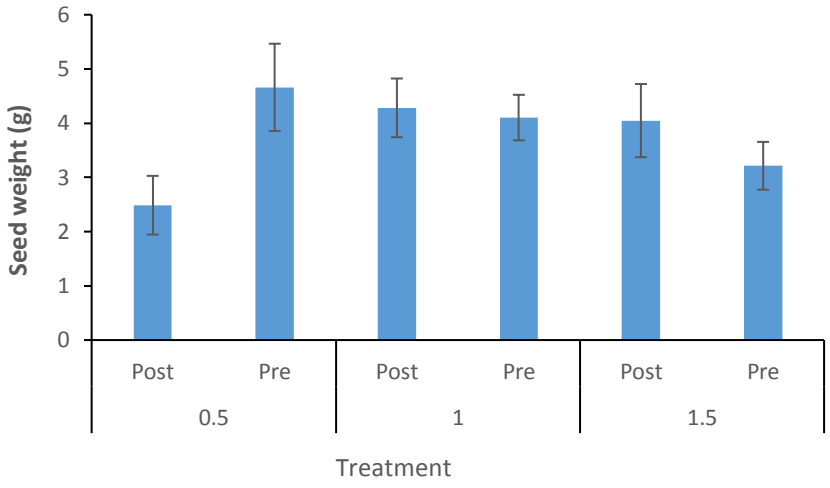

Figure 4-14. Effect of interaction of formulation rate and rate of neem on the dry weight of seeds of chickpea plants at 110 days. Error bars show standard errors.

Evaluation of reduced rates of neem

An experiment was done to test the effectiveness of neem pellets at rates below 0.5%. Inoculation with *M. phaseolina* reduced plant height by 30% in the controls (Figure 4-15). Neem pellets at the rate of 0.5% significantly increased the plant height so that it was the same as the uninoculated control treatment. There was no significant effect of using neem at the rates of 0.25% and 0.1% (Figure 4-15).

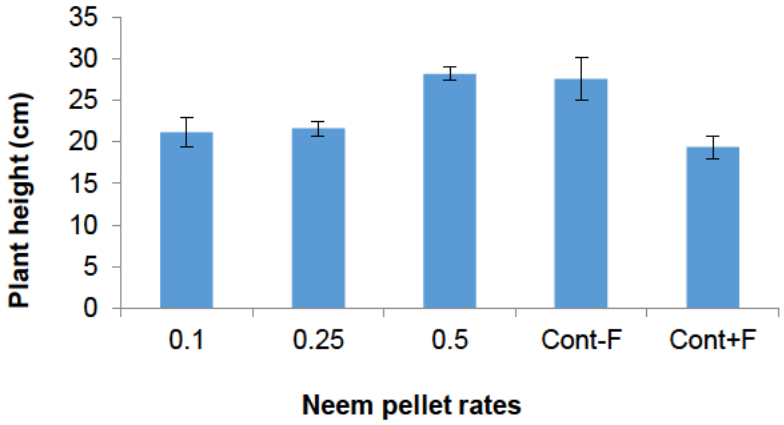

Figure 4-15. Effect of different rates of neem pellets on the height of chickpea plants reduced with *M. phaseolina*. Error bars show standard errors.

There was a highly significant effect of using neem pellets at the rate of 0.5% on the shoot dry weight of the plants compare with the inoculated control treatment. There was no significant effect on dry weight of using neem at the rates of 0.25% and 0.1% (Figure 4-16).

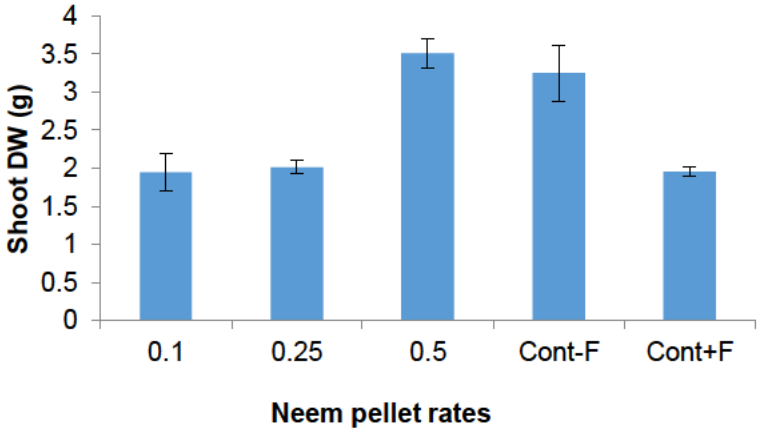

Figure 4-16. Effect of different rates of neem pellets on the shoot dry weight of chickpea plants reduced with *M. phaseolina*. Error bars show standard errors.

Seed coating

There was a highly significant negative effect of neem coating on the germination rate of the seeds during the five days of seedling emergence. The germination rate at 5 days was 49% compared with the uncoated treatment at 98% (Figure 4-17).

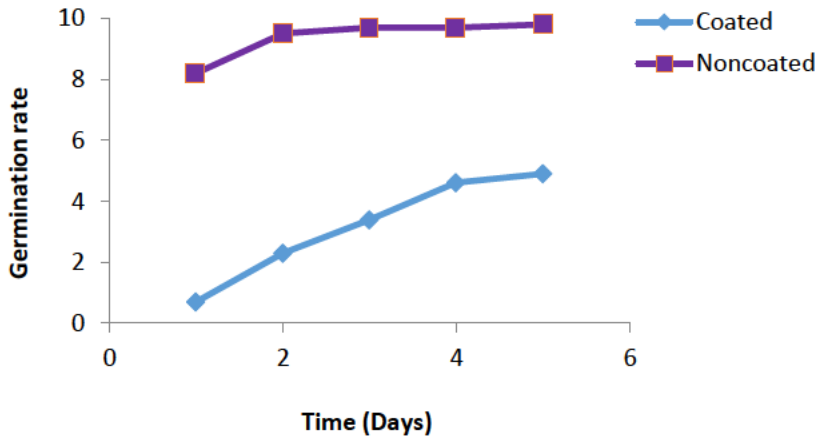

Figure 4-17. Effect of seed coating with neem on the germination rate of chickpea seeds

As well, seed coating reduced growth of shoots by 40% and roots by 32% compared with uncoated seeds (Figure 4-18). Because seed coating was phytotoxic, the effect on disease was not measured.

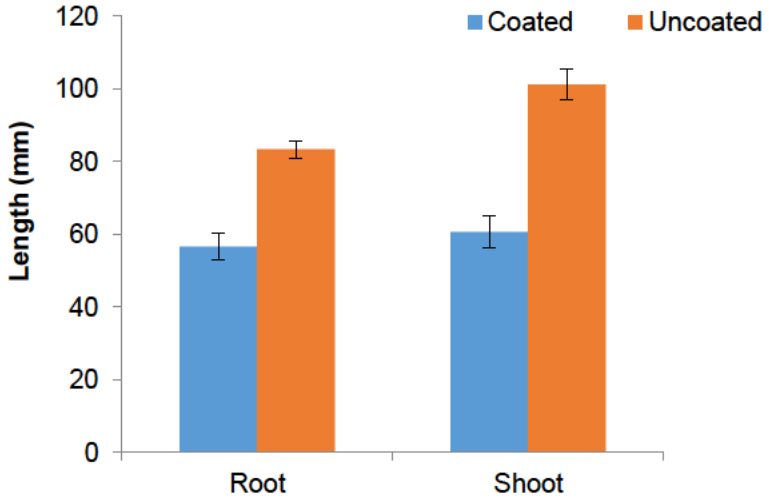

Figure 4-18. Effect of seed coating with neem on the shoot and root length of chickpea seedlings. Error bars show standard errors.

Discussion

The experiments in this chapter were conducted to find out the optimum formulation methods among neem products, the rate, and the application time. There have been no similar experiments with application of neem or other botanicals for soilborne diseases, so there is no literature for comparison.

There were four formulations: pellets, powder, capsules, and water extract with three rates 0.5%, 1%, and 1.5% for each formulation. All these formulations and rates were examined for their efficiency to control charcoal rot disease of chickpea. A pot trial experiment was conducted in the glasshouse. Formulations were applied at two different times (pre-emergence and post-emergence). Data were collected several times starting from day 10 (age of the plant) until harvest (day 110). Neem pellets gave the best results for disease reduction as measured by proportion of growth promotion followed by neem powder. The lowest rate of neem pellets that protected the plant from the pathogen effectively was 0.5%. In plants treated with 0.5% neem pellets, the application of the pellets five days before planting (pre-emergence), gave the greatest height (plant height), number of leaves, and number of leaflets. The data of harvest (110 days) showed that neem pellets with pre-emergence application increased the shoot dry weight of the plant. After the results of this experiment, neem pellets 0.5% in pre-emergence application was considered the optimum formulation of neem leaf for controlling charcoal rot.

In order to examine the effect of neem pellets at rates lower than 0.5% on the control of charcoal rot, a pot experiment was conducted with three different rates of neem pellets 0.5%, 0.25 % and 0.1%. The results of this experiment revealed that neem pellets at the rate 0.5% had a significantly positive effect on the plant height. However, that effect became lower when the rate of the pellets was 0.25 % and 0.1%. This experiment was considered the confirmation of the optimum rate of neem pellets which was 0.5%.

To explain the efficacy of neem pellets, the property of slowly breaking down and gradual release of the active ingredients enabled this kind of formulation to have a long-lasting effect which gives the plant a long-time protection from the disease.

There was evidence of some phytotoxicity because the number of leaflets was reduced at higher levels of neem. The differences in phytotoxicity between extract, pellets, powder and capsules were not measured directly. However, the slow degradation of pellets may have reduced the danger of phytotoxicity of neem to chickpea plants. In term of neem rates, the 0.5% was found to be the optimum rate because it had less phytotoxicity with a good range of reducing the disease. Also, the neem pellets were formed by using PVA which is an organic material and environmental friendly substance with no known toxic effects (Kegley, 2016). The PVA made up about 7% of the dry weight of the pellets, so was unlikely to have a large effect.

Time of application of the neem has the same importance as formulation. Adding neem product five days before planting with irrigation will allow the extract to dissolve and to be ready to control the disease by the time the fungus starts to germinate and the infection occurs. However, the post-emergence application will give the fungus time to spread and infect the plant. On the other hand, there were differences when using the other formulation methods. Neem pellets and capsules are easier to handle and apply to the soil than powder or aqueous extract. However, capsules were less effective than pellets especially on yield, which may because the gelatin cover of capsules dissolved more quickly than it needed to be. Powder was generally less effective than pellets but better than aqueous extract. However, powder is worth doing further testing on because it requires less processing than pellets so may be more economic.

To examine another formulation method, seed coating method was tested in another pot experiment. However, the results of the seed coating experiment showed there was high reduction of seed germination in the treated seeds. Also, seed coating reduced the growth and development of the plant shoots. This effect might come from the direct contact between neem extract and the seeds, which will be higher than if the neem extract is added to soil in which seeds are planted. An earlier study of U. Singh et al. (1980) showed that neem oil reduced rate of germination and rate of seedling growth in chickpea. Then the study of Kasarkar (2016) found that aqueous leaf extract of neem inhibited the germination of the legume crops mung bean and cowpea. Neem therefore needs to be applied in a way that avoids direct

contact with the germinating seed. It is possible that the seed coating process itself may have affected germination rate. In any case, this was shown to be not a suitable method for applying neem.

To sum up, the study in this chapter revealed that the optimum neem application was neem pellets at a rate of 0.5% which should be applied five days before planting (pre-emergence). Seed coating is not the recommended method to use neem extract. A field experiment should be conducted to confirm the results found in the glasshouse.

Chapter 5: Evaluation of neem in the field and against other fungi

Introduction

The previous chapters tested the effect of neem on charcoal rot of chickpeas. Chapter 3 showed that neem leaf powder could reduce charcoal rot in pot trials. Then Chapter 4 showed that neem pellets at 0.5% were the best formulation, applied before emergence. There is a need to test whether this works under field conditions, and also, to test the effects of neem on other potential pathogens and general soil biology.

In this chapter, neem pellets and neem powder were examined for their efficiency when they were used in field conditions. Plant dry weight and nodulation were measured at several points during growth. The effect of neem on mycorrhizal colonization was measured, as were bacterial and fungal populations in soil.

To further examine the effects of neem on soil fungi and bacteria that were found in the field trial, a laboratory test was done where neem was added to soil and bacterial and fungal populations were measured. An in vitro examination was conducted to evaluate the effect of neem extract on ten soil borne fungi including both common saprotrophs and plant pathogens.

A glasshouse experiment was conducted to evaluate the effect of neem pellets at different rates on development of *Phytophthora medicaginis* root rot, the major soilborne disease of chickpeas in Australia.

Neem pellets gave a growth promotion effect that was more than just preventing disease caused by *M. phaseolina*. To explore this, a pot experiment was conducted to evaluate the effect of neem pellets on root lesions of chickpea plants growing in field soil.

Material and Methods

Field trial

Field experiments were conducted at the Experimental Farm of Laureldale, University of New England, Armidale, New South Wales, Australia. The area is located at latitude 30.515° S, longitude 151.665° E and elevation: 980 m above mean sea level. Climatically, the area has a cold winter with minimum and maximum average winter temperatures of -1.8ºC and 11.5ºC. The temperatures in Armidale in summer average between 13.1ºC and 24.1ºC. Summer is also when Armidale receives most of its rainfall - an average of 800 mm annually. The soil was the cracking grey clay (vertosol) that was used in experiments in previous chapters.

The field area was surface cleaned from plant debris, large weeds were pulled off before tillage, and then the field area and surrounds were sprayed with glyphosate. In order to limit the spread of pathogen inoculum and reduce the quantity of neem material required, plants were grown in black polyethylene planter bags 2.5 litres (150 x 170 mm). The bags had large drainage holes in the bottom that allowed roots to penetrate deeper into the field soil. The bags were lined with paper bags to stop soil from leaking out of the holes while they were being prepared. Bags were filled with 1.5 kg sand and field soil mixed 1:1 and all bags were placed in holes in the field so that the soil surface in the bags was level with that of the field (Figure 5-1).

The experiment was laid out in a randomized complete block design with nine replicate blocks. The treatments were uninoculated control; inoculated with *M. phaseolina* only; and inoculated and treated with either neem pellets or neem leaf powder. In each block there were 5 plants for each treatment. Treatments of 0.5% w/w of neem pellets, and 0.5 % w/w neem powder (7.5 g of each product per bag) were applied onto the surface of the soil in the bags, five days before seeds of chickpea cv. Genesis 090 were planted and bags were irrigated regularly. The *M. phaseolina* inoculum was prepared in sand-cornmeal medium as described in the previous chapters and 10 g from that inoculum was added to each bag one day before seed planting. The seeds were sown on 2 November 2015 and thinned to one plant per bag after emergence. Plants were not inoculated with rhizobium.

Figure 5-1. Field site showing layout of planter bags.

From day 60 after sowing, one plant from each treatment was harvested randomly every 15 days until 120 days after sowing (4 March 2016). The plastic bag with the plant was pulled out of the soil carefully to avoid losses in the root system. The shoot of the plants was cut off at soil level then put in pre-weighed paper bags to dry in a 60° C oven for 48 hours, then dry weight was calculated as previously described. The root system within the plastic bags was soaked in water then gently removed from the soil. The steps of the previous experiment (Chapter 4) were followed to calculate the number and the dry weight of rhizobium nodules. At 60 days, samples of the roots were collected for the evaluation of mycorrhizal colonization. Roots were stained with the ink-vinegar method of Vierheilig et al. (1998) and percent colonization of root length calculated as described in Chapter 3.

Effect of neem on microbial abundance in the field

To estimate the effect of neem pellets and neem powder on the density of soil microbes two measurements were conducted in the field trial. The first was when the plants were at 75 days age and the second was in the last harvest at 120 days. Soil samples were

randomly collected from the soil within the plastic bags of each treatment from six replicates. The fungi colonization and bacterial colonization were estimated separately using soil dilution plating. Nutrient agar was used for bacteria, and Rose Bengal agar for fungi, according to the procedure of Sinclair and Dhingra (1995). Dilutions of 10^{-5} and 10^{-6} for fungi and 10^{-6} and 10^{-7} for bacterial were prepared in 0.01% agar in deionised water. 1ml of the soil dilution was spread on 9 cm Petri plates of the appropriate medium. All plates were incubated for 3 days at 30°C, then colonies of bacteria and fungi were counted.

The effect of neem on microbial abundance in vitro

To confirm the results from the field trial, the effect of neem on bacterial and fungal abundance was measured in an in vitro experiment. Soil was collected from the field site, sieved and cleaned from plant debris and large stones and particles and ground to fine particles. 50 grams of soil was put in a Petri-plate and mixed thoroughly with 0.5% neem powder (0.25 g) and moistened. All soil samples were incubated at 30°C for 30 days. After incubation, soil dilution method was conducted and Petri-plates with Rose Bengal medium and nutrient agar were prepared as above for estimation of fungus colonization and bacterial colonization respectively. There were six replicates of each treatment.

Effect of neem leaf extract on growth of soil fungi

Ten isolates of fungi representing a range of soilborne saprotrophs and pathogens were obtained from the Botany Laboratory, University of New England. The isolates were *Mucor genevensis, Sordaria fimicola, Rhizoctonia solani, Macrophomina phaseolina, Pythium irregulare, Trichoderma harzianum, Sclerotium rolfsii, Fusarium oxysporum, Phytophthora medicaginis, and Verticillium dahliae.* All isolates were reactivated on PDA medium before using them in the test.

Neem leaf powder was obtained from Neeming Australia. A suspension of 1:5 neem powder in sterilized deionized water was firmly mixed in a blender for 10 min. The

mixture was then poured through two layers of Miracloth then centrifuged at 5000 g for 10 min. The supernatant was then filtered through Whitman filter paper No. 1 (Mohana & Raveesha, 2007). The extract was sterilized by filtration through 0.22 µm filters. The extract was preserved in the freezer as crude concentrated extract for further use (Satish et al., 1999). 1 ml of the extract was added per 9 cm Petri plate before adding the culture media, and 1 ml of sterilized distilled water served as control. A PDA medium was prepared and autoclaved and then cooled down. 15 ml of this medium was added to each plate with gentle circular movement to mix the extract with PDA. An agar block 5 mm diameter which was taken from each isolate of the fungi was put in the centre of the PDA plate (with and without neem) with three replicates for each treatment. All plates were incubated at 25° C. Incubation time varied based on the fungus growth. Radial growth was measured for each fungus and percent inhibition of radial growth was calculated.

Effect of neem pellets on root lesions

This glasshouse experiment was conducted to measure the effect of neem pellets on organisms other than *M. phaseolina* which may have affected the growth of the root system of chickpea plants. Soil was prepared as in previous experiments (sand: field soil) (1:1), pots of 12 cm in diameter were filled with the soil. Then, neem pellets at 0.5% w/w were applied five days before planting seed of chickpea cv. Genesis 090 and pots were irrigated regularly. The treatments were neem pellets, and plants without neem as control. Plants were grown in the glasshouse as in previous experiments. Plant were harvested at eight weeks old. Pots were soaked in water until plants could be easily pulled out of the soil. The root system was cut off from the whole plant, and then gently washed several times with tap water to remove any adhesive articles like soil and plant debris. Roots for each plant were divided into two or three parts depending on the size of the root and transferred to Petri-plates. Then the percentage of root length showing discoloration was estimated under a stereomicroscope by using the grid line intercept method (Giovannetti & Mosse, 1980).

Effect of neem pellets on *Phytophthora medicaginis* root rot

An isolate of *Phytophthora medicaginis* was obtained from Tamworth Agricultural Institute. The isolate was reactivated on V8 juice agar prepared according to Stack and Millar (1985). 200 ml Campbell's V-8 juice, 3 g of $CaCO_3$, 15 g of agar and 800 ml of distilled water were autoclaved and poured in Petri-plates. Plates were incubated at 20°C in the dark.

In order to produce sporangia which produce zoospores of *P.medicaginis*, a V8 broth was prepared as above without agar, then poured in Petri-plates (10 ml/plate). Agar plugs (6 mm diameter from V8-agar) were removed from a young colony with hyphal growth and 5 plugs were placed in each Petri-plate. Plates were incubated at 20°C with continuous fluorescent light (Jeffers & Aldwinckle, 1988). A hyphal mat was formed and floated on the surface of the broth after one week incubation. All fungal mats were collected then blended in the blender to make a suspension which had sporangia and zoospores. The suspension served as inoculation solution and used directly after preparation.

Pots trials were set up in the glasshouse with 500 g soil in each 12 cm diameter pot. Neem pellets at rates 0.5%, 0.25%, and 0.1% were applied to the soil 5 days before planting seeds of chickpea cv. Genesis 090. *P. medicaginis* inoculation suspension was injected into the soil one day before planting in a proportion of 2 ml per 100 g soil at 3cm depth. The plants were grown in the glasshouse for 60 days then harvested to measure height and shoot dry weight as in previous experiments.

Statistical analysis

Data were analysed by ANOVA in SPSS version 22. Tukeys HSD was used for mean separation between treatments in the field trial. For experiments with only two treatments, t-tests were done in Microsoft Excel. The criterion for significance was $P < 0.05$.

Results

Field trial

Shoot dry weight

There was no significant effect of disease on shoot dry weight at the first three sampling times (60-90 days). However, at 105 days the shoot dry weight in the pathogen only treatment had declined as disease symptoms became apparent and leaf drop occurred (Figure 5-2). There was no further increase in shoot dry weight in the pathogen only treatment at 120 days and most of the plant canopy was dead (Figure 5.3).

Shoot dry weight of the plants treated with neem powder was not significantly different from the controls at the first three sample dates (60-90 days). Shoot dry weight in the neem powder treatment was significantly greater than the controls at 105 and 120 days (Figure 5-2). Shoot dry weight of plants treated with neem pellets was significantly higher than the control and pathogen only treatment at 60 and 75 days, and greater than all other treatments at 90-120 days (Figure 5-2). Plants treated with neem pellets had much larger, denser crowns than the other treatments (Figure 5-3). and were more than twice as large as control plants at 120 days.

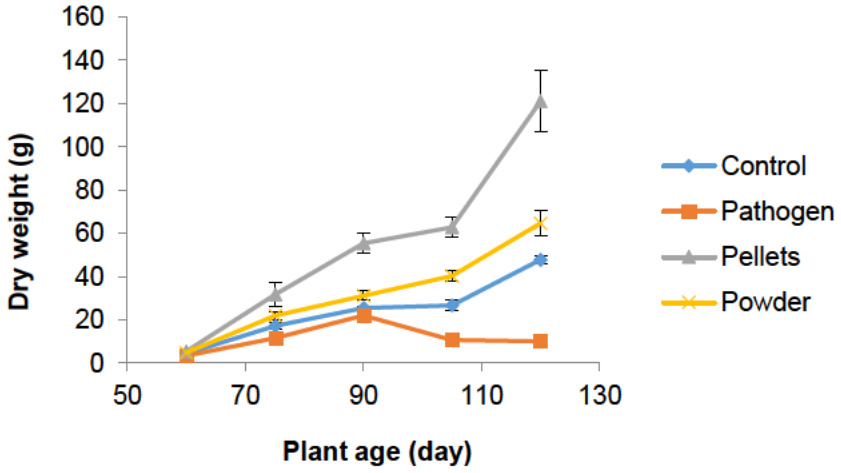

Figure 5-2. Effect of neem (pellets, powder) and *M. phaseolina* on shoot dry weight of chickpea plants. Error bars show standard errors.

Figure 5-3. Chickpea plants from field trial at final sampling (120 days), inoculated with *M. phaseolina* only (left) or inoculated with *M. phaseolina* and treated with neem pellets (right).

Nodule number

In the pathogen only treatment there was a reduction in the number of nodules per plant compared with the control (Figure 5-3). The number of nodules per plant increased greatly in the neem treatments between 60 and 75 days. There was very highly significant effect of neem pellets and powder to increase the number of rhizobium nodules compared with the pathogen treatment. Neem pellets gave a much higher number of nodules than all of the other treatments (Figure 5-3 and 5-4).

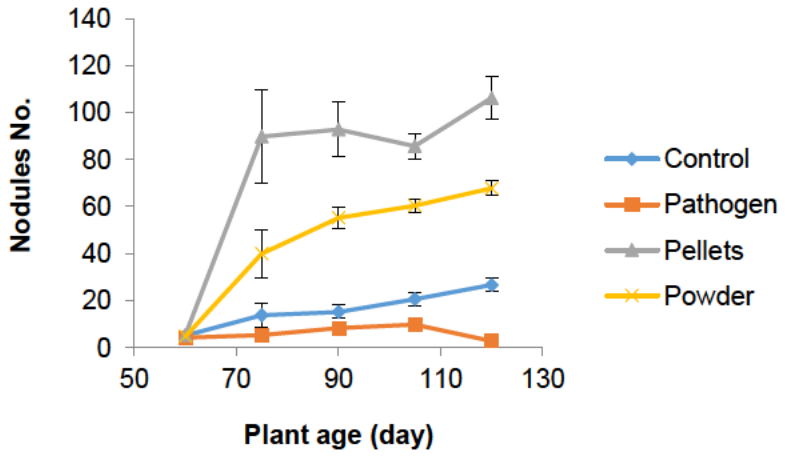

Figure 5-3. Effect of neem (pellets, powder) and *M. phaseolina* on number of nodules of chickpea plants. Error bars show standard errors.

Figure 5-4. Rhizobium nodules of 105 day old chickpea plants inoculated with *M. phaseolina* and treated with neem pellets (left) or untreated (right).

Nodule dry weight

There was a highly significant reduction in nodule dry weight in the pathogen only treatment compared with the control (Figure 5-5). On the other hand, there was a very highly significant effect of neem pellets and powder to increase the nodule dry weight compared with the pathogen only treatment and the control. There was a much higher dry weight of the nodules in the pellet treatment compared with all other treatments (Figure 5-5). Individual nodules in the pellet treatment were large and highly branched (Figure 5-6).

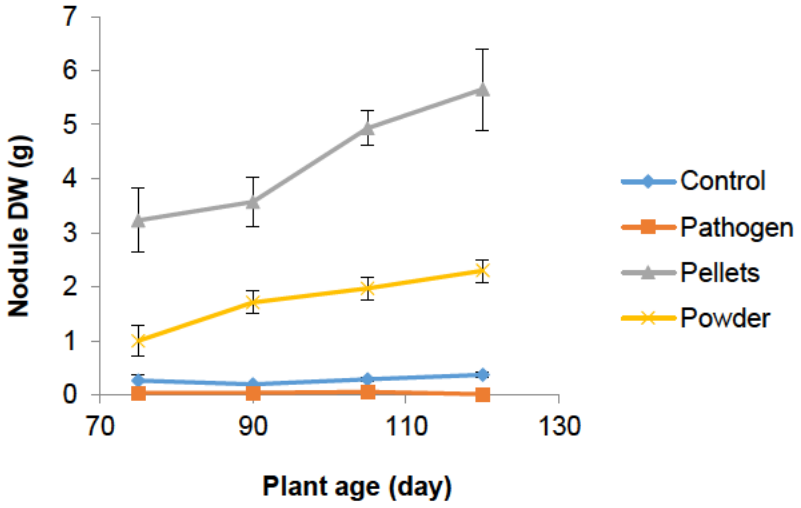

Figure 5-5. Effect of neem (pellets, powder) and *M. phaseolina* on dry weight of nodules of chickpea plants. Error bars show standard errors.

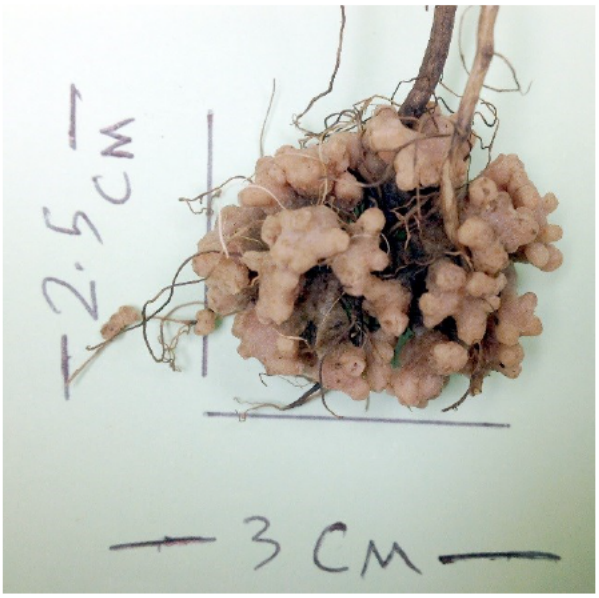

Figure 5-6. Large indeterminate rhizobium nodules on 120 day old chickpea plant inoculated with *M. phaseolina* and treated with neem pellets.

Mycorrhiza colonization

There was no significant effect of using neem pellets and powder on the mycorrhiza colonization proportion at 60 days (Figure 5-7).

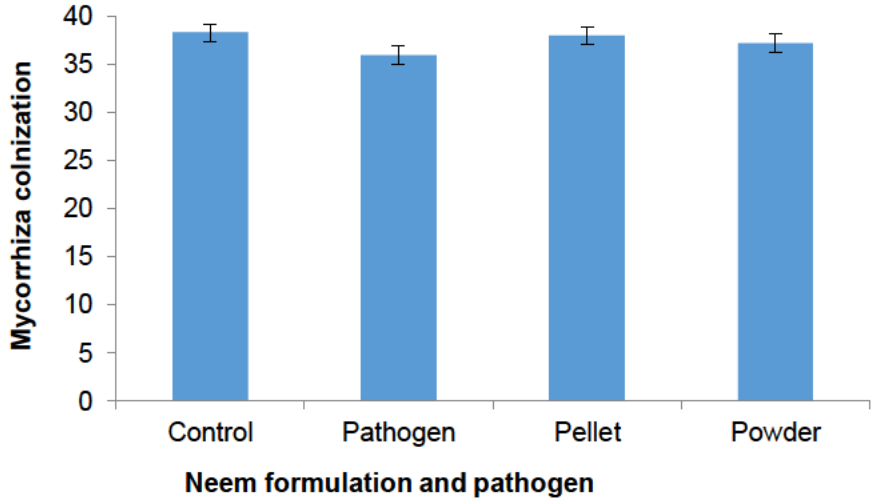

Figure 5-7. Effect of neem (pellets, powder) and *M. phaseolina* on percent length of chickpea roots colonized with *mycorrhiza* at 60 days. Error bars show standard errors.

Microbial abundance measurements in the field

In the soil microbe community assay at 75 days, there was a significant increase in fungal colonies in the pathogen only treatment compared with the control (Figure 5-8). There was a significant reduction in the number of fungal colonies in soil dilution 10^{-6} when using neem pellets compared with the pathogen treatment. The number of colonies in the pellet treatment was about half of that in the control which was a significant difference. However, there was no significant difference when using neem powder compared with the control treatment (Figure 5-8).

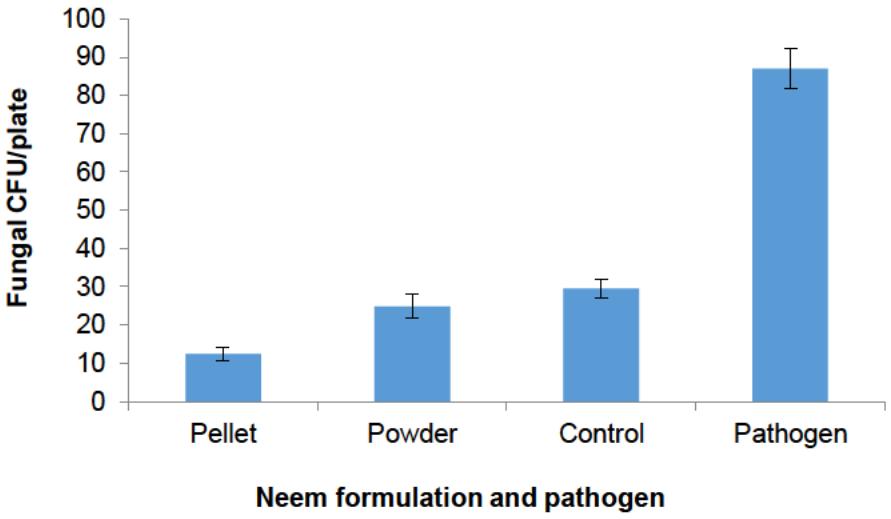

Figure 5-8. Effect of neem pellets, powder and the pathogen on fungal colonization at soil dilution 10^{-6}. Error bars show standard errors.

On the other hand, there was no significant difference in bacterial colony number at 75 days when using neem pellets and neem powder compared with controls or pathogen-only treatments in soil dilution assay at 10^{-7} dilution (Figure 5-9).

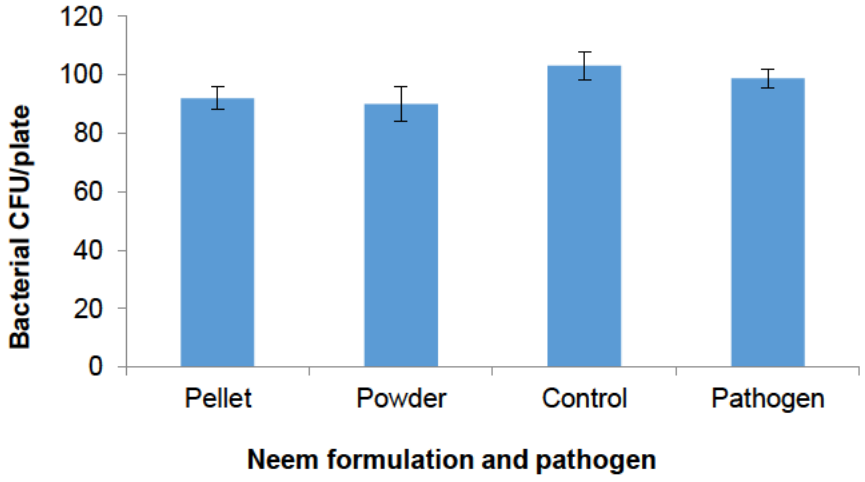

Figure 5-9. Effect of neem pellets, powder and the pathogen on bacterial colonization at soil dilution 10^{-7}. Error bars show standard errors.

The results of the fungi community assay at 120 days showed there was a significant reduction in the number of fungi colonies at the soil dilution 10^{-6} in the powder treatment compared with the pathogen treatment (Figure 5-10). However, none of the treatments were significantly different from the control.

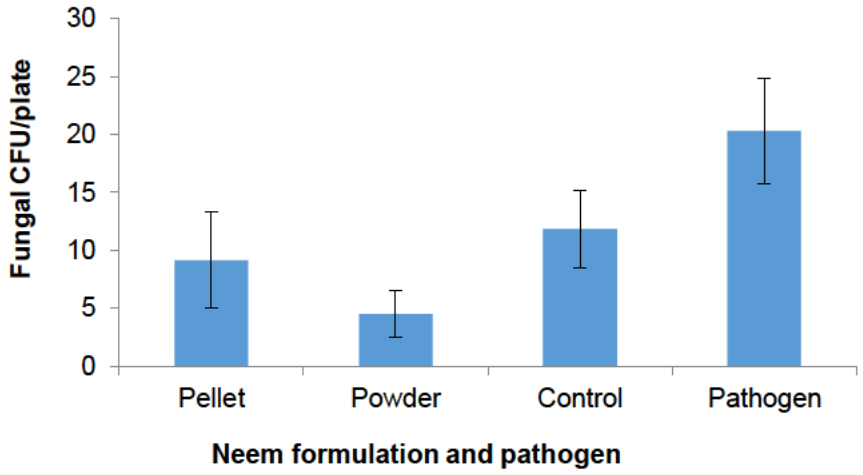

Figure 5-10. Effect of neem pellets, powder and the pathogen on fungal colonization at soil dilution 10^{-6}. Error bars show standard errors.

There was no significant difference between treatments in bacterial colonies at soil dilution 10^{-7} at 120 days (Figure 5-11).

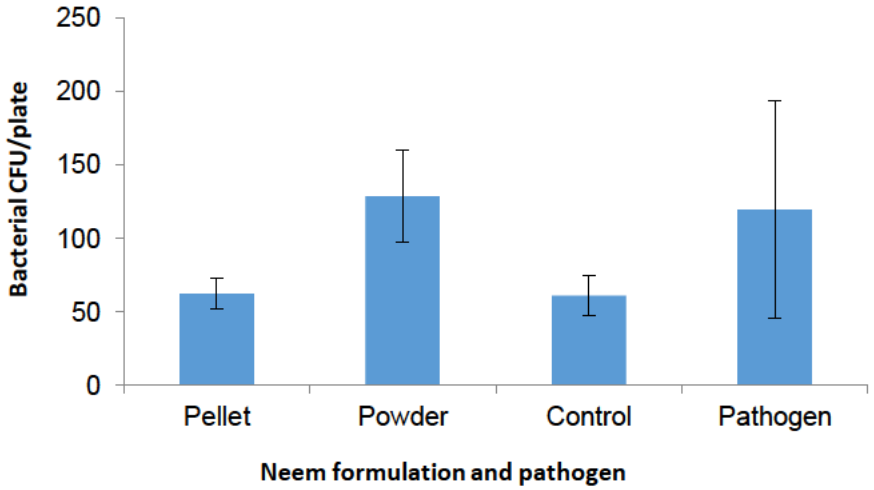

Figure 5-11. Effect of neem pellets, powder and the pathogen on bacterial colonization at soil dilution 10^{-7}. Error bars show standard errors.

Effect of neem on microbial abundance in vitro

The in vitro soil assay showed that fungal populations were 1.7 x 10^7 CFU/g in the untreated soil and 4.5 x 10^6 CFU/g in the soil treated with neem powder which is 74 % reduction. The difference was significant at $P < 0.001$. However, the population of bacteria was similar in both treatments, approximately 7 x 10^7 CFU/g, and there was no significant effect of neem on bacterial counts.

Effect of neem leaf extract on growth of soil fungi

There were various effect of neem extract on growth of the soil fungi. There was between 60 – 70% reduction in the growth of *Mucor genevensis, Rhizoctonia solani, Macrophomina phaseolina, Trichoderma harzianum,* and *Sclerotium rolfsii* compared with their controls (Figure 5-12). The growth inhibition of *Sordaria fimicola* and *Pythium irregulare* was about 30% compared with their controls (Figure 5-12). However, the least inhibition of growth was found with *Fusarium oxysporum, Phytophthora medicaginis*, and *Verticillium dahliae* which was between 5 – 20% compared with their controls (Figure 5-12).

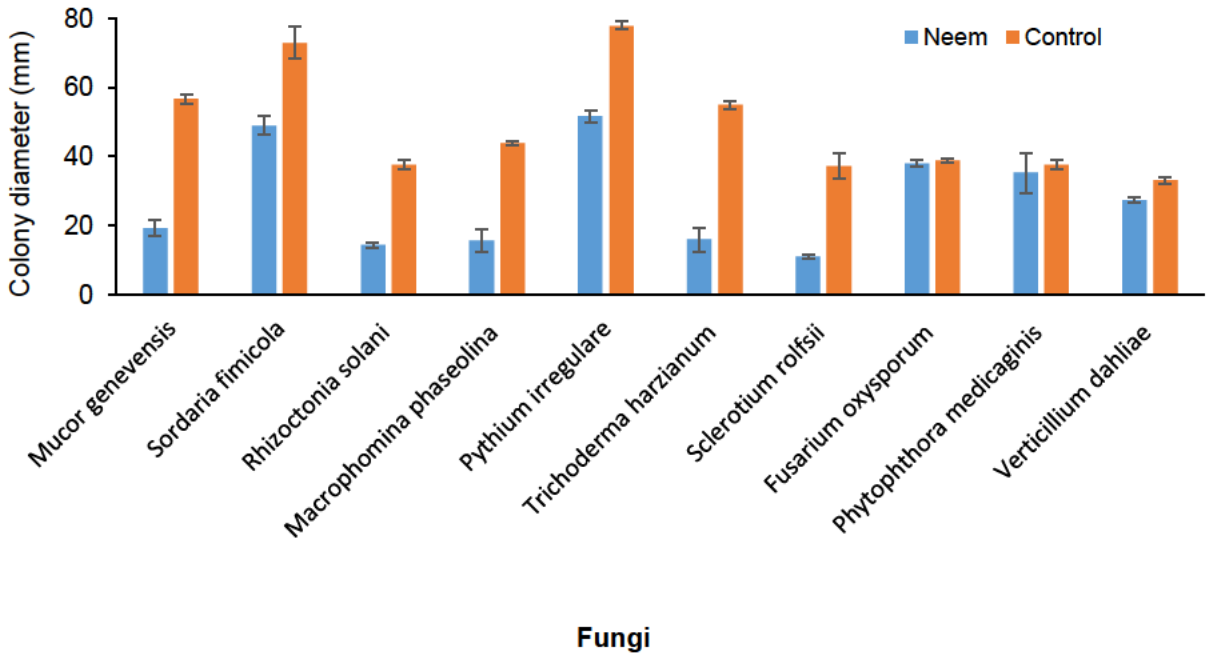

Figure 5-12. Colony diameter of soil fungi on media with and without neem extract. Error bars show standard errors.

Effect of neem on root lesions

There was no significant effect of using neem pellets on the percentage of root length with lesions in a pot trial. The proportion of root lesions with neem treatment was 38.9%, while in the control treatment it was 40.5%.

Effect of neem pellets on *Phytophthora medicaginis* root rot

Inoculation with *Phytophthora medicaginis* reduced the height of chickpea plants by 21% and the dry weight by 45% (Figures 5-13 and 5-14). There was no significant effect of using neem pellets in rates 0.5%, 0.25%, and 0.1% on the height and shoot

dry weight of the plants compared with the control plants inoculated with *P. medicaginis* (Figure 5-13 and 5-14).

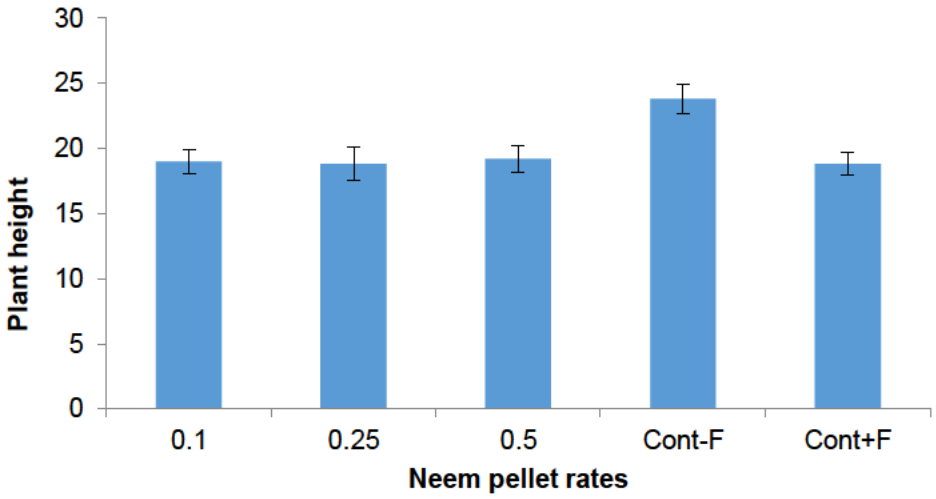

Figure 5-13. Effect of different rates of neem pellets on the plant height with and without *Phytophthora. medicaginis*. Error bars show standard errors.

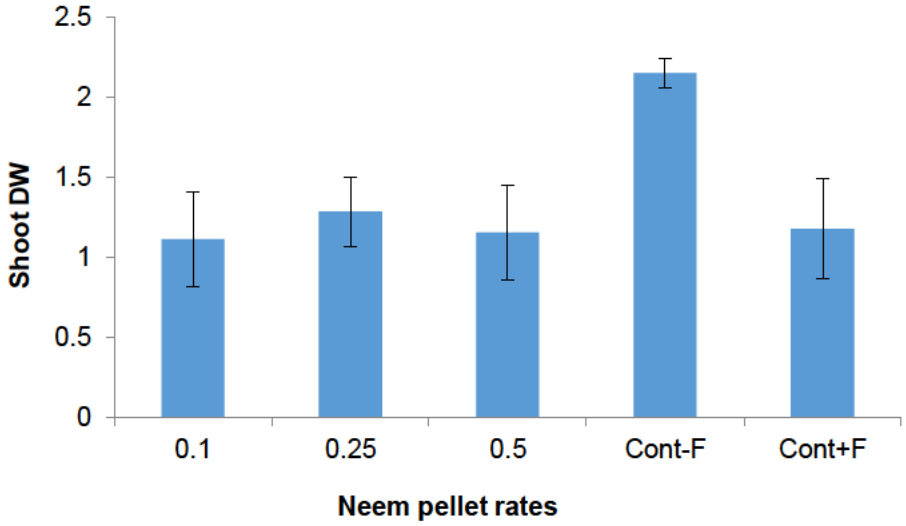

Figure 5-14. Effect of different rates of neem pellets on shoot dry weight of the plant with and without *Phytophthora. medicaginis*. Error bars show standard errors.

Discussion

In this chapter a field experiment was conducted to examine the effect of neem extract on charcoal rot under field conditions. The treatment pathogen only had less plant dry weight compared with the other treatments. The effect became obvious at 90 days (late summer) when temperatures were high and the plants were in reproductive stage. The timing and symptoms that were seen were typical of those reported for charcoal rot in chickpea in field crops (M. Sharma et al., 2015). The reduction in growth was an expected result because when symptoms appear on the inoculated plants, plant growth will be affected because the main supplier of water and mineral nutrients to the plant (the root system) is affected. In this case plants began to lose leaves and branches died.

Disease symptoms were not seen in the neem treatments. Neem powder treatments had a higher dry weight than the control. On the other hand, plant dry weight was double that of the control in the neem pellet treatment. A clear growth promotion had occurred when using the neem products. That promotion was slight in case of neem powder and it was very high in case of neem pellets. The greater effect of the pellets was probably due to their being slower to release the active ingredients and to degrade than the leaf powder.

One possible reason for the growth promotion effect of neem was inhibition of pathogens that were present in the soil, besides the *M. phaseolina*. The effect of neem in the number of root lesions on plants grown in untreated soil was examined in a glasshouse experiment. The results of that experiment showed that there was no effect of neem extract on the number of root lesions. This suggests that the effect of neem on other pathogens was not responsible for growth promotion.

Roots of young plants (60 days) were stained to estimate the colonization of mycorrhiza. Under field conditions, neem extract did not affect the proportion of root length colonized by mycorrhiza. This confirmed that neem does not appear to have a harmful effect on mycorrhiza, but also suggests that growth promotion was not due to interactions with mycorrhiza.

Neem pellets more than doubled the number of rhizobium nodules on each plant and greatly increased their size. It is possible that an increase in nitrogen fixation could have led to an increase in plant growth rates. This could explain why plants treated with the neem pellets had such profuse growth. The effect of neem on rhizobium has not been fully tested before, so there is little known about this phenomenon. Aeron et al. (2017) showed that inoculation with selected strains of rhizobium greatly increased the growth of plants of the legume *Mucuna pruriens* that were infected with *M. phaseolina,* so interactions between neem, pathogen and rhizobium that enhance plant growth are possible. However, the results of this test disagreed with the results of an earlier study by Sarawaneeyaruk et al. (2015) who found that neem extract reduced the number of nodules of mung bean, this may have been because their neem treatments were phytotoxic, indirectly affecting nodulation. The effect of neem on rhizobium nodulation, and the consequences for plant growth, need further study.

The effect of neem extract on the soil microbes in the rhizosphere of plants was estimated. Neem pellets reduced the number of fungal colony forming units in soil. However, bacterial colony number was not affected by neem pellets. The effect of neem pellets on the soil microbes was much stronger at the first soil assay than the last one. The reason was because of the effect of neem wearing off due to degradation.

Soil dilution was used to measure effects of neem on the soil microbiota**.** The study revealed there was a reduction of soil fungi population when the soil was treated with neem. However, there was no different in bacterial population. This has limitations because not all fungi or bacteria can be detected, and the number of colonies may not represent the true biomass or activity. Molecular methods of community analysis like high-throughput sequencing may give a better measure of effects on soil biota. Also, measures of soil respiration could give an estimate of the effect of neem on microbial activity. This is something that should be used in any future work.

To clarify the effect of neem extract on soil fungi, ten soil fungi species were chosen in a poison food test. The results showed there were a difference in that effect based

on the fungus species. Most species were sensitive to neem, which would explain why soil fungal populations would be reduced by treating with neem powder or pellets.

After confirming the effect of neem pellets on *M. phaseolina* the causal agent of charcoal rot, an experiment was conducted in the glasshouse to evaluate the effect of neem pellets on *Phytophthora medicaginis* which causes a major root rot disease in chickpea. The result of the experiment showed that there was no effect of neem pellets on Phytophthora root rot, which was consistent with the low inhibition of growth of *P. medicaginis* by leaf extract in the poisoned food test.

Neem had a large effect on growth of *Rhizoctonia solani* and *Sclerotium rolfsii*, so potentially it is useful against these pathogens. However, neem had little effect on growth of *Fusarium oxysporum* or *Verticillium dahliae*. As with *P. medicaginis*, neem may be expected to have little effect on diseases caused by these fungi. So neem can work against some pathogens, but not all. The study of U. Singh et al. (1980) found that neem aqueous extract completely inhibited the growth of *R. solani* and *S. rolfsii,* which agreed with results of the present study. However, they found that *F. oxysporum* was sensitive to high concentration (15000 ppm - 25000 ppm) of neem aqueous extract. This disagreed with result of the present study about the lack of sensitivity of *F. oxysporum* to neem aqueous extract. Later, Govindachari et al. (1999) found that neem leaf hexane extract completely inhibited growth of *F. oxysporum*. Therefore, strains of *F. oxysporum* may differ in sensitivity to neem extracts. There is no information of using neem extract before the present study against *V. dahliae* or *P. medicaginis*.

This the first time that neem has been tested against charcoal rot under field conditions, and clearly showed that neem treatment protected the plants against disease. Pellets gave an additional growth promotion effect and promoted nodulation.

Chapter 6: General discussion

The present study was conducted to find a reliable plant extract to control charcoal rot of chickpea plants caused by *M. phaseolina.* After reviewing many research papers and studies of previous people who worked in the same field of study, the neem plant with its different parts was chosen to use its extract to control the disease. The review also found that neem leaf extract and neem seed oil were the most common extracts that were tested to control many fungi diseases. Neem contains a wide variety of chemical compounds such as azadirachtin, azadirachtol, nimbocinol, nimolicinol, nimlinone, isolimolicinolide, nimocin, nimbocinone, etc. (Tewari, 1992). Azadirachtin was reported by Dubey and Kumar (2003) to have a fungicidal effect as good as the fungicides mancozeb and bavistin.

Initially, both leaf extract and seed oil were assessed and evaluated for their efficacy. The in vitro experiments examined the sensitivity of two strains of *M. phaseolina* to neem seed oil and leaf aqueous extract. The promotion of biomass yield of both fungus strains by neem oil was unexpected. That promotion increased with increase of the concentration of neem oil. The results were quite different from those of Dubey et al. (2009) who studied the effect of neem extract on *M. phaseolina*. There have not been many studies that examined the effect of neem oil on the biomass of *M. phaseolina* in liquid culture. Therefore, the explanation of the results of the present study is still uncertain. Although, neem oil in the present study was sterilized by filtration it seems that the fungus used neem oil as a substrate. Dubey et al. (2009) showed that autoclaving neem oil reduced its effect on *M. phaseolina*. However the oil used in this study was said by the manufacturer to be cold pressed, so was unlikely to have suffered from denaturation by heat. On the other hand, there was an inhibitory effect of leaf aqueous extract of neem on the mycelium yield (dry matter) of *M. phaseolina.* This is the same as what Dubey et al. (2009) found about the toxicity of leaf extract on the biomass of the fungus. These results are also supported by earlier work by Ashraf and Javaid (2007) who found inhibitory effect of neem aqueous extract on the mycelial biomass growth of *M. phaseolina.*

In the radial growth assay, neem leaf extract was also found to be toxic to *M. phaseolina* by reducing the growth of the fungus significantly. The results agreed with studies that reported the inhibition effect of leaf extract on the radial growth of *M. phaseolina* such as Dubey and Kumar (2003), Dhingani et al. (2013); (Dubey et al., 2009) and Meena et al. (2014).

The present study found that root exudates of both chickpea cultivars desi and kabuli increased the mycelium growth of *M. phaseolina*, but there were no differences between the varieties even though kabuli was more susceptible to the disease. Z. I. El-Gali (2015) found a stimulation effect of root exudates of the susceptible bean cultivar Libyan, but not the more resistant cultivar Giza-6, to mycelial growth of *M. phaseolina.* It was hypothesized that a similar result may be found in chickpea, but because it was not, the interactions of root exudates with control by neem were not examined further.

The first pot trial in the present study revealed the ability of neem extract to suppress the development of charcoal rot disease on both varieties of chickpea. This is the first time that neem extract has been shown to control charcoal rot of any plant in a pot experiment.

There were no negative effects of using neem on rhizobium nodulation and the percentage of mycorrhiza colonization of both chickpea varieties. This is the first time to prove that neem extract does not adversely affect these beneficial microbes. The results of this study are consistent with previous work of Gopal et al. (2007) who examined the effect of a neem product called azadirachtin (alcoholic extract of neem seed kernel mixed with China clay) on beneficial microbes. Their study found that neem increased the number of the free living nitrogen fixer *Azotobacter* which had an inhibition effect on the other microbes in the community. The promotion of activity of nitrogen fixers like *Rhizobium* and *Azotobacter* by neem could give a benefit to growth in addition to any effect on pests or diseases, as suggested in the field experiment.

The literature review indicated that there was a strong relationship between the nature of the plant extract (material) and the efficacy on the targeted pathogen in the soil. Therefore, neem leaf powder was modified into different formulations and

applied to the soil. The formulations were crude powder, aqueous extract, capsules, and neem pellets. These four formulations were evaluated for their effect to control charcoal rot in a glasshouse experiment at three rates. Neem pellets at 0.5% application pre-emergence achieved the best results. Neem formulated as a seed coating was phytotoxic. The new finding of this formulation experiment was that neem pellets gave a long lasting effect and the rate of 0.5% was the optimum. Also, the pre-emergence application time was the ideal time to treat the soil of the plant rhizosphere.

This finding was investigated further under field conditions. The results of the field matched the glasshouse results with proportion of growth promotion. Mycorrhiza colonization was detected during field experiments and there was no negative effect of using neem pellets on mycorrhiza. Treatment with neem pellets increased plant growth above that of the control. Part of this was due to suppression of the disease, but it is also possible that the amount of organic matter added could have an effect. This could be due to nutrients in the neem leaf, or to the effects of increased soil carbon on soil structure and soil biology. This is something that could be examined in future experiments.

The number and size of nodules was increased by the neem treatments in the field (powder and pellets) and this was associated with increase in plant growth. Although it was not demonstrated that improved nodulation was responsible for the growth increase, this is a likely explanation. Further work on this could include measuring the rate of nitrogen fixation and uptake into the plant. New findings of the research were that neem increased the number and the dry weight of rhizobium nodules. This the first time to show that neem promotes growth of rhizobium.

Soil was also analysed twice during the field experiment at 75 days and at harvest to evaluate the effect of neem pellets on the microbial community. Neem pellets were found to reduce the number of soil fungal colonies with no effect on the bacterial colony number. This was confirmed in a second experiment in vitro. Gopal et al. (2007) also showed that azidirachtin reduced fungal populations in soil more than bacterial populations. That may explain why the number of rhizobium nodules was not reduced by neem pellets. Neem pellets reduced a broad range of rhizosphere

microbes especially fungi. That could provide a less competitive environment for the beneficial microbes to grow which explained the promotion in number and in dry weight of nodules.

After it was confirmed that neem extract reduced soil fungi colonization, an investigation was conducted to evaluate the effect of neem on a range of soil fungi. Neem affected the growth of soil fungi in different degrees. Neem also did not affect root lesions which may indicate that neem does not affect soil nematodes.

Neem was also examined for control of *Phytophthora medicaginis* root rot in chickpea in a glasshouse experiment. The results showed there was no effect of using neem pellets on the root rot disease causes by *P. medicaginis.* This shows that neem will not control every disease that may be present in crop.

Conclusions

The new formulation of neem (pellets) with optimum rate 0.5% and pre-emergence application time was developed. This may be useful as protective method against charcoal rot disease of chickpea caused by *M. phaseolina*. The way is non-phytotoxic on seeds germination, as well, safe for the beneficial microbes. An important finding of this study is the promotion of number and the weight of nodules when using neem pellets which means high nitrogen fixation process occurs, that resulted in a healthy plant with high vegetation.

Pellets are a simple and cheap technique that can be considered a feasible way to use instead of chemicals. Plus, they have 100% organic compositions which are environment friendly.

Recommendations for future work

Further work is required on the development and use of neem pellets to control charcoal disease of chickpea under commercial conditions. This includes finding the best way of producing neem pellets with least cost. The rate of application of pellets in these experiments was equivalent to approximately 4 t/ha, and further field work is needed to test techniques like in-furrow applications that can reduce the amount of neem material used. Work is also needed on ways to integrate neem leaf production into farming system.

Further work is required to test the effect of neem pellets on charcoal rot disease in different types of soil and on other crops like sunflower. Although neem did not appear to be effective against Phytophthora root rot in chickpea, the in vitro experiments in the literature suggested activity against several other soilborne pathogens like *Sclerotium rolfsi*, *Rhizoctonia Solani* and *Pythium* species. More field experiments should be done to test control of diseases caused by these.

Neem pellets had a very large stimulatory effect on nodulation of chickpea. It is well worth doing more experiments to test neem extract as a growth enhancement agent of rhizobium and to explore the mechanisms for this effect.

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Appendix: ANOVA Tables

Figure 3-9

Dependent Variable: Rhizobium_no

Source	Sum of Squares	df	Mean Square	F	Sig.
Chickpea	325.125	1	325.125	11.816	0.001
Neem	47.531	1	47.531	1.727	0.191
Rhizobium	2112.5	1	2112.5	76.777	0
Repeat	40.5	1	40.5	1.472	0.227
Chickpea * Neem	9.031	1	9.031	0.328	0.568
Chickpea * Rhizobium	105.125	1	105.125	3.821	0.053
Neem * Rhizobium	11.281	1	11.281	0.41	0.523
Chickpea * Neem * Rhizobium	1.531	1	1.531	0.056	0.814
Error	3274.25	119	27.515		
Total	5926.875	127			

Figure 3-10

Dependent Variable: Log_weight

Source	Sum of Squares	df	Mean Square	F	Sig.
Chickpea	0.605	1	0.605	12.515	0.001
Neem	1.531	1	1.531	31.668	0
Rhizobium	6.006	1	6.006	124.249	0
Repeat	0.002	1	0.002	0.032	0.858
Chickpea * Neem	0.222	1	0.222	4.585	0.034
Chickpea * Rhizobium	0.175	1	0.175	3.624	0.059
Neem * Rhizobium	0.071	1	0.071	1.475	0.227
Chickpea * Neem * Rhizobium	0.013	1	0.013	0.263	0.609
Error	5.752	119	0.048		
Total	14.376	127			

Figure 3-11

Dependent Variable: Shoot

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Sterile	0.355	1	0.355	14.695	0
Variety	2.913	1	2.913	120.674	0
Fungus	2.479	1	2.479	102.696	0
Neem	0.301	1	0.301	12.451	0.001
Sterile * Variety	0.157	1	0.157	6.509	0.014
Sterile * Fungus	0.081	1	0.081	3.334	0.074
Sterile * Neem	8.17E-06	1	8.17E-06	0	0.985
Variety * Fungus	0.115	1	0.115	4.744	0.035
Variety * Neem	4.82E-05	1	4.82E-05	0.002	0.965
Fungus * Neem	1.124	1	1.124	46.558	0
Sterile * Variety * Fungus	0.001	1	0.001	0.041	0.841
Sterile * Variety * Neem	0.002	1	0.002	0.062	0.804
Sterile * Fungus * Neem	0.057	1	0.057	2.379	0.13
Variety * Fungus * Neem	0.178	1	0.178	7.366	0.009
Sterile * Variety * Fungus * Neem	0.008	1	0.008	0.343	0.561
Error	1.111	46	0.024		
Corrected Total	8.627	61			

Figure 3-12

Dependent Variable: Root

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Sterile	0.062	1	0.062	10.615	0.002
Variety	0.195	1	0.195	33.568	0
Fungus	0.362	1	0.362	62.098	0
Neem	0.062	1	0.062	10.615	0.002
Sterile * Variety	0.001	1	0.001	0.217	0.644
Sterile * Fungus	0.034	1	0.034	5.873	0.019
Sterile * Neem	0.001	1	0.001	0.093	0.762
Variety * Fungus	0.001	1	0.001	0.093	0.762
Variety * Neem	0.003	1	0.003	0.569	0.455
Fungus * Neem	0.297	1	0.297	51.008	0
Sterile * Variety * Fungus	0.006	1	0.006	1.022	0.317
Sterile * Variety * Neem	0.001	1	0.001	0.136	0.714
Sterile * Fungus * Neem	0.027	1	0.027	4.556	0.038
Variety * Fungus * Neem	0.011	1	0.011	1.95	0.169
Sterile * Variety * Fungus * Neem	0.011	1	0.011	1.95	0.169
Error	0.268	46	0.006		
Corrected Total	1.275	61			

Figure 4-5

Dependent Variable: Height

Source	Sum of Squares	df	Mean Square	F	Sig.
Formulation	84.111	3	28.037	2.8	0.05
Rate	91.75	2	45.875	4.581	0.015
Time	156.056	1	156.056	15.584	0
Formulation * Rate	27.472	6	4.579	0.457	0.836
Formulation * Time	65.611	3	21.87	2.184	0.102
Rate * Time	39.528	2	19.764	1.974	0.15
Formulation * Rate * Time	50.806	6	8.468	0.846	0.541
Error	480.667	48	10.014		
Total	996	71			

Figure 4-8

Dependent Variable: Leaves

Source	Sum of Squares	df	Mean Square	F	Sig.
Formulation	693.486	3	231.162	4.738	0.006
Rate	210.583	2	105.292	2.158	0.127
Time	276.125	1	276.125	5.659	0.021
Formulation * Rate	499.306	6	83.218	1.706	0.14
Formulation * Time	1024.153	3	341.384	6.997	0.001
Rate * Time	117.75	2	58.875	1.207	0.308
Formulation * Rate * Time	143.472	6	23.912	0.49	0.813
Error	2342	48	48.792		
Total	5306.875	71			

Figure 4-9

Dependent Variable: Leaflets

Source	Sum of Squares	df	Mean Square	F	Sig.
Formulation	30752.333	3	10250.778	4.351	0.009
Rate	34009.028	2	17004.514	7.217	0.002
Time	26912	1	26912	11.423	0.001
Formulation * Rate	11037.75	6	1839.625	0.781	0.589
Formulation * Time	49298.111	3	16432.704	6.975	0.001
Rate * Time	3227.25	2	1613.625	0.685	0.509
Formulation * Rate * Time	6877.972	6	1146.329	0.487	0.815
Error	113090	48	2356.042		
Total	275204.444	71			

Figure 4-11

Dependent Variable: ShootDW

Source	Sum of Squares	df	Mean Square	F	Sig.	
Formulation	570.09	3	190.03	4.408	0.008	
Rate	12.122	2	6.061	0.141	0.869	
Time	266.536	1	266.536		6.183	0.016
Formulation * Rate	259.063	6	43.177		1.002	0.435
Formulation * Time	105.535	3	35.178		0.816	0.491
Rate * Time	55.57	2	27.785		0.645	0.529
Formulation * Rate * Time	117.268	6	19.545		0.453	0.839
Error	2069.299	48	43.11			
Total	3455.482	71				

Figure 4-13

Dependent Variable: Seeds

Source	Sum of Squares	df	Mean Square	F	Sig.
Formulation	563.167	3	187.722	2.875	0.046
Rate	201.333	2	100.667	1.541	0.224
Time	46.722	1	46.722	0.715	0.402
Formulation * Rate	487.667	6	81.278	1.245	0.301
Formulation * Time	109.611	3	36.537	0.559	0.644
Rate * Time	274.111	2	137.056	2.099	0.134
Formulation * Rate * Time	66.222	6	11.037	0.169	0.984
Error	3134.667	48	65.306		
Total	4883.5	71			

Figure 4-14

Dependent Variable: SeedWt

Source	Sum of Squares	df	Mean Square	F	Sig.
Formulation	30.688	3	10.229	2.594	0.063
Rate	5.613	2	2.806	0.712	0.496
Time	2.703	1	2.703	0.685	0.412
Formulation * Rate	47.219	6	7.87	1.995	0.085
Formulation * Time	0.522	3	0.174	0.044	0.988
Rate * Time	30.017	2	15.008	3.805	0.029
Formulation * Rate * Time	4.18	6	0.697	0.177	0.982
Error	189.312	48	3.944		
Total	310.254	71			

Figure 4-15

Height

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*

Treatment	322.8	4	80.7	6.179173	0.002091
Error	261.2	20	13.06		
Total	584	24			

Figure 4-16

Dry weight

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment	12.081	4	3.02025	12.16029	3.57E-05
Error	4.9674	20	0.24837		
Total	17.0484	24			

Figure 5-2

Dependent Variable: Dryweight 60 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	19.819	3	6.606	12.718	0
Error	16.622	32	0.519		
Total	36.441	35			

Dependent Variable: Dryweight 75 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	2005.622	3	668.541	7.396	0.001
Error	2892.63	32	90.395		
Total	4898.252	35			

Dependent Variable: Dryweight 90 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	6198.248	3	2066.083	24.887	0
Error	2656.57	32	83.018		
Total	8854.818	35			

Dependent Variable: Dryweight 105 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	13253.344	3	4417.781	59.687	0
Error	2368.5	32	74.016		
Total	15621.844	35			

Dependent Variable: Dryweight 120 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	57490.593	3	19163.531	34.795	0
Error	17624.416	32	550.763		
Total	75115.01	35			

Figure 5-3

Dependent Variable: Nodules 60 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	6.889	3	2.296	1.378	0.267
Error	53.333	32	1.667		
Total	60.222	35			

Dependent Variable: Nodules 75 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	39058	3	13019.333	10.796	0
Error	38590.222	32	1205.944		
Total	77648.222	35			

Dependent Variable: Nodules 90 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	41280.556	3	13760.185	37.113	0
Error	11864.444	32	370.764		
Total	53145	35			

Dependent Variable: Nodules 105 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	33516.889	3	11172.296	95.728	0
Error	3734.667	32	116.708		
Total	37251.556	35			

Dependent Variable: Nodules 120 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	56235.639	3	18745.213	84.169	0
Error	7126.667	32	222.708		
Total	63362.306	35			

Figure 5-5

Dependent Variable: NoduleDW 75 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	57.388	3	19.129	19.327	0
Error	31.673	32	0.99		
Total	89.061	35			

Dependent Variable: Nodule DW 90 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	73.159	3	24.386	41.51	0
Error	18.799	32	0.587		
Total	91.958	35			

Dependent Variable: Nodule DW 105 days

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	137.255	3	45.752	137.484	0
Error	10.649	32	0.333		
Total	147.903	35			

Dependent Variable: Nodule DW 120 days

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	180.493	3	60.164	43.491	0
Error	44.268	32	1.383		
Total	224.761	35			

Figure 5-7

Dependent Variable: Mycorrhiza

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	29.651	3	9.884	1.284	0.297
Error	246.351	32	7.698		
Total	276.002	35			

Figure 5-8

Dependent Variable: Fungus6

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	19776.125	3	6592.042	94.725	0
Error	1391.833	20	69.592		
Total	21167.958	23			

Figure 5-9

Dependent Variable: Bacteria7

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	648.5	3	216.167	1.73	0.193
Error	2499.333	20	124.967		
Total	3147.833	23			

Figure 5-10

Dependent Variable: Fungus6

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	795.458	3	265.153	3.322	0.041
Error	1596.5	20	79.825		
Total	2391.958	23			

Figure 5-11

Dependent Variable: Bacteria7

Source	Sum of Squares	df	Mean Square	F	Sig.
Treatment	23749.792	3	7916.597	0.78	0.519
Error	203050.833	20	10152.542		
Total	226800.625	23			

Figure 5-13

Height

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment	94.64	4	23.66	4.255396	0.011851
Error	111.2	20	5.56		
Total	205.84	24			

Figure 5-14

Dry weight

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Treatment	3.828616	4	0.957154	2.882733	0.049095
Error	6.6406	20	0.33203		
Total	10.46922	24			