EFFECTIVENESS OF NEEM (*Azadirachta indica*) EXTRACTS FOR THE CONTROL OF *Fusarium oxysporum* IN PINES (*Pinus patula*)

BY

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DECLARATIONS

Declaration by the student

This research project is my original work and has not been presented for a degree in any other University. No part of this project report may be reproduced without the prior permission of the author and /or Moi University.

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Declaration by the supervisor

This project report has been submitted for examination with my approval as the University Supervisor

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DEDICATIONS

This work is dedicated to my dear husband, Mr. J.O Owira and children; Francis, Christine and Kevin.

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ABSTRACT

Fusarium wilt often leads to damping off and is an important disease of pine (*Pinus patula*) in the nursery. The use of chemicals as a control method is becoming less appealing because of the health implications as well as the high costs, which are often not within the reach of farmers in most of the developing world. Thus, neccesitates the evaluation of non-chemical methods of controlling the *Fusarium*.

This study was conducted to evaluate effectiveness of neem (*Azadirachta indica*) extracts for the control of *Fusarium oxysporum* in pines (*Pinus patula*). The specific objectives were to determine: (i) efficacy of *A. indica* extracts in controlling *F. oxysporum* growth (ii) effects of *A. indica* extracted from the leaves and bark on the germination of *P. patula* seed infested with *F. oxysporum* and (iii) effects of *A. indica* against *F. oxysporum* infesting the *P. patula* seedlings. Data were collected from KEFRI pathology laboratory between August and September 2006. *A. indica* was extracted from bark and leaves of neem tree using 99% ethanol followed by filtration while the seeds of *P. patula* were obtained from KEFRI, Muguga. Bioassay was done to test efficacy of *A. indica* extract on the growth of *F. oxysporum*. Seeds and seedlings of *A. indica* were first exposed to *F. oxysporum* followed by *A. indica* extracts applied at a concentration of 25%, 50%, 75% and 100% respectvely to test for germination of seeds and performance of seedlings.

Data collected were subjected to analysis of variance (One-Way ANOVA) and Tukeys's Post-Hoc comparisons. The results indicated that extracts of *A. indica* from the leaves were effective against *F. oxysporum* in concentrations above 25%. At that concentration (25%), *A. indica* extracts from the bark were more effective ($t = 118.3342$, $df = 1$, $P <$

0.001) against *F. oxysporum* as compared to leaves extracts. There was no significant differences ($P > 0.05$) in growth of *F. oxysporum* between the bark and the leaves at concentrations of 75% and 100%, of *A. indica*, resulted. There were significant differences in the germination percentages of *P. patula* ($P < 0.05$) among the leaf and bark extract treatments. Pre-emergence germination percentage was highest in the seeds treated with 25% *A. indica* extracted from leaves ($68.3 \pm 15.4\%$). Post-emergence germination of *P. patula* under 25% of the *A. indica* extract were highest both in the leaves and bark extracts. Death of *P. patula* seedling was significantly higher ($P < 0.05$) in untreated controls than all the other treatments. Among treatments, death of seedlings was lowest in 75% *A. indica* extracted from leaves and 100% *A. indica* extracted from the bark.

These results clearly demonstrate that *A. indica* extracts at suitable concentrations are apt to control *F. oxysporum* thus is appropriate in managing damping off disease of *P. patula*. Thus, it is recommended that farmers should use the *A. indica* extract as a suitable non-chemical control methods against damping off disease in pines subject to further research to streamline the methods of application of the chemical by farmers.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

Local communities are directly dependent on trees for their livelihood subsistence and income. As a result, many of third world rural households generate some of their income from selling timber and non-timber forest products (NTFPs). As pressure on the agricultural land base increase, leading to progressive fragmentation of farm holdings and overuse of arable land, the ability of farm households to achieve self-sufficiency from their land has been waning considerably (Fisseha 2007). Rural populations are increasingly becoming reliant on off farm and non-farm income in order to meet their food and other needs. Forest product activities have repeatedly been found to provide one of the main sources of non-farm income to rural households (Arnold and Dewees, 1995; Mead, 2003; Fisseha 2007). Marketable forest products provide the opportunity to supplement household income, as well as providing a relief source in times of seasonal and emergency food and cash shortages. Unfortunately, these forest products are under threats from several problems such as diseases that threaten to decimate their survival.

Fusarium wilts, caused by *Fusarium oxysporum,* is one of the most widespread and destructive diseases of many major forest trees seedlings causing damping off disease. This soil borne fungus causes vascular wilts by infecting plants through the roots and growing internally through the cortex to the stele (Tjamos and Beckman, 1998; Bruce *et al*., 2004; Becham, 2006). Initial symptoms appear as chlorosis and distortion of the lower leaves, often on one side of the plant. Foliar chlorosis, necrosis, and plant stunting become more pronounced as the disease progresses. Wilting occurs on the affected side of the plant, followed by vascular discoloration and stem necrosis. The entire plant wilts and dies as the pathogen moves into the stem.

Currently, preplant soil fumigation and fungicide applications are used to control wilts and other diseases caused by soilborne pathogens on high-value crops. However, the major fumigant used, methyl bromide, has been defined by the Montreal Protocol of 2001 as a

chemical that contributes to the depletion of the ozone layer. The U.S. Clean Air Act (Section 602) requires that production and importation of any substance defined as an ozone depleter by the Montreal Protocol be phased out. The Food Quality Protection Act of 1996 may further limit growers' options due to the requirement that all pesticides be reregistered with lower residue tolerances, which may result in label restrictions and pesticide losses. This has therefore necessitated the search for alternative means of treatment of the infestation by the *Fusarium*.

The long-term goal in the current research is to develop and evaluate new or existing alternative control methods for soilborne plant pathogens to replace methyl bromide and fungicides in forest systems. Biologically based and environmentally safe alternatives, such as biological control agents, natural plant products, and cultural methods, are being investigated for possible use as components in integrated management programs. Many plants and plant products have been reported to possess *Fusarium* control properties (Grayer and Harbone, 1994; Grange and Ahmed, 1998). Although much of the literature on natural products in the agricultural field concerns insect control, a smaller but emerging body of papers reports that plant extracts and plant essential oils are effective antimicrobials against plant fungi (Arras *et al*., 1993; Bishop and Thorton, 1997), foliar pathogens (Lawson and Kennedy, 1998; Northover and Schneider, 2003; Passini *et al*., 1997), soilborne fungi (Awuah, 1994; Bianchi *et al*., 1997 and Dubeyi and Kishore, 2006) and *Fusarium* (Chatarjee, 1989; Dixit *et al*., 1995; Montes-Belmont *et al*., 1998; Paster *et al*., 1995; Thompson, 2007; Wilson *et al*., 2007). In most reports, however, the efficacy of plant extracts and plant essential oils has been evaluated only in vitro, and efficacy data from trees extracts are lacking.

The neem tree *(Azadirachta indica*) is a member of the mahogany family (Meliacea) that is native to India and Burma, but it was introduced to other countries in the late 19th century (Bianchi *et al*., 1997). Six species in the family Meliacea have been studied for pesticidal properties in different parts of the world. They are *Azadirachta indica*, *A. excelsa*, *A. siamens*, *Melia azadirachta*, *M. toosendan* and *M. volkensii* (Backman, 2006). However,

the most promising phytochemical pesticides studied in recent years are those based on extracts of *A. indica*.

Various *A. indica* products have been researched extensively for their phytochemistry and exploitation in pest and fungal control programmes (Bowera and Locke, 1997; Backman, 2006). A number of bioactive components have been isolated from various parts of the tree. These chemical compounds have different designations, among which azadirachtin A is the major component. In addition to azadirachtin, a number of other active ingredients have also been isolated and identified from different parts of the *A. indica* tree, such as salannin, meliantriol and nimbin (Bianchi *et al.*, 1997). Two new triterpenoids (22,23-dihydronimocinol and des-furano-6-alpha-hydroxyazadiradione) were isolated from a methanolic extract of the fresh leaves of *A. indica* along with a known meliacin, 7-alpha-senecioyl-(7-deacetyl)-23-O-methylnimocinolide (Bishop and Thorton, 1997; Agbenin *et al*. 2005). Extracts contain active compounds that are biodegradable and are selective in their toxicity. Thus effectiveness of these against damping off disease can be quite vital for the management of forest tree plantations.

1.2 Statement of the problem

In Kenya, the forest cover is currently quite low at approximately 1.7% in comparison to 10% recommended national cover. At the same time, there is renewed interest by the government through Kenya Forestry Service, NGOs and other stakeholders to increase the forest cover in the country to meet the shortfalls. This has been achieved by encouraging plantations farming, afforestation as well as farm forestry that has gained more significance in Kenya in recent years (Koech, 2006). However, the main problem that has hindered the enhancement of forest development stems from the outbreaks of disease such as damping off in the nursery, which provides seedlings for plantations or farm forestry tree planters. This has led to massive losses among the farmers involved in the planting of trees in such ventures. There is, therefore a need to come up with the best method of preventing the spread of the diseases, which should be affordable to the farmers in terms of costs, efficacy and environmental concerns.

Though a number of chemicals have been used in the treatment of several tree diseases, there is still a matter of high costs involved in the purchase of these chemicals. Furthermore, the environmental concerns of the chemicals are also bringing about a serious debate to the environmentalists, chemical users and and other stakeholders as to the human health hazards involved in the use of these chemicals. Thus any method of treatment of the damping off using non-chemical methods can be able to receive considerable support from people who are the environmental consious.

1.3 Justification

Trees are important to the overall socio-economic, environmental and social responsibilities to any country. Having the appropriate number of trees at any given time often result in proper economic and environmental well being of the country. Besides, many citizens can be employed in numerous private forest plantations, and forest based industries, result in poverty reduction and creation of wealth among members of local communitiees. Considering that trees have to originatr from seeds, which if well tended will provide healthy seedlings and contributes forest tree plantations. Therefore, enhacing the germinationn of seeds as well as protecting the seedlings from nurous pathogens will enhance the integrity of forest ecosystems, which is is paramount to any forest resource manager. This study will therefore provide resourceful information to forest managers to come up with viable seedlings to enhance forest plantations.

There are a number of lines of defense against pest and disease, in tree nursery practices, among these include; practice of good husbandry and ensuring high standards of plantation hygiene. But no matter how diverse and healthy the plantation ecosystem may be, there will always be a degree of disease and pest present (Day *et al*., 2007). Damping off is a serous problem in nursery and there is need for a cheaper way of controlling it. Through such methods, Kenya Forest Service and several agro foresters can come up with cost effective ways of raising forest plantations that will save billions of shillings in purchase of environmentally hazardous chemicals.

Neem products have proved to be safe and easy to handle and apply. The extracts can be taken up by plants, (and thereby confer protection from within), and also has systematic

action in many plants. Treating soil with neem extracts can reduce the population of pest fungi in the rhizosphere that attack and feed on plant roots (NAP, 1992).

1.4 Objectives

1.4.1 Main Objective

The objective of the study was to come up with cheap, environmentally viable non-chemical treatment of plants infected by *Fusarium* that cause damping off using extracts from the miracle tree 'neem'.

1.4.2 Specific objectives

The specific objectives were to:

i). Determine the efficacy of *A. indica* extracts concentrations on the growth of *Fusarium* in *P. patula*.

ii). Determine the effects of *A. indica* extracts from the leaves and bark of *A. indica* on the germination of *P. patula* seed infested with *F. oxysporum.*

iii). Determine the effects of *A. indica* against *F. oxysporum* infesting the *P. patula* seedlings. effects of *A. indica* extracts from the leaves and bark on the growth performance of *P. patula* seedlings infested with *F. oxysporum.*

1.5 Hypothesis

1 H_0: *A. indica* extracts do not have significant effect on the growth of *F. oxysporum* in *P. patula*

H_1: *A. indica* extracts have significant effect on the growth of *F. oxysporum* in *P. patula*

2 H_0: *A. indica* extracts from the leaves and bark of *A. indica* do not significantly affect the germination of *P. patula* seed infested with *F. oxysporum*

H_1: *A. indica* extracts from the leaves and bark of *A. indica* significantly affect germination of *P. patula* seed infested with *F. oxysporum*

3 H_0: *A. indica* extracts from the leaves and bark of *A. indica* do not significantly affects growth performance of seedlings infested with *F. oxysporum.*

H_1: *A. indica* extracts from the leaves and bark of *A. indica* have significant effect effects of *A. indica* against *F. oxysporum*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The neem tree

Azadirachta indica, commonly referred to in many countries as the neem tree, is a member of the Meliaceae family. This broad-leaved evergreen can reach heights of 30 meters with a trunk girth of 2.5 meters and live for over two centuries. Its deep root system is well adapted to retrieving water and nutrients from the soil profile, but this deep root system is very sensitive to waterlogging (Shapiro *et al.*, 1994). The neem tree thrives in hot, dry climates where shade temperatures often reach 50°C and annual rainfall ranges from 400 to 1,200 millimeters.

The tree can withstand many environmental adversities including drought and infertile, stony, shallow, or acidic soils. The neem produces ellipsoidal drupes that are about two centimeters in length, borne on axillary clusters. These fruits contain kernels that have high concentrations of secondary metabolites (National Research Council, 1992). There is evidence, but no scientific correlation, that trees grown in climates with lower rainfall produce kernels with higher content of metabolites (Schmutterer, 2004).

The neem tree is believed to have originated in Assam and Burma of South Asia, but other reports suggest various areas of Pakistan, Sri Lanka, Thailand, Malaysia, and Indonesia (National Research Council, 1992). The tree also grows well in other tropical and subtropical areas around the world (Verkerk *et al.*, 1993). Neem trees have successfully been established in Australia, Haiti, West Africa, the Dominican Republic, Ecuador, Puerto Rico, the Virgin Islands, and in the continental United States in Florida, California, Oklahoma, and Arizona (Jacobson, 1990; Schmutterer, 1990a; Verkerk *et al.*, 1993). The trees growing in Arizona are part of a breeding and selection program aimed at developing a variety that will be frost tolerant to temperatures as low as 18 degrees below zero celsius. Such a development would allow this tree to be established in many more regions. The seed for this project was obtained from natural tree populations growing in northern India where the climate is cooler than most areas where neem grows (Jacobson, 1990).

Cultivation of the neem tree is also an important consideration as the tree is established in new regions. Very little problems arise in vegetative propagation. Transplanting seedlings, saplings, or root suckers achieves a high success rate (National Research Council, 1992). Seeds are more desirable to use when transporting a long distance for ease of packing, however, minor problems have been observed when growing these trees from seeds. It was found that dry or unripe seeds would rot in soil. Large scale establishment of neem trees required germination in sand, transplanting to clay pots after a month, and then planting in the field when the seedlings reached 30 to 45 centimeters in height (Jacobson, 1990).

2.2 Neem chemistry

The chemicals that have pesticidal activity can most efficiently be extracted from neem seed kernels. Neem trees begin their reproductive stage at about three to five years of age but don't become fully reproductive until they are ten years old. From this time on, the tree will yield an average of about 20.5 kilograms of fruit per year, with maximum production reaching 50 kilograms per year (National Research Council, 1992).

Of the fruit yield, only about 10% is attributed to seed kernels, and desired biologically active compounds comprise only ten grams per kilogram of kernel weight. This means that an adult neem tree will only produce about 20 grams of pesticidal compounds in a season (Schmutterer, 1990b). Many biologically active compounds can be extracted from neem, including triterpenoids, phenolic compounds, carotenoids, steroids, and ketones. The tetranortriterpenoid azadirachtin has received the most attention as a pesticide because it is relatively abundant in neem kernels and has shown biological activity on a wide range of insects. Azadirachtin is actually a mixture of seven isomeric compounds labeled as azadirachtin-A to azadirachtin-G with azadirachtin-A being present in the highest quantity and azadirachtin-E regarded as the most effective insect growth regulator (Verkerk *et al.*, 1993). Many other compounds have been isolated that show antifeedant activity as well as growth regulating activity on insects. Polar and non-polar extractions yield about 24 compounds other than azadirachtin that have at least some biological activity (Schmutterer, 1990b; Jacobson, 1990). This cocktail of compounds significantly reduces the chances of tolerance or resistance developing in any of the affected organisms. However, only four of

the compounds in neem have been shown to be highly effective in their activity as pesticides: azadirachtin, salannin, meliantriol, and nimbin (Jacobson, 1990; National Research Council, 1992). These compounds can be extracted by many methods. Leaching with water is the oldest method and is still used by some firms to selectively extract azadirachtin. On the other hand, most companies are using more non-polar solvents to obtain a more varied mixture of chemicals. Hexane, pentane, ethanol, methanol, esters, and dichloromethane are used in extractions as well as mixtures of these solvents with water (Lee *et al.*, 1988; National Research Council, 1992; Schmutterer, 1990b). Once extracted, several separation techniques are often incorporated to isolate compounds. For instance, in the isolation and identification of 7-deacetyl-17½-hydroxyazadiradione, researchers used insect bioassays to guide reverse phase HPLC fractionation, IR spectrum analysis, 13C NMR and 1H NMR spectrum analysis, and mass spectrum to determine the structure of the active compound (Lee *et al.*, 1988).

By using laboratory techniques, it is possible to closely mimic azadirachtin, as it has been identified from the neem tree. Anderson *et al.* (1990) and Kolb *et al.* (2001) each describe processes in which they synthesized roughly half of separate ends of the azadirachtin molecule. These subunits form a compound that has similar but less activity than the natural molecule. The activity of synthetic azadirachtin compares close enough to natural products to verify that azadirachtin is the primary toxic compound in neem (Verkerk *et al.*, 1993). Because of the great number of reactions involved in each process, synthetic azadirachtin will be very costly to produce. For this reason, companies developing azadirachtin as a commercial pesticide are working with natural products. Research is discovering that initial by-products of azadirachtin extraction have significant efficacy on pests also. Neem seed oils have detrimental effects on viruses, mites, and early larval stages of some insects, while the solid seed residue has enough residual chemical content to have activity on soilborne fungal pathogens and plant parasitic nematodes (Larew, 1990; Locke, 1990; Schmutterer, 1990b).

2.3 Neem effects

The mode of action of neem extracts is not understood very well. It is quite possible that the different chemicals or different ratios of chemicals found in neem trees have varied effects on plants growths. There is also evidence given in many research studies that plants infected by fungi react quite differently to compounds from the neem tree. More research has been conducted to find the primary mode of action of azadirachtin than of any other chemical in the neem tree. This is because of interest in it as a product for commercial use. Azadirachtin alone probably has several modes and sites of action (Koul, 2001). Primary of which is an interference with the nutrient uptake system in plant pathogens, which controls the synthesis of ecdysone and hormone. It has been indicated by Schmutterer (2004) that interference involves the inhibition of the release of these hormones. Indication of this was an accumulation of large quantities of stainable neurosecretory material in the cardiaca of most moulds. This and other research has convinced many people that azadirachtin definitely has antihormonal activity. However, other evidence indicating that control of hormone concentrations is controlled indirectly leads to the conclusion that azadirachtin is not a true antihormone.

There have been a number of clinical studies showing that Neem has significant effects on several bacterial strains. Among some of the more prominent strains studied were staphylococcus aureus, streptococcus pyogenes, cornebacterium, *E. coli*, and salmonella typhosa. These bacteria's can cause meningitis, cystitis, sore throats, typhoid, blood poisoning, and food poisoning. Neem's ability to exert significant effects over the above mentioned bacterial strains indicate its ability to resolve the aforementioned conditions (Subapriya, 2005).

Tropical climate especially in the coastal regions creates the kind of humid hothouse atmosphere that funguses thrive in. Traditionally, in Ayurveda, Neem seed oil, aqueous extracts of Neem leaf, Neem leaf powder, the smoke from burning dried Neem leaves, and Neem leaf pastes have been used for the prevention and treatment of fungal conditions in India. Athlete's foot, ringworm, and Candida, which cause vaginal yeast infections and thrush, are some of the more common fungi that attack humans. There are two medicinal

compounds in the Neem leaf, gedunin and nimbidol, which have been clinically proven to control these fungi. Jock itch, other fungi that attacks humans, has been treated traditionally in India for thousands of years with Neem seed oil and aqueous extracts of Neem leaf. Creating medicinal smoke by burning dried Neem leaves is an ancient practice in Ayurveda for purifying the atmosphere around a seriously ill patient. A clinical study examining the efficacy of this ancient practice found that smoke from burning dried Neem leaves exerted an extreme suppression of fungal growth and germination. Amongst the thousands of Ayurvedic medicinal plants in India, modern clinical research has now proven that Neem extracts contain some of the most powerful antifungal compounds against certain fungi.

The effect of azadirachtin as an antihormone on juvenile hormone titer was also investigated in the variegated cutworm by Koul *et al.* (2001). Their goal was to either eliminate or reproduce the effect of azadirachtin on metamorphic abnormalities by artificially raising the concentrations of juvenile hormones I and II or BEPAT, a juvenile hormone esterase inhibitor. They were unable to achieve any desired outcome, but ligation experiments did indicate that the region of activity was in the head capsule. The possibility was proposed that an inhibition of the synthesis of a neurosecretory protein could alter titer levels (Koul *et al.*, 2001). So while azadirachtin has activity on hormone levels, it may be an indirect relationship indicating that azadirachtin is not a true antihormone. Evidence at this time is not conclusive on the matter of primary mode and site of action, and researchers involved admit that much more investigation is necessary to unwind the mystery (Schmutterer, 1990a).

Other research has indicated a more direct role in the inhibition of molting. Direct cytotoxic effects on imaginal discs and epidermal cells result in primary lesions that prevent molting (Koul *et al.*, 2001). Azadirachtin has also been proved to be a chitin synthesis inhibitor, but the role of this inhibition as the primary mode of action has not been investigated (Schmutterer, 2004).

2.4 Efficacy studies

Research has shown that many organisms are sensitive to neem extracts. These include insects from several orders, mites, nematodes, snails, fungi, and viruses (Bhatnagar *et al.*, 1990; Locke, 2005). The efficacy of leaf extracts of basil (*Ocimum basilicum*), bitter leaf (*Vernonia amygdalina*), lemon grass (*Cymbopogen citratus*), neem (*Azadirachta indica*) and paw-paw (*Carica papaya*) on major seed-borne fungi: *Aspergillus niger, Aspergillus flavus, Botryodiplodia theobromae* and *Fusarium moniliforme* of African yam bean (*Sphenostylis stenocarpa*) seeds, and on seed germination and seedling emergence have been studied *in vitro* and *in vivo*. All the plants leaf extracts (crude and aqueous) significantly reduced the incidence of seed-borne fungi tested and increased seed germination and seedling emergence except lemon grass leaf extract when compared with the untreated control. Neem extract was the most effective while lemon grass extract was the least. Crude extracts from all the plant leaves tested increased seed germination and seedling emergence of African yam bean seeds and gave significant reduction of mycelial growth of all the fungi tested when compared with their aqueous extracts. Leaf extracts of neem, basil, bitter leaf and paw-paw, which are cheap and environmentally safe, are promising for protecting African yam bean seeds against major seed-borne fungi and in the improvement of the crop.

Information relating to the antifungal activities of compounds from neem is limited (Ako *et al.*, 2003). Neem leaves have been shown to possess antifungal activity either by direct soil amendment or as extracts of them (Aldhous, 2002) active against a number of phytopathogens (Aldhous, 2002; Ghandhi *et al.*, 2007). They reduced radial growth and spore germination of *Curvularia lunata*, successfully controlled fruit rots of cucurbitaceous plants caused by *Fusarium equiseti* and *F. semitectum* and significantly reduced fruit rot of tomatoes caused by *Aspergillus flavus* and *A. niger* (Locke, 2005). Aqueous neem leaf extracts controlled foliar diseases of groundnut, *viz., Puccinia arachidis* and *Mycosphaerella berkeleyi* (Koul *et al.*, 2001).

The use of plant products for control of *Fusarium* wilt in crops is rather limited, the effect of neem products have been reported to have significant controlling effects on some other

fungi, for example on conidial germination of *Sclerospora sacchari* isolates from maize (Poswal and Akpa 2001) and mycelial growth of *Colletotrichum gloeosporoides* isolates from pawpaw (Shimfe 2004).

One of the main problems of using neem treatments is the durability of azadirachtin in field conditions. The activity of neem-based products subsides rapidly, lasting four to eight days, meaning that many applications will likely be needed in a season. The primary means of this is photodegradation by ultra-violet light. But leaf pH can also affect detoxification rates, and rain can wash residue off leaf surfaces. Derivation of natural product stabilizes azadirachtin and may provide an avenue for greatly increasing its residual activity (Wood, 1990). Also, activity can be extended in plants, such as potato and tomato that demonstrate systemic activity. This protects azadirachtin from light and through translocation enables protection of new growth, which is often preferred by insects (Klocke *et al.*, 2001, Verkerk *et al.*, 1993).

Systemic activity in plants also relates to a greater chance of phytotoxicity. Potato, onion, cabbage, and chrysanthemum have demonstrated various types and extent of phytotoxicity. In most instances this is undesirable, but the stunting that occurs on chrysanthemums can actually take the place of plant growth regulators that are sprayed for the same effect on plants grown in greenhouses (Oetting *et al.*, 1990; Schmutterer, 1990a). Azadirachtin content in neem kernels and quickness of activity are further considerations in the commercialization of neem extracts. To provide a consistent product, refining kernels with similar levels of compounds is essential. On the contrary, a Canadian company discovered that samples of neem oil from Indian sources ranged from undetectable amounts, less than 50 ppm, of azadirachtin to 6,800 ppm (Isman *et al.*, 1990). Farmers using synthetic pesticides also are used to quick acting chemicals. They may not be patient enough to wait for the activity of neem-based products to produce results (Schmutterer, 1990b).

2.5 Conclusion

While neem tree products have some shortcomings as a conventional alternative, they can fit well as a tool to be used in management of forestry especially in *P. patula*. Their use in

control of *Fusarium* needs further reaserch. As more and more synthetic chemicals are being pulled from the market, neem is an environmentally benign alternative. Toxicology studies have indicated it to be quite safe to mammals also (Schmutterer, 1990b). Subsequent to the isolation of azadirachtin from neem seed kernels in 1968, extensive work has been done on the chemistry and pesticidal properties of compounds from the neem tree, *Azadirachta indica*. A number of compounds have been isolated from neem leaves, including protomeliacins (triterpenoids) and limonoids. Cyclic tri- and tetrasulfides have been obtained from the steam distillates. Whereas insect antifeedant properties of some of the triterpenoids and limonoids are known, no information is available on the antifungal activities of these compounds or the fractions containing them and hence the current study.

CHAPTER THREE

3.0 METHODS AND MATERIALS

3.1 Study Area

3.1.1 Geographical locations

The study was conducted at KEFRI's pathology laboratory and in the glass house. KEFRI is in Kikuyu division, Kiambu district in Central Province of Kenya. It lies at an altitude of between 2096m -2100m a.s.l, level $1^0$12S, $36^0$37^{0}E approximately 25km North-West of Nairobi- Naivasha road.

3.1.2 Climate

The area has a mean rainfall of 950mm. There are two peak periods of rains, the long rains which account of an average of 50% of the total rainfall, and the short rains account for a further 235mm. The dry season may be prolonged and drought conditions can be severe. The maximum temperature is 21.4^{0}C and the minimum temperature is 4.7^{0}C.

3.1.3 Geology and Soils

Most of the soils are less well drained and are referred to as Muguga orange brown. The dark-brown clay loam typically shallow (about 1.2m) is derived from "tuffs" present on deposits and is usually contaminated with eroded red soils. These soils are less fertile than soils derived from lava. Drainage is seasonally imbedded, the pH is approximately 6.2 in the top 18cm and 6.2 in the 0 – 108 depth range

3.1.4 Geology

The basement complex of Archees gneisses is covered by volcanic series spread in tertiary and quaternary times and of considerable depth. The age of the volcanic succession is still uncertain, but it is known that the volcanic activity was intermitted with long dormant periods when soils were formed, only to be subsequently burned known as the Limuru Quartz Frachyte which is a highly prophylitic rock, which weathered to a soft grey stone often to a considerable depth.

3.2 Experimental procedure

3.2.1 Field procedure

In the greenhouse the experiment was complete randomized design, replicated three times. The pots were arranged randomly at a distance of 15cm from each other. 30 pots were labeled A, B, C, D, E, F, G, H, Co. There were five treatments replicated three times variable (bark and leaves).

Table 3.1: Randomization of the treatments

A_3	D_2	G_1	C_2	B_3	F_2	D_1	CO_4
B_1	E_3	F_3	1	D_3	CO_3	C_3	$C0_6$
A_2	H_2	A_1	C_1	E_2	G_3	G_2	
F_1	CO_1	B_2	H_3	CO_2	CO_5	H_1	

Key

Treatment CO pots with fungi but not extract.
Treatment A pots with 25% leave extracts concentration and fungus.
Treatment B pots with 50% leaf extract concentration
Treatment C posts with 75% leaf extract concentration
Treatment D pots with 100% leaf extract concentration
Treatment E pots with 25% Bark extract concentration
Treatment F pots with 50% Bark extract concentration
Treatment G pots with 75% bark extract concentration
Treatment H pots with 100% bark extract concentration.

3.2.2 Preparation of soils and pots

The soil was collected under mature *Croton megalocarpus* tree in Muguga Natural Forest. It was mixed with sand in a ratio of 3:1 respectively. The soil sand mixture was heated at 85°C for a period of eight hours in an oven to ensure no fungi exists before inoculating the *F. oxysporum*. The pots were sterilized by dipping in 10% sodium hypochlorite in water

and allowed to air dry. The sterilized soil was then left to cool for twelve hours in the laboratory and thereafter inoculated with the fungus by dissolving the fungus (*F. oxysporum*) in distilled water and sprayed using a watering can until the soil mixture was wet coupled with regular shoveling to ensure complete mixing. The inoculated soil mixture was then potted and left for four days to ensure the fungal growth was maximum before introducing the crude extract. A total of four concentrations that formed the treatments were extrated in triplicates and used during this experiment. The treatments concentrations ranged from 0%, 25% 50% 75% and 100% for both the leaves and barks; 0% being the control. After this treatment the soil was left for seven days before the beginning of the experimental setup. At the onset of the experiment, *P. patula* seeds were watered using distilled water to retain the soil moisture.

3.2.3 Raising of the seedlings

P. patula seeds obtained from Kenya Forestry Research Institute, Muguga having a germination capacity of 95% were sown into the thirty pots 1cm deep. In each pot atotal of 250 seeds sown. The seeds were allowed to germinate under normal seedbed conditions. Such conditions are conducive for the development of of *F. oxysporum* (Marois *et al.*, 2005).

3.2.4 Collection and extraction of *Azadrachta indica*

The leaves and stem bark of *A. indica* were collected from Malindi, Kenya and air-dried in the laboratory for one and a half months before grinding into powder. Half a kilogram of the ground *A. indica* were soaked in 99% analytical ethanol for a period of 48 hours and then filtered using muslin clone and a filter paper. The resulting solution was concentrated at reduced pressure using solutions rotary evaporator and a set temperature in a water bath of 80^0C to remove ethanol. The crude of the bark and the leaves were then divided into portions, which were later used in the laboratory and field experimentation.

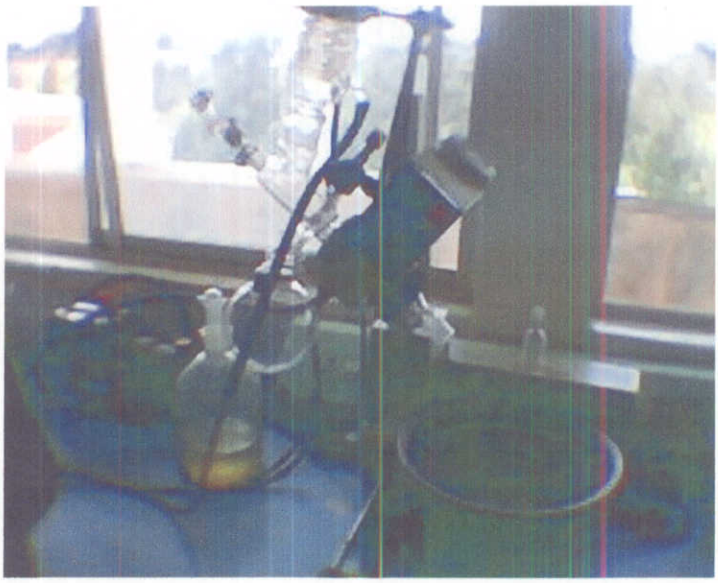

Plate 1: Extraction of *A. indica* extracts

5g of malt and 5g of Agar was weighed and dissolved into 500ml of distilled water in a beaker and stirred to dissolve using a stirring rod. The solution was then transferred into a conical flask and the top sealed using aluminum foil. This was sterilized by autoclaving at 120°C at one atmosphere pressure for 20 minutes. The sterilized media was then allowed to cool and then poured from the flask into petri dishes, which had been oven sterilized at 160° C for 2 hours.

3.2.5 Isolation of Pathogen from seeds

Infected *Prunus africana* seeds collected from KEFRI seed Centre were used. The seeds were sterilized in 70% ethanol for 2 minutes and washed in distilled water and then dump chambered. Fungus was then cultured in a 2% malt extract medium, which was later purified by sub-culturing to eliminate bacteria. After staining the slides using Alanine, the fungus were examined under an Olympus compound microscope at a maganification of X400 using several keys and illustrations.

3.2.6 Bioassay

Diffusion method (Thapiyal, 1979) was used to assess the antifungal activity against *F. oxysporum*. Four concentrations of *A. indica* extracts were prepared, 25% 50%, 75%, 100%, and control without any extract. 0.02 ml of each concentration of *A. indica* extracts were applied to 10mm diameter filter paper discs in triplicate. The culture medium (2% malt extract agar) was prepared by adding 10g of malt extract agar in 500ml of distilled water and stirred to dissolve the contents. The media, distilled water and petri dishes were then sterilized by autoclaving at 120°C at 1 atmosphere pressure for 20 minutes. 1 ml of distilled water was used to dissolve the test microorganism. This was then introduced into the media before the media solidified and inverted several times to evenly distribute the spores. The media was then poured into the sterile petri dishes and allowed to solidify. The air-dried discs were then applied on the medium in the petri dishes and incubated for three days at a temperature of 26 ± 0.1°C. The growth inhibition dimater around the discs was then measured and recorded.

3.2.7 Collection and extraction of *Azadirachta indica* (bark and leaves)

The leaves and stem bark of *A. indica* were collected from Malindi Kenya. The materials were air dried in the laboratory for 1.5 month before grinding (natural drying). The ground material produced 1 kg of bark and ½ kg of leaves. The ground material was soaked in analytical ethanol for a period of 48 hours. The solution was then filtered using muslin cloth and a filter paper. This was then concentrated using ratorrvopor machine to drive off the solvent (Ethanol) at 35°C-45°C (Plate 1). The crude of the barks and leaves were the divided into portions, which were used in the laboratory and in the field.

3.2.8 Germination tests

In the nursery experiment the number of germinated and dead seed and were counted and recorded in data collection sheet for 17 days (Appendix 2 and 3).

The germination percentage was calculated using the fomula $\%\,\text{germination} = \frac{\text{Number of germinated seeds}}{\text{Total number of planted seeds}} * 100$. The dead seedlings were later cultured in the laboratory to determine presense of any pathogen growth on their surfaces.

3.3 Data analysis

All Data collected were entered, organized and managed using EXCEL spreadsheet for Windows XP. All statistical analyses were performed with a version of SPSS 13.5 Statistical packages. Normality of data distributions were checked by means of the skewness and kurtosis to determine any need for applying appropriate data transformation procedures as described in Zar (2001).

Mean differences in the inhibition diameters among concentration treatments were analyzed using a one-way ANOVA. In cases where significant differences occurred, Duncans Multiples Range test (DMRT) was used to discriminate between the means that were actually different from each other (Michael and Douglas, 2004). Significant differences in inhibition diameters at a given concentration between bark and leaves were tested by 2-tailed significance Student t-test.

Both pre-emergence and post emergence germination data were calculated as percentage germination. Upon, which cumulative germination percentage was determined for the seeds of *P. patula*. Data of theh germination percentage was Arcsine transformed to conform to normality of percentage data (Michael and Douglass, 2004) before subjecting them to ANOVA tests. In all the statistical analysis, results were accepted as significant at $P < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Efficacy of neem extracts concentrations on the growth of *Fusarium* in *P. patula*.

Results of the efficacy trials of *A. indica* extracts on *F. oxysporum* are presented in Plate 2. The plate indicates that *A. indica* extracts controlled the growth of *F. oxysporum* when it came into contact with it (pointed arrows). The inhibition diameters covered by the *A. indica* extracts are shown in Table 4.1. There were significant differences in inhibition diameters of *A. indica* extracted from the bark ($F = 1254$, $df = 5$, $P = 0.000$) and leaves ($F = 4218$, $df = 4$, $P = 0.000$) on growth of *F. oxysporum*. At 50% concentration, *A. indica* extracted from the bark, resulted to significantly higher inhibition diameter against *F. oxysporum* than *A. indica* extracted from leaves ($t = -61.000$, $df = 4$, $P = 0.000$). At a concentration of 75% and 100% of *A. indica* extracts, inhibition diameters were not significantly different ($t = 9.334$, $df = 1$, $P = 0.002$) between *A. indica* extracted from the leaves and bark.

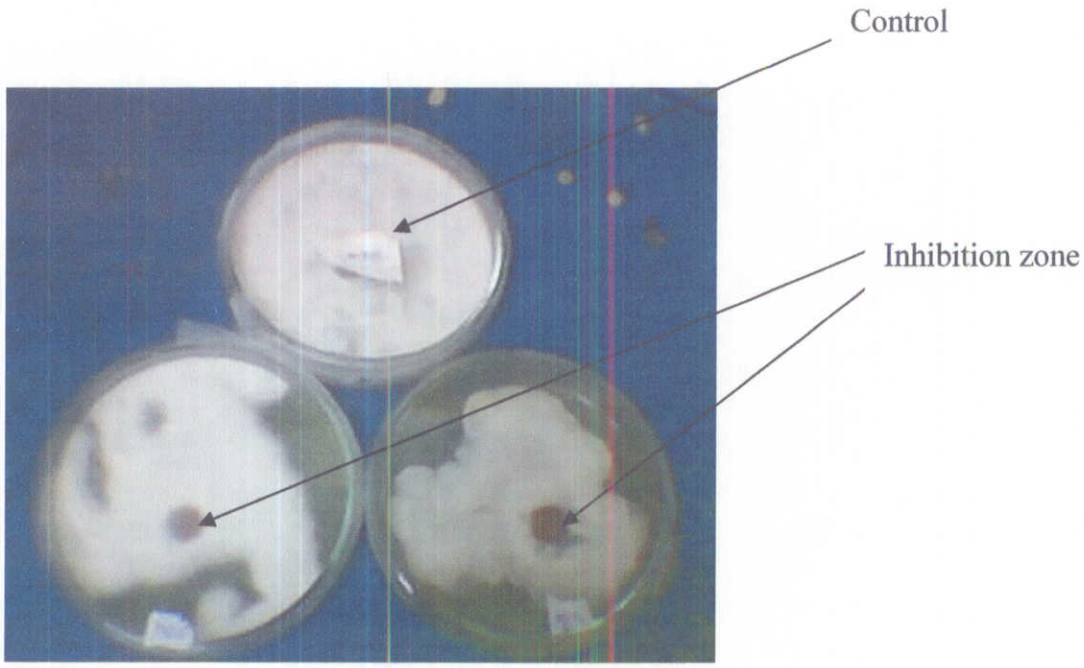

Plate 2: Inhibition zones of *A. indica* in *Fusarium* colonies.

Table 4.1: The efficacy of neem extracts on control of *Fusarium*

	Concentration of *A. indica*				
	Control (0%)	25%	50%	75%	100%
Leaves	0.0^{a}	0.0^{a}	2.1^{b}	10.2^{c}	10.2^{c}
Bark	0.0^{a}	10.2^{c}	10.2^{c}	10.4^{cd}	10.7^{d}

Values followed by similar superscripts are not significantly different at $\alpha = 0.05$

4.2 Effects of *A. indica* extracts on the germination of *P. patula* seed infested with *F. oxysporum.*

4.2.1 Pre-emergence germination of *P. patula* under *A. indica* extracted from the leaves

Results of the overall pre- emergence germination percentage of *P. patula* under varying concentration of *A. indica* extracted from the leaves is presented in Figure 4.1. Analysis of Variance (One-Way ANOVA) indicates that there were significant differences in the germination percentages ($F_{4, 80} = 28.845$, $P < 0.001$) in *P. patula* seeds treated with leaves extracts of *A. indica*. Post-Hoc, Duncan's Multiple Range Test (DMRT) indicate that germination percentage was highest in leaves treated with 25% *A. indica* extract (68.3 ± 15.4%) which did not differ significantly from the germination percentage of leaves treated with 50% *A. indica* extracts (65.7 ± 15.6%) but were were significantly ($P < 0.05$) in comparison to the untreated controls (35.2 ± 8.7%). At higher concentration of (75% and 100%) of *A. indica* extract, the germination percentages were generally lower than germination of leaves without any treatment (control).

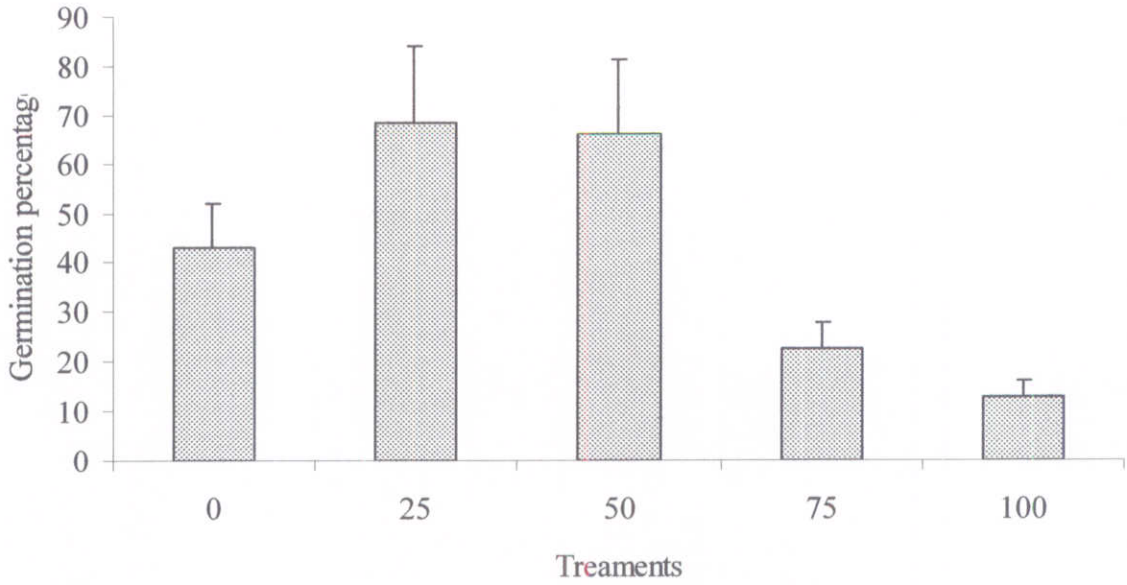

Figure 4.1: Overall germination percentage of *P. patula* seeds after 17 days of experimental period

4.2.2 Post emergence-germination of *P. patula* under *A. indica* extracted from the leaves

Results of cumulative post emergence germination percentage from the onset of germination to the end of the experimental period under various treatments with *A. indica* extracted from leaves are presented in Figure 4.2. Germination of *P. patula* under 25% of the *A. indica* extract maintained the highest post emergence germination throughout the experimental periods, which was followed by post emergence germination of *P. patula* treated with 50 % leaves extracts from *A. indica*. Post emergence germination of *P. patula* under 75% and 100% leaves extracts of *A. indica* were all significantly ($P < 0.05$) lower than those of untreated controls. In all treatments, post emergence stagnation in germination started to gain prominence after the 8th day of germination when increase in germination in all treatments were not significantly different ($P > 0.05$).

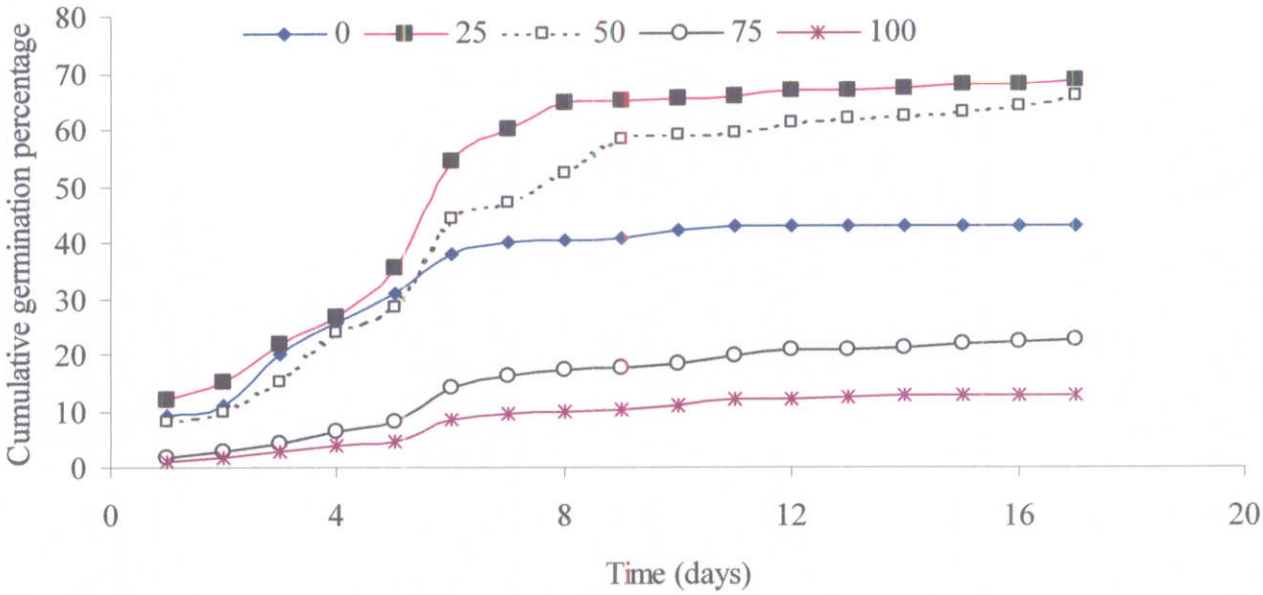

Figure 4.2: Cumulative germination percentage of *P. patula* seeds from the onset of germination to the end of the experimental period under various treatments with *A. indica* extracted from leaves

4.2.3 Pre-emergence germination of *P. patula* under *A. indica* extracted from the bark

Pre-emergence germination percentage of *P. patula* under varying concentrations of *A. indica* extracted from the bark is presented in Figure 4.3. One-Way ANOVA indicates that there were significant differences seed germination percentages ($F_{4, 80} = 31.853$, $P < 0.001$) among the bark extract treatments. DMRT, indicated that germination percentage was highest in *P. patula* treated with 25% *A. indica* extract (48.7 ± 11.4%) which did not differ significantly from the germination percentage of leaves treated with 50% *A. indica* extracts (48.6 ± 15.6%). *A. indica* concentrations of 25% and 50% resulted to significantly ($P < 0.05$) higher germination percentage in comparison to the untreated controls (41.2 ± 8.2%). At a concentration of 75% and 100% respectively of the *A. indica* extract, the germination percentages were generally much lower, being significantly ($P < 0.05$) lower than germination of leaves without any treatment (control).

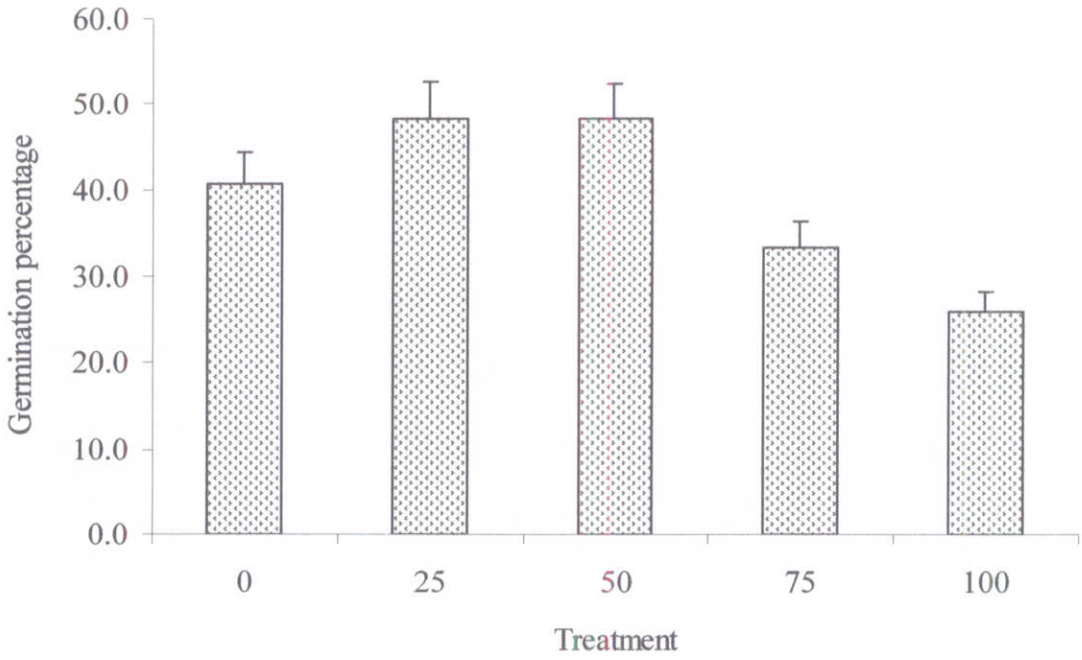

Figure 4.3: Pre-emergence germination percentage of *P. patula* under various *A. indica* treatments extracted from the bark.

4.2.4 Post emergence-germination of *P. patula* under *A. indica* extracted from the bark

Results of cumulative post emergence germination percentage from the onset of germination to the end of the experimental period under various treatments with *A. indica* extracted from bark are presented in Figure 4.4. Germination of *P. patula* under 25% and 50% of the *A. indica* extract maintained the highest post emergence germination throughout the experimental periods, which was followed by post emergence germination by *P. patula* in untreated controls. Post emergence germination of *P. patula* under 75% and 100% leaves extracts of *A. indica* were all significantly ($P < 0.05$) lower than those of untreated controls as well as 25% and 50% concentrations. In all treatments, post emergence stagnation in germination started to gain prominence after the 6th day of germination when the increase in germination in all treatments was not significant ($P > 0.05$).

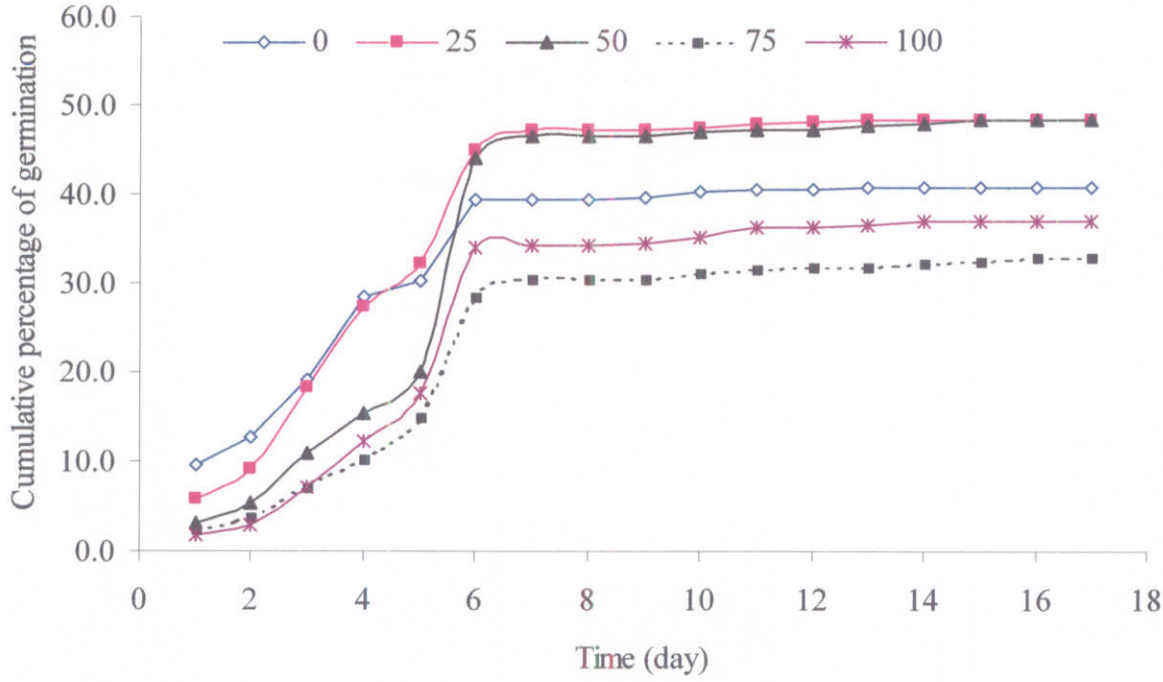

Figure 4.4: Cumulative post emergence germination percentage P.patula seeds treated with various concentration of *A. indica* extracted from bark.

4.3 Effacacy of *A. indica* against *F. oxysporum* infesting the *P. patula* seedlings

4.3.1 Efficacy of *A. indica* extracted from leaves in controlling *F. oxysporum* on *P. patula* seedlings

Results of the efficacy of different concentrations of *A. indica* extracted from the leaves in controlling *F. oxysporum* that have infested *P. patula* seedlings are shown in Figure 4.5. Deaths of *P. patula* seedlings were significantly higher ($P < 0.05$) in untreated controls than all the other treatments throughout the experimental period. Death of seedlings treated with 25% and 50% leaf extracts of *A. indica* maintained a statistical similarity ($P < 0.05$) throughout the experimental study but resulted to higher seedlings deaths than *P. patula* seedlings treated with 100% and 75% *A. indica* extracts.

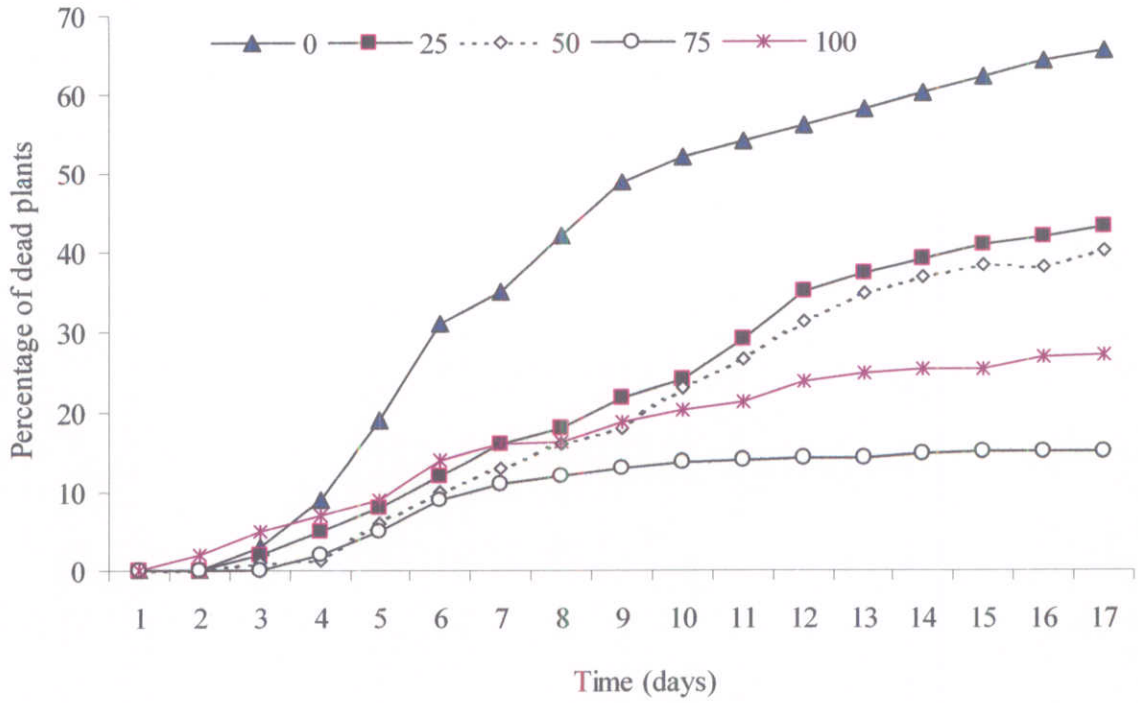

Figure 4.5: Cumulative percentage of dead seedlings under varying neem treatments

4.3.2 Efficacy of *A. indica* extracted from bark in controlling *F. oxysporum* on *P. patula* seedlings

Results of the efficacy of *A. indica* extracted from bark on controlling the *F. oxysporum* infesting the seedlings of *P. patula* are shown in Figure 4.6. Death of *P. patula* was significantly higher ($P < 0.05$) in untreated controls than all the other treatments throughout the experimental period. Death of leaves treated with 25% was statistically higher than deaths at 75% and 50% concentration of *A. indica* bark extracts. Deaths of leaves at 100% bark extract of *A. indica* was the lowest throughout the experimental period.

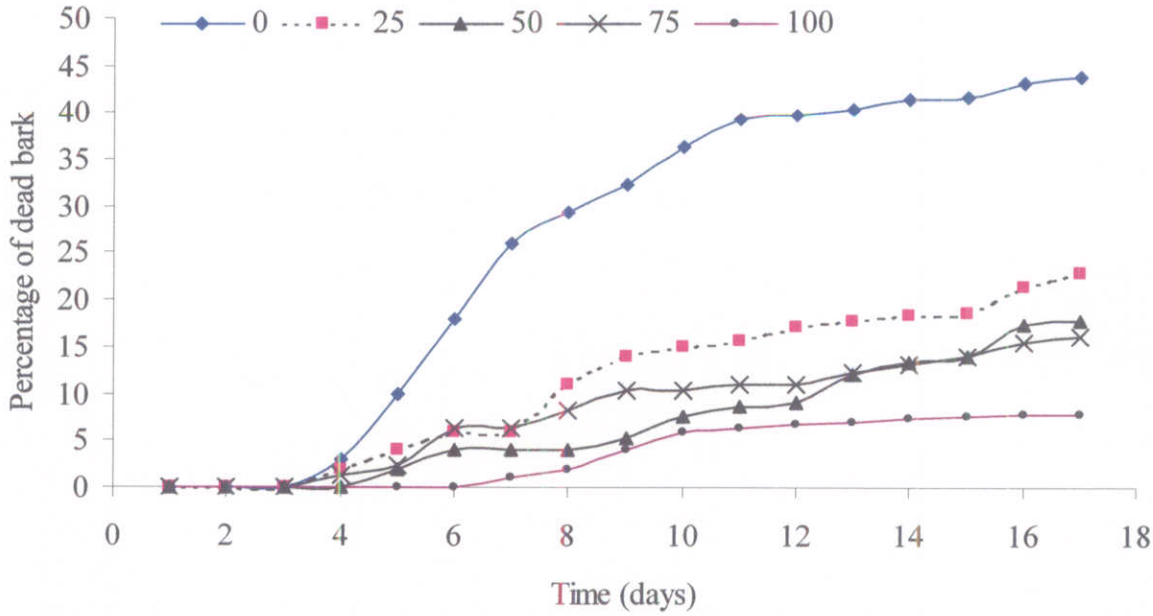

Figure 4.6: Cumulative percentage of dead seedlings under varying neem extract treatments

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

Although the neem tree has been known to be useful in soil enrichment and for insect, pest control and to some extent of disease control, its potential for the control of forest tree diseases especially, *Fusarium* wilt causing damping off has not been fully exploited. The present results show clearly the potential of neem extracts for control of *Fusarium* wilt in pines. Several plant extracts have been shown to effectively reduce populations of *F. oxysporum* and increase symptomless seedling stand in controlled experiments. The suppression of wilt development in the greenhouse corresponds with the ability of these extracts to reduce populations of *Fusarium* in leaves of trees. In the present study, extracts of *A. indica* reduced the population density of *Fusarium* in the laboratory trial bioassays in comparison to untreated controls. Thus the laboaratory bioassay results indicated that *A. indica* extract was actually efficient against *F. oxysporum*. The effective concentrations that prooved efficacious against *F. oxysporum* were highly variable but as a general rule, effectiveness of *A. indica* extracts increased with increasing concentration. At moderate concentrations of 50%, bark extracts were more effective than leaves extracts in controlling *F. oxysporum* in the laboratory. Simular results were also observed in the field conditions in controlling *F. oxysporum* infesting the seedlings of *P. patula*. In the field trials, *A. indica* effectively controlled *F. oxysporum* resulting to less number of seedlings deaths in comparison to untreated controls. Seedlings deaths were reduced at higher concentration of *A. indica* extarcts treatments.

These results agree with those obtained by several researchers key among them, Olufolaji (1999) on wet rot disease of *Amaranthus sp.* and *Choanephora cucurbitarum* using neem root bark and leaves extracts. Similarly, Shimfe (2004) observed inhibition of mycelial development of *Colletotrichum gleosporiodes* by extracts of neem leaves and fruit. Ogechi and Marley (2006) established that neem extracts were effective in controlling *Fusarium* causing tomato wilt. Dry neem seed extract completely inhibited mycelial growth of *F. oxysporum* at all concentrations. Bruce *et al.* (2004) established that application of *A. indica* oil in sesame (*Sesamia calamistis*) resulted in control of fungal pathogens that inhibited growth and oviposition in the plant. The efficacy of neem leaf extracts against

major seed borne fungi of African yam bean seeds showed that the five plant leaf extracts significantly inhibited the radial growth of all the test fungi with inhibition varying from one extract to another (Nwachukwu and Umechuruba, 2005). The findings from the present study suggest that the compound, azadirachtin found in the plant extract have antimicrobial effects. Further deducations points to the directon that extracts of neem probably have some fungicidal properties that inhibit the growth of the tree borne fungi. Aqueous, methanolic and ethanolic extracts of Neem show biological activity in the laboratory and the field, although at a varying extent to different target organisms.

Inhibitory activities of plant extracts vary with the plant part used. This explains the difference in toxicity to the fungus exhibited by the dry neem bark and fresh leaf extracts. According to Sardsurd *et al*. (1995), extracts from the bark of *A. indica* was found to completely inhibit the growth of *Botryodiplodia obromae*, while leaves extracts showed complete toxicity against *Pythium debaryanum*, *Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii*. Though, in this study trials were not conducted with seeds extracts, many researchers seem to point to the direction of seeds of *A. indica* having more effectiveness against pathogens than both the barks and leaves. It has been shown by Jacobson (1990), Schmutterer (1990a) and Koul and Isman (2001), that seeds of the neem tree contain the highest concentration of azadirachtin than all other biologically active chemical compounds present in *A. indica.* Other tissues of *A. indica* known to contain these compounds at lower levels are the bark, leaves and heartwood (Awuah, 1999). The neem terpenoids are present in all parts of the plant, in the living tissues. In recent studies by Shimoni *et al*. (2007), the site of synthesis and accumulation of the neem chemicals has been identified as secretory cells, which are more abundant in the seed kernels.

Effectiveness of plant extracts in controlling pathogens are highly variable at variable concentrations. The present study indicated that *A. indica* extracts were effective in enhancing germination of pine seeds and seedlings. However, the effective concentrations that were determinate to germination of the test trees were highly variable. Pre-emergence and post emergence germination were better at moderate concentration of *A. indica* but significantly reduced at higher concentration of the extracts. The crude extracts were more

effective in reducing the incidence of fungi thus enhancing seeds germination. This is an indication that dilution of the extracts reduced effectiveness of the extracts on the seed-borne fungi. This result agrees with the findings of Zaman *et al* (1997) that the efficacy of neem, extracts on seed borne fungi of mustard declined with increased dilution.

Studies carried out by Hussaini and Deeni (2001) established that the neem extracts at certain concentration were not phytotoxic to seeds rather they improved seed germination and seedling emergence significantly more than the untreated control seeds. Presently, the extracts from neem were effective in improving seed germination and seedling emergence. The ability of the extracts to increase seed germination and seedling emergence could be attributed to the suppression of the incidence of the seed borne fungi that could have killed the embryo of the seeds. This result is consistent with that of Homer *et* al. (1990) who established that leaf extracts of *A. indica* increased seed germination and improved seedling development of rice seeds. Neem oil inhibited the growth of *S. oryzae* under *in vitro* conditions. Inhibition of *S. oryzae* by neem leaf extract and of *D. oryzae* by pungam leaf extract and control of rice sheath rot with neem seed kernel extract as foliar spray with reduction in disease incidence and increase in yield.

Data in this study also strongly suggested that extracts of *A. indica* are capable of pathogen growth inhibition in pre emergence and post emergence seeds. The effect of an extract on the target pathogen population over time is also an issue when determining whether repeated plants treatment is necessary throughout the growing season. For example, soil populations of *F. oxysporum* were lowest after 3 to 7 days of incubation when the soil was treated with 50% and 75% aqueous emulsions of *A. indica* extract. Population numbers then maintained constancy over time. In greenhouse experiments, the *A. indica* extract controlled disease initially, but disease incidence increased as the experiments progressed, especially with the 25% aqueous emulsion. *Fusarium* populations may have increased, as in the laboratory studies, to sufficient levels in the greenhouse over time to cause symptom development. Observation of the lack of background microflora on dilution plates suggests that the *A. indica* extract may act as a general biocide. At this concentration, the extract may affect a wide range of plant microorganisms and thus may create a biological vacuum.

Fusarium has been shown to rapidly colonize fumigated soil in the absence of competition (Marois *et al.*, 2005). It can be hypothesized that the extract may break down rapidly in soil, and a small surviving population of *Fusarium* may have flourished in the soil environment with the natural microflora at low population levels once the active ingredient in the extract was no longer present. Treatment of soil with a 50% aqueous emulsion may not have reduced *Fusarium* populations enough, thus allowing rapid recolonization with reduced microbial competition, to effectively control disease over a long time frame. Populations of *Fusarium* also increased numerically over time when the *A. indica* extract was added as a 10% aqueous emulsion. However, disease control was less than 100% during the 17 day of the experiment in the greenhouse.

Treatment of the soil with a 75% - 100% extracts of *A. indica* may have reduced *Fusarium* populations sufficiently to suppress disease over a longer period of time. A very low *Fusarium* population may not compete as well over time with other soil populations during recolonization (Pant *et al.*, 1986). Thus, any threshold needed for observable symptom development may be delayed. It is within this time frame that the addition of biological control microorganisms may be most effective (Bowers and Locke, 2006). Retreatment of soil during the growing period may not be feasible with the *A. indica* extract because of potential phytotoxicity (Isman *et al.*, 2004). Potentially beneficial fungi may not be affected and may actually increase in numbers. These groups of organisms could suppress disease development in the presence of the pathogen. Further evidence of this phenomenon may be evident in treatments when the neem extract was added as a 25% aqueous emulsion. Soil populations of *Fusarium* were not reduced compared with the untreated, infested soil; yet disease was suppressed by evidence of an increase in symptomless plants. Biological factors may be involved.

The organism may need only to be capable of rapid colonization of the treated soil in order to suppress reestablishment or proliferation of the pathogen, or the organism may interact with the pathogen and/or host in some manner, thus achieving disease control (Singh and Handique, 1997). Initial experiments in the greenhouse to test this strategy have had favorable results, where the addition of a biological control agent in combination with the

A. indica extract resulted in increased symptomless seedling stand over either the biological agent or the *A. indica* extract used alone.

CHAPTER SIX

6.0 RECOMMENDATIONS

This present study has shown that leaf extracts of neem, can be used as fungicidal seed treatments for the control of seed-borne fungi of *P. patula* and for increasing seed germination and seedling emergence. However, the following recommendations would enhance further effectiveness of the *A. indica*.

1. The extract formulations should be developed depending on the particular cropping system after effective research outputs since several authors have reported toxicity of the extract to certain crops at inneficient concentrations.
2. Since the neem extracts are effective antifungal agents in controlling growth of fungi, it would be advisable to prepare effective concentrations of the extracts and apply them as foliar sprays. Such information can be disseminated to the farmers who are planting the pines at home in order to reduce the effective cost of chemical treatment.
3. Considering that neem is also threatened by depletion just like other forest trees, it is recommended that the Kenya Forest service and other stakeholders shouls be actively involved in the plantation of this tree in as many part of the country as well as involving as many farmers as possible to encourage sustainable exploitation of the tree.
4. Several parameters need to be researched on and effectively developed in order to scale up to field or nursery conditions, such as delivery method, appropriate formulation for delivery and soil type, rate, cultural practices, and economic factors involved. Consideration will need to be given to the mechanism of the interaction of the product with the pathogen population and the host plant.
5. Further research could also be conducted in future involving the seeds extracts of *A. indica* on the effectiveness of biological agents. This could also be extended to other forest trees especially those being adopted currently by farmers such as improved *Eucalyptus sps* supplied to the farmers by the Tree Biotechnology Projects (TBP).
6. *A. indica* reduced *Fusarium* populations in soil or reduced disease development. The observed reduction in the *Fusarium* population and increased symptomless

seedling stands in the greenhouse suggests that natural plant extracts may have important roles in biologically based management strategies. One plausible scenario is that a natural extract be incorporated into soil to initially reduce the pathogen population. This would be followed 1 to 2 weeks later by application of a biological agent, which may or may not need to be compatible with the extract

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APPENDICES

Appendix 1: Raw data showing inhibition diameter of A.indica extracts

Concentrations	Leaves	Bark
0	0.0	0.0
0	0.0	0.0
0	0.0	0.0
25	0.0	10.3
25	0.0	10.3
25	0.0	10.1
50	2.0	10.2
50	2.3	10.3
50	1.9	10.1
75	10.4	10.5
75	9.9	10.4
75	10.8	10.3
100	10.3	10.9
100	10.5	10.6
100	9.9	10.5

Appendix 2: Raw data on germinationof seeds and death of seedlings treated with *A. indica* extracted from the leaves

Day	Treatment	Replicate	Number of seeds	Germinated seeds	% Germination	Deaths
1	0	1	250	13	5.2	0
1	0	2	250	46	18.4	0
1	0	3	250	9	3.6	0
1	25	1	250	25	10	0
1	25	2	250	11	4.4	0
1	25	3	250	53	21.2	0
1	50	1	250	23	9.2	0
1	50	2	250	14	5.6	0
1	50	3	250	23	9.2	0
1	75	1	250	4	1.6	0
1	75	2	250	7	2.8	0
1	75	3	250	2	0.8	0
1	100	1	250	1	0.4	0
1	100	2	250	5	2	0
1	100	3	250	3	1.2	0
2	0	1	250	6	2.4	0
2	0	2	250	3	1.2	0
2	0	3	250	5	2	0
2	25	1	250	13	5.2	0
2	25	2	250	2	0.8	0
2	25	3	250	10	4	0
2	50	1	250	6	2.4	0
2	50	2	250	1	0.4	0
2	50	3	250	7	2.8	0
2	75	1	250	6	2.4	0
2	75	2	250	2	0.8	1
2	75	3	250	0	0	0
2	100	1	250	1	0.4	0
2	100	2	250	0	0	0
2	100	3	250	3	1.2	0
3	0	1	250	24	9.6	0
3	0	2	250	34	13.6	0
3	0	3	250	11	4.4	0
3	25	1	250	10	4	0
3	25	2	250	34	13.6	0
3	25	3	250	5	2	0
3	50	1	250	12	4.8	0

3	50	2	250	12	4.8	0
3	50	3	250	15	6	0
3	75	1	250	7	2.8	0
3	75	2	250	3	1.2	0
3	75	3	250	0	0	0
3	100	1	250	0	0	0
3	100	2	250	4	1.6	0
3	100	3	250	3	1.2	0
4	0	1	250	27	10.8	0
4	0	2	250	3	1.2	0
4	0	3	250	11	4.4	1
4	25	1	250	13	5.2	3
4	25	2	250	18	7.2	9
4	25	3	250	25	10	4
4	50	1	250	39	15.6	6
4	50	2	250	8	3.2	9
4	50	3	250	20	8	5
4	75	1	250	9	3.6	0
4	75	2	250	5	2	0
4	75	3	250	3	1.2	0
4	100	1	250	0	0	0
4	100	2	250	2	0.8	4
4	100	3	250	7	2.8	5
5	0	1	250	24	9.6	0
5	0	2	250	10	4	2
5	0	3	250	5	2	2
5	25	1	250	2	0.8	4
5	25	2	250	8	3.2	0
5	25	3	250	1	0.4	2
5	50	1	250	9	3.6	7
5	50	2	250	16	6.4	10
5	50	3	250	9	3.6	13
5	75	1	250	2	0.8	0
5	75	2	250	5	2	5
5	75	3	250	5	2	0
5	100	1	250	1	0.4	0
5	100	2	250	0	0	0
5	100	3	250	4	1.6	5
6	0	1	250	34	13.6	8
6	0	2	250	23	9.2	4
6	0	3	250	16	6.4	20

6	25	1	250	33	13.2	7
6	25	2	250	48	19.2	16
6	25	3	250	59	23.6	8
6	50	1	250	50	20	23
6	50	2	250	32	12.8	28
6	50	3	250	35	14	13
6	75	1	250	22	8.8	5
6	75	2	250	28	11.2	19
6	75	3	250	26	10.4	10
6	100	1	250	10	4	1
6	100	2	250	6	2.4	10
6	100	3	250	14	5.6	2
7	0	1	250	0	0	0
7	0	2	250	0	0	0
7	0	3	250	0	0	0
7	25	1	250	0	0	0
7	25	2	250	0	0	0
7	25	3	250	0	0	0
7	50	1	250	8	3.2	0
7	50	2	250	7	2.8	0
7	50	3	250	7	2.8	0
7	75	1	250	2	0.8	0
7	75	2	250	0	0	0
7	75	3	250	0	0	0
7	100	1	250	0	0	0
7	100	2	250	6	2.4	0
7	100	3	250	0	0	0
8	0	1	250	0	0	0
8	0	2	250	0	0	0
8	0	3	250	0	0	0
8	25	1	250	0	0	0
8	25	2	250	0	0	0
8	25	3	250	0	0	1
8	50	1	250	0	0	0
8	50	2	250	0	0	0
8	50	3	250	0	0	5
8	75	1	250	0	0	0
8	75	2	250	0	0	0
8	75	3	250	0	0	1
8	100	1	250	0	0	0
8	100	2	250	0	0	1

8	100	3	250	0	0	0
9	0	1	250	0	0	3
9	0	2	250	0	0	1
9	0	3	250	0	0	2
9	25	1	250	1	0.4	2
9	25	2	250	1	0.4	3
9	25	3	250	0	0	6
9	50	1	250	0	0	8
9	50	2	250	0	0	4
9	50	3	250	0	0	8
9	75	1	250	2	0.8	3
9	75	2	250	1	0.4	3
9	75	3	250	0	0	2
9	100	1	250	1	0.4	2
9	100	2	250	1	0.4	0
9	100	3	250	1	0.4	5
10	0	1	250	3	1.2	13
10	0	2	250	3	1.2	1
10	0	3	250	4	1.6	1
10	25	1	250	0	0	1
10	25	2	250	2	0.8	0
10	25	3	250	0	0	0
10	50	1	250	1	0.4	0
10	50	2	250	3	1.2	0
10	50	3	250	1	0.4	0
10	75	1	250	1	0.4	3
10	75	2	250	2	0.8	1
10	75	3	250	3	1.2	1
10	100	1	250	2	0.8	5
10	100	2	250	1	0.4	0
10	100	3	250	2	0.8	0
11	0	1	250	2	0.8	5
11	0	2	250	2	0.8	
11	0	3	250	0	0	2
11	25	1	250	2	0.8	2
11	25	2	250	0	0	6
11	25	3	250	0	0	7
11	50	1	250	2	0.8	2
11	50	2	250	2	0.8	0
11	50	3	250	0	0	3
11	75	1	250	6	2.4	2

11	75	2	250	2	0.8	3
11	75	3	250	2	0.8	5
11	100	1	250	1	0.4	1
11	100	2	250	4	1.6	0
11	100	3	250	4	1.6	2
12	0	1	250	0	0	11
12	0	2	250	1	0.4	0
12	0	3	250	0	0	3
12	25	1	250	0	0	2
12	25	2	250	0	0	2
12	25	3	250	0	0	14
12	50	1	250	0	0	0
12	50	2	250	0	0	0
12	50	3	250	0	0	2
12	75	1	250	0	0	4
12	75	2	250	0	0	2
12	75	3	250	0	0	3
12	100	1	250	0	0	0
12	100	2	250	0	0	4
12	100	3	250	1	0.4	3
13	0	1	250	1	0.4	5
13	0	2	250	0	0	2
13	0	3	250	0	0	4
13	25	1	250	1	0.4	2
13	25	2	250	0	0	1
13	25	3	250	0	0	4
13	50	1	250	1	0.4	0
13	50	2	250	2	0.8	0
13	50	3	250	2	0.8	3
13	75	1	250	1	0.4	4
13	75	2	250	0	0	1
13	75	3	250	1	0.4	2
13	100	1	250	2	0.8	0
13	100	2	250	1	0.4	1
13	100	3	250	0	0	2
14	0	1	250	0	0	5
14	0	2	250	0	0	1
14	0	3	250	0	0	0
14	25	1	250	1	0.4	1
14	25	2	250	0	0	0
14	25	3	250	0	0	4

14	50	1	250	0	0	0
14	50	2	250	3	1.2	2
14	50	3	250	0	0	2
14	75	1	250	0	0	1
14	75	2	250	0	0	0
14	75	3	250	0	0	0
14	100	1	250	1	0.4	0
14	100	2	250	1	0.4	0
14	100	3	250	0	0	2
15	0	1	250	0	0	3
15	0	2	250	0	0	0
15	0	3	250	0	0	1
15	25	1	250	0	0	0
15	25	2	250	0	0	0
15	25	3	250	0	0	5
15	50	1	250	0	0	0
15	50	2	250	0	0	1
15	50	3	250	0	0	5
15	75	1	250	0	0	0
15	75	2	250	0	0	0
15	75	3	250	0	0	2
15	100	1	250	0	0	0
15	100	2	250	0	0	0
15	100	3	250	0	0	0
16	0	1	250	0	0	12
16	0	2	250	0	0	3
16	0	3	250	0	0	6
16	25	1	250	1	0.4	2
16	25	2	250	0	0	0
16	25	3	250	0	0	1
16	50	1	250	0	0	0
16	50	2	250	0	0	0
16	50	3	250	0	0	18
16	75	1	250	0	0	1
16	75	2	250	0	0	3
16	75	3	250	0	0	0
16	100	1	250	3	1.2	0
16	100	2	250	0	0	0
16	100	3	250	0	0	4
17	0	1	250	0	0	0
17	0	2	250	0	0	4

17	0	3	250	0	0	8
17	25	1	250	0	0	1
17	25	2	250	0	0	0
17	25	3	250	0	0	3
17	50	1	250	0	0	0
17	50	2	250	0	0	1
17	50	3	250	0	0	3
17	75	1	250	0	0	0
17	75	2	250	0	0	0
17	75	3	250	0	0	1
17	100	1	250	0	0	1
17	100	2	250	0	0	3
17	100	3	250	0	0	1

Appendix 3: Raw data on germinationof seeds and death of seedlings treated with *A. indica* extracted from the bark

Day	Treatment	Replicate	Number of seeds	Germinated number	% Germination	Death
1	0	1	250	19	7.6	0
1	0	2	250	26	10.4	0
1	0	3	250	27	10.8	0
1	25	1	250	17	6.8	0
1	25	2	250	18	7.2	0
1	25	3	250	8	3.2	0
1	50	1	250	10	4	0
1	50	2	250	3	1.2	0
1	50	3	250	10	4	0
1	75	1	250	4	1.6	0
1	75	2	250	9	3.6	0
1	75	3	250	5	2	0
1	100	1	250	8	3.2	0
1	100	2	250	2	0.8	0
1	100	3	250	4	1.6	0
2	0	1	250	5	2	0
2	0	2	250	11	4.4	0
2	0	3	250	7	2.8	0
2	25	1	250	11	4.4	0
2	25	2	250	8	3.2	0
2	25	3	250	7	2.8	0
2	50	1	250	2	0.8	0
2	50	2	250	2	0.8	0
2	50	3	250	13	5.2	0
2	75	1	250	3	1.2	0
2	75	2	250	3	1.2	0
2	75	3	250	4	1.6	0
2	100	1	250	5	2	0
2	100	2	250	0	0	0
2	100	3	250	3	1.2	0
3	0	1	250	12	4.8	0
3	0	2	250	13	5.2	0
3	0	3	250	23	9.2	0
3	25	1	250	29	11.6	0
3	25	2	250	31	12.4	0
3	25	3	250	7	2.8	0
3	50	1	250	4	1.6	0
3	50	2	250	8	3.2	0
3	50	3	250	30	12	0
3	75	1	250	11	4.4	0

3	75	2	250	5	2	0
3	75	3	250	10	4	0
3	100	1	250	13	5.2	0
3	100	2	250	10	4	0
3	100	3	250	8	3.2	0
4	0	1	250	8	3.2	0
4	0	2	250	25	10	0
4	0	3	250	37	14.8	0
4	25	1	250	19	7.6	1
4	25	2	250	26	10.4	6
4	25	3	250	24	9.6	5
4	50	1	250	3	1.2	0
4	50	2	250	7	2.8	1
4	50	3	250	23	9.2	3
4	75	1	250	6	2.4	0
4	75	2	250	13	5.2	4
4	75	3	250	4	1.6	0
4	100	1	250	14	5.6	0
4	100	2	250	12	4.8	0
4	100	3	250	13	5.2	0
5	0	1	250	11	4.4	0
5	0	2	250	2	0.8	0
5	0	3	250	1	0.4	0
5	25	1	250	16	6.4	0
5	25	2	250	3	1.2	6
5	25	3	250	17	6.8	0
5	50	1	250	7	2.8	1
5	50	2	250	11	4.4	0
5	50	3	250	17	6.8	10
5	75	1	250	5	2	0
5	75	2	250	9	3.6	1
5	75	3	250	20	8	2
5	100	1	250	1	0.4	2
5	100	2	250	14	5.6	0
5	100	3	250	24	9.6	0
6	0	1	250	5	2	7
6	0	2	250	53	21.2	0
6	0	3	250	10	4	6
6	25	1	250	17	6.8	3
6	25	2	250	34	13.6	0
6	25	3	250	44	17.6	3
6	50	1	250	73	29.2	11
6	50	2	250	73	29.2	16
6	50	3	250	34	13.6	18
6	75	1	250	41	16.4	3

6	75	2	250	51	20.4	5
6	75	3	250	10	4	4
6	100	1	250	45	18	8
6	100	2	250	45	18	9
6	100	3	250	34	13.6	14
7	0	1	250	0	0	0
7	0	2	250	0	0	0
7	0	3	250	0	0	0
7	25	1	250	12	4.8	0
7	25	2	250	5	2	0
7	25	3	250	0	0	0
7	50	1	250	0	0	0
7	50	2	250	4	1.6	0
7	50	3	250	15	6	0
7	75	1	250	13	5.2	0
7	75	2	250	2	0.8	0
7	75	3	250	0	0	0
7	100	1	250	0	0	0
7	100	2	250	0	0	0
7	100	3	250	2	0.8	0
8	0	1	250	0	0	0
8	0	2	250	0	0	0
8	0	3	250	0	0	0
8	25	1	250	0	0	5
8	25	2	250	0	0	0
8	25	3	250	0	0	4
8	50	1	250	0	0	9
8	50	2	250	0	0	1
8	50	3	250	0	0	0
8	75	1	250	0	0	3
8	75	2	250	0	0	0
8	75	3	250	0	0	3
8	100	1	250	0	0	2
8	100	2	250	0	0	2
8	100	3	250	0	0	0
9	0	1	250	1	0.4	1
9	0	2	250	0	0	0
9	0	3	250	0	0	3
9	25	1	250	0	0	4
9	25	2	250	0	0	2
9	25	3	250	1	0.4	10
9	50	1	250	0	0	3
9	50	2	250	0	0	15
9	50	3	250	0	0	11
9	75	1	250	1	0.4	2

9	75	2	250	0	0	4
9	75	3	250	0	0	0
9	100	1	250	0	0	6
9	100	2	250	1	0.4	1
9	100	3	250	0	0	5
10	0	1	250	3	1.2	2
10	0	2	250	0	0	2
10	0	3	250	3	1.2	3
10	25	1	250	0	0	1
10	25	2	250	1	0.4	1
10	25	3	250	0	0	1
10	50	1	250	1	0.4	1
10	50	2	250	1	0.4	1
10	50	3	250	0	0	1
10	75	1	250	0	0	0
10	75	2	250	2	0.8	0
10	75	3	250	2	0.8	0
10	100	1	250	1	0.4	2
10	100	2	250	3	1.2	0
10	100	3	250	2	0.8	1
11	0	1	250	0	0	1
11	0	2	250	1	0.4	0
11	0	3	250	0	0	2
11	25	1	250	0	0	0
11	25	2	250	2	0.8	2
11	25	3	250	1	0.4	0
11	50	1	250	0	0	0
11	50	2	250	1	0.4	0
11	50	3	250	1	0.4	0
11	75	1	250	1	0.4	1
11	75	2	250	2	0.8	0
11	75	3	250	1	0.4	1
11	100	1	250	2	0.8	0
11	100	2	250	1	0.4	1
11	100	3	250	4	1.6	0
12	0	1	250	1	0.4	1
12	0	2	250	0	0	0
12	0	3	250	0	0	0
12	25	1	250	0	0	1
12	25	2	250	2	0.8	2
12	25	3	250	0	0	1
12	50	1	250	0	0	0
12	50	2	250	0	0	0
12	50	3	250	1	0.4	1
12	75	1	250	0	0	0

12	75	2	250	2	0.8	0
12	75	3	250	0	0	0
12	100	1	250	1	0.4	1
12	100	2	250	0	0	0
12	100	3	250	0	0	0
13	0	1	250	0	0	7
13	0	2	250	0	0	1
13	0	3	250	1	0.4	1
13	25	1	250	0	0	1
13	25	2	250	1	0.4	0
13	25	3	250	0	0	1
13	50	1	250	1	0.4	0
13	50	2	250	1	0.4	2
13	50	3	250	1	0.4	0
13	75	1	250	0	0	2
13	75	2	250	0	0	0
13	75	3	250	0	0	2
13	100	1	250	1	0.4	0
13	100	2	250	0	0	0
13	100	3	250	0	0	1
14	0	1	250	0	0	2
14	0	2	250	0	0	0
14	0	3	250	0	0	2
14	25	1	250	1	0.4	0
14	25	2	250	0	0	2
14	25	3	250	0	0	0
14	50	1	250	1	0.4	0
14	50	2	250	0	0	2
14	50	3	250	1	0.4	1
14	75	1	250	0	0	1
14	75	2	250	0	0	1
14	75	3	250	2	0.8	0
14	100	1	250	2	0.8	1
14	100	2	250	1	0.4	0
14	100	3	250	0	0	0
15	0	1	250	0	0	0
15	0	2	250	0	0	2
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15	25	1	250	0	0	1
15	25	2	250	0	0	0
15	25	3	250	0	0	0
15	50	1	250	1	0.4	0
15	50	2	250	0	0	1
15	50	3	250	1	0.4	0
15	75	1	250	2	0.8	2

15	75	2	250	0	0	1
15	75	3	250	0	0	0
15	100	1	250	0	0	1
15	100	2	250	1	0.4	0
15	100	3	250	0	0	0
16	0	1	250	0	0	3
16	0	2	250	0	0	2
16	0	3	250	0	0	5
16	25	1	250	0	0	1
16	25	2	250	0	0	1
16	25	3	250	0	0	2
16	50	1	250	0	0	1
16	50	2	250	0	0	2
16	50	3	250	0	0	1
16	75	1	250	3	1.2	1
16	75	2	250	0	0	0
16	75	3	250	0	0	3
16	100	1	250	0	0	0
16	100	2	250	0	0	1
16	100	3	250	0	0	3
17	0	1	250	0	0	0
17	0	2	250	0	0	1
17	0	3	250	0	0	0
17	25	1	250	0	0	6
17	25	2	250	0	0	0
17	25	3	250	0	0	2
17	50	1	250	0	0	2
17	50	2	250	0	0	0
17	50	3	250	0	0	0
17	75	1	250	0	0	0
17	75	2	250	0	0	0
17	75	3	250	0	0	2
17	100	1	250	0	0	0
17	100	2	250	0	0	0
17	100	3	250	0	0	0