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Morphological Characterization of Fungal Infestations on Road-side Tree Nursery Seedlings and the Influence of Environmental Factors on their Spread

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Abstract Road-side tree nurseries are a major source of planting materials for small-scale agroforestry and plantation programmes. However, these nurseries are potential breeding grounds for many tree diseases. Buying seedlings from roadside tree nurseries is one of the easiest routes through which these diseases get into farms. This study sampled road-side tree nurseries along several agro-ecological zones to assess fungal pathogen infestations and the influence of environmental factors on their spread. All seedlings were morphologically assessed for symptoms and the infested samples collected for laboratory analysis. Potting soil samples were analyzed for physical-chemical characteristics while sources of water for irrigation were analyzed for pH and salinity. Identifying sources of seeds and other propagation materials was done using a survey. *Fusarium*, *Rhizopus*, *Pestalotia* and *Trichoderma* were the most common fungal pathogens in all the nurseries. Leaf spot and leaf blight were the main symptoms that reduced the aesthetic quality and commercial value of the seedlings. The most affected seedlings were fruit trees but with no significant differences in pathogen infections ($P \geq 0.05$). Agro-ecological zone II and III had the highest susceptibility of pathogens probably due to humid environmental conditions. The soils used for potting were acidic with high amount phosphorus probably due to fertilizer application. Water sources had an optimum pH with a high exchange capacity. Some nursery owners (7%) planted seeds obtained from their farms rather than from certified centres. About 70% of the nursery vendors used water from the nearby rivers while 80% bought or collected soils from the forests. All nursery vendors used narrow-spectrum fungicides that were ineffective. These factors may have contributed to the infestations and spread of the pathogens among the nurseries. Various strategies are recommended for proper nursery management.

Keywords Roadside tree nurseries, Agro-ecological zones, Fungal pathogens, Environmental factors

1. Introduction

Tree seedlings are propagated, managed and grown to appropriate size in a tree nursery before being planted out in the field. These nurseries require healthy and disease-free nursery stock for sustainable management and utilization [1]. However, pathogens can easily escape through transplants into plantations or farmers' fields. Major causes of seedling mortality on-farm include poor health of the seedlings at the time of planting [2]. After trans-planting, the weak plants are immediately exposed to different environmental conditions where they struggle to cope. Poor seedlings are likely to have slower growth, less able to compete with weeds or drought and are more susceptible to damage from insects and pests [2]. Early detection of diseases in tree seedlings can facilitate their control through proper management strategies and consequent production of high quality tree seedlings of various uses. Nearly 50% of artificial forests worldwide are planted with nursery-produced seedlings [3]. Retail tree nurseries especially along the roadsides supply seedlings to potential buyers that eventually generate wood and non-wood forest products, which have immense contributions to the living

standards of humankind [4]. However, shortages of quality tree seedling constrain land restoration activities such as afforestation, reforestation and agroforestry [5].

Plant diseases limit the production of quality seedlings within nurseries [6]. This is because environmental conditions within tree nurseries such as humidity and high nutrient levels are often ideal for proliferation of disease causing pathogens [7]. Once the symptoms appear, the infection spreads rapidly and many therapeutic treatments are not often effective or become very expensive. Naturally, mature trees have evolved structural and chemical defenses against plant pathogens [8] but the virulence factors secreted have greater potency to young seedlings [9]. Fungal infections are the most common and destructive of all plant diseases. Upon infection, a plant develops symptoms that appear on different parts of the plants. Important symptoms of fungal infections include damping off, spots, rot, die-back, grey mould and needle cast [10]. These symptoms may be detected when seeds fail to emerge, after germination or when the seedlings turn woody and can be on the plant roots, stems or leaves. Some fungal inocula can stay for long period in the soil where they are stimulated by root exudates which initiate the disease [11]. With time, the diseases can spread over a larger area mainly through infected plant materials [12].

Variations in environmental factors are known to exacerbate tree nursery diseases [13] since the composition and structure of sources of infection such as soil, air, water and plant debris vary with prevailing conditions [5]. Environmental changes affect susceptibility of both the host plants by predisposing it to infection and the activity of the pathogen by providing suitable conditions for inocula germination. For instance, if environmental temperature is optimum to the host plant and unfavorable to the pathogen, the disease cycle is slowed down and vice versa [9]. Soil inhabiting fungi are more virulent to young plants when soil moisture is high since the tissues become more succulent making it easier for activated pathogens to penetrate and cause infection [7]. Host plants become weakened and more susceptible under unfavorable soil pH while the pathogen may be activated or inactivated depending on its pH requirements [14].

Nursery cultural management practices such as water sprinkling, manure application, pruning of infected plants, hand weeding and spacing significantly affect spread and susceptibility of seedlings to fungal infections [9]. Water sprinkling causes rapid spread since water facilitates the entrance of pathogen into wounds while propagation with infected plant material is another major way that pathogens are moved over great distances [10]. Other practices such as use of non-pasteurized potting media, reuse of dirty containers, lack of sanitation practices in nursery production and bringing infected plants from other sources into the nursery contribute to the spread of these pathogens [15]. Planting diseased seedlings in nursery stock can permanently infest both cultivated and native habitats with exotic pathogens and these can adversely affect the long-term health, management and sustainability of these habitats [16].

In Kenya, most retail tree nurseries are situated on roadsides for attraction of potential buyers. According to Kenya Forestry Research Institute [17], roadside tree nurseries are major sources of tree infections, which may eventually cause devastating effects on plantations, farmers' fields or forests. Although Kenya Plant Health Inspectorate Service (KEPHIS) has a core mandate to contribute to forest productivity improvement programmes by ensuring that planting material is free from pests and diseases [18], small scale roadside tree nurseries have continued to emerge, negatively affecting sustainability of such programmes. The most commonly practiced technology by the roadside is the container-grown tree nursery through seed or vegetative propagation. This technology is exposed to greater risks of disease-spread since the cultivation soil and irrigation water are often re-circulated for economic reasons [10]. However, it remains to be determined if the seedlings supplied to farmers from nurseries along the highways are diseased, and if the varying environmental conditions affect the rates of infestation, thereby creating possible conduits of infections into end-users farms. Furthermore, nursery phyto-sanitary management practices to wade off infestation and sources of seeds used to raise the seedlings are still unclear. This study therefore identified fungal pathogens responsible for road-side tree nursery diseases and determined the influence of environmental factors on their spread along agro-ecological zones for proper decision making process and improved disease management.

2. Materials and Methods

2.1. Description of the study site

The study was carried out on tree nurseries found along a highway that covers a distance of 197 Km. The highway was purposively selected because it hosts many tree nurseries and passes through six counties along diverse agro-ecological zones. The nurseries supply the greater part of Mount Kenya region with tree and fruit seedlings. The agro-ecologies along the road vary in topography, soil type, rainfall and temperature. The road was stratified based on the different agro-ecological zones according to Sombroek *et al*. [19] to ensure that all climatic zones were covered (Table 1). In each stratum, tree nurseries were samples on both sides of the road to avoid bias.

Table 1: Agro-Ecological Zones of the Study Area

Road	Zone	Ecology	Average annual temp (ºC)	Average annual rainfall (mm)	No of sampled Nurseries
Nairobi -Kenol	V	Semi-arid	20-22	450-900	4
Kenol-Sagana	IV	Semi-humid to semi-arid	18-20	600-1100	4
Sagana-Karatina	III	Semi-humid	16-20	800-1400	3
Karatina-Nyeri	II	Sub-humid	14-16	1000-1600	4
		Total samples			15

2.2. Sample collection

All the nurseries selected were described in details including the size, location, surrounding vegetation, type and number of tree species planted and the distance from the road. All the seedlings were then morphologically assessed for the presence of diseases according to Horst [20]. Nursery stock were assessed for disease symptoms, including parts affected and the severity (if the entire plant is involved or only a part of the plant).Signs of the presence of plant disease agents such as mycelia, fungal spores and spore-producing bodies were assessed. Samples were obtained from infected seedlings and preserved in khaki bags with silica gel until laboratory analysis.

2.3. Pathogen isolation

Diseased samples were surface-sterilized using 30% hydrogen peroxide for one minute and then rinsed three times in sterile water and air dried. They were then damp chambered and incubated on a laboratory bench at room temperature for 5 days to allow fungus to grow and then morphologically identified. They were then cut into small pieces, surface sterilized with 30% hydrogen peroxide for one minute and then rinsed three times in sterile water. The isolates were then transferred onto Malt Extract Agar (MEA) medium, tightly sealed with parafilm and incubated at 23°C for 5 days.Pure cultures of the pathogen were obtained by transferring hyphal tips onto new MEA for morphological description. Colony characteristics were assessed visually or with a dissecting microscope. The shape and size of conidia were determined using a compound microscope.

2.4. Soil and water samples collection and analysis

Soil samples were collected from the source. The samples were put in Khaki bags, which were then tightly sealed and labeled. The soils were then taken to the laboratory for physical-chemical analysis as outlined by Okalebo *et al.* [21]. The samples were analyzed for soil organic carbon (SOC) {Walkly-Black method}, pH (1:2 soil water suspension), % total nitrogen (N) {Kjeldahl acid digestion method} and phosphorus (P) {Olsen method)}. Water used for irrigation in the nursery was collected in clean sterilized bottles, which were then labeled and tightly sealed. The samples were taken to the laboratory for pH and salinity analysis.

2.4. Identification of the seed sources and other Propagation Materials

A formal survey of the nursery vendors was carried out using a semi-structured questionnaire. They were administered to the owner or representative. The respondents were asked about the sources of seeds, soil and

water, disinfection of tools before and after use, use of pesticides and fertilizers and sources of information on nursery management. A pilot baseline survey to pre-test the questionnaire was carried out in one of the nurseries.

2.5. Data Analysis

Data was analyzed using R- programme. One-way analysis of variance (ANOVA) was performed at $P \leq 0.05$ to show the differences in tree nursery diseases among the zones and associated plants. Mean separation was done using the least significant difference (LSD). Data for questionnaires was coded, cleaned and analyzed using MS Excel and the IBM Statistical Package for Social Sciences (SPSS) version 20 to generate descriptive statistics in form of frequencies, plots, tables and charts and lists.

3. Results

3.1. Morphological characterization of pathogens

Through morphological characterization, nine fungal pathogen species were clearly identified in the laboratory. These were species of *Fusarium*, *Pestalotia*, *Cladosporium*, *Botryosphaeria*, *Nectria*, *Alternalia*, *Trichoderma*, *Rhizopus* and *Aspergillus*. *Fusarium* was the most common among the nurseries and had the highest occurrence of 53% followed by *Rhizopus* (37%), *Pestalotia* (29%) and *Trichoderma* (17%) (Fig. 1). The pathogens with the lowest occurrence were *Aspergillus*, *Alternalia* and *Nectria*. Unidentified species of bacteria formed 5% of the pathogens. Diseases were restricted mainly on the aerial part of the seedlings. Leaves were the main parts affected with leaf spot and leaf blight as the most common symptoms observed (Table 2).

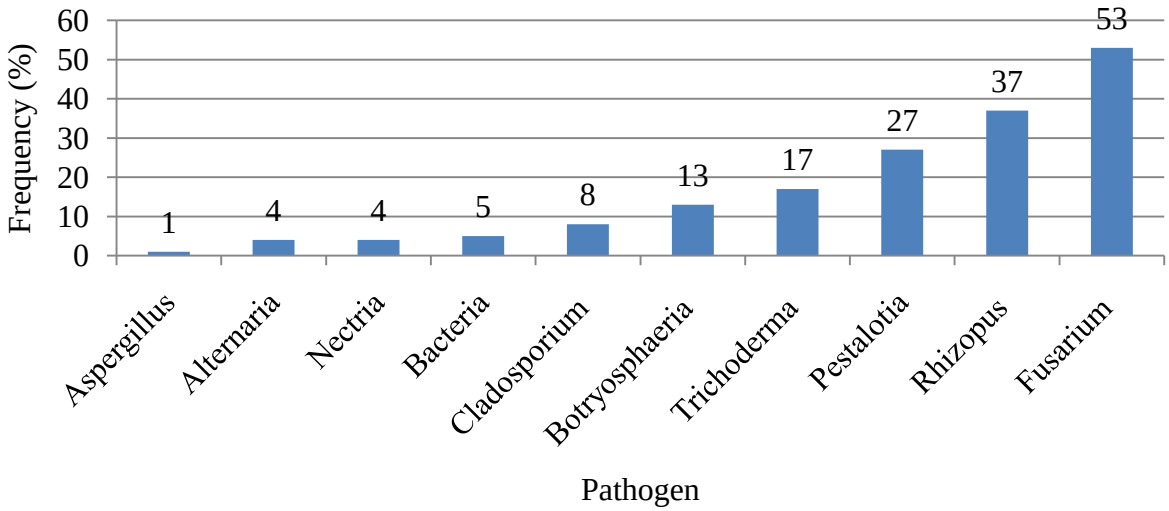

Figure 1: Pathogens Identified from the Roadside Tree Nurseries

Table 2: Pathogens identified, parts affected, symptoms and morphological characteristics

S/No	Pathogen	Parts affected	Symptoms	Morphological characteristics
1.	*Fusarium*	Leaves, stems and roots	Wilting, leaf blight, root rot	Extensive mycelium cottony-like with some taints of yellow, variable and slender spores
2.	*Pestalotia*	Leaves	Leaf spots on the leaf blade	Dark cushion-shaped mycelium, short conidiophores with hyaline pointed end cells.
3.	*Botryosphaeria*	Leaves	Leaf blight	Dark mycelium and spores; slender and hyaline conidiophores with numerous lateral branches
4.	*Cladosporium*	Leaves	Leaf spots and leaf lesions	Dark upright and branched conidiospores

5.	*Trichoderma*	Leaves and stems	Die back	White cottony mycelium with green conidia, hyaline and much branched spores
6.	*Alternaria*	Leaves	Leaf spots and leaf blight	Initial white colony then developed a shade of grey to brown
7.	*Rhizopus*	Leaves and stems	Soft rot	White mycelia and black sporangia.
8.	*Nectria*	Leaves and stems	Canker and dieback	Pink to black mycelia
9.	*Aspergillus*	Stems	Stem rot	Yellow mycelia covered by a thick layer of large black conidial heads.

Many seedlings showed symptoms of plant diseases. The most affected were fruits trees with *Malus domestica* (apple) having the highest infestation by pathogens (67%), followed by *Persea americana* (avocado), *Citrus limon* (lemon) and *Mangifera indica* (mango) with 55% each (Fig. 2). The apples were mainly affected by *Alternaria, Botryosphaeria, Fusarium* and *Rhizopus* species in all the sampled nurseries. Avocado and lemon were mainly affected by *Pestalotia, Trichoderma* and *Fusarium* species. *Cupressus* and *Ficus* were the most affected tree species by *Botryosphaeria*, *Fusarium*, *Pestalotia* and *Rhizopus* species. Other tree species were *Casualina* and *Glaveria* both only affected by *Rhizopus* species. However, the differences in pathogen infections among the trees were not significant (P≥ 0.05). Based on the symptoms, infected plants per nursery were identified and counted and they ranged from six to 29.

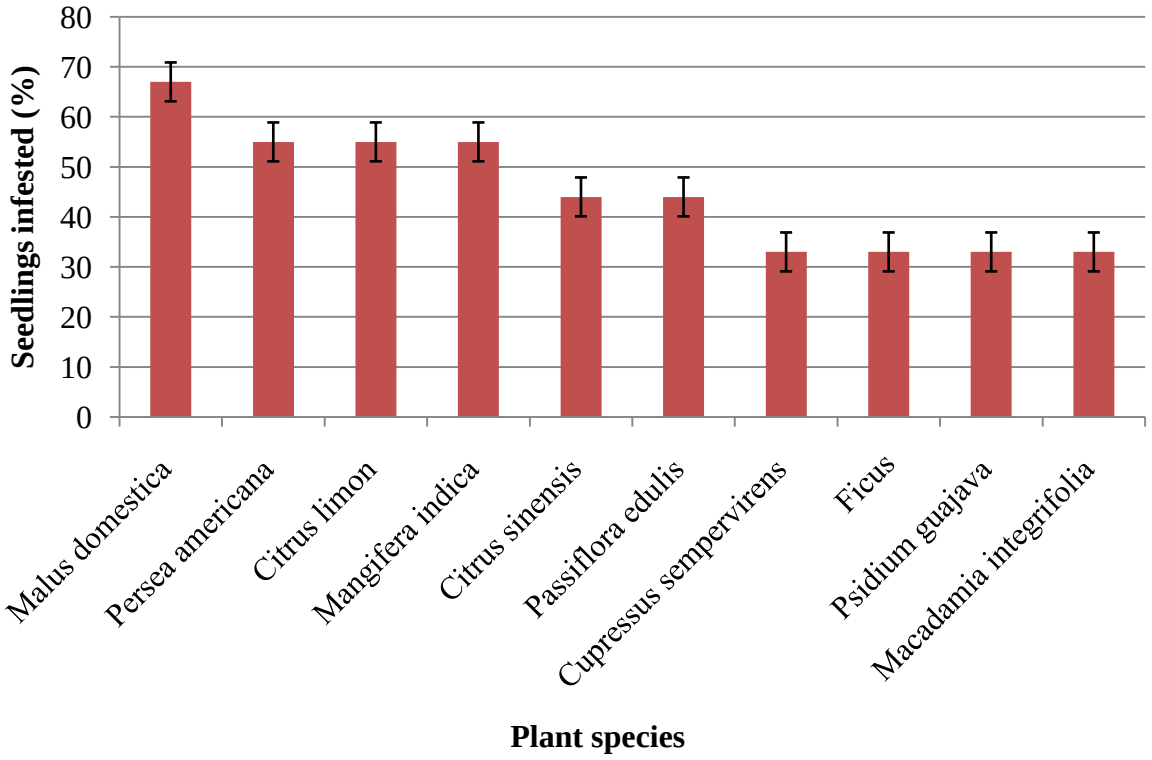

Figure 2: The Most Affected Seedlings in the Tree Nurseries

3.2. Environmental Factors Contributing to Pathogens infestations and Spread

Based on the agro-ecological zones, Zone II and III (humid zones) had the highest infestation of pathogens (34% and 32% respectively) while Zone IV and Zone V had the same prevalence of 18%. The most common

pathogen species in all the zones were *Fusarium, Pestalotia, Trichoderma, Aspergillus, Alternaria* and *Rhizopus* while *Nectria* and *Cladosporium* species were not found in the semi arid zones (Table 3). However, the differences in tree nursery diseases among the zones were not significant (P≥ 0.05).The soils used for planting had a mean phosphorus content of 27.88ppm and a mean nitrogen content of 0.16 ppm. The carbon content had a mean of 13.16ppm. They were slightly acidic (pH 6.7) with exchange capacity (EC) of 0.09 ppm. There was a linear relationship between carbon, nitrogen and phosphorus of the sampled soils (Fig. 3). There was a significant variation in water pH and EC with a mean of 7.5 and 0.27 respectively.

Table 3: The most common pathogen species present in each agro-ecological zone

Zone	Species present
Zone II (sub-humid)	*Fusarium, Pestalotia, Trichoderma, Aspergillus, Nectria, Rhizopus, Botryosphaeria, Cladosporium,*
Zone III (semi-humid)	*Fusarium, Pestalotia, Trichoderma, Aspergillus, Nectria,Rhizopus, AlternariaBotryosphaeria, Cladosporium,*
Zone IV (semi-humid to semi-arid)	*Fusarium, Pestalotia, Trichoderma, Aspergillus, Alternaria, Rhizopus, Botryosphaeria*
Zone V (semi-arid)	*Fusarium, Pestalotia, Trichoderma, Aspergillus, Alternaria, Rhizopus, Botryosphaeria*

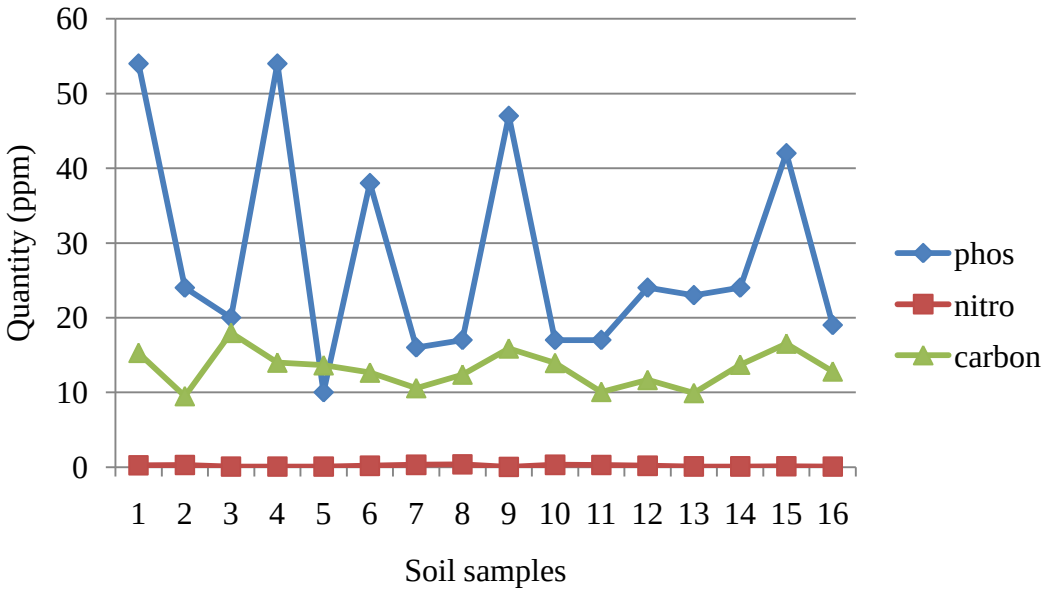

Figure 3: Nutrient Contents of the Soils Used for Seedling Propagation

3.3. Sources of Seeds and other Propagation Materials

Results of the questionnaires showed that almost all the respondents (nursery owners) sourced their seeds and seedlings from the Agrovets, KEFRI and seed centres approved by KEPHIS. They also got information about nursery management from these centres. However, 7% of the respondents replanted seeds that were harvested from their own farms (Fig. 4). On the sources of soil for planting, 20% of the respondents sourced from their own farms while those who bought the soil and also collected from the forest were 80%.While determining the sources of water used in the nursery, more than 70% of the nursery owners sourced their water from the nearby rivers. About 20% of the respondents sourced their water from the dams and piped water. In assessing the phytosanitary condition of the nurseries, none of respondents disinfected the tools before and after use. All the nursery owners used pesticides and only 13% of them did not use fertilizers. The respondents reported that seed dressing and spraying seedlings were done using narrow spectrum fungicides, which were ineffective.

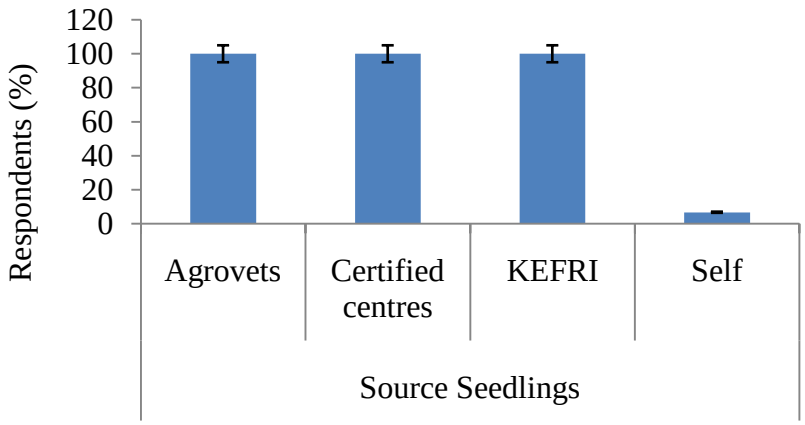

Figure 4: Sources of Seeds and Other Vegetative Materials Used for Propagation

4. Discussion

4.1. Pathogen characterization

Most of the fungal pathogens identified in this study cause diseases in nursery seedlings and affect mature tree species in Kenya. *Fusarium* was the most common in all the nurseries. The species is one of the most common causes of diseases in nursery seedlings in the world and with a wide range of environmental conditions. *Fusarium* wilt is a soil and seed-borne disease that affect many different horticultural plants and is the most important pathological problem of plants grown in artificial growing media [22]. The fungus also prefers warmer temperatures. *Fusarium oxysporium* has been reported on several tree seeds where it causes seed decay, germination reduction and seedling wilt [23]. *Rhizopus* species were also among the most common fungal species in this study. *Rhizopus* is a very-fast-growing, spreading type of mold which has white mycelia and black sporangia. The most common of the *Rhizopus* species is *R. stolonifer*. A rhizopus soft rot caused by *R. stolonifer* is reported mainly to occur in apples [24] as was observed in this study. *Pestalotia* is reported to cause leaf spots and petiole blights and attacks all parts of the leaf from base to tip [25]. Disease development can be restricted to only the leaf blade or only the petiole. In this study it was restricted only on the leaf blade. Spots begin as very small yellow, brown or black spots. Under optimum environmental conditions, the spots may expand and increase in number until they merge to form a leaf blight or rachis blight [26]. *Cladosporium* species are common in many areas of the world and are known to cause scab diseases [27]. In this study,*Cladosporium* was found to cause leaf spots and lesions. The symptoms of the disease can affect leaves, petiole, stems and fruits. The leaves turn from grey to white. The spots can be invaded by secondary rotting bacteria which cause the spots to smell [28]. Their spores can be found in air, water and soil [29]. *Alternaria* contributed to formation of leaf spots in many seedlings. It survives from crop to crop as mycelium or spores in infected plant material are left on the ground and within the seeds from infected plants. The elongated spores allow for better wind dispersal, which increases the distances travelled. Healthy plants pick up the fungal spores from the ground or in the air [30]. Under favourable conditions, the spores quickly mature into fungal leaf spots that release more spores, which can be carried to other plants by wind or water. *Nectria* on the other hard cause several common canker and dieback diseases especially in hardwood trees. The disease can cause considerable damage as the cankered area is weakened and susceptible to breakage hence reducing the quality and quantity of tree products [31].

4.2. Tree seedlings affected by the pathogen

This study encountered a total of 34 tree species infected by various pathogens. *Malus domestica* (apple) had the highest infestation of pathogens followed by avocado, lemon and mango. Several species of fungi and oomycetes including *Fusarium, Rhizoctonia,* and *Pythium* have been reported as pathogens of apple where they contribute to a phenomenon known as apple replant disease [32]. Furthermore, a survey of fungi associated with avocado found several pathogens including *Alternaria, Aspergillus, Fusarium,* and *Rhizopus* [33]. Dieback

disease affects mangoes and is caused by *Lasidioplodia theobromae*. Symptoms at early stages include general wilting, appearance of mango trees and dieback of twigs [34]. Symptoms are extremely useful in the diagnosis of a disease and identification of the agent causing the disease [20]. The most common symptom observed in this study was leaf spot and leaf blight. The spots vary in size and color depending on the plant affected, the specific organism involved and the stage of development [31].

4.3. Environmental Factors and pathogens

Zone II and III had the highest seedlings infestations by pathogens probably due to their humid conditions. These zones are generally restricted to the highlands of Kenya surrounding Mt Kenya (parts of Meru, Embu, Kirinyaga and Nyeri), between 1980 and 2700 m with a minimum rainfall of 1000 mm [35]. Since nursery plants are normally maintained under moist and humid conditions, they somehow mimick these zones and this may provide ample moisture for sporangium production. For instance, *Cladosporium* require cool, damp, weather conditions for vivid growth, sporulation, spore release, germination and disease development. These fungi are active at low temperature and high humidity [28] and this explains why this pathogen was not common in high temperature zones. Others such as *Aspergillus* are highly favoured by high temperatures (25–40°C) and high relative humidity. *Aspergillus niger* causes rapid decay and spreads very fast at 30–35°C. The most favourable conditions for *Alternaria* is heavy rain or heavy dew occurring when temperatures are between 25-28°C [27].

The soils used for potting in this study were acidic soils. Soil acidification tends to be worse in areas that are annually cropped or those that were historically forested [36]. These conditions are similar to those observed in the sampled nurseries. Almost all nursery owners used fertilizers in planting seedlings. Direct seeding can result in a stratification of soil pH in which the top few inches of soil are more acidic. This is because acidification caused by fertilizer application in the top soil layers is not diluted by mixing with the more alkaline soil below the fertilizer zone [36]. The soils also had very low nitrogen content. Plants suffering from a lack of nitrogen are weaker, slow growing and faster aging. Such plants are susceptible to pathogens that are best able to attack weak, slow-growing plants. Reduced availability of nitrogen may also increase the prevalence of *Fusarium* wilt and *Alternaria solani* and most seedlings to damping-off [37]. In the current study, the main source of water for irrigation was from the nearby rivers. Surface water supplies such as lakes, ponds, rivers and streams contain pathogens in the bottom sediment [38]. Water pH also influences disease management and is an important factor that needs to be monitored and adjusted during seedling production. Notably, pH levels greater than 6.5 can encourage the development of damping off diseases during germination and early seedling development [39].

4.4. Sources of Seeds for planting

The major sources of seeds and seedlings were from certified centres. This is a good indication of the quality of seeds planted. Seed sources should represent the best available genetic material for planting as exhibited by the parental material [40]. A seed source should be selected based on growth performance, size of the mother trees, age, health and suitability of the species in the environment where the seed is to be planted. A few of the respondents sourced seeds from their farms. This affirms the reason why some tree planting materials of trees around Mt. Kenya are of poor quality in terms of physical, genetical and physiological qualities [41]. The poor quality germplasm leads to poor performance of most trees and tree populations, and therefore provision of adequate high quality seed is critical in promoting agroforestry and tree planting in general.

4.5. Control and management of fungal diseases

Fungal pathogens are one of the most common resistant types of pathogens. They produce abundant spores that can be easily dispersed by wind and water movement, so sanitation and water management are critical [32]. Wounds or plant damage often seems to be a prerequisite for disease development, naturally or from insects, other pathogens, nutrient deficiencies or human activity. Water management requires limiting the length of time when the leaves are wet or exposed to high humidity levels [18]. This includes elimination of overhead irrigation or irrigating in the early morning hours when dew is already present [40]. Although fungal spores of seedling pathogens are ubiquitous, good sanitation potentially decreases the spore population in the nursery

[10]. In a nursery situation, severely diseased leaves should be pruned and destroyed to reduce spores available to infect healthy tissue. While fungicides may be useful to prevent further spread of the disease, they are merely a supplement to water management, sanitation, injury prevention and good nutrition [42]. Fungicides alone cannot solve the problem of fungal pathogens. It is critical to understand that fungicides do not cure the leaf spot or petiole blight already present. Once a leaf spot or petiole lesion occurs, it will remain for the duration of the life of that leaf. Fungicides are used to prevent further spread of the disease by protecting leaf tissue that has not been infected by the fungal pathogen. Systemic fungicides with post- infection activity can also be used as a protectant to prevent resistance [43].

5. Conclusion and Recommendations

Fungal pathogens are one of the most common resistant types of pathogens and their control and management is of great importance. *Alternaria*, *Botryosphaeria*, *Fusarium* and *Rhizopus* were the most common in all the nurseries and are reported to cause diseases in tree nurseries all over the world. However, molecular characterization is necessary to identify all the pathogens affecting roadside tree nurseries and relating them with the immediate environment. Leaf spot and leaf blight were the main symptoms observed and can reduce aesthetic quality and commercial value of seedlings. The soils used for planting were acidic mainly due to fertilizer application. Zone II and III had the highest susceptibility of pathogens probably due to humid conditions and low temperatures. This study recommends the following to minimize infections and spread of tree nursery diseases:

i. Use of sterilized soils for potting
ii. Use of treated water for irrigation
iii. Use of certified seeds and buying seedlings from certified centres
iv. Awareness creation on proper planting hygiene

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