SEED-BORNE FUNGAL ORGANISMS ASSOCIATED WITH GERMINATION SUCCESS OF *Terminalia brownii* (Fresen, 1837) IN DRYLANDS OF KENYA

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Master of Science Degree in Natural Resources Management of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this University or any other for the award of a degree.

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DEDICATIONS

Special gratitude goes to my parents and my siblings for all the support they accorded me. Thank you, my beloved ones, God bless. And above all, I thank the Almighty God for having been faithful in this journey.

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ABSTRACT

Terminalia brownii (Fresen, 1837) is one of the important species distributed throughout the tropical and sub-tropical regions of the world. It is highly valued in the Kenyan drylands for its products such as timber for wood carving, medicine, and charcoal production, among others. Due to this high value, followed by overutilization, its population in the drylands is dwindling. Concern regarding its regeneration capacity has been raised, as shown by low germination both in situ and ex-situ. The main aim of this research was to assess seed-borne fungal organisms associated with germination success of *T. brownii* seeds in Kenyan drylands, which will contribute to the conservation of the species and in turn improve livelihoods of the dependent communities. The specifics objectives were to analyze germination success of *T. brownii,* assess seed-borne fungal organisms associated with *T. brownii* success and compare seed-borne fungal organisms associated with germination of *T. brownii* in Baringo, Kendu Bay, and Kitui areas. Sites with a high density of *T. brownii* trees were chosen for fruit probing and maturity confirmation. Random sampling was used in selecting thirty trees, fifty meters apart, from which fresh fruits were collected from the crowns at three height levels. A complete randomized block experimental design where one hundred *T. brownii* seeds from each of the three sites, were subjected to a germination test. Seeds that failed to germinate were assessed for the presence of fungal organisms. The present organisms were cultured, DNA extracted and sequenced for identification. The results were displayed an insignificant difference between the number of seeds that germinated and those that did not germinate (N=12, P=0.0000, t=16.29), and fungal organisms identified associating with germination failure were *Fusarium equiseti*, *Pestalotia sp,* and *Alternaria alternata*. Seeds that germinated were also tested for fungal infestation and *Penicillium sp* were found to be present. A Paired t-test between the number of seeds that did not germinate, and the number of fungal infected seeds gave N=12, a p-value of 0.000, t=-8.78. Fungal organisms associating with the germination success of *T. brownii* do not significantly affect the germination of freshly extracted seeds from the field, hence for maximum germination achievement, seeds should be sown while still fresh.

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LIST OF ABBREVIATIONS AND ACRONYMS

CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organization
ICRAF	International Centre for Research in Agroforestry (World Agroforestry Centre)
IUCN	International Union for Conservation of Nature
ISTA	International Seed Testing Association
KEFRI	Kenya Forestry Research Institute
KFS	Kenya Forest Service
MEA	Malt Extract Agar
NaCl	Sodium chloride
NaOAc	Sodium acetate
SDGs	Sustainable Development Goals
TBE	Tris Borate Edta
PCR	Polymerase chain reaction
rDNA	Ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
RAPD	Random Amplified Polymorphic DNA
ITS	Internal transcribed spacer
PCR	Polymerase Chain Reaction
CIA	Chloroform and Iso Amyl
TOC	Total Organic Carbon

CHAPTER ONE

INTRODUCTION

1.1 Background information

Terminalia brownii (Red pod *Terminalia*) belongs to the family Combretaceae comprising more than two hundred species distributed throughout the tropical and sub-tropical regions of the world. Approximately fifty species of *Terminalia* are naturally distributed in western, eastern, and southern Africa. These species, which are semi-deciduous range from small shrubs or trees to large deciduous forest trees (Beentje et al., 1994; Gibreel et al., 2013). Tree species heights in the Terminalia genus range 20m to 30m high with a densely layered crown of drooping foliage.

Many *Terminalia* species are drought tolerant occurring in woodlands, bush lands, and wooded savannah within arid and semi-arid regions. They are also found more often along river valleys in dry areas. Species in the genera *Terminalia* include several indigenous trees and shrubs mainly in Africa, such as *T. brownii*, *T. spinosa*, *T. prunioides*, *T. kilimandscharica,* and *T. mollis*, from East Africa; *T. superba* and *T. ivorensis* from West Africa and *T. catappa* which is cultivated in coastal areas as an ornamental (Beentje et al., 1994). *T. brownii* is native to Eritrea, Ethiopia, Kenya, Somalia, Sudan, Tanzania, and Uganda.

Species biodiversity is one of the important aspects of any ecosystem. Consequently, species in the genera *Terminalia* have their unique roles in the ecosystem and thus the need to conserve them. Practices that have been geared towards the conservation of this species, include on-farm planting. In Uganda, it is a good agroforestry tree (Okullo & Waithum. 2007) while sections with *Terminalia species* have received protected area status under Yemen law (Hall et al., 2008). *T. brownii* species are also being used in the rehabilitation of drylands in West Pokot, Kenya (Kitalyi et al., 2002). The species are left on land during habitat conversion into agricultural lands, mainly because of the medicinal value (Njoroge et al., 2010). In Ethiopia, the species is being afforded highest conservation in Babile elephant sanctuary (Belayneh et al., 2011), and it is also being conserved in a programme dealing with synergizing conservation and adaptation to climate change in Kitui Kenya, (Eriksen et al., 2006).

T. brownii is not listed among endangered species in Kenya, and hence it is not in the IUCN Red List of Endangered plant species (IUCN Red List Committee, 2013). However, its populations are reported to be reducing in Kenya due to selective harvesting for various uses such as timber, charcoal production, woodcarving, house construction, fencing poles and posts, hand-tools, for medicinal purposes, dye and tannin making among others (De Leeuw et al., 2014; John et al., 2015). This reduction in the number of *T. brownii* trees in its natural habitats in Kenya (Mutinda, 2014) is also being exacerbated by the few numbers of seedlings available in private, and public nurseries. The few numbers are due to the unexplained poor seed germination and low germination percentage.

Reports according to Bewley (1997) and Yongesha (2005) indicate that seeds may fail to germinate due to poor pollination and synchronization of flowering in dioecious plant species or when harvested before maturity. Additionally, poor handling during harvesting, processing, and storage may also lead to poor seed germination. Seeds also may fail to germinate due to unfavorable conditions such as sowing media, temperature, and day length or because they are dormant (Baskin & Baskin, 2004; Turner et al., 2005). Seed infested by insects and diseases may also fail to germinate.

Seed-borne diseases are those alterations that arise either from the internal or external parts of seeds, carried from the mother tree, affecting survival of tree seedlings at their initial stages of growth. Diseases are generally caused by pathogens, fungi, bacteria, viruses, and nematodes. They affect plant growth, and it has been reported that its only fungi that attack seeds, interfering with germination and establishment (Cram & Fraedrich, 2010). Some signs of the pathogens include fungal fruiting bodies in fungi, bacterial ooze in case of bacteria, and nematode cysts for the nematodes.

Terminalia seeds have in the past reported to heavily been infested by disease-causing organisms and insect pests (Mosango, 2013). For example, seeds collected from Kitui were reported to be highly infested by insects (Mosango, 2013), while those from other drylands of Kenya have not been researched. As such, there has been a need to work on ways of promoting the propagation of *T. brownii* before it becomes threatened or endangered. Whereas Kenya Forestry Research Institute (KEFRI), together with its stakeholders such as the World Agroforestry Centre (ICRAF) and the Kenya Forest Service (KFS) has been researching appropriate spacing and management practices for efficient propagation of this

species (KEFRI, 2016), little research has been done on disease-causing organisms that are attacking the species in the field. Consequently, *Terminalia* continues to exhibit a poor germination percentage and is on the verge of being threatened as its numbers dwindle. Therefore, this study sought to establish if fungal organisms affect the germination of *Terminalia brownii* seeds and the emergence of the seedlings.

1.2 Statement of the problem

The germination percentage of extracted *Terminalia brownii* seeds is 30%, which is low (Mosango, 2013), leading to few seedlings in the government and private nurseries for its regeneration. The population of *T. brownii* has been reported to be reducing drastically to the extent that it might become endangered, due to overutilization of timber and charcoal in the dry land regions, yet its regeneration is hindered by low germination. The role of seed-borne fungal organisms associated with germination success of *T. brownii* is not well understood. This study, therefore, sought to assess the role of seed-borne fungal organisms in the germination success of *Terminalia brownii* in the drylands of Kenya.

1.3 Objectives

1.3.1 Broad objective

To assess seed-borne fungal organisms associated with germination success of *T. brownii* seeds in Kenyan drylands, to contribute to the conservation of the species which will, in turn, improve livelihoods of the dependent communities.

1.3.2 Specific objectives

i. To analyze germination successes of *T. brownii* seeds.

ii. To assess seed-borne fungal organisms associated with *T. brownii* germination success

iii. To compare seed-borne fungal organisms associated with germination failure and germination success of *T. brownii* in the three sites.

1.4. Hypotheses

i. Germination successes of *T. brownii* seeds is not high.

ii. There is no association between seed-borne fungal organisms and germination failure of *T. brownii* seeds.

iii. There is no relationship between seed-borne fungal organisms associated with germination failure and germination success of *T. brownii* in the three sites.

1.5. Justification

Human beings interact with natural resources daily for their survival. For example, they rely on natural resources such as water for domestic and commercial use, wildlife for tourism, forests for timber, among others. In the drylands of Kenya, local communities derive various uses from different tree species. *T. brownii* is one of the indigenous species growing in dryland areas with numerous benefits such as providing medicine and wood for carving industries. This has made the species highly preferred over other *Terminalia* species such *Terminalia prunoides* and *Terminalia spinosa* growing in the same area. The uses have led to higher rates of extraction of the species, more than it can regenerate, leading to a reduction in the population of the species. One of the reasons for low regeneration was reported to be physical dormancy, which was solved by extraction attaining 30% (Mosango, 2013).

In determining whether fungal organisms found affecting flowers and fruits could be associating with the germination success of the seeds of *T. brownii* through this research, valuable information on how to carry out seed treatment has been generated. The information is necessary for the management and control of the fungal organisms by treatment, which will help increase *T. brownii* seed germination percentage and reduce seedling losses. Seed treatments on the other hand should only be used when the gain in germination and seedling survival is greater than the potential loss to avoid negative impacts of seed treatments such as damage of seeds, seed toxification in the case of chemical treatments, which should also be used with caution (Cram & Fraedrich, 2010). Hence, such discoveries on the specific type of organisms infesting seeds help in making such specific decisions on whether to use chemicals or not and the specific appropriate chemicals to be used.

The research contributes to the attainment of Sustainable Development Goal (SDG) number 15, and in achieving Kenya's National Forest Programme goal which is to develop

and sustainably manage, conserve, restore and utilize forests and allied resources for socio-economic growth and climate resilience**.** In addition, these findings address Kenya's constitution chapter 5 part 2 (1a) which touches on management and conservation of the environment and natural resources, and Kenya's vision 2030, social pillar, on a clean and safe environment.

1.6 Scope and limitation of the study

1.6.1 Scope

Terminalia brownii is widely distributed in the Kenyan drylands but the research was carried out on seeds collected from three provenances, namely, Ndumoni in Kitui, Kimose in Baringo, and Gendia in Homa Bay counties, from November to December 2018. These three provenances were selected because they represent the different agro-ecological regions (along with the Lake Region, which is III, eastern and Northern Kenya which IV) where *T. brownii* is naturally distributed in Kenya (Useful trees and shrubs in Kenya). In Kenya, *T. brownii* is found in deciduous woodland, wooded grassland, and riverine vegetation. It grows well in Agro-ecological zones IV and V, with an altitudinal range of 600-2,000 m. with a mean annual rainfall of 500-1,300 mm in deep, sandy soils, and is widespread on loam soils (Orwa et al., 2009).

The research focussed specifically on fungal organisms, identifying them at the species level, as most studies have reported that its mostly fungi associating with germination successes of tree seeds.

1.6.2 Limitation

Extracted DNA stored for an extended period undergoes false amplification results. This was avoided by immediate amplification of the extracted DNA samples, and remaining samples stored at 3^0C in a fridge.

1.7 Assumption

Seed samples collected captured all the fungal organisms which were associating with the *T. brownii* seeds. This was done by collecting the fruits from three sections of the selected sample collection trees, and sites chosen from the different ecological zones of species' provenance in Kenya.

1.8 Operationalization of terms

Dioecious: Plants with female and male reproductive flowers parts on separate trees.

Germination failure: Imbibition and lack of registered radicle and or shoot growth.

Germination capacity: Total number of seeds that germinate from viable seed lot when exposed to suitable conditions over a given time.

Germination: Protrusion of radicle and plumule from the seed coat.

Physical dormancy: Dormancy caused by one or more water-impermeable layers of palisade cells in the seed coat.

Provenance: The original geographic locality of a stand of trees (source) from where the seed is collected

Viability: The capacity of the seed to germinate and produce a normal seedling under optimum conditions

Seed-borne organism: Organism growing from the internal or external part of seed structures.

Culture: Provide suitable media for fungal organisms' growth

CHAPTER TWO

LITERATURE REVIEW

2.1 The genus *Terminalia*

The genus *Terminalia* is found in the family of trees Combretaceae and is the second largest genus in the family comprising of twenty genera and 475 species (Thiombiano et al., 2006). Species in this family range from shrubs, trees, and deciduous tree of heights ranging from 1.5 to 75m tall, and they are distributed throughout tropical and sub-tropical regions (Schmidt et al., 2002). *Terminalia* is distributed in western, eastern, and southern Africa (Smith et al., 2004). Most species in this genus are propagated by seeds or natural vegetative methods such as plant stumps, wildings, striplings, and seedlings. Some of the identified uses derived from the species in this genus include medicinal value, spiritual value, social and economic benefits in terms of timber for carpentry, joinery, plywood manufacture, and flooring (Schmidt et al., 2002; Smith et al., 2004).

A review on species found in this *Terminalia* genus by (*Chapter 1 Literature Review Terminalia Spp in Africa with Special Reference to Its Health Status*, n.d.) identified that many insect species have been reported to associate with the species. The insects do not contribute to the spreading of pest problems except for dangerous species reported to be potentially affecting the species at initial stages of the plant such as flowering and fruiting. However, some feed on the seed leaving them hollow and thus recording no germination. Few studies have been done on disease-causing organisms on these species, despite the species being proven to be potential hosts for many fungal pathogens. The research was done on some of the disease-causing agents and the organisms identified morphologically, but were not identified at the species level (*Chapter 1 Literature Review Terminalia Spp in Africa with Special Reference to Its Health Status*, n.d.).

Flowers of the species in this genus are pollinated by insects from the following genera: *Coleoptera, Diptera, Hemiptera, Hymenoptera,* and *Lepidoptera* (*Chapter 1 Literature Review Terminalia Spp in Africa with Special Reference to Its Health Status*, n.d.). Some of the diseases identified to be affecting the species include those that attack the roots where we have *Armillaria mellia* reported in Ghana (Coetzee et al., 2000). Other fungal species found affecting the tree species' roots include *Rosellinia* and *Phytopthora*. Stem diseases on the other hand include dieback and canker caused by *Spaeronaema* spp and

Endothiella spp reported in Ghana too. Another stem canker has been reported to be identified in India, a pink disease caused by *Erythricium salmonicolor* (Thomson & Evans, 2006). The Disease *Rostraureum tropicale* has also been identified to be associated with the dying T. ivorensis in Ecuador (*Chapter 1 Literature Review Terminalia Spp in Africa with Special Reference to Its Health Status*, n.d.).

Diseases causing organisms genera identified so far to be affecting Terminalia species' leaves include *Cercospora, Ramularia, Irenina,* and *Spaceloma* which have been reported to be affecting *T. superba* in Africa. Some of the disease-causing organisms identified so far *Korinomyces terminaliae* causing leaf spots in younger seedlings and young plants on *T. ivorensis,* and *Auerswaldiella parvispora* which aused black botches on the leaves of the tree in Brazil. *(Chapter 1 Literature Review Terminalia Spp in Africa with Special Reference to Its Health Status*, n.d.) Other disease-causing organisms found attacking debris of fallen *T. superba* are blue stains caused by *Lasiodiplodia theobromae.* The fungus is reported to be capable of spoiling the lustrous creamy white color of the *T. superba* log even if the sheen of the wood is altered.

2.2 *Terminalia brownii* (Fresen, 1837) species

2.2.1 Ecology and Biology of *Terminalia* species

In Kenya, *T. brownii* is found in deciduous woodland, wooded grassland, and riverine vegetation. It grows well in Agro-ecological zones IV and V, with an altitudinal range of 600-2,000 m. The annual rainfall range for this species is 500-1,300 mm, and deep, sandy soils, with a widespread on loam soils (Orwa et al., 2009).

The inflorescence of the tree species is axillary spikes 9.5-12 cm in length, peduncle 1.5-2 cm long, tomentose. Each inflorescence contains female and male flowers, the male ones towards the apex and female ones towards the base. Inflorescences are long with white to cream flowers, 0.5 mm wide, glabrous, calyx lobes acuminate, unpleasantly scented. Fruits are woody samara containing one and rarely two oblong, delicate seeds inside (Beentje et al., 1994; Mosango, 2013). In Kenya, *T. brownii* trees are found growing with other species such as *T. Spinosa*, *Tamarindus indica*, *Acacia* spp, *Balanites* spp, among others.

A screening trial of some indigenous and Australian *Acacia* tree species at Loruk in Baringo, Kenya (mean annual rainfall of 500 mm), reported a mean annual increment of 0.65 m in height for *T. brownii* within the first three years of establishment. Out of the twenty-eight tree species comprising of 10 Australian *Acacia* spp. and twelve indigenous tree species, *T. brownii* had a remarkably high survival of 97 % within two years of establishment (Kimondo, 1991). These trials though preliminary, clearly indicate the growth potential of *T. brownii* in the dry land which is yet to be exploited.

2.2.2 *Terminalia brownii* (Fresen, 1837) population status

The numbers of this tree species have been reported to be reducing, because of few seedlings in various nurseries in drylands of Kenya where the tree does best (Okeyo et al., 2020). Farmers in Gendia, village located in Kendu Bay, who had seedlings of this species in their nurseries identified the problem to be low germination percentages from the fruits they were using to raise seedlings (Okeyo et al., 2020). Alternatively, farmers depended on the wildlings of the tree species as they preferred it to raising the seedlings from collected fruits which is time-consuming. This has, however, been solved by the idea of extracting the seeds from the fruits using secateurs, in which the hard fruit endocarp was hindering the faster emergence of seed embryos. The number of seedlings of this species in the surveyed nurseries ranged from 30 to 50, against hundreds of other species. Seedlings of this species thus were being sold at the prices of KES 30 to 50 per seedling, against KES 10-20 for other species in the nurseries.

Farmers in Ndumoni in Kitui on the other hand did not know extraction of seed from the fruit. They waited for the seeds to germinate from fruits on their own, after which they transplanted the seedlings to their preferred sites. A similar case was noted in Kimose, where there were no attempts at all in line with propagating the species by use of seeds as the residents lacked information seed extraction from fruits.

The Kenya Forestry Research Institute Centre in charge of the Northern drylands situated in Baringo also reported propagation of seeds using fruits, and that germination percentages were incredibly low. Seedlings emerged from the planted fruits after 3 months and at low percentages of below 10%. This translates to the incredibly low population of *T. brownii* being established yearly compared to other species in restoration or establishment of

forest plantations. The main contributing factor as noticed in Kendu Bay's case is lack of planting materials, as the sources such as wildlings in the range and seedlings raised in nurseries are scarce.

Despite the reported low germination cases of this species on farms, it is one of the species preferred for wood carvings over other species and thus is over-utilized in Eastern Kenya. Mutinda (2014) reports that the species is also among those whose status stands as having been wiped out and not found in places such as Wamunyu-Machakos, in Eastern Kenya Other species that have been reported to be wiped out in the area include *Olea europaea, Terminalia spinosa, Afzelia quanzensis, Dalbergia melanoxylon,* and *Brachyleana huillensis*. Species associating with this species which were reported to be diminishing also include *Combretum schumanii, Balanites aegyptica, Rhamnus staddo, Boscia angustifolia, Prunus africana, Ficus natalensis, Spirostachy africana, Croton megalocarpus, Acacia seyal, Adonsonia digitata, Lannea scheifurthii,* and *Tamarindus indica.* To add to that, *T. brownii* is being preferred especially in Machakos County as its workability is preferred over other species in the genera *Terminalia* (Mutinda, 2014). This, therefore, helps in justifying the reduction in the population of the species as it is being over-utilized.

2.2.3 Conservation status of *Terminalia brownii* (Fresen, 1837)

There are efforts that have been geared to conservation of this species. On-farm planting in Uganda where it is being adopted as an agroforestry tree for ex situ conservation (Okullo & Waithum. 2007). Increased encroachment of forests by the expansion of agricultural land through forest clearing and conversion of the forest into plantations for timber has led to diminishing access to woody resources. This, therefore, led to the adoption of on-farm woody plants (OFWPs) adaptable to specific agro-ecological regions. Some of the benefits derived from the on-farm woody plants included goods and services such as poles, shade, fruits, and medicine (Okullo et al., 2007). The species were integrated or conserved on farms by directly planting the seeds or stem cuttings on the farms and raising other tree seedlings in a tree nursery. However, there are some tree species that are challenging in propagating using seeds. These species were therefore left on farms for natural regeneration. Species in this category included: *Tamarindus indica Erythrina abyssinica, Combretum molle, Acacia hockii, Terminalia brownii, Combretum collinum, Vangueria apiculate, Acacia*

senegal, Albizzia coriaria, Piliostigma thorningi, Grewia mollis, Acacia tortilis, Vitellaria paradoxa Balanites aegyptica, Kigelia africana, Balanites orbicularis, Bridelia micrantha, and Teclea nobilis. Terminalia brownii is one of the listed species with no information on its propagation, therefore studies should be carried out on how its maximum germination can be achieved (Okullo et al., 2007).

In another conservation effort, a forest in Saud Arabia was under threat and a section of it was put under conservation in the valley forest. This later was reported to be the home of several regionally rare species (Hall et al., 2008). Tree species discovered to be conserved in the forest include *Ficus ingens, Breonadia salicinawith Ficus sycomorus, Acacia commiphora Ficus vasta, Acacia asak, Commiphora myrrha, Acacia mellifera, Adenium obesum, Berchemia discolor Combretum molle, Mimusops laurifolia, Trichilia emetic, Actiniopteris radiata, Commelina erecta, Aneilema forskalei, Tamarindus indica* and *Terminalia species.*

Continued conservation works have been reported in Yemen where a forest also received protection status under Yemen law (Hall et al., 2008) to promote species existance. *T. brownii* species were among the prominent species in the area recorded. Some of the other prominent species in the forest included: *Combretum molle (Combretaceae), Carissa spinarum (Apocynaceae), Grewia schweinfurthii (Malvaceae), Ruellia patula (Acanthaceae), Cissus quadrangularis (Vitaceae), Pavetta longiflora (Rubiaceae), Acacia asak (Leguminosae), Phoenix caespitosa (Arecaceae), Tamarindus indica (Leguminosae), Mimusops laurifolia (Sapotaceae), Cissus rotundifolia (Vitaceae), Mimusops,* and *Acalypha fruticosa (Euphorbiaceae).*

In Kenya, *T. brownii* species has been used in the rehabilitation of drylands of Kenya, especially in Northern parts (Kitalyi et al., 2002). Characteristics considered in choosing tree species for the rehabilitation process included: medicinal value, shade for both human and livestock, deep tree roots extract nutrients from the deep layers of soil, wood for construction fuel and charcoal, cultural conservation, scenery, and landscape beauty leaves as vegetables for human consumption, nectar source from the flowers for bee foraging, litter for improving soil fertility and fodder for animals. These characteristics were therefore the gate pass for a species to be conserved. Some of the tree species that were conserved during the rehabilitation in the drylands of Kenya, therefore, include *Balanites aegyptica, Acacia*

tortilis, Terminalia brownii, Zizyphus mucronata, Kigelia africana, and Grewia bicolor (Kitalyi et al., 2002). They are also used to mark boundaries of farms as they are preferred indigenous tree species in the region.

A study by Njoroge et.al. (2020) identified medicinal values preferred mostly by the Kamba community in Eastern Kenya. Among them were *Albizia amara, Aloe secundiflora, Acalypha fruticose, Salvadora persica, Zanthoxylum chalybeum, Warbughia ugandensis, Terminalia brownii, Azadirachta indica, Ricinus communis, Ficus sycomorus, Ajuga remora. Croton megalocarpus, Commiphora aerythrea, Carissa edulis, Albizia anthelmintica, Verninia amygdalina,* and *Agave sisalana*, *T. brownii* being one of them. Some of these have been over-exploited leading to concerns of conserving them before their extinction. These important trees are left on land during habitat conversion into agricultural lands, mainly because of the medicinal values which are well recognized in Eastern Kenya (Njoroge et al., 2010).

Reduction of vegetation cover and degradation of species have been reported to be at an alarming rate and are some of the most serious environmental issues affecting humans today (Belayneh et al., 2011). This is a result of the increase in population as more land is converted to agricultural use. Due to this, a sanctuary was established to promote conservation measures of some tree species in Ethiopia in an area known as Babile elephant sanctuary. Species conserved in this sanctuary were identified per floristic composition, plant community types in which *Terminalia brownii* was classified as one of the dominant species in a community that grow between 1250 and 1350 m a.s.l. Other classifications were based on floristic diversity, floristic similarity, basal area, and important value indexes wood species density. Species found in community five, especially the dominant ones had high evenness and diversity but less species richness. *T. brownii* is, therefore, one of the few species being afforded the highest conservation in Babile elephant sanctuary (Belayneh et al., 2011)

Efforts towards the restoration of woody vegetation species in Ukambani and Maasai land were implemented using technologies and approaches that ensured success in rehabilitation in the chosen areas (John et al., 2003). This is a step that was taken to address the pressure that arose from migration to the drier parts despite its ecological conditions. This led to the degradation of vegetation and soils thus calling for the need to rehabilitate the area.

The restoration programme by John et al. (2003) initially proposed to perfect the rehabilitation tools' intervention had the following three objectives namely to: establish methods of seedling management and tree establishment, ii) select, develop and promote high-value timber and food tree that would fit into alternative livelihood commodity development options, iii) improve the capacity of extension service and farmers in dryland forest rehabilitation. Other aspects included to develop a social forestry extension model for arid and semi-arid areas and establish restoration possibilities for degraded woodlands, soils, and soil moisture regimes. High-value timber and fruit trees were also used in this rehabilitation programme and this included: indigenous *Melia volkensii* proposed for its fast-growth and resistance to termites, slow-growing *Dalbergia melanoxylon* for its highly valued wood for carving, *Terminalia brownii* for its good form, and resistance to termites, *Senna siammea* an exotic species which was selected for its fast growth and thus quick provision of fuel wood, *Mangifera indica* for its fruits, and *Citrus sinensis* for its adaptability and fruits.

Another method that was used in the conservation of the woody species was the natural restoration of the degraded woodlands which were exploited due to overgrazing, and charcoal production. This was due to difficulty and the slow recovery of vegetation. An area was then excluded from grazing for a minimum of two years, and some of the species that were closed are of *Commiphora, Terminalia,* and *Acacia* which showed signs of regeneration immediately. This is based on a passive ecological approach in the restoration of an area where the species are left to regenerate on their own without any external silvicultural support. This, therefore, benefits from factors such as shading, best light requirement achieved on their own as well as soil conditions being restored, and vertical structure with original species achieved on their own.

2.2.4 Germination performance of *Terminalia brownii* (Fresen, 1837)

Terminalia brownii fruit is a samara, and the covering is reported to be hard thus making it difficult to extract the seed from the fruit. This makes the germination percentage to be low, as the embryo in most cases fails to find its way easily out of the endocarp. Another reason based on the uses of the seed parts is that the micropyle is not able to absorb water easily for the germinating embryo. Before invention of the extraction of seeds from fruits, this species used to be propagated at first by the maintenance of wildlings found where

the mother plants existed and transplanted for fair establishment rates at nurseries. This was then followed by transplanting them back to the field in appropriate sites after successful seedling establishment (Mosango, 2013). This is because the rates of survival in the initial stages in the field are low compared to when raised in a nursery, thus this ensured maximum transition from the initial stages to the sapling stage.

The moisture content of this species at harvesting time is usually about 32%. However, the seed being orthodox needs to be dried first before storage and sowing to a moisture content of about 10% to 12%, depending on the temperatures and humidity of the air. This recommended storage temperature, however, has a disadvantage which is that seed-borne fungal organisms that thrive well in such temperatures get the best opportunity to attack the seeds quickly.

An initial cutting test is also recommended to be done on randomly picked one hundred fruits to confirm the viability of the fruits. This is done by cutting the fruits in the middle and checking for fruit emptiness, shriveled seeds, insects' infections, or any symptoms of fungal structures. An approximate number of above five viable seeds out of ten is a good go-ahead for seed collection. This is easily achieved if the fruit is dried well in the sun, hardening the samara, hence secateurs penetrate easily into them. This is effective when the test is first done on seeds found on the forest floor immediately after the species drops the fruits and the fruits have not overstayed on the floor. This helps in minimizing external cases such as seed-borne fungi getting to infest the sample seeds which could also contribute to germination failure.

Nipped *T. brownii* fruit at the proximal end was then recommended to be put facing upwards on the seedbed during sowing and covered with a centimeter of sand or soil around the fruit. This ensures that the plumule faces up and the radicle down, hence hastening germination. Results were best achieved in seeds that were dusted by the fungicide captan, compared to the undusted ones, suggesting that the seeds are susceptible to attack by fungi and various insects (Mosango, 2013; Specht et al., 2005)

According to Specht et al. (2005), germination of this species is reported to be irregular and slow, leading to poor germination results. Some of the proposed methods include pretreatments methods applied to the fruits. This involves the use of mechanical, water, or acidic pretreatment that would soften or open the hard and sturdy endocarp without

damaging the target young plant which is the embryo. Some of the proposed methods include:

- Soaking in water for about seven days or hot water and left to cool overnight at room temperature.
- Stratification in which the seed is subjected to damp sand for about 30 days in a refrigerator of about 7^0C.
- Mechanical stratification where the fruits are rotated in a concrete mixer together with sand for some hours which helps in removing the wings and nipping the radicle end by crosscut forming the V-shaped which is done by secateurs.
- Burning using grass.
- Soaking in chemical solutions such as potassium nitrate, hydrogen peroxide, and concentrated sulphuric acid for about 30 minutes

However, the untreated seed sample did not produce a single germinant, and that most of the pretreatments involved such as burning, mechanical, soaking in water resulted in only a few germinants. The only treatment among the proposed methods that produced acceptable results of 25% to 55% was of soaking in sulphuric acid for an hour and on those that were nipped in V shape.

Despite the indicated treatments above, *Terminalia brownii* germination performance has been reported to be low (Omondi 2011; Mosango 2013) in which those collected from Kitui attained a better percentage of 80% only after being separated from the highly insect-infested seeds. However, from other sites which were Embu and Makueni, the seeds germination still recorded a range of 25%-30%. The insects are being identified for a better solution. However much has not been done on the disease-causing organisms whereas those on insects are already being done. Insects greatly infest seeds in Kitui (Mosango, 2013).

Recent studies of disease-causing organisms that could be affecting the seeds at their development stage of flowering which could be leading to poor germination have been carried out. Some of the fungal organisms that have been found associating with the floral phenology of the species identified at genus level include *Aspergillus sp*, *Botryosphaeria sp*, *Cladosporium sp*, *Fusarium sp*, *Pestalotia sp*, *Rhizopus sp*, *Trichoderma sp,* and *Penicillium sp* (Okeyo et al., 2019). The disease-causing organisms showed constancy in the various

development stages of the fruits which ranged from the flowers, fruits, immature, and mature fruits. This, therefore, confirmed that the effect of fungi could have been transmitted by the seeds to germination of the seeds.

Interest in this species also as an agroforestry tree has led to the interest in detailed research on disease-causing organisms which could also be contributing to its low propagation, by attacking the floral phenology. The organisms that have been identified so far are *Alternaria sp*, *Aspergillus sp*, *Botryosphaeria sp*, *Cladosporium sp*, *Epicoccum sp*, *Fusarium sp*, *Pestalotia sp*, *Rhizopus sp*, Rot fungi, and *Trichoderma sp* (Okeyo et al., 2019), with pathogenicity test confirming the signs of *Botryosphaeria sp*, *Aspergillus sp*, *Fusarium sp*, *Pestalotia sp,* and *Cladosporium sp*.

2.3 Factors that contribute to species loss or extinction.

Plant species can be become vulnerable or extinct depending on internal and external factors (Chen et al., 2014). The internal factors include reproduction, seed viability, plant biological characteristics which will enable it to survive, or failures in heritability and adaptability of the species that qualify to survive in the changing environment. Eternal factors on the other hand include human and natural factors (Chen et al., 2014). These natural factors include topography in which environment that has under gone changes will influence the type of the species that will survive in an area; soil determined by its components in which if it cannot support certain species, the species will be wiped away, the ecological environment where the species in the neighboring vicinity have a great influence on the adjacent species in terms of species competition for nutrients, water, and even light, for example, a climate where any changes also lead to wiping out of certain species not used to the prevailing climate, together with other biological factors found in the newly created ecosystem.

Natural factors, for example, contribute to the determination of species existence where many rare and endangered species of plants are only found in a narrow area with a specific special microclimate. Other species occur in some temporary habitats with the required characteristics and fragmented habitat, or areas described as ecological islands.

Human factors on the other hand include land-use types, to begin with. Here, changes are encountered and these in the end affect the species composition thus altering the ecosystem, leading to loss of certain species sensitive to such changes. The second human-

induced factor is roads, with the same effect in altering the habitat. Roads also introduce other external factors such as materials used in its construction thus altering soil composition in the area. Lastly is human disturbance. This entails a lot, including the first two discussed factors alongside habitat encroachment, deforestation, overutilization without putting into consideration regeneration of the over-utilized species and introduction of alien species. These alien species impact negatively in many ways where they compete for resources such as nutrients and producing allelochemicals that affect growth and survival of other species in their vicinity. The human-induced factor of environmental pollution also alters the environment that the species thrive well thus compromising their existence. Most of these factors have therefore been reported to be impacting negatively on the identified rare and endangered plant species in general (Chen et al., 2014)

It takes a long time to notice the diminishing numbers of tree species. This is because one must concentrate on the specific species and follow up on the population's status and other factors such as its population structure. The International Union of Conservation of Nature (IUCN) gathers data regarding the population of various species, both plants and animals, and groups them into nine categories. The categories are data deficient, least concern, near threatened, threatened, vulnerable, endangered, critically endangered, extinct in the world, and extinct. Various data are therefore needed to beef up the grouping of the species in any of the groups (IUCN Red List Committee, 2013).

Scatz (2009) argues that plant diversity will in the future impose a profound impact on the human population than any other biodiversity loss. As such, species conservation should be considered to protect the future for the coming generations. Some of the main causes of biodiversity loss include habitat fragmentation, deterioration of the specific environments, the introduction of exotic species, and over-exploitation of the species. An example is in the case of *Butia eriospatha*, a threatened palm species in Southern Brazil. In addition, lack of recruitment and mortality has been documented to be accounting for almost 50% reduction, and this calls for the implementation of effective policies (Nazareno & Dos, 2014). Deforestation on the other hand has been reported to be contributing much to the extinction of local species. Other causes of decline in species populations include firstly, hunting and taking of the species out of their habitats. Secondly, the introduction of diseases giving rise to some emerging infectious diseases whose solutions have not been identified thus risking wiping species fast and easily. Thirdly, environmental pollutants alter environment

composition. Lastly, lack of appropriate prey for predatory species and habitat loss destroys the habit thus affecting the survival of the species in question as they will not have their conducive environment (Scatz, 2009).

2.4 Sampling of fruit trees for the germination test.

Trees for seed collection should be more than fifty individuals to results in the greatest proportion of alleles (genetic codes specifying certain traits) present in the field population, while still being practical. However, thirty can be used in fully outbred sexual species, or fifty-nine randomly chosen trees in self-fertilizing species. This will help in capturing all alleles. The samples, of not more than 20%, are then to be collected from all parts of the crown top, bottom, sides of the selected trees. Selected trees should be at least 150ft (50m) apart (Luna & Wilkinson, 2009), which ensures that the same disease-causing organisms are not collected and that the risk of carrying all infested seeds is reduced.

2.5. Seed germination and emergence

2.5.1 Seed growth

There are two basic units of production in plant life, and they are through seedlings or vegetative propagation where we have cuttings and use of tissues, among others. The seed being the focus in this study is defined as the embryonic plant enclosed in a seed coat with endosperm that is produced by the mother plant (Tsedaley, 2015). The young plant, which is the embryo sources its energy for growth from the endosperm to enable it to germinate and emerge from the seed coat. Seeds’ main germination requirements are oxygen absorbed from the environment, the warmth that can also be altered in an incubator or cotton and any other covering materials, and moisture absorbed from the media which needs to be watered. This will trigger the radicle’s protrusion out to look for more requirements for continued development. The root will then source nutrients needed, which then will stimulate the growth of the shoot for the continued growth.

Detailed functions of the parts of the seeds which are necessary for development therefore are: First, embryo comprising of radicle and plumule which gives rise to the young plant thus initiating germination process. The second one is the endosperm which stores food for the

embryo's development. The third one is the seed coat which protects the embryo and everything in it from external injuries. Forth is the hypocotyl which connects the plumule and radicle for translocation of what the root will have absorbed and vice versa after photosynthesis will have kicked off. Fifth is the cotyledon which becomes the first leaves to initiate photosynthesis. Sixth is the hilum which controls water content in the seed at later stages of seed formation and micropyle which allows water into the seed during germination. All these once affected interfere with the germination process.

2.5.2 Seed germination and emergence

Seed is said to have started germinating on the onset of uptake of water by the seed which is confirmed by the visible sign of penetration of radicle from the structure surrounding it (Bewly, 1997). What follows next depends majorly on the storage reserves of the seed, and other internal factors such as the effect of seed-borne diseases. Germination occurs in three phases. In the first phase there is uptake of water, followed by a plateau in which water is not absorbed. Then there is a final stage where water is absorbed for elongation of the embryonic axes which is thus visible.

Seeds on the other hand undergo dormancy after the first stage, where water is not absorbed for the completion of the third phase of germination (Bewly, 1997). This is where the internal seed-borne fungi affect the seed for the last time and thus during the last phase of water uptake, the seed does not continue germinating. Dormancy is addressed by species and thus treatments are not standard. *T. brownii* fruits were reported to be exhibiting physical dormancy, in which seed germination rate registered a significant increase when they were extracted from the fruits (Mosango, 2013), and soaked in water for 24 hours. Solution to this, therefore, has been agreed to be extraction of the seeds from the fruits, followed by soaking in cold water for 24 hours as for those that were soaked for the recommended time got destroyed.

Developments that follow onwards are therefore dependent on the energy reserves of the seed, which is followed by elongation of the shoot from the seed coat. This confirms the halting of the process when the food reserve gets destroyed by other competitors. The seed germination at this stage will be in the last stages of consumption of the reserves and thus the root will commence its work of water uptake for continued development of the seedling.

Emergence is thus regarded complete on protrusion of the whole embryo from the seed coat, which again is subject to external factors that might bring everything to a halt. The first step of the seed germination is said to be successful on completion of these two steps.

However, there are other factors that contribute to the failure of germination such as internal ones like diseases and insect infection which act both externally and internally. Other external factors are hard seed coat and right germination conditions. Germination conditions are hence addressed by the provision of the required factors and the process just commences. These factors are collectively grouped as factors affecting seed viability.

2.5.3 Factors affecting seed viability

A seed can be rendered not viable when it is still in its dormancy period or is dead. Dormancy could be endogenous (originating from inside, related to the seed embryo), or exogenous, related to seed coat or surrounding tissues. Endogenous could be because of physiological or morphological conditions of seed embryo, or both. Exogenous on the other side could be as a result of physical, chemical, or mechanical characteristics of the seed coat or fruit (Fraedrich, 2001).

Dead seed can be a result of bacterial or fungal disease infection. A pathogen can establish on and inside seeds during the development of the seed. Fungal propagules get into the plant system at any stage of flowering thus infection of seeds by pathogens can occur in all phases of seed production. These infections could be i) by the destruction of the petals of flowers, thus interfering with pollination, ii) during fertilization where the pathogens destroy the male and female parts of the flowers, iii) after final fruit production while still on the tree or when it falls onto the ground with the fungi inoculum, iv) and during collection, processing, transit, and storage (Gure et al., 2005). The seed-borne pathogen spreads to seeds before germination and can result in germination infection (Fraedrich, 2001).

There are a variety of seed-borne pathogens which include a viruses, bacteria, nematodes, and fungi, but the most pathogenic and destructive organisms have been noted to be fungi. Fungi can be carried on the seeds internally or externally, and thus have the potential to cause disease in either seeds or the developing plants. Pathogenic fungi can therefore be classified as those that infect seeds internally and destroy the endosperm and the embryo or contaminate the seeds and affect seedling germination and development. Some of

the seed-borne fungi are *Aspergillus spp*., *Mucor spp*., *Penicillium spp*., *Rhizopus spp*., and *Trichoderma spp* which have been reported to be reducing germination of conifers in some laboratory tests (Fraedrich, 2001). Research on disease-causing organisms that could be affecting germination of *T. brownii* seeds has not been carried out, except for those associating with its floral phenology.

2.6 Seed-borne fungi.

2.6.1 Eukaryotic group of seed borne Fungi

These are a group of eukaryotic non-photosynthetic organisms that reproduce both sexually and asexually and have no chlorophyll. The yeast cell or mold filament is surrounded by a true cell wall. Some are dimorphic in which they exist in two forms of mass of yeast cells or a filamentous mat of mold. These micro-organisms have two types of nutrition which are parasitic and saprophytic. They also reproduce in various forms both sexual and asexual, where sexual means include isogamy, anisogamy, heterogamy, gametangial copulation, and somatogamy.

On the other hand, asexual means by use of fission, budding, fragmentation which then ends in either arthrospore, sporangiospores, aplanospores, conidiospores, arthrospores or oidia, chlamydospores, and blastopores. These microorganisms can be classified into genera as Myxomycetes, Phycomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes. Most of the seed-borne fungi are thus found in the group ascomycetes, which reproduce by conidia (Gure et al., 2005).

2.6.2 Effects of seed-borne fungi on seed germination

Seed-borne diseases mainly affect the seed in two ways which are either internally where the endosperm or embryo is destroyed or externally where the seed is simply contaminated on the seed coat (Fraedrich, 2001). Symptoms of these disease infections can also be in two forms. The first form is where they can affect the seed at the pre-emergence causing reduced emergence or death of radicle. The second form is where fungi on the seed coat cause root rot, cotyledon rot, basal stem rot after seedling emergence at the post-emergence stage (Franke et al., 2014). Emergence is marked successful upon the appearance

of cotyledon, as the shoot will have finally gotten out of the seed coat (Masangwa et al., 2017)

Results by Solorzano and Malvick (2011), showed that seed-borne fungi affected germination of the seeds, and upon treatment by a phytoextract, the seeds managed to germinate. Another seed-borne related study is to do with cotton, in which a diverse number of seed-borne fungi associating with the cottonseed have been identified (Khatun et al., 2018). Some of the identified effects of the borne fungi on seedling early growth include no or reduced pre germination where the radicle isn't produced, interference of germination after radicle protrusion, and development of abnormal seedlings (Gure et al., 2005). Aspergillus has also been identified to be producing aflatoxin, hence hindering germination.

Some of the other genera of pathogenic fungi that have been reported to be affecting the seed hindering germination completely include those of Fusarium. Others like *Alternaria sp* and *Pestalotia sp* have also been discovered to be affecting seedling growth after protrusion of the radicle in which they affect the root tips thus barring further germination (Gure et al., 2005).

Seed-borne fungi effects on germination have also been identified in other species such as maize and wheat, where there are similarities in the type of species. A related study was carried out in identifying some of the seed-borne fungi associating with germination of rice in Bangladesh and they included: *Aspergillus niger, Fusarium sp, P. oxalicum, R. stolonifera, Aspergillus flavus,* and *C. lunata*. The highest germination failure was recorded on seeds with the highest prevalence of various seed-borne fungi recorded. Other seed-borne fungi that have been identified to be associating with germination successes include *Alternaria alternata*, *Bipolaris sorokiniana*, *Fusarium avenaceum* which were successfully managed by supernatants Epichloe (Li et al., 2017).

2.7 Tree seed-borne fungal organisms

Tree seeds are not exceptional of the effect of the seed-borne fungi as seen in crops. The same way seed-borne fungi affect seeds of crops is the same way some of the identified fungi affect tree seeds. These seed-borne diseases have been classified per their effects which are: those that are pathogenic to germlings only, those that are pathogenic to both seeds and

emerging seedlings, those less harmless to seeds and seedlings, and those that are germination promoters (Gure et al., 2005).

Some of the fungi that have been identified to be dangerous to tree seeds as per studies in line with tree seed-borne fungal pathogens include specific species of *Fusarium sp*, *Aspergillus sp*, *Pestalotia sp*, *Cladosporium sp,* and *Trichoderma sp* (Lezcano et al., 2015). Seeds being the most propagation materials are therefore the most agent for the spread of diseases to farms, and eventually to crops in cases of agroforestry trees. Effects of the spread of these seed-borne diseases have been emphasized whereby the consequences are not only seen on the population of the affected species, but it causes irreversible effects in agricultural systems, as trees and crops must in one way or another interact with the crops nearby, in an ecosystem.

2.8 Seed health testing

Seed health testing is the determination of the freedom of seeds from pathogens. The term seed health, therefore, is used to determine the presence or absence of fungi, bacteria, viruses, nematodes, and insects (Tsedaley, 2015) which contribute in one way or another in affecting the health status of the seeds. This can be done by visual examination of the seeds internally or externally, microscopically, or macroscopically, and by incubating the seeds on agar or moist blotter papers followed by identification of the pathogens microscopically. Seed testing is important for various purposes in that they are: a) for determination of the quality of the seeds, b) in the provision of a basis for price and consumer, c) discrimination among a group of seed lots and the source, d) determination of the origin of a seed problem which therefore guides and gives leads on ways of finding the corrective measure of addressing identified problem, e) and also helping in conservation or protecting of certain species in long run, both in the wild and also in agroforestry. This precaution helps in preventing the spread of diseases in agroforestry system as seed-borne fungal diseases can easily spread to crops integrated with tree species which has been affected.

2.8.1 Pathogen determination techniques.

There are several ways of determining pathogen but the standard ways that have been approved include:

- Incubation test

  The seeds are inoculated on agar or blotter test under specific conditions and pathogens allowed to grow, which are then identified using their features such as length, form, and size, and septation.

- Inspection (Direct examination)

  This is by identification of fruiting bodies of the fungi, such as smut balls and fungal spores under the stereomicroscope, or by identification of fungal pathogens on the physical appearance of the seed.

- Seedling symptom test/blotter test

  This is a type of germination test, where seeds can be germinated in conditions favorable for fungi growth, and the effects and symptoms of the fungi on the germination of seeds are monitored per seed. Symptoms could include mycelium and fruiting bodies. On the other hand, germination could be suppressed deliberately to allow the pathogen to grow.

- Agar plate method

  This involves placing seeds onto sterile potato dextrose or malt agar, which encourages the growth of the seed-borne fungi. This method can be employed to determine quantitatively or qualitatively the species composition. In this technique, there are two methods of determining the pathogens which involve the dilution method and direct plating method. The latter method has been identified as the best in determining the composition of grain as to genera and species. Bacterial colonies do inhibit fungal growth and so in this procedure, antibiotic streptomycin is added to autoclaved agar medium after it cools to around 500c to 55oc, and acidic media added to reduce bacterial contaminants.

- Embryo count method/Embryo examination method

Dry seeds are examined by naked eyes and at a magnification of 10 to 30 times that reveal the count of pathogens. Staining methods are also used in this procedure for those pathogens that cannot be detected by direct examination or incubation method.

- Immunoassays/Enzyme-Linked Immunosorbent Assays test

  This is where an antigen to a specific protein in the pathogen is added to the sample in the test and reaction between the two reflects colour change which indicates infection. DNA fungal organisms' identification can be identified using this method.

- Molecular methods

This involves polymerase chain reaction in which the pathogen's DNA is amplified, followed by electrophoresis separating DNA into varied sizes and then stained for comparison with known samples. Further identification of a species then includes the next step of DNA extraction, polymerases chain reaction, DNA sequencing and lastly analyzing for identification.

2.8.2 DNA extraction

A cell contains various proteinous organelle materials inside it and the in membranes. These organelles have got different functions such: i) nucleus for DNA storage, ii) ribosomes for protein synthesis, iii) Golgi apparatus responsible for modification and export of proteins, iv) lysosomes for protein destruction, v) cytoplasm which houses the organelles, vi) mitochondria to produce energy, vii) vacuoles, viii) plastids, ix) rough endoplasmic reticulum for protein production, x) smooth endoplasmic reticulum for lipid production. The nucleus organelle is the one responsible for housing chromosome, while the chloroplast contains some genes for photosynthesis (Kelly, 2013)

DNA extraction, therefore, is the process by which the deoxyribonucleic acid (DNA) is separated from these other cell components. The phosphate group of DNA makes it highly negatively charged, and the charge is stabilized by the magnesium in the cell when unwound. The negatively charged DNA chromosomes are coiled around histones which are negatively charged thus forming nucleosomes. Therefore, for the DNA to be separated successfully from these other cell organelles and components of the nucleus, general three steps are involved

which are lysis of the cell, separation of DNA from other cell components, and isolation of DNA (Butler, 2012).

There are four methods of DNA extraction:

- Differential extraction is mainly applied in DNA extraction from an animal cell.
- Organic method, or chloroform/phenol procedure. This is reported to be labour intensive and yields a remarkably high and exceptionally clean double-stranded DNA sample, as it involves the use of multistep liquid processes.
- Silica methods or inorganic chelax. This is a simple and cheap one-tube extraction in which Magnesium iron in the reagent binds to resin beads and yields only the needed single DNA product.
- Solid extraction methods. In this, the DNA binds to paramagnetic or silica beads and that is how the DNA strands are obtained.

Butler (2012) also reported the last two procedures.

DNA of woody plants has been proven to be difficult to extract because of the polysaccharides and polyphenols. Due to this complication, woody plants' DNA extraction process must include some reagents such as isolation buffer, lysis buffer, L sarcosine, CTAB solution, TE saturated phenol, PCI which is the TE saturated phenol of CIA 24:1, Ethanol Isopropanol or ethanol, and sodium acetate (NaOAc) (Hanaoka et al., 2013). However, the basic method for DNA extraction involves the use of isolation buffer, CTAB solution, CIA of chloroform, and isoamyl alcohol at a ratio of 24:1, 3M of sodium acetate, and 70% of ethanol (Hanaoka et al., 2013). This is done in various methods which involve mixing the reagents and centrifuging them at various temperatures and different periods till when the DNA is obtained.

2.8.3 Polymerase Chain Reaction (PCR)

This is an in-vitro nucleic-based method, where primers are detected, and nucleic acid enzymes amplified. In this process, small primers are annealed to specific DNA sequences in the target organism chromosome DNA or RNA, and directly amplified for millions of copies of the target sequence. Amplified DNA can then be seen after electrophoresis in buffered agarose gel (Tsedaley, 2015). Fungal organisms through this process can therefore be

identified at the species level by performing sequence analysis of internal transcribed spacer/5.8S ribosomal DNA (rDNA), which are the ITS (Badotti et al., 2017). This internal transcribed spacer has been identified to be with the highest probability of correct identification (PCI) of numerous fungal lineages (Badotti et al., 2017).

Despite ITS spacer being identified with the highest PCI, as per the results of Badotti (2017), it has been discovered not to be effective in the identification of the organism in the *genera Atlernaria, Penicillium, Fusarium, Aspergillus, Cladosporium,* in the International Nucleotide Sequence Database (INSD) which are GeneBank, EMBL, and DDBJ. This, therefore, led to the development of another separate gene bank specifically for the fungal sequences which are User-friendly Nordic ITS Ecto mycorrhiza (UNITE) database. This gene bank mainly aims at unifying fungal taxonomic identification and correcting any annotation associated with the taxonomic names (Badotti et al., 2017). Apart from the developed UNITE gene bank for efficient fungal sequences annotation, there is also NCBI which has been proved to be efficient too for fungal identification and these two have minimized problems of reliability and technical quality varying significantly. This is because only 50% of fungal species could be annotated at the gene banks and some cases have been reported to be more than 10% fungal ITS sequences, being annotated incorrectly at the species level. Another gene bank apart from the above-mentioned one is the Barcode of Life Data system (BOLD). This however is not preferred in the identification of fungi as it underrepresents the sequences.

The approved and accepted DNA barcode for Fungi is the rDNA ITS region. ITS is recognized as a fungal barcode because it is the most sequenced region of fungi and is routinely used for systematics, phylogenetics, and identification (Badotti et al., 2017). This has been used successfully in the identification of fungal organisms by various researchers, some being the work of Gure et al. (2005) and Machua et al. (2016).

One of the precautions in this process is that one must be keen in making the master mix, in which the solution should be closed as quickly as possible to avoid non-specific replication of non-genes which would lead to failure of the process of sequencing. This will be facilitated by the external temperature, as this should be carried out in a thermocycling machine. When preparing the electrophoresis gel which is organism-specific, combs should

be used to create the bases of the DNA samples. Buffer provides the electrons which will expel the negatively charged DNA. Electrophoresis should then be done in a covered box.

In summary, polymerase chain reaction involves four steps in which involve thermal cycling consisting of repeated heating and cooling of reaction. The steps are:

- Denaturation. This is where the sample mixture is heated to a temperature of 95ºC of about 5 minutes for a polymerase that requires heat activation. This is followed by further heating at a longer period which will then separate the DNA template by breaking the hydrogen bonds found between complementary bases resulting in single strands of the DNA molecules.
- Annealing. The temperature here is lowered to about fifty -65 ºC and time reduced too., which allows the primers for forward and reverse primers to bind onto the single DNA templates denatured in the first step.
- Elongation. Here, the temperature is then raised to about 72ºC depending on the polymerase used, and the polymerase attaches to each priming site and synthesizes new strands of DNA complementary to the initial DNA templates from denaturing phase. This is achieved by adding dNTPs that are the DNA templates.
- Final extension and elongation. This step is carried out at a temperature of 72 ºC for about 15 minutes to make sure that any remaining single-stranded DNA is fully extended.

For the process to be successful, at least two primers must have annealed successfully in the forward and reverse annealing (Hanaoka et al., 2013). Each of these primers on the other hand operates at different temperatures and times but all are set for thirty-five cycles. Some of the primers include restriction fragment length polymorphism (RFLP), Simple Sequence Repeat (SSR), Expressed Sequence Tag-SSR (EST-SSR), Cleaved Amplified Polymorphic Sequence (CAPS), Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Diversity Arrays Technology (DarT), TaqMan and Snapshot (Hanaoka et al., 2013).

This polymerase chain reaction is followed closely by electrophoresis, which is a method that separates macromolecules (DNA, RNA, and proteins) based on their size and charge (Hanaoka et al., 2013). This method applies the technique of the properties of like

charges repelling and unlike charges attracting one another. This is seen where the buffer used in preparing the gel is to provide electrons which will, in turn, repel negatively charged nucleic acid (DNA or RNA), to positive sides in the electric field of the gel. Most used reagents are agarose or acrylamide (Hanaoka et al., 2013), as the macromolecules move in them with ease. Smaller molecules are reported to move faster. Buffers used in the preparation of this gel mainly are two which are TAE (Tris/acetate/EDTA) or TBE (Tris/borate/EDTA). Stains or dyes added in this gel also mainly are ethidium bromide, SYBR Green and SYBR safe which are always light sensitive.

2.8.4 DNA sequencing

The PCR products are sequenced for the identification of the sequences. This can be in two ways, which are sanger or next-generation sequence (NGS).

- Sanger sequencing/Chain termination method

This involves the incorporation of chain-terminating dideoxynucleotides by DNA polymerases during the polymerase chain reaction step for DNA replication (Stranneheim & Lundeberg, 2012). This is made complete by reliance on capillary electrophoresis. It involves denaturation of the DNA double strands, single strands of the DNA annealed to oligonucleotide primers, the product is then elongated using dNTPs which provide the needed bases of Arginine (A), Cytosine (C), Tyrosine (T), and guanine (G) which builds the new double-stranded structure. After this, a small quantity of chain-terminating dideoxynucleotide is included for each of the bases. Sequencing will therefore proceed with the dNTPs until ddNTPs attach, as the two have equal chances of attaching to the sequence which also terminates at varying lengths.

These ddNTP of ddATP, ddGTP, ddCTP, ddTTP have got fluorescent markers. These ddNTP, when attached to the elongating sequence, will be able to display the sequences in form of colours, where T is shown by red, G is shown by black, and C is shown by blue and as indicated by the green fluorescence. A laser from an automated machine is used to read the sequence in which the machine detects the fluorescent intensity that is translated into a peak (Stranneheim & Lundeberg, 2012).

Some of the benefits of this technology, therefore, are that mutations or small deletions/duplication can be detected, and so primers can be created to cover several regions

of interest. It is therefore the best in sequencing specific genes or when fragments of specific genes are needed. Its limitation on the other side is that the process is costly compared to multiplex testing systems, specifically NGS.

- **Next-Gen Sequencing (NGS)**

The principle used in this method also relies on capillary electrophoresis as in the case of Sanger sequencing. In this method, a genomic strand is fragmented, and the bases are identified by emitted signals when the fragments in the study are ligated against a template strand. One of the differences between NGS and sanger is that Sanger uses different steps in sequencing, separation, and detection, while NGS employs array-based sequencing which combines techniques developed in Sanger sequencing to process millions of reactions in parallel. This, therefore, results in remarkably high speed thus reducing the cost. This is mainly for genome sequencing. The general steps followed in this method are library preparation where random fragmentation of DNA is used in creating libraries and then followed by ligation with custom linkers.

The second step is amplification in which the library created is amplified using clonal amplification methods and PCR. The third and last step is sequencing where the DNA is sequenced using one of several different approaches. The different approaches include the following: i) pyrosequencing where a complementary strand is synthesized in presence of a polymerase enzyme. This approach detects the release of pyrophosphate when a nucleotide is attached to the DNA chain instead of the addition of the chain terminator as in the case of Sanger sequencing. ii) iron torrent semiconductor sequencing where the DNA strand complementary to the target strand is synthesized one base at a time. A semiconductor chip then detects the hydrogen ions produced. dNTP is incorporated into a new strand if the two are complementary with the nucleotide on the targeted strand, which if successfully added, hydrogen ions are produced, iii) Sequencing by ligation (SOLiD). This is an enzymatic method that uses DNA ligase to ligate double-stranded DNA strands. PCR is used in the process to amplify the DNA primer binding region and then deposited onto the glass surface.

Other NGS methods include reversible terminator sequencing (illumine), 3'-O-blocked reversible terminator, 3'-unblocked reversible terminator, micro electrophoretic method, hybridization sequencing, and real-time observation of the single molecules (Dey P). The most widely used gene is 16SrRNA, best known for phylogenetic marking (Srinivasan

et al., 2015). Some of the advantages are that they are cost-effective and done at a remarkably high speed and throughout. Disadvantages are that the RNA (rRNA) is predominant, and it, therefore, limits detection of more informative messenger and regulatory RNAs (Wheeler et al., 2018). This method is used to screen more samples cost-effectively and in detecting multiple variants across the targeted area of the genome.

2.9 Studies on pathogenic fungi

Seed transmitted pathogens are mainly fungi compared to bacteria, viruses, and nematode transmissions. Various species of fungi are associated with seed germination of various seeds, some being pathogenic and others nonpathogenic. Some are associated with the seeds but rarely produce symptoms such as *Rhizopus stolonifer*; *Cladosporium sphaerospermum* and *Aspergillus wentii* (Lezcano et al., 2015). Others identified in the study that showed symptoms include *P. expansum*, *A. flavus*isolate, *A. niger, F. oxysporum*, *Trichoderma* sp. and *Pestalotia* sp, with *Penicillium expansum* showing the highest rate of symptoms of infections. From this, we can conclude that some pathogens are easily detected from the symptoms they produce, while others are not easily detected but can be revealed by some conventional testing procedures.

Pathogens survive in seed because they can tolerate dehydration especially for the storage of seed-borne fungi, mainly known as xerophilic fungi and examples include *Cercospora, Curvularia, Drechslera,* and *Stemphylium.* Other fungi are hydrophilic which are constantly dependent on humid environments such as *Peronospora manshurica, Sclerophthora macrospora, Phytophthora parasitica* var. *sesame,* and *Sesamum orientale.* Activities of these various fungi are accelerated by environmental conditions, and climatic changes that have been discovered to be contributing significantly to the rates of their activities.

The term pathogenic means disease-causing whereas its opposite is non-pathogenic. Some of the pathogenic fungi include *Fusarium oxysporum*, which is soil borne and is reported to be causing root rot or inducing wilt thus affecting germination of seeds. Biocontrol for the pathogen was proposed in which its nonpathogenic stains can be used in controlling the pathogenic strain, whereby a competitive strategy is adopted in which the non-

pathogenic strain competes for nutrients and infection sites thus hindering the pathogenic strain from affecting the seed (Serig, 2011).

2.10 Importance of detection of seed-borne pathogen infestation and management

The detection of seed-borne pathogenic fungi and diseases affecting seeds is an important aspect of disease management. Determining the presence of seed-borne pathogens allows managers to apply the appropriate controls or modify management practices to avoid the problem in the future. Seed-borne pathogens can also be present on seeds without obvious disease symptoms or signs. The presence of pathogenic fungi on seeds is most often determined through laboratory culture and identification, which will be useful in determining management strategy.

Strategies for the management of seed and seed-borne diseases focus on the prevention of disease spread and contamination reduction. The type of problem and the causal agent determine the applicability of various pest management approaches. Generally, no single method will provide complete control of any specific seed-borne disease; control is best achieved through an integrated pest management approach (Cram & Fraedrich, 2010).

2. 11 Drylands in Kenya

Kenya as a country experiences varied weather patterns in different areas. It is divided into agro-ecological zones ranging from AEZ I to AEZ IV, which are experiencing different weather patterns. Agricultural ecological zones of the ranges 1V, V, VI, and V11 are sub-humid zones that are classified as drylands (Pratt et al., 1977; Sombroek et al., 1982). The differences are majorly on the amount of rainfall received in the regions, with a small section of the Country in the Rift valley region and central area recording the highest rainfall, while most parts being classified as arid and semi-arid regions.

Classification of an area as dryland is determined by the climate experienced and in this case, they receive erratic rainfall, as they are characterized by inconsistent low annual rainfall that ranges between 100-600 mm (Pratt et al., 1977). Alternatively, Food and Agriculture Organization, FAO, classifies regions having crop growing days ranging from 1-74 days as arid, and those experiencing 75-119 days as semi-arid regions. Muok et al. (2007) further describe dryland to be regions characterized by hot and dry unfavorable for

conventional forestry, with cloudless sky and daytime temperatures which range between mean minimum temperatures of 16-20^{0}C, and maximum mean of 18-28^{0}C. Rainfall precipitation received in these regions is therefore low, erratic, and distributed poorly with maximums being around 750mm. The rains occur in one or two peaks around April or May, and November or December which are the long and short rain seasons respectively in the semi-arid regions.

Kenya comprises arid and semi-arid areas which cover about 80% of its total land surface and hold approximately 25% of the human population. These regions are reported to be unique and thus require special attention to strengthen not only the economic base of the inhabitants but also the national economy through all possible ways. Apart from that, they also offer the greatest potential for intensifying afforestation in achieving the proposed objective of 10% tree cover.

Despite ASAL regions being frequently stressed by drought, they are rich in biodiversity and have the potential to provide marketable commodities on a sustainable basis such as edible fruits, aloe, and resins, charcoal, gums, essential oils, silk, fruits, timber, and honey. However, due to population pressure, there is migration from wetter regions and medium potential areas into the drylands resulting in degradation of the tree/forest resources. The specific woody vegetation in these arid and semi-arid areas provides useful cover to the state of fragile and highly erodible soils in the regions, shelter for people and livestock in the harsh environment, and habitats for wildlife. In as much as the few trees in these regions can supply the commodities on a sustainable basis, much effort needs to be put into the conservation of the species native to the area, as the regions comprise the bigger part of Kenya.

2.12 Policies and legal frameworks on conservation of plant species

United efforts towards conservation and ecologically sustainable use of natural resources worldwide started with World Conservation Union in 1948, which changed its name to the International Union for Conservation of Nature in 1956. From this, the convention on biological diversity treaty was then agreed upon. The treaty's three main objectives are: Conservation of biological diversity, Sustainable use of its components and fair and equitable sharing of the benefits arising out of the utilization of genetic resources. The sharing includes appropriate access to genetic resources and appropriate transfer of relevant technologies, considering all rights over those resources and to technologies, and

having appropriate funding. Currently, there is a draft policy and law relating to the conservation of plant species in Kenya: National Forest policy 2014, and Forest Conservation and Management Act, act no 34 of 2016, respectively.

2.12.1 National Forest Policy 2014.

Part four of the policy addresses sustainable forest management of indigenous forests. Indigenous forests in this chapter's policy, represent some of the most diverse ecosystems found in the country. They supply important economic, environmental, recreational, scientific, social, cultural, and spiritual benefits. However, many of these forests have been subjected to land-use changes such as conversion to farmlands, urban centers, and settlements, reducing their ability to supply forest products and serve as water catchments, biodiversity conservation reservoirs, wildlife habitats, and carbon sinks.

Sustainable multiple-use and management of forests is critical. Where appropriate, forest management should include adequate provisions for wildlife conservation. The Government will manage all indigenous forests for water and soil conservation, provision of forest goods, and services and biodiversity conservation. Policy statements and one of the eight government goals to achieve this sustainable forest management is to promote ex-situ and in-situ conservation of forest genetic resources.

Under the key challenges facing forestry development in the policy, some of the factors mentioned are namely: small genetic base of the tree crops, low investments in technology development, poor investment in forest-based industry, climate change, and the emerging pests and diseases. The proposed suggestion from this, therefore, is that research and development should major in basic disciplines such as forest productivity health, crop diversification, value addition, intellectual property rights, indigenous knowledge, crop diversification, and health of the forest. This research, therefore, contributes information on the health of the tree species which is a concern that affects its regeneration.

Objectives of the policy on the other hand include i) Supporting forestry research, education, training, information generation and dissemination, and technology transfer for sustainable development. ii) Establishing and enabling legislative and institutional framework for the development of the forest sector iii) Promoting Investment in commercial tree growing, forest industry and trade iv) Enhancement of forest resources for conservation of soil, water biodiversity, and environmental stability, v) Increasing and maintain tree and

forest cover of at least ten percent of the land of Kenya. By working on ways of improving the regeneration of *Terminalia brownii* species, will help in contributing to the achievement of the 10% forest cover as is required by one of the objectives of the policy.

The policy also emphasizes how dryland forests are to be managed and they include: a) promoting sustainable management of dryland forest, b) promoting sustainable production of charcoal c) promoting the production of wood and non-wood forest products, d) promoting commercial tree growing of suitable tree species e) promoting sustainable management of dryland forests f) creating a conducive environment for the establishment of forest-based enterprises, and g)supporting the rehabilitation of degraded forests and encouraging tree planting in the arid. Conservation of *T. brownii* therefore will help in promoting sustainable management of the forests, conserving the genetic resources, and in supporting the rehabilitation of the degraded forests using the indigenous tree species as people will be encouraged in tree planting with the available planting materials.

2.12.2 Forest Conservation and Management Act no 34 of 2016

This act addresses the management of indigenous forests where the forest and woodlands are to be managed for:

i. Conservation of water, soil, and biodiversity
ii. Culture use and heritage
iii. Recreation and tourism
iv. Riparian and shoreline protection
v. Sustainable production of wood and non-wood products
vi. Carbon sequestration and other environmental services
vii. Education and research purposes, habitat for wildlife in terrestrial forest and fisheries in a mangrove forest.

This Act is in line with the research in that the *T. brownii* species has been reported to be reducing in numbers, calling for ways to help in conserving it. One of the ways, therefore, is to establish the root cause of the reduction in its regeneration performance as is in this research.

2.12.3 Environmental Management and Coordination Act, 2015

Part iv, article 50 states that measures to ensure the conservation of biological diversity should be prescribed. For the guidelines listed, activities of this research will mainly undertake measures intended to integrate the conservation and sustainable utilization ethic concerning biological diversity in existing government activities by private persons.

2.13 Knowledge gaps

Seeds are usually prepared, and various properties determined such as the number of seed in a kilogram, moisture content, and even seed quality tested and assured to farmers before dispatch to them. In Kenya therefore, Kenya Forestry Seed Centre is responsible for the collection of the tree seeds and preparing them which in one way helps in conserving the species by keeping their seeds. An interim recommendation for the pre-sowing treatment of *Terminalia brownii*, therefore, stated that seed germination was extremely low when not treated with the general fungicide captan and that they need to be treated (Specht, 2005). Propagation at this time was done by planting the dewinged fruits, in which the hard endocarp also played a crucial role in contributing to the low germination percentages ranging from 25% to 35%.

Other studies done confirming the causes of the low germination of *Terminalia brownii* seeds by physical dormancy is the one done by Mosango (2013), in which the seeds recorded low germination percentages too. Omondi (2011) researched how to solve the physical dormancy problem, by extracting seeds fully from fruits and separating them from the hollow insect-infested ones. The seeds collected recorded a better germination capacity with those collected from Kitui attaining 80% within two weeks. A recent study on this *T. brownii* species in Kimose, Kendu Bay, and Ndumoni have shown that there is a high number of fungal organisms associated with the flowers, fruits, and seeds of this species (Okeyo et al., 2019). The gaps are summarized in Table 2.1 on page 6. Seed's germination performance in the three sites is not attaining the recommended percentage too, and thus the need to find out other causes for poor germination and emergence, in which this research will establish if the fungal organisms found in flowers and fruits are associated with the germination success of the seeds. This research assessed seed-borne fungal organisms associated with non-germination and germination of *T. brownii* seeds.

Table 2.1: Knowledge gaps on *Terminalia brownii* low germination percentages

Authors	Results	Gaps / Further research
Specht et al. (2005)	The germination percentage of *T. brownii* seeds recorded ranged between 25% and 35%, and this was only for those that were treated with the general fungicide captan	Research should be carried out on the specific fungi that are affecting the germination of *T. brownii* seeds
Mosango (2013)	*T. brownii* exhibits poor and slow germination because of the physical dormancy caused by the wood endocarp. Germination of *T. brownii* varies across provenances	There should be detailed studies on *T. brownii* germination and dormancy. Research should be done on the causes of the germination differences across the provenances.
Omondi (2011)	Germination capacity of extracted seeds collected attained 80%, after being subjected to cutting test in which most were infested by an insect pest.	Research should be carried out on the effect insects associating with *T. brownii* could be having on the germination of the seeds.
Okeyo et al. (2019)	Fungal organisms found attacking floral phenology of *Terminalia brownii* in Kenyan drylands include: *Alternaria sp*, *Aspergillus sp*, *Botryosphaeria sp*, *Cladosporium sp*, *Epicoccum sp*, *Fusarium sp*, *Pestalotia sp*, *Rhizopus sp*, Rot fungi, and *Trichoderma sp*.	Research should be done on whether the identified fungal organisms contribute to low seed germination percentage.

2.14 Theoretical framework

Seed-borne fungi can affect germination of a seed in diverse ways. There are various seed-borne fungi in different genera, and they affect seeds in separate stages too. Some of the fungi affect the seed by interfering with the embryo while still in the seed coat thus hindering germination. Another phase of infection is when the radicle protrudes and dies during the initiation of germination, and then some affect the seed once it has become a seedling thus killing it and stopping its further development (Gure et al., 2005).

Fungi affect seeds either at the seed state or once planted in the soil The fungi inoculums in the soil affect the seeds once they come into contact with it. The seeds are therefore either exogenously or endogenously placed, where the germinating seedling can be affected at different stages of the establishment (Cram & Fraedrich, 2010)

Plant diseases may find entry into fruits and seeds via damaged insect seeds and other insects may be vectors of fungal and bacterial diseases (Wubneh et al., 2016), and the effect of insect infestation may not necessarily cause immediate damage to the seeds and fruits during development, but their effects are seen during seed storage and germination. However, even minor damages to the vital part of the embryo such as the radicle or/and hypocotyl may rapidly cause the death of seed resulting in poor germination. Seeds will germinate readily within a period of 3 to 21 days when all the conditions are met unless infested with pests or/and diseases.

This research used the seed-borne fungi effect theories by Gure et al. (2005) and Cram & Fraedrich (2010), which state that seed-borne fungi can potentially affect seed germination at various stages, right from - storage, to the initial germination phase of root protrusion, final germination phases and seedling stage level. Apart from this, it followed principles to be met for a seed to germinate which are: alternating temperatures, destruction of the germination inhibitors, light, and oxygen.

2.15 Conceptual framework

Negative effects of pathogenic organisms contribute to poor or no seed germination. They may either be endogenous or exogenous pathogenic organisms. The organisms have been reported to be mainly fungi (Gure et al., 2005; Cram & Fraedrich, 2010), as shown in Figure 2.1 on page 38.

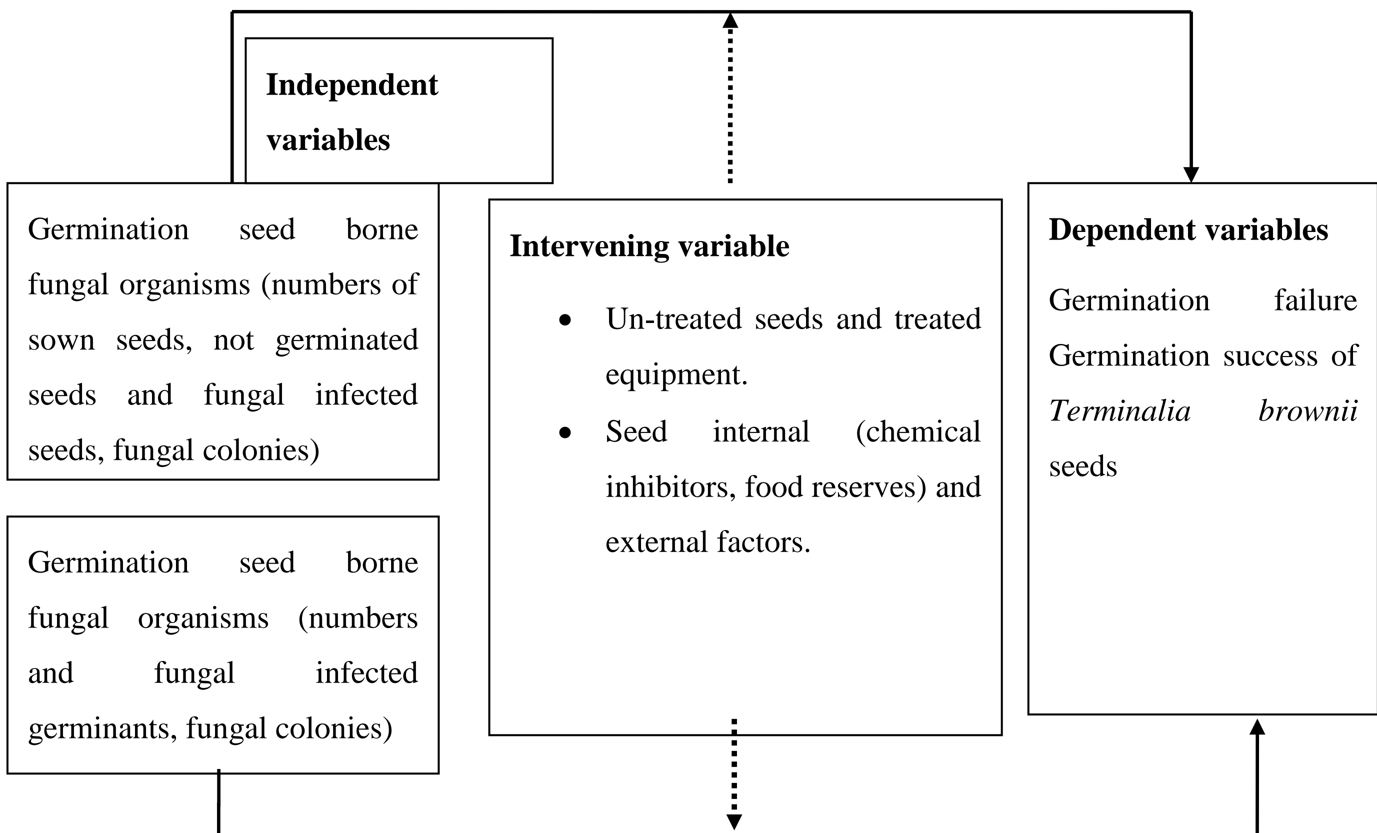


Figure 2.1: Conceptual framework

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of the study areas.

The seeds were collected in three selected provenances which are Ndumoni in Kitui, Kimose in Baringo, and Gendia in Homa Bay Counties (Figure 3.1). The sites represent distinct ecological regions where the species is naturally distributed in Kenyan drylands (Beentje et al., 1994).

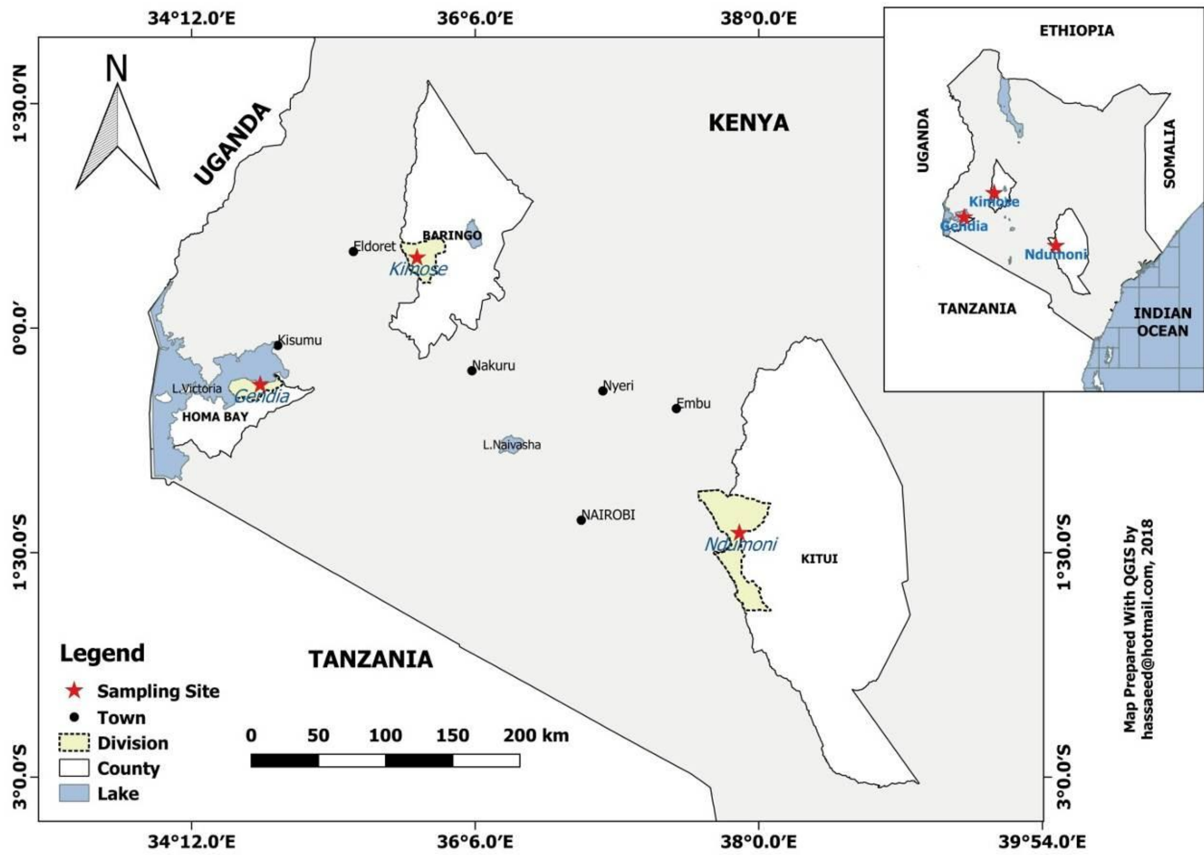


Figure 3.1: Map showing sample collection sites. (Source: Kenya Topographical GIS shape files, modified using ArcMap)

3.1.1 Location

Ndumoni is in Kyangwithia East location of Kitui Central sub-County within Kitui County at a latitude of 0.97° S and longitude of 37.47° E in Eastern Kenya. While Kimose is in Kimose location of Mogotio Sub County within Baringo County at a Latitude of 0.47° N

and a Longitude of 35.71° E in the Rift Valley. Kendu Bay is in North Karachuonyo location in Karachuonyo sub-County within Homa Bay County at a latitude of 0.38° S and longitude of 34.66° E within the Lake Victoria Basin. Topography varies from well undulating land at Ndumoni in Kitui and Gendia in Kendu Bay to small hills with rocky outcrops at Kimose in Baringo. The average altitude for Ndumoni, Kimose, and Gendia is 1,141, 1,563, and 1,217 m above sea level, respectively.

3.1.2 Climate characteristics

The study sites receive bi-modal rainfall during April/May and October/November annually. However, rainfall is erratic with frequent crop failures. Summaries of the temperatures experienced, and amount of rainfall received in the three sites are as follows:

Ndumoni in Kitui County is found in the drylands of Eastern Kenya. Annual temperatures experienced in this County ranges between 14°C to 34°C, with the hot months being between September – January, and the wet months being between late February to April. Precipitation recorded for the County ranges between 250 mm and 1050 mm per annum. In a summary, the specific site where the fruits were collected receives a mean annual rainfall of 816 mm with mean temperatures of 22.11^{0}C, falling within the brackets for the County's weather parameters recorded.

Kimose in Baringo County, which has two distinct weather patterns. The temperatures in the southern part range between 25°C during the cold months of June and July, and 30°C temperature during the hot months of January and February. Northern parts on the other hand have temperatures ranging between 30°C to 35°C throughout the year. The county receives precipitation of between 1000mm and 1500mm of rainfall annually in the highlands, and 600mm in the lowlands. In general, Baringo experiences two rainy seasons, where long rains are between March to June and short rains being between November to December. (http://www.kenya-information-guide.com/baringo-county.html). In a summary, Kimose receives a mean annual rainfall of 889 mm with mean temperatures of 22.59^{0}C. Gendia is found near Lake Victoria, and thus receives mainly convectional rainfall. It is in Homabay County which is used as the standard site for data collection for the weather. An average of daily temperature ranges has been recorded to be about 26°C during the coldest months which start from May to August, and a temperature of 34°C during the hottest months which

are usually from the end of December to early or mid-March. The county receives precipitation ranging from 250 mm and 1200 mm of rainfall annually, with the average annual rainfall estimated at 1,100 mm. The area experiences two rainy seasons. The first one is from mainly March to May which is long rains, while the second season of short rains is between September and December (http://www.kenya-information-guide.com/homa-bay-county.html). Specific sites in Gendia where the fruit samples were collected therefore receive a mean annual rainfall of 1,199 mm with a mean annual temperature of 21.56^{0}C from the data collected in the previous consecutive three years, which falls well in the summarized County's climate measured parameters.

3.1.3 Soil characteristics

These sites within the Kenyan drylands are characterized by shallow loam sand soil with a depth of 20 cm. Ndumoni soils are deeply weathered, moderately deep with a pH ranging from moderately acid (5.67) to moderately alkaline (8.27). Soil organic matter content ranges from low (0.48% Total Organic Carbon (TOC) to moderate (1.4% TOC) (Batjes & Gicheru, 2004).

Soils in Kimose are commonly shallow and rocky, with a pH ranging from strongly acid (4.5) to strongly alkaline (8.59). Soil organic matter content ranges from low (0.48%) to moderate (1.4%) (Batjes & Gicheru, 2004).

Soils at Gendia are dark with a pH ranging from strongly (4.76) to slightly acid (6.32). Soil organic matter content ranges from 0.51% Total Organic Carbon (TOC) to 1.88 %.) (Batjes & Gicheru, 2004).

3.1.4 Land use

Kendu Bay is along Lake Victoria, and the major source of livelihood is fishing and small-scale farming, where they grow maize, sugar cane, millet, potatoes, and vegetables. These activities are supplemented by small-scale farming for other needs such as vegetables and grains. Farming is therefore considered mainly during rainy seasons which usually are twice a year, from March to June, and September to December. In Baringo, pastoralism is the main source of livelihood as the area is semi-arid, followed by subsistence farming which is mainly beekeeping, aloe vera plant cultivation, and fishing on small scale. This area usually

receives one rainy season, and they do plant maize and millet mainly both for commercial and subsistence. Lastly in Kitui, the area is classified as semi-arid too. Occupants of the area are mainly known for business and crafting. However, due to changes in climatic conditions and tough economic times, the occupants have adopted farming mainly for subsistence, growing cotton, tobacco, sisal, mangoes, maize, beans, cassava, sorghum, millet, and pigeon peas, and keeping livestock.

3.2 Sampling and Experimental design

3.2.1 Sampling

Sections of forests where *T. brownii* trees were in high numbers were chosen. Fruits were first probed to confirm their maturity, after which thirty trees that were fifty meters apart were selected and samples collected uniformly from their crowns (Luna & Wilkinson, 2009), as shown in Plate 3.1. The seeds were collected from the three parts of the tree which are the top, middle and bottom, to maximize sampling of the various microorganisms as most fungi tend to prefer warm (25^0C-30^0C) and poor ventilated areas with good moisture and oxygen

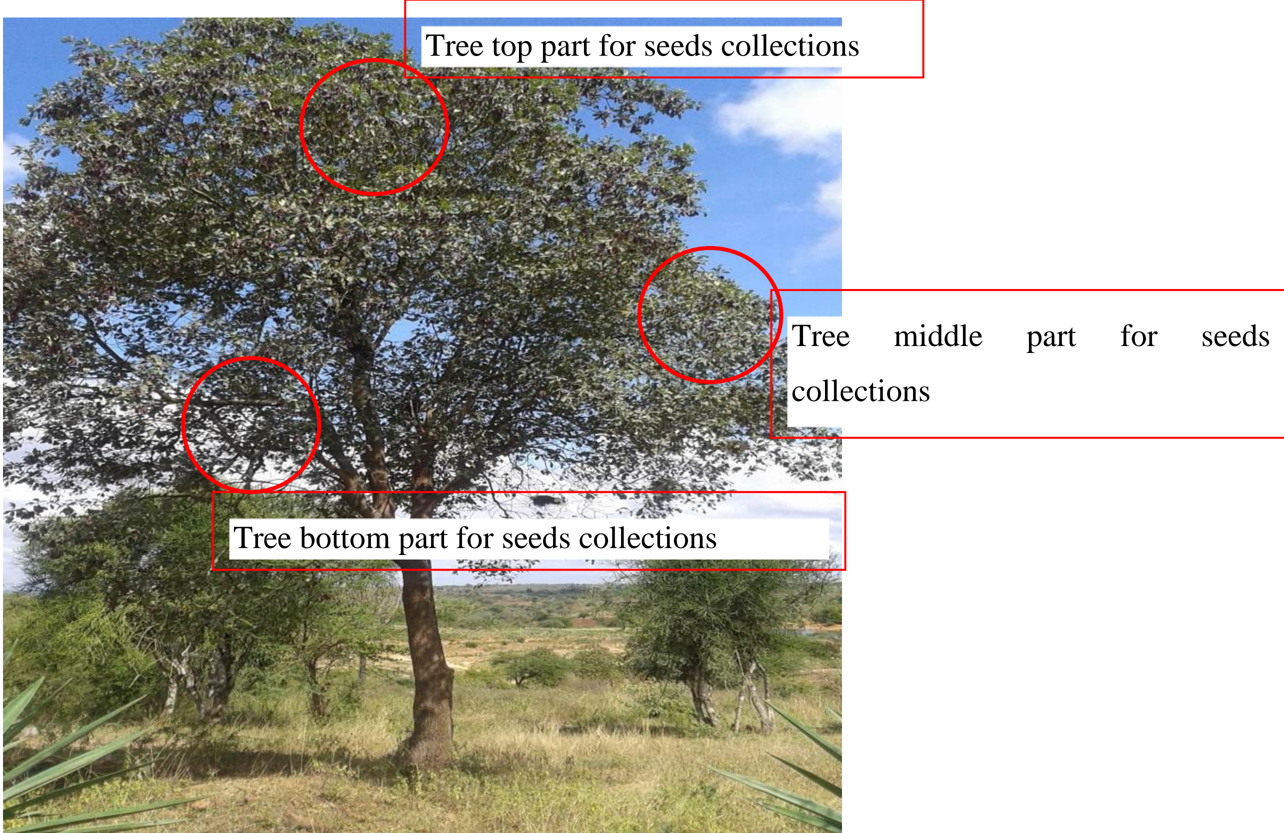


Plate 3.1: Parts of a tree crown from which fruit samples were collected

3.2.2 Experimental design

The research adopted a complete random experimental design where one hundred seeds each from the different lots collected from the three sites, i.e., Kimose, Kendu Bay, and Ndumoni, were soaked in cold water for 24 hours and then incubated in filter paper. They were therefore in groups of twenty-five of four replicates per provenance, each with its control of *Senna siamea* as shown in Plate 3.2. These seed samples were then arranged in an incubator as in Plate 3.2.

Plate 3.2: Germination seeds test arrangement in groups of 25 on filter paper

This design was appropriate as the seeds from the different sites need to be treated separately to identify the fungal organisms per the sites and compare the degree of the effect of the fungal infections as shown in Table 3.1 on page 44.

Table 3.1: Experimental design adopted

Provenance	Controls	Rep one	Rep two	Rep three	Rep four
Gendia in Homa Bay	○	X	X	X	X
Kimose in Baringo	○	X	X	X	X
Ndumoni in Kitui	○	X	X	X	X

Key

X-25 *T. brownii* seeds

○ -25 Treated *Senna siammea* seeds

3.2.3 Laboratory work

Germination success tests were carried out in KEFRI Kitui laboratories, where other projects on the species are being executed. Kitui is found in Eastern Kenya where various dryland tree species' seedlings are being raised. The last part of the experiment on DNA extraction of the seed-borne fungal pathogens right from culturing was done at KEFRI headquarters laboratories. Lastly, DNA sequencing was done at the Inqaba laboratories in South Africa for clear nucleotides as this was Sanger sequencing as opposed to NGS sequencing which could be done locally.

3.3 Germination success tests

Terminalia brownii fruits from the three sites were first air-dried separately for a day by being left open spread in the sun. The seeds were then immediately (while still dry) extracted

from the fruits using secateurs by cutting the fruits on both sides slightly and removing the seed, as a whole without, cutting it.

One hundred *T. brownii* seeds from each site were soaked in cold water for 12 hours in separate containers. Twenty-four filter papers were moistened and placed inside the glass petri dishes in pairs, to provide a base worth retaining the moisture for some time. *T. brownii* seeds were distributed on a moistened filter paper in Petri dishes with tweezers in neat rows as shown in Plate 3.2(page 43), making sure that they are kept at a distance between one another. The seeds arranged in the Petri dishes were incubated at a constant temperature of 30°C. Monitoring followed for any developments which were then recorded for some seeds from the third day. The seeds were considered to have germinated only upon the radicle protrusion, as shown in Plate 3.3, and their numbers recorded.

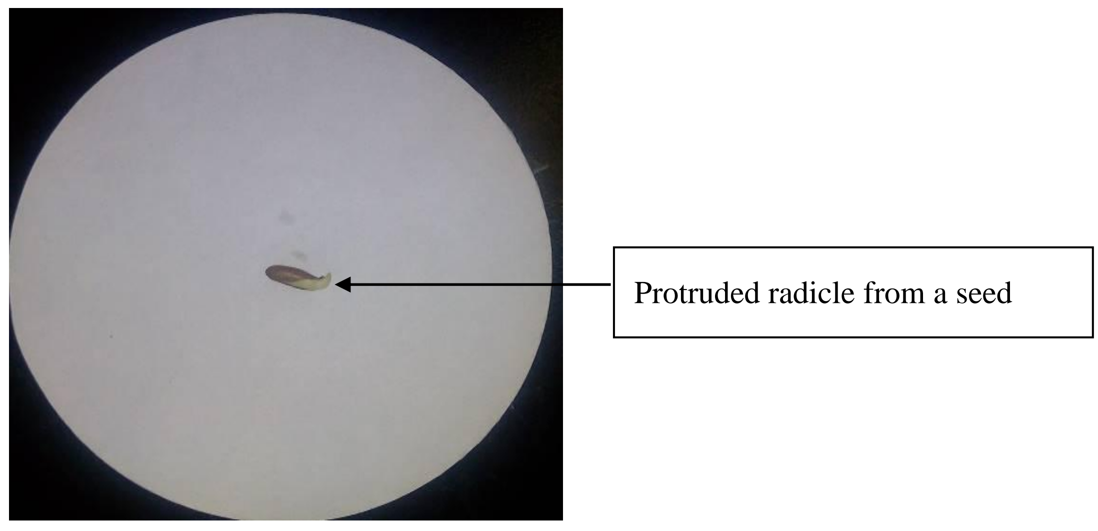


Plate 3.3: Germinated *Terminalia brownii* seed

3.4 Evaluation of *T. brownii* seeds that initiated germination.

Developments were observed on the seeds till the fifth day when some seeds that had produced radicles on the third day had started producing the shoot. The numbers of *T. brownii* seeds that had not germinated by this day, therefore, were counted and recorded. The seeds were then given more time for germination, while those that did not germinate were monitored up to 30 days.

3.5 Evaluation of *T. brownii* seedlings that germinated.

Terminalia brownii seeds were considered to have germinated on protrusion of radicle and plumule, as shown in plate 3.4. Germinants were counted and recorded, together with the numbers of those that had not germinated. Seeds that did not germinate and those that germinated were blot dried using filter papers and processed for fungal culturing being labeled clearly per replicate and collection site.

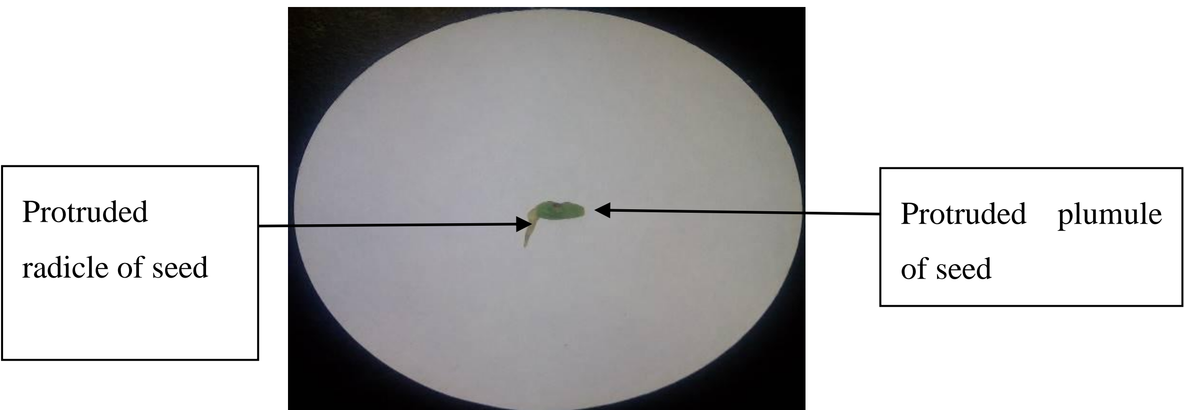


Plate 3.4: Fully germinated *Terminalia brownii* seed

3.6 Assessment of Fungal pathogens

Terminalia brownii seeds that did not germinate and those that germinated were washed with distilled water, then sterilized using 2% hypochloric acid, and rinsed with distilled water. Growth media of malt extract agar was mixed with 0.1% of streptomycellia antibiotic to suppress bacterial organisms' growth which could hinder the growth of fungi. *T. brownii* test sample seeds were then placed on the growth media as in the earlier experiment with the cover petri dishes tightly sealed using para films. All these were done in a hood, to exclude external contamination. The samples were then incubated at 28±^{o}C for four days. Fungal growth was then identified at the genus level using cultural, morphological, and biochemical characteristics (Binyam & Girma, 2016).

Colonies showing typical fungal characteristics were sub-cultured on Malt Extra Agar until pure cultures were obtained. The pure cultures were preserved in MEA slants at 4°C and glycerol for DNA extraction.

3.7 Seed-borne fungal organisms' identification

Fugal organisms associated with the non-germination and germination were identified through DNA analysis. The process involved DNA extraction, amplification, and sequencing.

3.7.1 DNA Extraction from mycelium

Selected mycelium isolates from the pure culture were grown in 2% liquid MEA for four days and the isolates later freeze-dried. Genomic DNA was extracted using Cetyl trimethyl ammonium bromide (CTAB) (3 %), which is also known as the Chloroform method described by Gardes and Bruns, (1993) with modification according to Ihrmark et al. (2002). 0.1 M Tris solution was prepared by mixing 50g of Tris base, 0.5 M EDTA in one litre of water. CTAB buffer on the other hand was the prepared by mixing 1% of CTAB, 0.05 MTris, HCl, 0.7 M NaCl, and 0.5% ethanol. 0.2 g of freeze-dried isolated mycelia were grinded differently using a mortar and pestle, mixed with five hundred µl isolation buffer (CTAB) and then transferred into a 2ml microfuge tube. The components were mixed to help in separating the contaminants in the organic phase. The combination thereafter was mixed by vortexing to release the DNA structure. This was then followed by centrifugation at 4ºC for 3 minutes, which helped in removing insoluble particles. To each of the mixtures made, 250 µl of chloroform: Iso Amyl was added and vortexed for one minute.

Supernatant isolation buffer was removed, and a new 800 µl isolation buffer was added fresh and remixed by vortexing. This procedure of replacing isolation buffer and remixing was repeated until the supernatant became less viscous. The supernatant buffer was then removed and 800µl of CTAB solution added. This was followed by incubation at 65 ºC for 60 minutes, and then at 37 ºC for another 60 minutes. 800 µl mixture of chloroform and isoamyl alcohol was then added to the solution mixed by inversion for 10 minutes and centrifuged at 37 ºC for 10 minutes. The upper aqueous phase of the solution was transferred to a new 2.0 ml microfuge tube and another fresh volume of a mixture of chloroform and isoamyl (CIA 0f 42:1) mixed and inverted for another 10 minutes. This again was centrifuged at 37 ºC for 10 minutes. The upper aqueous phase was again transferred to a new 1.5 ml microfuge tube, 0.1ml of NaOAc added, together with 2.2 ml of isopropanol which was then mixed by inversion and centrifuged at 4 ºC for 5 minutes until a clear solution was achieved.

All the supernatants were discarded, 800 µl of 70% ethanol was added and flipped to wash the DNA. The solution was then centrifuged at 4 ºC for 5 minutes, discard the supernatant and dry the DNA pellet, and then 100 µl of DNase-free water was added to dissolve the DNA.

3.7.2 DNA Amplification and purification

The DNA solutions were mixed with various primers together with Taq polymerase and mixtures of water, Magnesium chloride ($MgCl_2$) PCR buffer, dNTP, Magnesium Chloride ($MgCl_2$) in different concentration till lear sequence was achieved. A fragment of the internal transcribed spacer (ITS) rDNA was amplified using fungal–specific primers, ITSIF (5'-CTTGGTCATTTAGAGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGGSTTATTGATATGC-3') (White et al., 1990). Part of the translation elongation factor 1-alpha (Tef 1-alpha) gene was elongated with primers EFI-986R (5'-TACTTGAAGGAACCCTTACC 3') to confirm the identity of fungal groups. A mixture for the polymerase reaction was prepared in which Random Amplified Polymorphic DNA (RAPD) primers were used. A solution of were mixed by vortexing. DNA solution from DNA extraction process loaded then onto the pipettes, followed by addition of the master mix solution, EFI, BT2, ITS 1, and ITS 4 into the pipette. All these in different five pipettes. Distilled water in a separate pipette was added in separate pipette and everything stirred.

All these solutions and the water were then loaded onto the thermocycler at RAPD primers temperature profile, for 45 minutes. Electrophoresis gel on the other hand was prepared by use of agarose and TBE. A dye was mixed with the buffer to which helped in the sinking of the DNA so as not to float on the buffer. Once the thermocycler was off, the DNA samples together with the mix were loaded onto the gel where the combs left holes and electrophoresis set for 45 minutes. The product was then viewed using the computer after it was loaded. Optimization was done for the correct primers in that primers that worked for the fungal DNA samples in question were ITS 1 and ITS 4. This then aided in the next process of PCR.

PCR products were then transferred into a 1.5 ml microtube, sodium chloride solution of 6 ml added into it, the solution incubated for 10 minutes at 37ºC. This was followed by centrifuging the solution at room temperature (37ºC). Supernatant removed was then removed and 500 µl of 70% ethanol was added into it and centrifuged again at room

temperature for 5 minutes. The final supernatant was again removed and now dried with ethanol, after which 30µl of sterile water was added onto it and taken for sequencing.

3.7.3 Sequencing analysis of fungal isolates

Amplified and purified PCR products were sequenced in both directions twice using the PCR Big Dye Terminator v.1 cycle sequencing kit. The PCR reaction mix was prepared according to Inqaba's instructions. The sequence was analyzed using ABI 310 genetic analyzer. Seqman (DNASTAR Inc., USA) was used to edit the nucleotide sequences. The edited nucleotide sequence was submitted to the national center for biotechnology information (NCBI) Gene bank sequence database and was identified by comparing with a relevant sequence of ITS region rDNA and (Tef-α) using BLASTN algorithm (www.ncbi.nlm.nih.gov/BLAST/blst.cgi) (Altschul et al., 1997). Sequences that showed 98-100 % similarity were closest matches to the sequence and were assigned the names with the highest species identity from Gene bank. The nucleotide sequences obtained from this study were deposited in the Gene bank. Data collected in this research were recorded in datasheets attached in the appendix.

3.8 Data analysis

Data were analyzed per objective as summarized in Table 3.2 on page 49, in which paired t-test was used in analyzing the number of seeds that did not germinate versus the number of sown seeds. The second objective t-test was used in which the variables were the number of fungal infected germinants versus the sown number of twenty-five seeds. This comparison test would then prove that the fungal organisms affected the seeds in a significant number. This was chosen as the changes were to be monitored on the same samples. For the third objective, the Mann-Whitney U test was used in analyzing the number of seeds that germinated versus that of fungal-infected germinants. This was to determine whether there was a change between the two independent variables, thus determining whether the infection was significant. This was done to determine whether there was a significant difference between the two, brought about by fungal infection. The fourth objective which is the last was analyzed using descriptive statistics, basically displaying the types and frequencies of the fungal pathogens.

Table 3.2: Analysis tools per study objective.

No.	Objective	Variable	Analysis tool
1	To analyze the germination success of *T. brownii* seeds	• The number of germinated seeds. • Number of seeds sown	One sample t-test
2	To assess seed-borne fungal organisms associated with *T. brownii* germination success.	• Number of fungal infected seeds • The number of seeds sown.	Paired t-test
3	To compare seed-borne fungal organisms associated with germination failure and germination success of *T. brownii* in the three sites	• Names of fungal organisms	Descriptive test

CHAPTER FOUR

RESULTS

4.1 Analysis of germination success of *Terminalia brownii* seeds

The performance of *T. brownii*'s seed germination was determined by noting the numbers that germinated from the sown lots. The seeds were subjected to a germination test and the number of seeds that did not germinate recorded as shown in Table 4.1.

Table 4.1: Number of *T. brownii* seeds that did not germinate versus number of sown seeds

Site	No. of seeds that did not germinate	No. of seeds sown
1	8	25
1	8	25
1	9	25
1	10	25
2	13	25
2	9	25
2	12	25
2	18	25
3	9	25
3	9	25
3	12	25
3	6	25

Key (Site numbers)

1-Kitui 2-Baringo 3-Kendu Bay

Data of the number of seeds that did not germinate were subjected to a normality test which tested normal (N=12, P=0.063). One sample t-test between the numbers of none germinated seeds against 25 seeds for significant change in terms of germination, gave (N=12, P value=0.000, t=-16.29). There is evidence therefore to suggest that there is a significant difference between the number of none germinated seeds versus the number of seeds sown which was 25. A significant did not germinate in this case.

Germination percentages recorded from germination tests of seeds collected from the three study sites, purely on those that fully germinated are as shown in Table 4.2.

Table 4.2: Germination percentages of *T. brownii* seeds

Site	**Germination percentages**				
	1st Replicate	**2nd Replicate**	**3rd Replicate**	**4th Replicate**	**Average**
Gendia	68%	68%	68%	60%	**66%**
Kimose	56%	60%	56%	32%	**51%**
Ndumoni	64%	72%	52%	80%	**67%**

4.2 Seed-borne fungal organisms associated with *T. brownii* germination success.

Data of the infested seeds were subjected to a normality test and gave (N=12, P=0.149). Paired sample t-test was performed on the number of seeds that did not germinate and fungal infested seeds (Table 4.3 on the following page) which were the main variables in this objective giving a result of (N=12, P value=0.000, T value=-8.78).

Table 4.3: Number of *T. brownii* seeds that did not germinate versus number of fungal infested seeds

Site	No. of seeds that did not germinate	No. of fungal infested seeds
1	8	2
1	8	0
1	9	0
1	10	3
2	13	1
2	9	1
2	12	3
2	18	1
3	9	3
3	9	2
3	12	1
3	6	2

Key (Site numbers)

1-Kitui 2-Baringo 3-Kendu Bay

This shows that there is evidence to suggest there is no significant difference between the data of the two variables. Fungal organisms therefore did not affect germination significantly.

The evidence, therefore, confirms that fungal organisms identified did not contribute significantly to registered germination failure. Seed-borne fungal organisms associated with germination failure of *T. brownii* as identified in this experiment were mainly *Alternaria sp* (47.40%) *Fusarium sp* (42.10%) and *Pestalotia sp* (10.50%), as shown in Figure 4.1, with the morphological differences depicted in Plate 4.1.

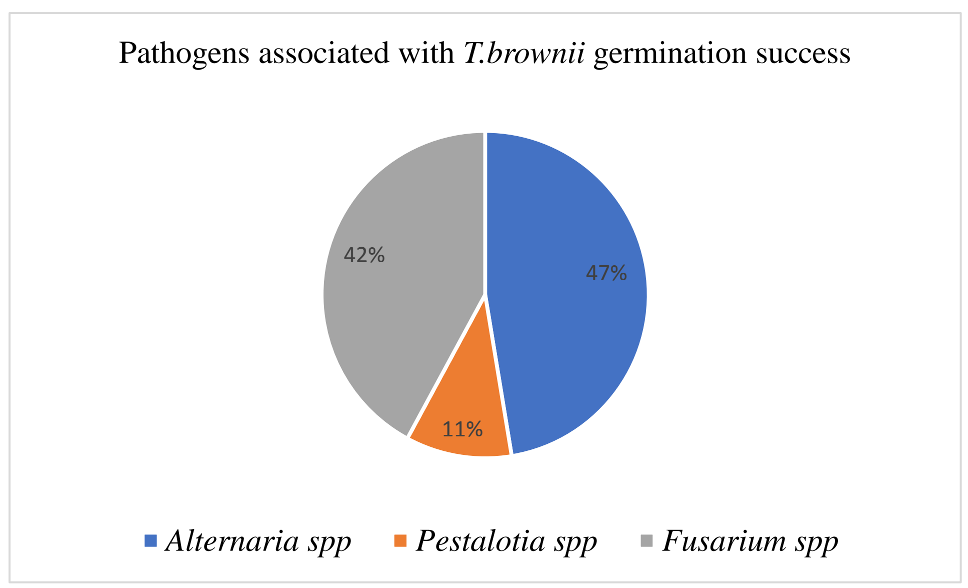
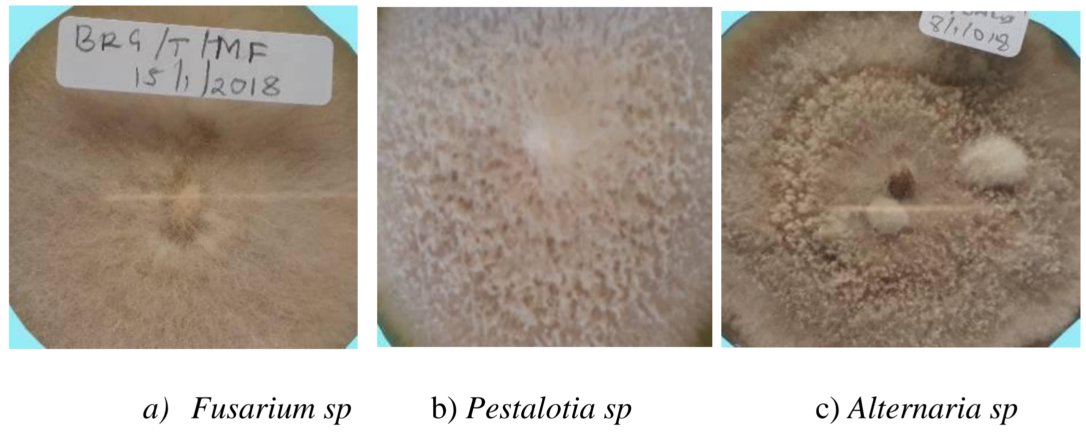


Figure 4.1: Fungal genera of pathogens associating with germination success of *T. brownii*



a) *Fusarium sp* b) *Pestalotia sp* c) *Alternaria sp*

Plate 4.1: Morphological differences between genera *Fusarium sp*, *Pestalotia sp,* and *Alternaria sp*

Fusarium has been identified to be one of the dangerous pathogens that affect seedling growth, but the frequency in the lot that was collected back in November-December 2018 was not high enough to contribute negatively to the germination of the *T. brownii* species.

Pathogens that were identified to be present on the seeds collected from the three sites were as shown in Figure 4.2 on the following page. Additionally, *Terminalia brownii* seeds that germinated and those that did not were left for more hours in a germination incubator.

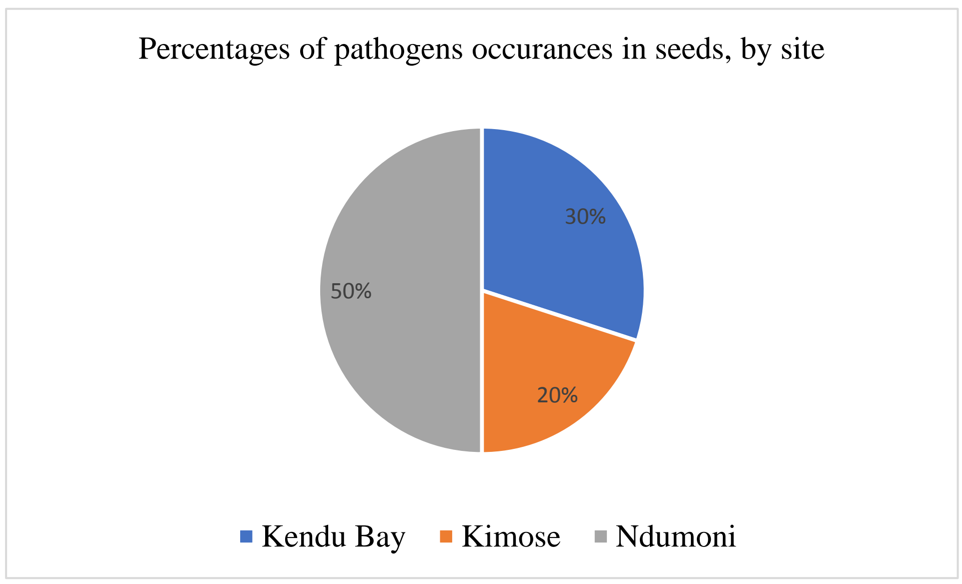


Figure 4.2: Pathogens occurrences in seeds by study sites

In determining the difference between germinants and infested germinants, data of the number of germinants which was normal (N=12, P-value= 0.063), and data of the number of fungal infected germinants was not normal (N=12, P value<0.005) were analyzed using a non-parametric test of Mann-Whitney U test of null hypothesis test stating that the two were not equal. The results showed that the test was significant at 0.000, thus suggesting that there is a relation between the two variables.

Identified fungal organisms therefore associated with a lot of the germinants but did not contribute negatively to progressive germination of the seeds. Seed-borne fungal organisms associated with germination of *T. brownii* as identified were mainly *Penicillium*.

Terminalia brownii seeds were subjected to a germination test under required conditions for germination where some seeds germinated while others did not. Conditions presented were warmth, oxygen, and water. General types of fungal organisms that were identified on surface non-sterilized full seeds generally included: *Alternaria sp* of 12%,

Pestalotia sp of 2%, *Rhizopus sp* of 81%, *Penicillium sp* of 5%, and *Fusarium sp* of 0% in as in Figure 4.3 on page 56.

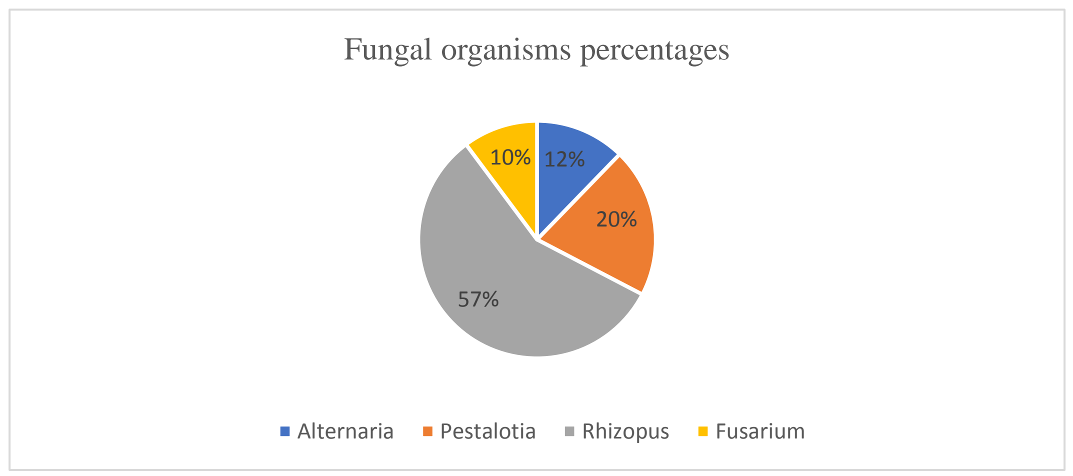


Figure 4.3: Identified *T. brownii* seed-borne fungal organisms

Terminalia brownii seeds sampled from Kimose on the other hand showed rapid shoot growth (Plate 4.2) in the germination experiment, as opposed to what was observed in the case of those sampled from Gendia and Ndumoni as in Plate 4.3 The differences in the seed sources could therefore be one the factors contributing to the differences in the rates of seedlings grown from the three sites observed.

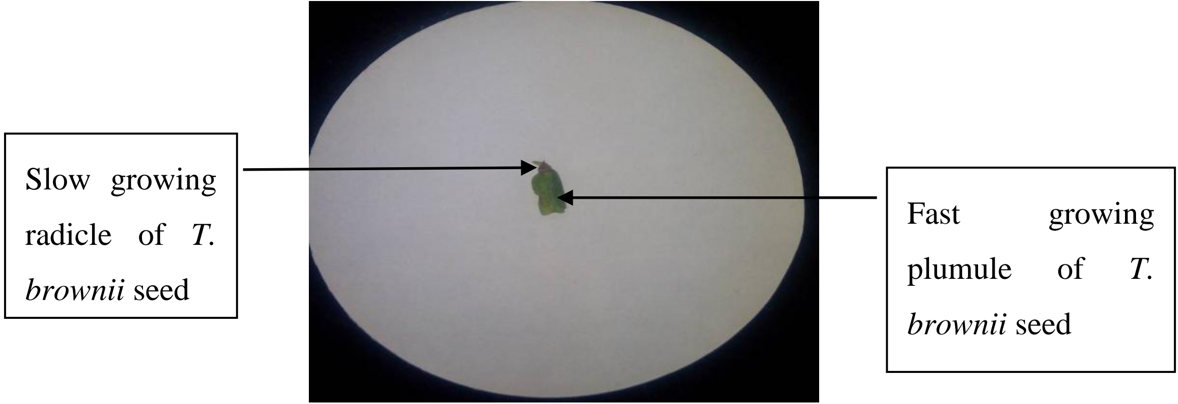


Plate 4.2: Unique germination exhibited by *T. brownii* seeds collected from Kimose

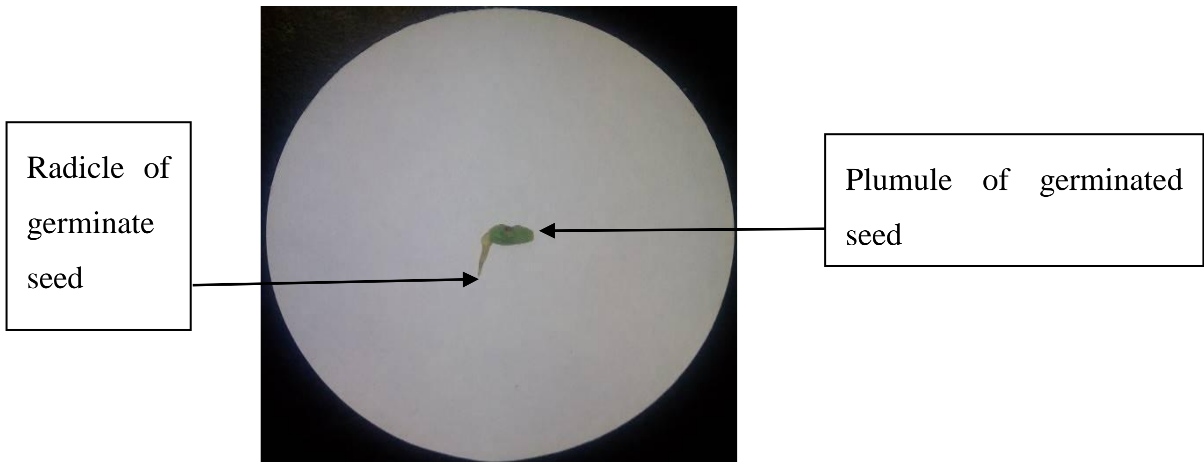


Plate 4.3: Germination exhibited by *T. brownii* seeds collected from Kendu Bay and Ndumoni

4.3 Comparison of seed borne fungal organisms found associating with germination failure and germination success of *T. brownii* seeds.

Terminalia brownii seeds that proceeded to the fungal identification stage, were keenly monitored which ensured that seeds from the three sites of seed collection did not mix. The amplified aqueous solution of the DNA samples gave the results as shown on Plate 4.4 where the first solutions did not amplify the DNA bases clearly when primers EF1 and BT2 primers were used, at sequence 5' to 3' (GGT AAC CAA ATC GGT GCT TTC) bases arrangement.. The sequence in this case was Plate 4.5 is the clearer version of the DNA bases when primer ITS was used, at a sequence 5' to 3' (CTT GTT CAT TTA GAG GAA GTA A) bases arrangemnent. The consequent sequences are shown in Figure 4.4 which indicates both the infectoius organisms and non infectious fungal organisms derived from the PCR.

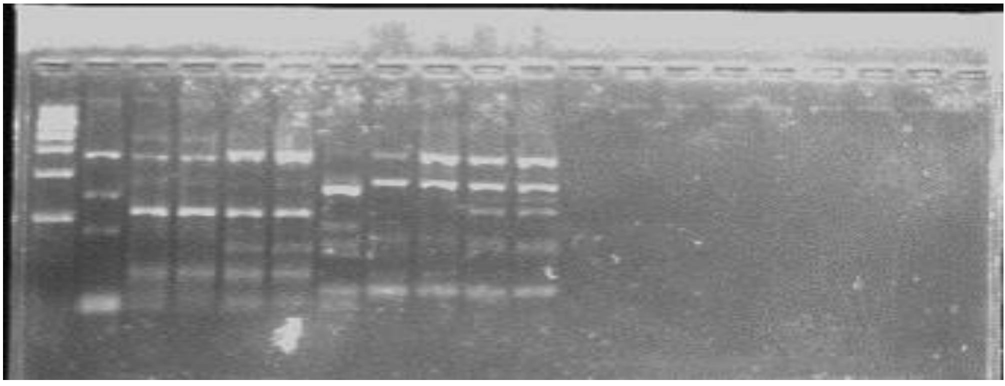


Plate 4.4: PCR Test for *T. brownii* seeds with EF1 and BT2 respectively for the four genera samples at sequence 5’ to 3’ (GGT AAC CAA ATC GGT GCT TTC) bases arrangement.

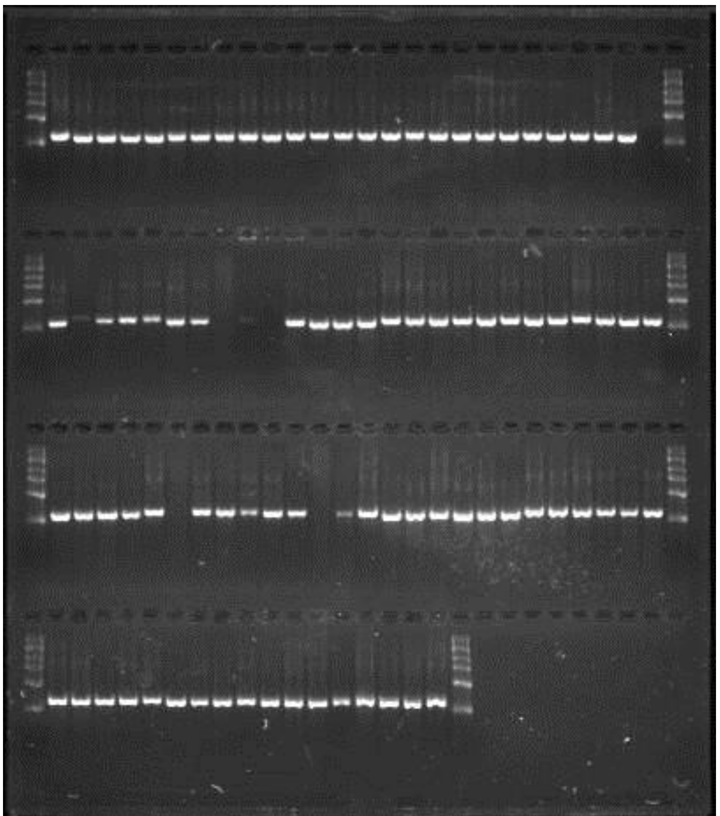


Plate 4.5: *T. brownii* seed-borne fungal DNA amplification stages with ITS 1 primers at a sequence 5’ to 3’ (CTT GTT CAT TTA GAG GAA GTA A) bases arrangemnent.

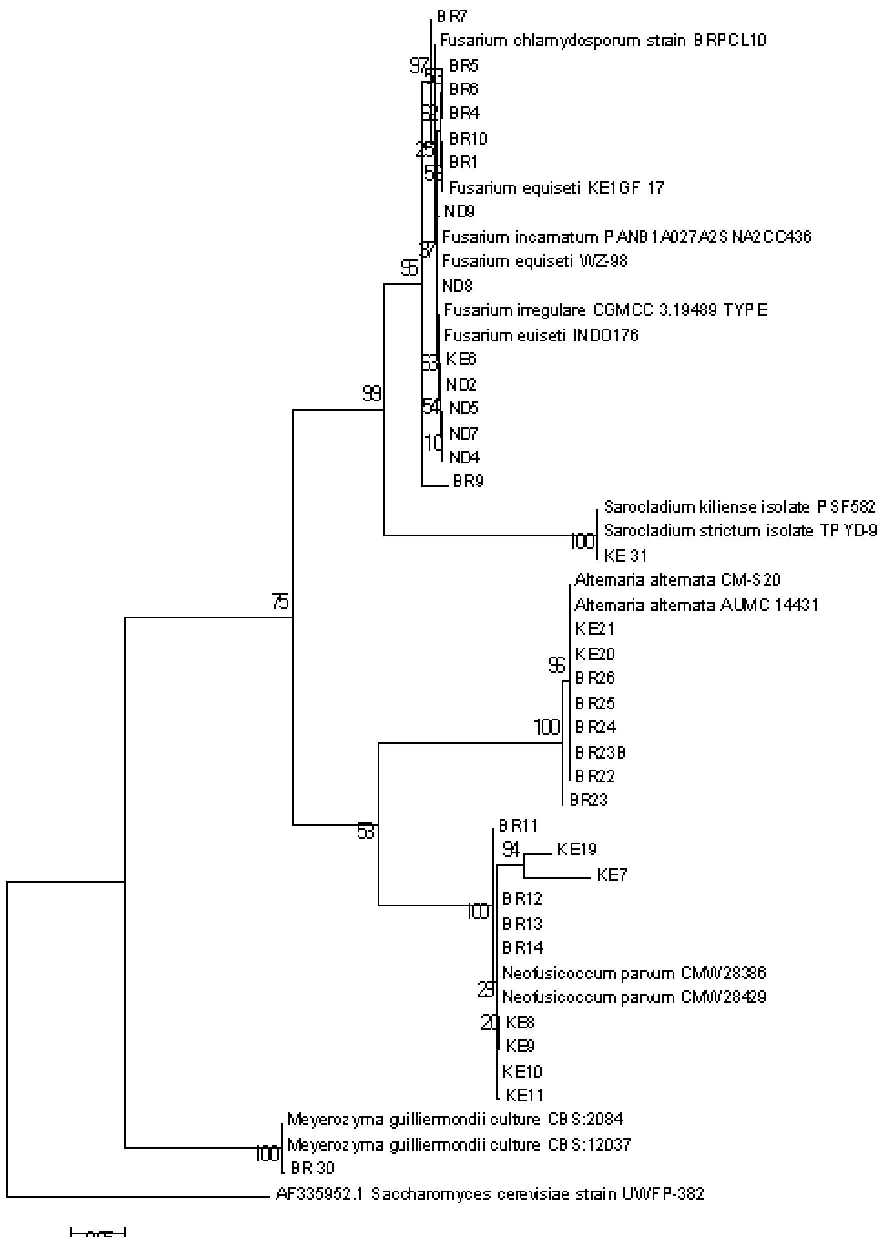


Figure 4.4: Sequenced DNA samples of fungal organisms associating with *T. brownii* germination success

The fungal organisms were then identified and recorded as in Table 4.4 on page 60, indicating their stages of identification during the germination of the experiment.

Table 4.4: Identified fungal organisms

Type of fungal organisms	The number at germination failure stage	No fungal infected germinants
Pestalotia sp	1	0
Alternaria alternata	9	0
Penicillium sp	0	9
Fusarium equiseti	8	0

Pearson correlation value of 0.958 for the two variables of non-germinated seeds versus germinants, showed there was a positive correlation, with an R sq. value of 91.8 for regression analysis.

CHAPTER FIVE

DISCUSSION

5.1 Analysis of germination success of freshly harvested *Terminalia brownii* seeds.

Terminalia brownii seeds put to germination test in this research displayed a significant difference between the sown seeds and germinants meaning there is a significant number that did not germinate. This confirms the root cause of seed germination failure which could be exogenous or endogenous factors. The endogenous factor could be a physiological or morphological condition of the embryo or both and leaving exogenous factors of chemical-physical and mechanical characteristics out of the question as the seeds were treated by soaking in water.

These endogenous factors could also be a result of infection by bacteria, fungi, or viruses interfering with the condition of the embryo (Gure et al., 2005) Alternatively, some of these pathogens also interfere with the development of the embryo by competing for the food reserved for development. In this research therefore with the seeds having been collected directly from the treetop, the transmission of the pathogens is ruled out.

Results of this germination test varied, whereby results of seeds that were extracted immediately showed the highest germination percentages, which again reduced over time depending on the length of storage period while in the fruits before being extracted. In-field conditions too, the seeds are likely to be highly affected by the fusarium pathogen during dry conditions when the dormant phase is over (Franke et al., 2014).

5.2 Seed-borne fungal organisms associating with *T. brownii* germination success

There was a significant difference recorded in *T. brownii* seeds that failed to germinate and those that germinated successfully. However, the difference was not contributed to by the identified fungal organisms thus confirming the specific association of the identified seed-borne fungal organisms where they affect the seeds at various stages. This is confirmed by research done by Fraedrich (2001) and Gure et al. (2005) where they found that there are seed-borne fungal organisms that destroy the young plant by competing for the food reserves for the young embryo which is to be used during the initial stages of the development. For the seeds, whose embryo get attacked while still in the seed coat, that

marked the end of their germination process, while the ones which proceed to the final stages of germination germinated successfully, hence ready for nursery management activities. However, other seed-borne fungi associate with seedlings after germination as discovered in this study and they did not affect the germination of the seeds.

Seed-borne fungi identified in this study to be associating with the germination success of *T. brownii* is *Penicillium*. This confirms a report done by Noelting et al. (2011), where a detailed pathogenicity test carried out depicted the effect of discoloration on infected amaranth seeds which registered low germination values compared to the seeds that had no discolorations symptoms. This agrees with the results of a study done by Khatum, Shamsi, and Bashar (2018), whereby seed germination was affected by *Aspergillus niger, Aspergillus flavus, Chaetomium globosum, Penicillium sp, Rhizopus stolonifera, and Aspergillus fumigatus.* An interesting finding of this is that the prevalence in seed also increases directly with an increase in storage time unlike for some seed-borne diseases such as *Syncephalastrum recemosum.*

Some of the seed-borne fungi identified to be associating with the germination process of *Leucaena leucocephala* include *Penicillium expansum, Aspergillus niger, Trichoderma sp, Cladosporium sphaerospermum, Aspergillus wentii, Pestalotia species, Fusarium oxysporum, and Aspergillus flavus* (Lezcano et al., 2015). However, among these species, only six of them produced symptoms making identification easy while the rest needed extra examination tests to be identified. The six that easily produced the symptoms include *Pestalotia sp*, *Aspergillus niger*, *Fusarium oxysporum*, *Trichoderma sp*, *Aspergillus flava,* and *Penicillium expansum* which showed the highest percentage of infection.

Penicillium spp on the other hand has been identified to be associating with other seed-borne fungi of tomatoes in Bangladesh among others which are: *Alternaria solani, Helminthosporium solani, Penicillium spp, Aspergillus niger, Fusarium moniliforme, and Curvularia lunata* (Raju, 2017)

However, some genera species have been identified to be a dangerous pathogen that affects seedling growth and emergence, but the frequency in the lot that had been collected back in November-December 2019 was not high enough to contribute negatively to the emergence of the species' seeds. Research findings by Lezcano et al. (2015), also showed that infection increased with an increase in storage time.

5.3 Comparison of seed borne fungal organisms found associating with germination failure and germination success of *T. brownii* in the three sites.

Seed-borne fungal organisms that have been identified in this experiment at the various stages are *Fusarium equiseti*, *Pestalotia sp, Alternaria alternata* at the initial germination stage, and *Penicillium sp* at the final germination stage. From this study, therefore, diseases associating with different germination success are not the same.

This has been confirmed by Gure et al. (2014), where the report says that most of the diseases identified as seed-borne are mainly fungal, and their effects are associated with different phases of the growth of the seedling. Some of these fungi attack the seeds at storage, some at initial stages, others after the germination phases, and others at the final stage of the establishment which is at the seedling phase. Diseases that have been identified at the species levels to be affecting the development of the seedling's growth at the various stages therefore include:

- Those attacking these seeds to be listed seed-borne fungi include *Cercospora* sp. *Phoma* sp, *Guignardia* sp, *Ulocladium botrytis U. chartarum, U. chartarum.*
- Those attacking the seeds both at the seed and seedling stages mainly being *Fusarium oxysporum* and *Polyporus* sp.

This, therefore, confirms the two fungal species identified in this experiment of *Fusarium oxysporum* and *Aspergillus flavus* affects the various stages.

Some pathogens are very dangerous in that they can penetrate seed wall and infect the seeds from the internal side by destroying the embryo and endosperm like in the case of fusarium and *Penicillin expansum* (Franke et al., 2014; Lezcano et al., 2015) Once they destroy the endosperm, the effect is impacted on germination process where if extreme, damages on the endosperm lead to total loss of germination as the embryo won't find sources of energy for its growth.

Pathogens sometimes affect the seed by affecting the germination upon initiation of germination as is in the case of *Fusarium tricintum* and *Aspergillus flava* (Franke et al., 2014; Lezcano et al., 2015). These, therefore, indicate clearly that some seed-borne fungi attack seeds after germination initiation, where they attack the radicle mainly and thus can be controlled by doing away with them from the coat. While some serious ones like Fusarium

reported being an internal fungus originating from within the seed, destroy the seed completely leaving them with no signs of germination at all. Such studies on the effect of seed-borne fungi on germination failure and germination of seeds have been confirmed in the germination of grains which subsequently led to a reduction in seedling growth vigour and reduced germination percentages (Tsedaley, 2015).

Hamim et al. (2013) also reported species of fungal diseases that affected germination processes, and the identified species include" *Colletotrichum dematium, Aspergillus flavus, Fusarium spp*, *Macrophomina phaseolina, Penicillium spp.*, and *Aspergillus niger.* Similar species from this report is the *Fusarium spp*.

Findings from this study indicate that there were seed-borne fungal organisms associated with germination of *Terminalia brownii* seeds, which were *Fusarium equiseti*, *Pestalotia sp,* and *Alternaria alternata*. The effects of the seed-borne fungi identified, however, increased with the storage time of the seed, in which the effects were minimal in freshly harvested seeds as compared to those stored for some time. This confirms the result where *Fusarium equiseti* was reported by Suthar R., Bhatt D, and Bhatt P (2014), where the *Fusarium equiseti* had an inhibitory effect on germination of *Cuminum cyminum L*, whereby the effect increased with the concentration of the filtrate, recording 8% at 50% concentration. Similar cases of the total loss of seed germination and root rot in seeds are where inoculated seeds with various seed-borne diseases in a pathogenicity test (Omukhua & Egein, 2011) reported that ten isolated soil-borne organisns which are *C. albidum, I gabonesis, P. americana, D. edulis,* and *A. muricata and Susarium oxysporium,* significantly deterred germination.

Gure et al. (2005) also reported the effects of seed-borne fungi on *Prunus africana* and classified the effects on the seeds into four categories as per their impacts which are: i) isolates pathogenic only to seeds with no obvious impacts on the germlings; ii) isolates pathogenic to germlings only, iii) isolates pathogenic both to emerging germlings and seeds, iv) isolates more or less harmless both to seeds and seedlings; and iv) germination promoters isolates. With much concern on the i), iii), categories which are in relation with the study, listed seed-borne fungi include: *Cercospora sp*. *Phoma sp Guignardia sp Ulocladium botrytis U. chartarum, U. chartarum reducing germination percentage by 50% for the organisms identified in category i. In category iii, identified fungal species included Fusarium*

oxysporum, Polyporus sp. Effect of *Fusarium oxysporum* therefore is identified into details in this study.

A similar report about the effects of *Alternatia alternata* on germination of seeds is the one reported by Tylkoeska et al. (2003) showed the fungi's toxin resulted in a lower percentage of normal seedlings and increased necrosis of the seedlings.

Comparably, reports by Hamim, et al. (2013) show that *Colletotrichum dematium, Aspergillus flavus, Fusarium* spp, *Macrophomina phaseolina, Penicillium sp*, and *Aspergillus niger,* associated with the germination of the various seeds which were of red amaranth, okra, eggplant, cucumber, spinach, which were used as samples in the experiment, and their germination percentages were significantly reduced.

A similar fungus of the same genus but different species *Fusarium tricintum* has been reported to be pathogenic as *Fusarium oxysporum* and that it showed that the pathogen caused total mortality in seeds especially under conditions of water stress (Franke et al., 2014). This, therefore, suggests that the effects of *Fusarium oxyporum* could be as low as indicated in the results above, as the fruits were subjected to germination test immediately after harvesting. This is different in seeds stored for some time due to the difference in water levels which is high in the freshly harvested fruits hence slowing the effect of fungi infestation. To support that, the pathogen had not found enough time to penetrate the seeds as they were still fresh from the collection (Lezcano et al., 2015), thus confirming the insignificant damage caused. This is because this fusarium pathogen, for example in this case it enters the seeds first through the wall, then to the embryo, and lastly to the endosperm, thus completely killing the seed (Franke et al., 2014).

Another form in which these fungi affect germination when the embryo manages to survive and imbibes water for germination is that the exudates released by the seed where the fruit was attached to the flowers during germination will trigger the development of a layer of this fungi around the seed, thus killing the root upon emergence (Franke et al., 2014). This, therefore, leaves us with no doubt that this fungus is both an internal and external fungi, as it attacks the seeds from both inside and from outside. *Terminalia brownii* seeds germinate from the proximal end where the roots were therefore definitely killed upon the emergence of the seed's embryo and endosperm were not killed while still inside the coat.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. Analysis of germination success of *T. brownii* seeds

Freshly harvested *T. brownii* still faces challenges in achieving a hundred percent germination according to this research's first objective results. However, germination performance of the freshly harvested seeds is average.

ii. Seed borne fungal organism associated with *T. brownii* success

Seed-borne fungi identified to be associated with the *T. brownii* seeds that did not germinate did not significantly affect the seeds' germination though if they could be in high number their effects could have been identified in the research. There are identified fungal organisms associating with germination success of *T. bownii* in reports though in this research, the organisms did not display significant impact on the stage.

iii. Comparison of seed borne fungal organisms associated with germination failure and success of *T. brownii* in the three sites.

Seed-borne fungi found on the germinant do not interfere with seeds' germination. Out of the identified fungi, the most dangerous seed-borne to germination is *Fusarium equiseti* and *Alternaria alternata*. *Fusarium equiseti* and *Alternaria alternata* is a species that hinders germination of even freshly harvested seeds, both internally and externally, as it was identified on the affected specific seeds meaning it destroys the seed's embryo fully while still in the seed coat. The results, therefore, showed that fungal infections that affect the seeds at the initial stages of germination are most serious as compared to those affecting the seeds after complete germination. *Fusarium equiseti* and *Alternaria alternata* are therefore the two main seed-borne fungi with identified effects on germination while other seed-borne fungi identified to the genera level did not affect the germination of the *Terminalia brownii* seeds. Identified fungal organisms identified at the various stages of germination vary and associate with them seeds differently.

6.2 Recommendations

i. Analysis of germination successes of *T. brownii* seeds

Seeds of *T. brownii* species recorded an average germination percentage hence seeds propagation should be done using freshly harvested seeds.

ii. Seed borne fungal organisms associated with *T. brownii* germination success.

Internal seed-borne fungal organisms as per the effect of *Fusarium equiseti* need to be addressed earlier before storing the seeds, followed by relevant external seed fungicide for the organisms which wait for the emergence of the embryo.

Propagation of tree species by seeds on the other hand needs keen monitoring at the earlier stages of germination in which seed-borne, reported to be soil-borne, greatly influences the stage. This can be achieved by dressing the seeds with the appropriate fungicide, concerning the pathogen causing the disease infection in specific cases.

iii. Comparison of seed borne fungal organisms associated with germination failure and germination success of *T. brownii* in the three sites.

Fungicides specific for *Fusarium equiseti* need to be invented. On the other hand, anti-pathogenic strains of these fungi can be discovered and applied to the soil during planting.

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APPENDICES

Appendix A: Germination experiment set on date: 11th January 2019

Genus's level fungal organisms coding: *Rhizopus sp*=1, *Pestalotia sp*=2, *Aspergillus sp*=3, *Penicillium sp*=4, *Fusarium sp*=5

Site	Replicate	No of seeds sown	No of germinants	No of fungal infected none germinants with their names	No of fungal infected-germinants
1	1	25	17	2 (*Pestalotia sp*)	2(*Pestalotia sp*)
1	2	25	17	0	1(*Pestalotia sp*)
1	3	25	16	0	4(Pestalotia sp)
1	4	25	15	3(*Fusarium sp*)	1 (*Pestalotia sp*)
2	1	25	12	1(*Alternaria sp*)	0
2	2	25	16	1(*Alternaria sp*)	1 (*Penicillin sp*)
2	3	25	13	2(*Fusarium sp*) 1(*Alternaria sp*)	0
2	4	25	7	1 (*Alternaria sp*)	0
3	1	25	16	2 (*Fusarium sp*)	0
3	2	25	16	1 (*Fusarium sp*) 2(*Alternaria sp*)	0
3	3	25	13	1(*Alternaria sp*)	0
3	4	25	19	2(*Alternaria sp*)	0

KEY: (Site numbers)

Kendu=1; Kimose =2; Ndumoni =3; Dates for fruit collection: 16th Dec 2018 14th Dec 2018, 13th Dec 2018, respectively

Appendix B: Research permit

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

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Ref. No. **NACOSTI/P/19/33924/29225**

Date: **16th July, 2019.**

Omondi Jackline Atieno
Egerton University
P.O. Box 536-20115
NJORO.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Assessment of seed borne fungal organisms associated with germination success of Terminalia brownii in drylands in Kenya."*** I am pleased to inform you that you have been authorized to undertake research in **selected Counties for** the period ending **16th July, 2020.**

You are advised to report to **the County Commissioners, and the County Directors of Education, selected Counties** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

DR. STEPHEN K. KIBIRU, PhD.
FOR: DIRECTOR-GENERAL/CEO

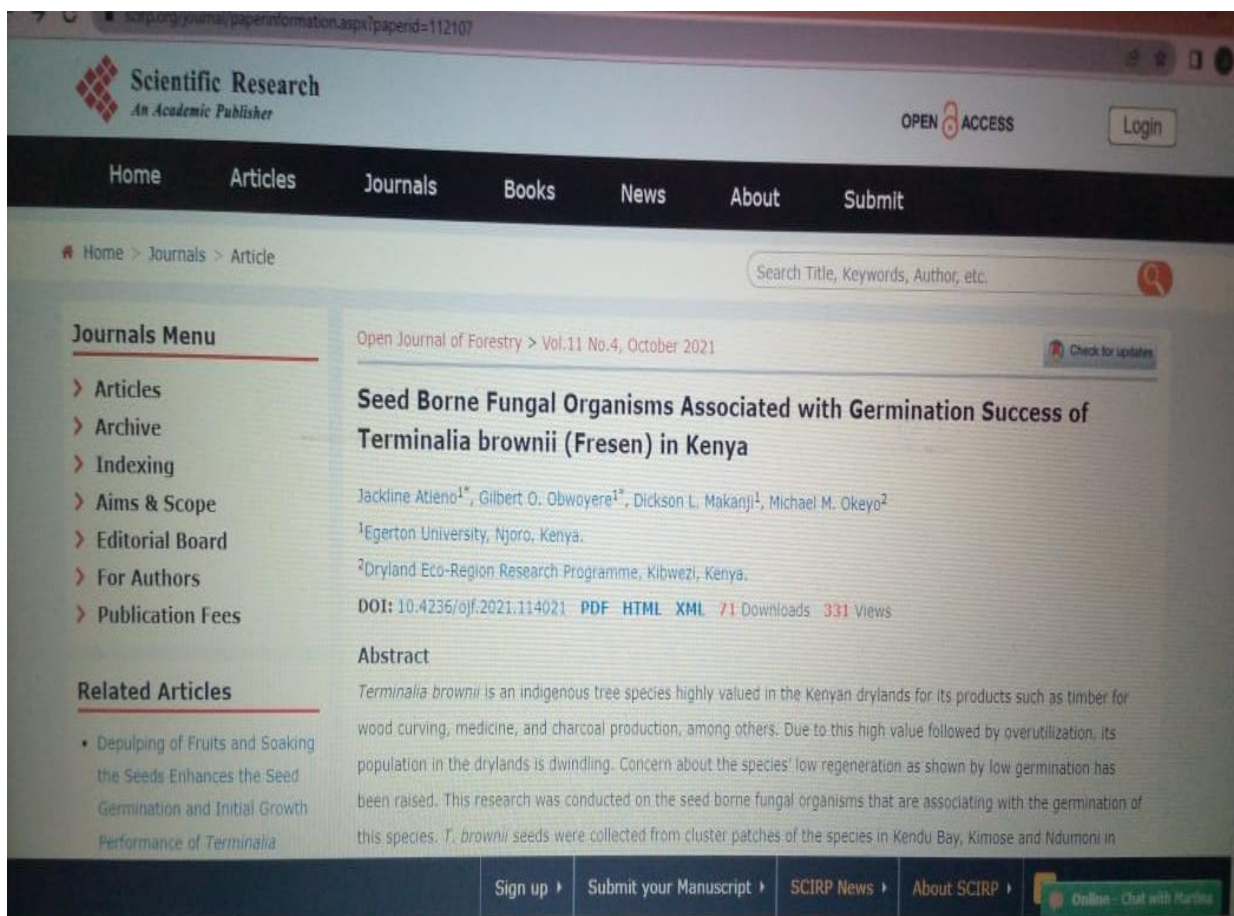
Copy to:

The County Commissioners
Selected Counties.

The County Directors of Education
Selected Counties.

National Commission for Science, Technology and Innovation is ISO9001: 2008 Certified

Appendix C: Snapshot of abstract of the publication



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Seed Borne Fungal Organisms Associated with Germination Success of Terminalia brownii (Fresen) in Kenya

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Abstract

Terminalia brownii is an indigenous tree species highly valued in the Kenyan drylands for its products such as timber for wood curving, medicine, and charcoal production, among others. Due to this high value followed by overutilization, its population in the drylands is dwindling. Concern about the species' low regeneration as shown by low germination has been raised. This research was conducted on the seed borne fungal organisms that are associating with the germination of this species. *T. brownii* seeds were collected from cluster patches of the species in Kendu Bay, Kimose and Ndumoni in

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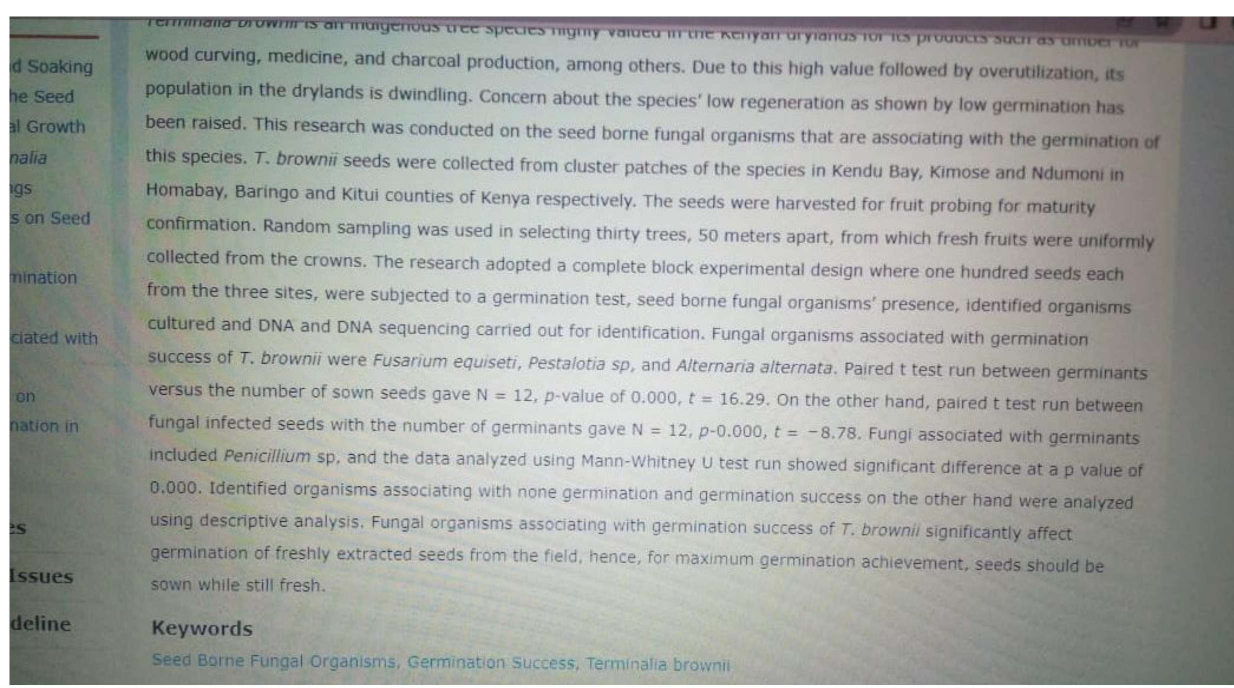

Terminalia brownii is an indigenous tree species highly valued in the Kenyan drylands for its products such as timber for wood curving, medicine, and charcoal production, among others. Due to this high value followed by overutilization, its population in the drylands is dwindling. Concern about the species' low regeneration as shown by low germination has been raised. This research was conducted on the seed borne fungal organisms that are associating with the germination of this species. *T. brownii* seeds were collected from cluster patches of the species in Kendu Bay, Kimose and Ndumoni in Homabay, Baringo and Kitui counties of Kenya respectively. The seeds were harvested for fruit probing for maturity confirmation. Random sampling was used in selecting thirty trees, 50 meters apart, from which fresh fruits were uniformly collected from the crowns. The research adopted a complete block experimental design where one hundred seeds each from the three sites, were subjected to a germination test, seed borne fungal organisms' presence, identified organisms cultured and DNA and DNA sequencing carried out for identification. Fungal organisms associated with germination success of *T. brownii* were *Fusarium equiseti*, *Pestalotia sp*, and *Alternaria alternata*. Paired t test run between germinants versus the number of sown seeds gave N = 12, *p*-value of 0.000, *t* = 16.29. On the other hand, paired t test run between fungal infected seeds with the number of germinants gave N = 12, *p*-0.000, *t* = −8.78. Fungi associated with germinants included *Penicillium* sp, and the data analyzed using Mann-Whitney U test run showed significant difference at a p value of 0.000. Identified organisms associating with none germination and germination success on the other hand were analyzed using descriptive analysis. Fungal organisms associating with germination success of *T. brownii* significantly affect germination of freshly extracted seeds from the field, hence, for maximum germination achievement, seeds should be sown while still fresh.

Keywords

Seed Borne Fungal Organisms, Germination Success, Terminalia brownii