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Fungal Pathogens Affecting Terminalia Brownii Flowers, Fruits and Seeds in Drylands, Kenya

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ABSTRACT

Terminalia brownii Freshen (Combretaceae family) is a multipurpose agroforestry tree species widely grown in Eastern Africa's dry lowlands. However, it needs to be more utilised, which has led to concern about its regeneration, which is hindered by low seed germination percentages. The study was carried out in three sites representing distinct ecological regions in agrosilvopastoral agroforestry systems where T. brownii trees are naturally distributed in the drylands of Kenya. These sites are Nduumoni, Kimose and Kendu Bay, in Kitui, Baringo and Homa Bay counties. Farmers practice subsistence crop farming mixed with livestock and T. brownii trees within the same farming system. The study investigated incidences of fungal disease infection in flowers and fruits during their growth. Flower buds, flowers, and immature and mature fruits were sampled for culturing from 30 reproductively mature trees selected randomly within 5.0 ha in an agrosilvopastoral agroforestry system in each site. Samples of 100 flower buds, flowers, and immature and mature fruits were surface sterilised using 40 % hydrogen peroxide for 2 minutes and rinsed in distilled water. These were plated on Malt Extract Agar (MEA) media and incubated at 28 ± 20C for seven days; fungal colonies were evaluated and subcultured to obtain pure cultures, and pathogens were identified using morphological characteristics. Statistical analyses were done using the Kruskal-Wallis and Bonferroni multiple comparison tests to determine the differences among the sites. Common fungal pathogens isolated were Fusarium spp (42-49%), Alternaria spp (29-33%), Bostryosphaeria spp (4-12%), Cladosporium spp (38%) and Pestalotia spp (7%). There were significant differences ($p<0.05$) in fungal infection between flower buds, flowers, immature, mature fruits and extracted seeds but not across sites. Flower buds had minor fungal diseases, thus indicating that infection took place during and after flowering. The author hypothesises that these fungi could affect seed germination by either causing seed deterioration or affecting the germinants, lowering seed quality.

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Introduction

Terminalia brownii Freshen belongs to the Combretaceae family and is a multipurpose tree species distributed across drylands of East, Central and West Africa [1]. Terminalia brownii flowers and fruits prolifically, but its regeneration could be better in most of its habitats, and its seeds record poor germination of less than 30% [2]. Despite its many uses, there is a need for more seedlings to meet afforestation and agroforestry purposes. Poor seed germination has been attributed to seed dormancy problems and infestation by diseases and insect pests.

The effect of fungal pathogens that affect the reproductive biology of T. brownii needs to be better understood. In drylands, T. brownii grows up to a height of 20 ± 5 m high. They are drought-resistant trees which prefer deep sandy soils and can occur in woodlands, wooded savannahs in semi-arid areas and near rivers in dry areas up to 2,000 m altitude with an annual rainfall of 500 – 1300 mm. Terminalia brownii has many local names depending on different communities that reside in different geographical regions in Kenya: Baresa (Borana), Onera/Manera (Luo), Muuku/Kyuku (Kamba), Koloswo (Pokot), Koloswet (Tugen), Mururuku (Embu) and Mbarao/Mwalambe (Swahili) [1,2].

Pathogens are disease-causing organisms that are microscopic and difficult to see or recognise with our naked eyes. They vary significantly in their mode of infection; some can infect several kinds of plants, while others require a specific host. Harmful diseases are found in plantations, woodlands and natural forests in temperate and tropical zones. Fungi grow best in warm and moist environments because they require water to survive and a pH of 5.6 for optimal growth [3]. They are known to have effects on both flora and fauna and can cause a significant amount of economic damage. Many resources have been employed in researching fungi and their effects on flora and fauna.

The mortality of trees is higher in areas with a higher number of fungi populations than in areas with considerably low fungi populations [4]. The survival and health of tree flowers are pivotal to the quality of the resultant fruits and seeds. Fungal pathogens

may affect flower buds or parts of a flower, causing it to wither out [5]. This, in turn, results in the formation of non-viable or low-quality fruits and seeds, thus affecting the future generations of the tree plant. On the other hand, pathogens might attack leaves, causing them to wither, rust or swell, interfering with the flower nutrients reservoirs. This might also result in fewer flowers in a tree that matures to fruits and seeds.

Fungal pathogens vary from species, genus and family; hence a plant may be attacked by various fungal pathogens with the same or different resultant characteristics. Some of these fungal pathogens include Fusarium sp, Botryosphaeriaceae, Cladosporium and Alternaria, which are known to affect roots, leaves, barks, flowers, seeds and seedlings of trees which have a significant impact on the survival and quality of seeds and fruits [6]. Identifying the fungal pathogens affecting T. brownii can contribute significantly to its conservation biology in the dry lands of Kenya.

Fungi of the genus Cladosporium are reported to affect leaves, branches, stems and fruits leading to reduced or stunted growth [7]. Some fungus diseases result from the rot of seeds during germination through storage and are caused by Alternaria and Fusarium species [5]. Fusarium species are soil-borne fungal pathogens that account for a great deal of economic damage to crops and trees [4]. They affect nearly every part of a plant, from the roots to the crown and the reproductive parts. It has a wide range of hosts and is responsible for several plant diseases, e.g. the crown rot disease on wheat caused by F. pseudograminearum [4,6]. A study by on Fusarium seed pathogens on Bromus tectorum seeds highlighted that Fusarium is reported to cause seed rot diseases often as part of a complex of diseases that affect different stages of the host, such as seeds, seedlings, and the crowns of developing plants [6]. The fungus of the genus Pestalotia causes several diseases, including fruit and seed rots in T. brownie [7].

The study determined fungi pathogens that infest T. brownii flowers and fruits during the growing period. Infected flowers, fruits, stems, and leaves exhibit localised symptoms. Symptoms such as rusts, anthracnose, powdery mildew, blights, spots and short holes are associated with leaf fungal diseases. Grain discolouration, head, capsule and fruit rots are some of the common fungi symptoms found in flowers and fruits.

Methodology

Study Sites

The study was undertaken in three sites: at Nduumoni in Kitui, Kimose in Baringo and Kendu Bay in Homa Bay counties (Figure 1). The selected sites represent distinct ecological regions where T. brownii is naturally distributed in Kenyan drylands.

Table 1: Location

County	Sub-County	Average Altitude	Latitude	Longitude
Kitui	Kitui Central	1,141	0.969915° S	37.471730° E
Baringo	Mogotio	1,563	0.473887° N	35.707631° E
Kendu Bay	Karachuonyo	1,217	0.381885° S	34.655023° E

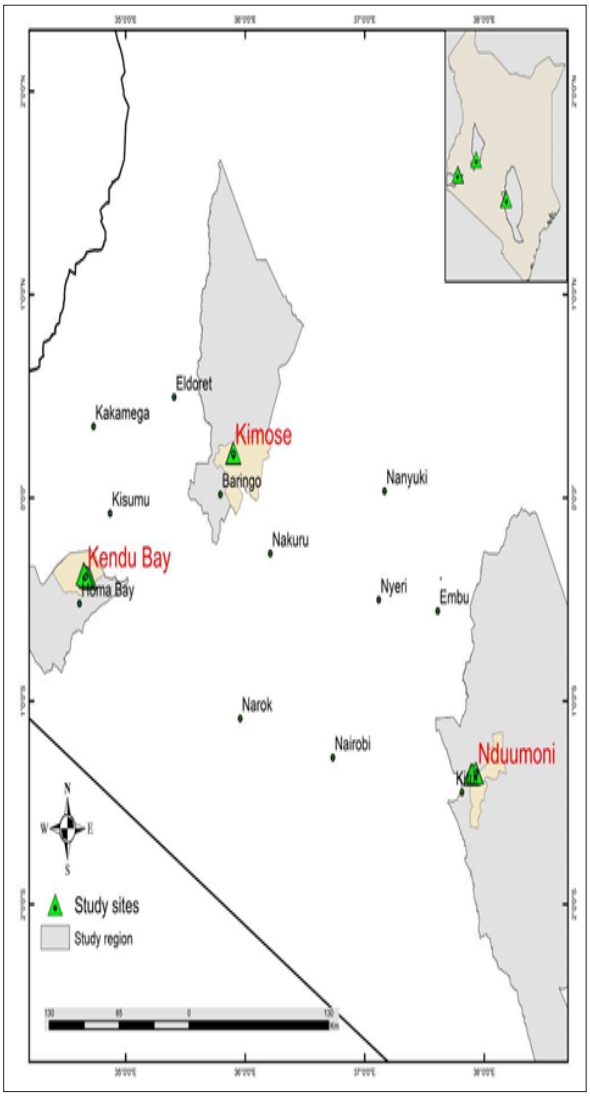

Figure 1: Terminalia Brownii Sample and Data Collection Sites

Sampling and Study Design

The research adopted an observational, descriptive and experimental design of Randomised Complete Block Design (RCBD), which entailed naturalistic observations carried out at different stages of floral and fruiting phenophases. The phenological traits of tree samples selected in the three sites were observed and recorded over time. Thirty reproductively mature trees were randomly selected in their natural habitat, marked and geo-referenced within the study site in an area measuring 200 by 200 metres in each provenance [8].

Individual study trees were randomly selected in each study site using a grid system. Each grid measuring 20 by 20 metres (400 m2) was demarcated, and 30% of the grids with reproductively mature T. brownii trees were selected randomly out of 100 grids by use of random numbers from 1 to 100 [9]. Plant samples were collected from the top, middle and lower crowns of 20 trees randomly selected from each site. In addition, samples of flower buds, full-bloom flowers, and immature and mature fruits were collected once a week. All the samples were labelled and transported in ice-cold boxes to KEFRI pathology laboratories for culturing and identification. Excess plant samples were preserved in a deep freezer at -200C.

Fungal pathogens were isolated using Potato Dextrose Agar (composition; peeled potato 100.0 g, glucose 20.0 g, agar 15.0 g, water 1000.0 ml) obtained from Oxoid. In surface sterilisation, to sterilise the surface, the samples were placed in 10 % sodium hypochlorite for 2 minutes, followed by rinsing in sterile distilled water. The samples were blot-dried using sterilised Whatman filter papers and directly plated on Potato Dextrose Agar (PDA) media. The plates were incubated at 28oC for up to 7 days. Colonies showing typical fungal characteristics were sub-cultured on PDA until pure cultures were obtained. The pure cultures were preserved in PDA slants at 4oC and glycerol until further processing. The fungal pathogens and colonies were identified using cultural, morphological and biochemical characteristics [10]. Data on types of fungal pathogens, number of colonies and frequency of the pathogens fungal diseases infesting flower buds, flowers and fruits samples were collected and recorded.

Pathogenicity Test

Once the fungi were isolated to the species level, the fastest growing isolates were chosen for inoculation of seedlings and grown in MEA for some days before inoculation. Pure fungi isolates of each pathogen were tested on the healthy-looking fruit tree species Terminalia brownii raised from seeds collected from fruit harvested during studies. These seedlings were raised and maintained in the glasshouse for six months before inoculation, and temperatures were recorded daily at 2.00 pm during the study period. Seedlings were watered to field capacity during growing and experimental periods as needed. There were two experiments to determine pathogenicity, germination of inoculated T. brownii seeds in petri-dish and inoculation of six-month-old seedlings. Pathogenicity of pure fungi isolates was evaluated using canker development, lesion length and seedling mortality after six months.

Petri-Dish Experiment

Four replicates of 25 extracted T. brownii seeds were sterilized before being inoculated in a solution containing spores of pure isolates from four fungal pathogens [11]. These seeds germinated on agar in Petri-dishes at constant temperatures of 28 ± 2 0C. Germination tests were done using 25 seeds per replicate in four replicates in a randomized complete block (RCBD) experimental design. After five incubation days, germination was scored after every two days because T. brownii seeds take a long period to germinate. Incubated seeds were considered to have germinated when the radicle or plumule protruded by 2.0 mm [12]. Germination tests were monitored for 20 days after incubation or until there were no more germinations. Seeds that failed to germinate were evaluated for various pathogenic fungi to confirm whether seed failure was due to specific fungal pathogens that seeds were inoculated with [11,13]. Data captured included number, germination capacity, speed, seedling mortality, seed colouration and texture, pathogenic mycelia growth rate and colouration.

Inoculation of Seedlings

Healthy T. brownii seedlings, approximately 30-35 cm in height and root collar diameter of approximately 0.8 - 1.0 cm, were chosen for pathogenicity tests conducted at the Kitui greenhouse. Single mycelium cultures of fungal species were grown in 2 % MEA at 25 °C and used to inoculate T. brownii healthy seedlings. The part of the stem 10 cm above the soil was sterilised with 70 % ethanol. A vertical incision of approximately 1 cm was made using a sterile blade on the stem of each seedling, and the bark was carefully lifted off. Mycelia plugs of 5 mm2 were excised from four-day-old cultures using a cork borer and placed at the center of the incision between the bark and woody tissues with the mycelial side facing the wood and covered with Parafilm. In each treatment, ten (10) T. brownii seedlings were used in four replicates, bringing 200 seedlings, including the control that was not inoculated. The experiment was laid out in a completely randomised block design in four replicates. The control and inoculated T. brownii seedlings were placed in the glass house and quarantined during the study period of six months. Experimental seedlings were watered once daily at 4.00 pm with 100 ml of distilled water in wash bottles.

Monitoring, assessment and data recording were done weekly for the first three months. The following parameters were recorded: exudates/resin and its colouration, formation of cankers, necrosis around points of inoculation, lesion length and seedling mortality. Time taken by each fungal species to show disease symptoms was recorded, and after six (6) months, the number of seedlings showing various disease symptoms types and the number of dead seedlings were counted in each treatment. Five (5) seedlings were sampled randomly from each treatment, slit longitudinally, and the length of internal lesions measured upwards and downwards from the point of inoculation, and recordings shall be made.

Estimating Virulence of Identified Fungal Species

Fungal pathogens were isolated using Potato Dextrose Agar (composition; peeled potato 100.0 g, glucose 20.0 g, agar 15.0 g, water 1000.0 ml) obtained from Oxoid. In surface sterilisation, to sterilise the surface, the samples were placed in 10 % sodium hypochlorite for 2 minutes, followed by rinsing in sterile distilled water. The samples were blot-dried using previously sterilised Whatman filter papers and directly plated on Potato Dextrose Agar (PDA) media. The plates were incubated at 28 0C for up to 7 days. Colonies showing typical fungal characteristics were sub-cultured on PDA until pure cultures were obtained. The pure cultures were preserved in PDA slants at 4oC and glycerol until further processing. The fungal pathogens and colonies were identified using cultural, morphological and biochemical characteristics [10]. Data on types of fungal pathogens, number of colonies and frequency of the pathogens fungal diseases infesting flower buds, flowers and fruits samples were collected and recorded.

Ranking of pathogenicity from the petri-dish and inoculated seedlings experiments, respectively, were estimated from the seed inhibited to germinate due to fungal infestation and percentage mortality on inoculated seedlings. Occurrences under each variable were categorised on a scale of 1-4 scale from the highest to the lowest for each fungal species on T. brownii seeds and seedlings.

Re-Isolations of Fungal Pathogens

To confirm if isolated pathogens were responsible for disease in T. brownii and fulfil Koch's postulates, samples from the five randomly un-germinated seeds and seedlings from each treatment and control were selected for re-isolation of inoculated pathogens. Seeds were used directly, and from each seedling, 2 cm pieces were cut off from the discoloured regions between infected and healthy tissues (disease leading edge) of each sample, sterilised and plated on 2 % malt extract agar. The plates were incubated at 28 ± 2 °C for 14 days, after which individual fungal pathogen colonies and conidia morphology confirmed fungal identity.

DNA Extraction from Mycelium and Amplification

Selected mycelium isolates were grown in 2 % liquid MEA for four days and freeze-dried for two weeks. Genomic DNA was extracted using 3% Cetyl trimethyl ammonium bromide (CTAB), a chloroform method described by Gardes and Bruns with modification according to [14,15].

The PCR reaction was prepared according to the manufacturer's instructions. The samples were sequenced in both directions using the PCR Big Dye Terminator v. one-cycle sequencing kit, followed by appropriate optimisation. A fragment of the internal transcribed spacer (ITS) rDNA was therefore amplified using fungal-specific primers, ITSIF (5' CTTGGTCATTTAGAGAAGTAA-3') Gardes and Bruns, and ITS4(5'-TCCTCCGGSTTATTGATATGC-3') [14]. Part of the translation elongation factor 1-alpha (Tef 1-alpha) gene was amplified with primers EFI-986R (5'-TACTTGAAGGAACCCTTACC 3') to confirm the identity of fungal groups. The amplified PCR products were purified with 6 % Sephadex G-50 columns with 50-150-mm bead size (sigma - Aldrich) following the manufacturer's instructions.

CTAB Fungal DNA Extraction

100 mg of each plant tissue was homogenised using 500 µl of CTAB Extraction Buffer. This was mixed and thoroughly vortexed before the transfer of the homogenate to a 60°C bath for 30 minutes. After incubation, the homogenates were centrifuged for 5 minutes at 14,000 xg. The supernatant was transferred to a new tube, and 5 µl of RNase solution was added before incubation at 37°C for 20 minutes.

An equal chloroform/isoamyl alcohol (24:1) was added and vortexed for 5 seconds. The samples were centrifuged for 1 minute at 14,000 xg to separate the phases. The aqueous phase was transferred to new tubes and repeated until the upper phase was clear. The clear upper aqueous phase was transferred to a new tube, and DNA precipitated by adding 0.7 volume of cold isopropanol, then incubated at 20°C for 15 minutes. Samples were centrifuged at 14,000 x g for 10 minutes. The supernatant was decanted without disturbing the pellet and washed with 500 µl ice-cold 70% ethanol. Ethanol was decanted, and the pellet dried long enough to remove alcohol without completely drying the DNA. DNA was dissolved in 20 µl T.E. buffer (10 mM Tris, pH 8, 1 mM EDTA).

UV Gel DNA Detection

Detection of DNA was determined through agarose gel electrophoresis to allow visualisation. The PCR products were electrophoresed in an agarose gel stained with ethidium bromide. The gel was prepared by dissolving one gram of agarose in 100 ml of TAE. This was heated to dissolve the agarose and cooled on an electrophoresis tray with combs inserted to form wells. 4ul of the PCR product was mixed with one µl of loading dye and added into the wells. A 100 bp marker ladder was added to one of the wells to indicate the size of the amplified product.

Sequencing and Sequencing Analysis

PCR products were sequenced and analysed with ABI 310 genetic analyser. Seqman (DNASTAR Inc., USA) was used to edit the nucleotide sequences. Edited sequences were submitted to the national center for biotechnology information (NCBI) Gene bank sequence database. They were identified by comparing with the relevant sequence of ITS region rDNA and (Tef-α) using the BLASTN algorithm (www.ncbi.nlm.nih.gov/BLAST/blst.cgi). Sequences that showed 98 - 100 % similarity were considered the closest matches to the sequence and were submitted and assigned the names with the highest species identity from Genebank. The sequences were downloaded from the database for further analysis. The nucleotide sequences obtained from this study were deposited in the Genebank, and accession numbers are shown. Selected cultures of identified fungal species were deposited at the Forestry and Agriculture Biotechnology Institute (FABI), ICRAF and the Department of plant pathology at KEFRI.

To assess the phylogenetic relationship between the sequenced fungi species, study sequences and references from Genebank were used for phylogenetic analyses. The sequences were aligned using MUSCLE with a maximum number of 100 iterations. The phylogeny tree was built on Geneious v8.1.9 using Tamura-Nei genetic distance model and the Neighbour-joining method with no outgroup. The tree was resampled using Bootstrap with 1000 replications with a support threshold of 50%.

Results

Fungal organisms isolated from flowers collected from the three study sites: Kimose in Baringo, Ndumoni in Kitui and Kendu Bay in Homa Bay, included: Aspergillus, Penicillium, Rhizopus, Fusarium and Alternaria. These fungal organisms infected 71.11% of the trees in the sample.

Table 2: Organisms Isolated from T. Brownii Flowers and Fruits in Nduumoni, Kimose and Kendu Bay During 2018

Provenance	Crown level	Organisms Isolated
Kendu Bay	Bottom	Aspergillus, Penicillium
	middle	Aspergillus, Penicillium
	Top	Penicillium
Kimose	Bottom	Aspergillus, Rhizopus
	General	Penicillium
	middle	Aspergillus, Rhizopus, Penicillium
	Top	Aspergillus, Penicillium, Rhizopus,
Nduumoni	Bottom	Aspergillus, Penicillium
	middle	Penicillium
	Top	Alternaria, Fusarium, Rhizopus, Aspergillus, Penicillium

For the pathology experiment, there were no significant ($p \leq 0.05$) differences in the mean number of Alternaria isolates between Kendu Bay (2.4 ± 0.4) and Kimose (2 ± 0.0) sites, but also between the bottom (4.3 ± 0.0), middle (3.3 ± 0.0) and top (3.4 ± 0.4) crown levels. There were no significant ($p \leq 0.05$) differences in the mean number of Botryosphaeria isolates between Kimose (2.2 ± 0.8) and Nduumoni (2.3 ± 0.0) sites and also, between the middle (4.3 ± 1.3) and top (4 ± 2.7) crown levels. There were no significant ($p \leq 0.05$) differences in the mean number of Cladosporium isolates between Nduumoni (1.3 ± 0.7), Kimose (2.6 ± 0.0) and Kendu Bay (0.6 ± 0.0) sites and also, between the top (1.3 ± 0.7), middle (2 ± 0.0) and bottom (1.3 ± 0.0) crown levels. There were no significant ($p \leq 0.05$) differences in the mean number of Fusarium isolates between Kendu Bay (11.9 ± 2.7) and Nduumoni (11.8 ± 5.3) sites and also, between middle (21.8 ± 7.5) and top (17.2 ± 5.8) crown levels. There were no significant ($p \leq 0.05$) differences in the mean number of Pestalotia isolates between Kimose (56.1 ± 17.4), Kendu Bay (44.3 ± 13.0) and Nduumoni (36.2 ± 24.1) sites and also, the top (44 ± 20.8), middle (53.2 ± 14.3) and bottom (39.5 ± 19.4) crown levels. There were no significant ($p \leq 0.05$) differences in the mean number of Alternaria isolates between buds (2.3), flowers (2.3), Immature fruits (2.7) and Mature fruits (1). There were no significant ($p \leq 0.05$) differences in the mean number of Botryosphaeria isolates between buds (2),

flowers (4.8), immature fruits (3.3) and mature fruits (1). There were no significant ($p \leq 0.05$) differences in the mean number of Cladosporium isolates between buds (2.7), flowers (1.3), immature fruits (1.3) and mature fruits (0).

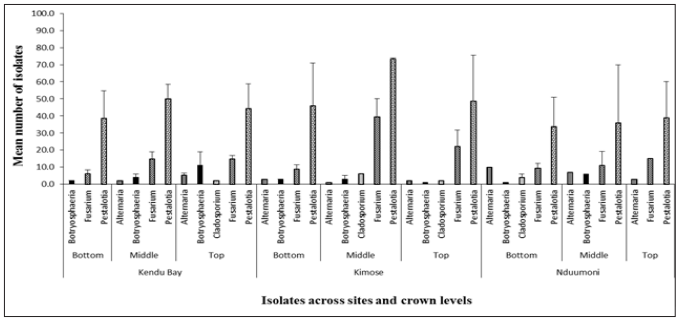

Figure 2: Mean Numbe of Isolates Per Site

Percentages of the fungal pathogens isolates from flower buds, open flowers and immature fruits of T. brownii were as follows: Pestalotia spp (53 – 57%), Fusarium spp (12 – 19%), Rhizopus (16 – 21%) and Cladosporium (1 – 5%). While that of Bostryosphaeria, Trichoderma and Alternaria species were less than 2.5% in all sampled floral phenophases. Below are some of the images of the fungal isolates from the three floral phenophases.

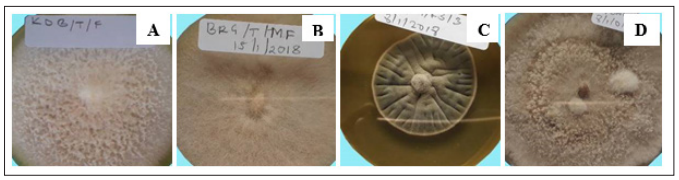

Figure 3: Some of the Common Fungal Pathogens Isolated from T. Brownii Floral Phenophases: Pestalotia (A), Fusarium (B), Cladosporium (C) and Alternaria (D) Species

Common fungal organisms isolated were Pestalotia, Fusarium, and Cladosporium species (Plate 5.1). Other organisms were Bostrosphaeria and Alternaria species were in very small amounts across all the study sites. On the other hand, disease infestations across the sites recorded presence of each Alternaria, botryospaheria, Cladosporium, Fusarium and Pestalotia.

Effect of Fungal Pathogen Isolates on Seed Germination

In the pathogenicity experiment, seed of T. brownii samples inoculated with Botryosphaeria, Fusarium, Alternaria and Pestalotia recorded germination of 9 ± 3.4%, 22 ± 1.4%, 62.5 ± 8.1% and 69.5 ± 11.1% respectively, while the control had a germination of 96.8 ± 1.6% germination capacity. There were significant ($p \leq 0.05$) differences between germination of seed samples inoculated by different pathogen isolates.

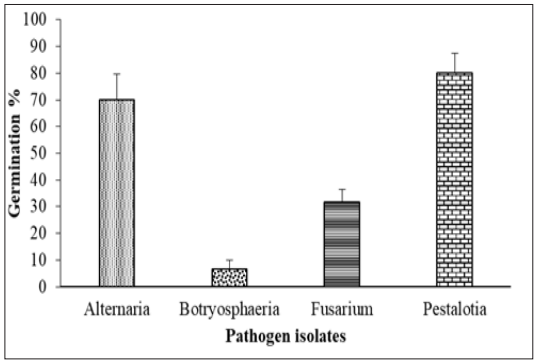

Figure 4: Germination Capacity of Terminalia Brownii Extracted Seeds Inoculated by Fungal Pathogens

Seed samples inoculated with a combination of two pathogen isolates; Alternaria and Botryosphaeria, Alternaria and Fusarium, Alternaria and Pestalotia, Botryosphaeria and Fusarium, Botryosphaeria and Pestalotia, Fusarium and Pestalotia depicted a germination capacity of; (20.5 ± 11.2)%, (28.5 ± 11.4)%, (55.5 ± 8.5)%, (6.5 ± 1.9)%, (27 ± 8.3)% and (33.5 ± 7.9)% respectively.

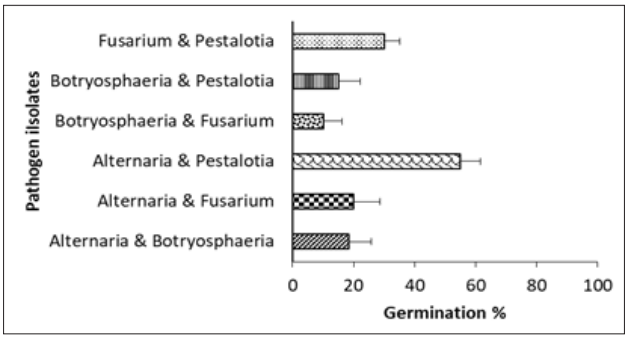

Figure 5: Germination Capacity Across Pathogens Inoculated

Effect of Fungal Pathogens on Seedlings

Pathogens were inoculated on seedlings of T. brownii. The control had 100% survival rate, seedlings inoculated with; Alternaria sp 90% survived, Botryosphaeria sp 48.3% survived, Pestalotia sp 86.7% survived and those inoculated with Fusarium sp 61.7% survived (Table 4).

Table 3: Survival Percentage of Seedlings Inoculated with Various Pathogen Isolates

Pathogen inoculated	Survival %	Std. Error of Mean
Alternaria sp	90.0	3.651
Botryosphaeria sp	48.3	4.014
Control	100.0	0.000
Fusarium sp	61.7	3.073
Pestalotia sp	86.7	3.333

Molecular Identification

PCR products showed bands that were distinctive. Molecular size of the amplicons was approximately 500 bp to 1,000 bp indicating that there was little variation in size. The gel was electrophoresed at 80volts for fifty minutes then imaged under UV light to show the bands.

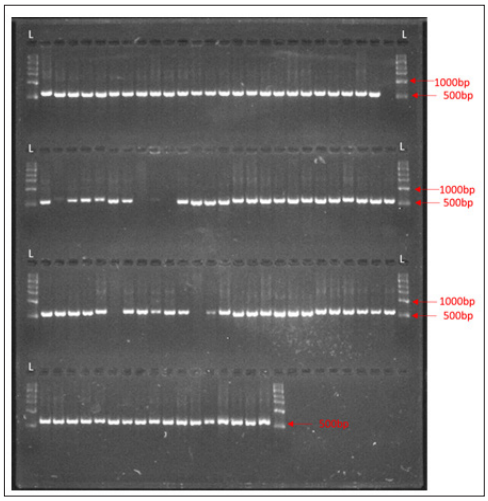

Figure 6: Agarose gel showing PCR Fragments of Fungal DNA Amplification of the ITS Region

The phylogenetic tree showed diverse relationship among the recovered species. Sample BR30 identified as Meyerozyma guilliermondii were the most divergent followed by KE31 identified as Sarocladium strictum. Samples KE 6 and ND2 identified as Fusarium incarnatum had 96% support to the consensus and are closely related to BR 2 and BR3 identified as Fusarium equiseti. Apart from sample BR2 and BR3, the rest formed distinct clades showing evolutionary changes between them and the closest species identified in Genbank.

Six samples were successfully analyzed using molecular analysis. 3 genera and 4 distinct species identified: Fusarium equiseti, Meyerozyma guilliermondii, Fusarium incarnatum and Sarocladium strictum. Table 1 shows the samples and the closest match in genbank.

Table 4: Sequence Identity from BLAST

Sample number	Species	Accession number	Sequence length	Identity
BR2	Fusarium equiseti	MK922069	461	100%
BR3	Fusarium equiseti	MK922069	461	100%
BR30	Meyerozyma guilliermondii	MT635314	568	98.77%
KE6	Fusarium incarnatum	MT032390	461	99.8%
KE31	Sarocladium strictum	MN544916	435	98.6%
ND2	Fusarium incarnatum	MT032390	461	99.8%

The phylogenetic tree showed diverse relationship among the recovered species. Sample BR30 identified as Meyerozyma guilliermondii were the most divergent followed by KE31 identified as Sarocladium strictum. Samples KE 6 and ND2 identified as Fusarium incarnatum had 96% support to the consensus and are closely related to BR 2 and BR3 identified as Fusarium equiseti. Apart from sample BR2 and BR3, the rest formed distinct clades showing evolutionary changes between them and the closest species identified in Genbank (Figure 4).

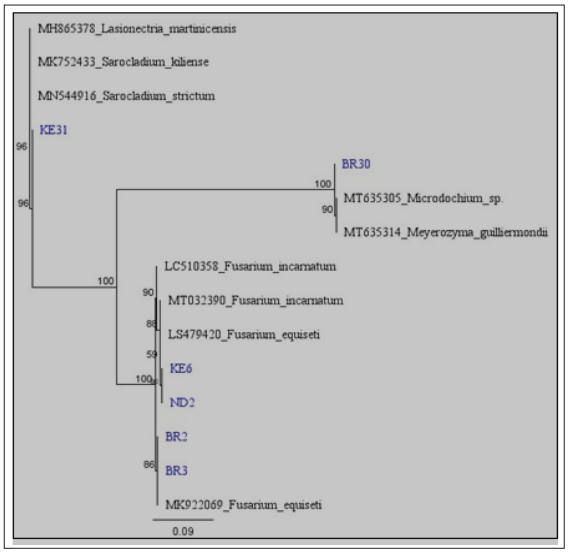

Figure 7: Fungal Molecular Phylogenetic Tree by Maximum Likelihood (ML) within Study samples (in blue) and Reference Sequences Obtained from Genbank.

Image from Geneious version 8.1 created by Biomatters. http://www.geneious.com

Discussion

Alternaria spp, Cladosporium spp, Fusarium spp, Botryospaheria spp, and Pestalotia spp were isolated from flowers and fruits in all three study sites and crown levels. In flower buds, open flowers and immature fruits, the Pestalotia spp and Fusarium spp pathogens were most evident, while Rhizopus spp and Cladosporium spp were not as evident. Fungal pathogens can affect various plants as diseases or hosts. Fusarium is a genus of many species which cause plant diseases, such as F. oxysporum, F. moniliforme, and F. proliferatum [16]. Some diseases from Fusarium infestation include collar rot of seedlings, vascular wilts and cob rots. Similarly, observed Fusarium moniliforme in Pinus elliottii, Pinus taeda, and Pseudotsuga menziesii tree species caused seed disease and damping-off [17,18]. Studies by also indicated that Fusarium invades the endocarp and mesocarp of the seed causing abortion, seed deterioration and locule emptiness [19]. Some fungi and bacterial diseases affect tree seedlings in nurseries, while others attack older trees in various ways.

Fusarium spp and Bostryosphaeria spp affected seed germination; seeds inoculated with Bostryosphaeria spp only 9% germinated, while for Fusarium spp, only 22% germinated. Fungi of Cladosporium spp are reported to affect leaves, branches, stems and fruits leading to reduced or stunted growth [7]. Studies by indicated that Fusarium species were primarily found in the soil and accounted for a great deal of damage to trees and crops, even though sometimes they affect all parts of the plant [4]. Seeds experienced poorer germination by 6% when seeds were inoculated with a combination of Bostryosphaeria spp and Fusarium spp pathogens. Studies done by Begoude indicated that dying back in Terminalia tree species can be caused by Botryosphaeriaceae fungi comprising Diplodia, Lasiodiplodia, Neofusicoccum, Pseudofusicoccum, Dothiorella and Sphaeropsis. Similarly, found that Fusarium pathogens on Bromus tectorum seeds caused seed rot diseases in developing plants [6].

Alternaria spp was observed across all sites, and it mainly occurred on the flowers, flower buds and immature fruits. Alternaria and Fusarium species can also cause fungal diseases, resulting in seeds rotting during germination through storage [5]. Seeds inculcated with Alternaria spp had a 63% germination implying that Alternaria spp affected the germination of T. brownii. Studies by Okeyo confirmed the presence of Alternaria spp in East Africa, causing damping off [7]. Pestalotia was the pathogen that occurred most across all the study sites and all the crowns and had the highest germination approximately 70% implying that it did not affect seed germination. Even though prior studies found Pestalotia genus to cause several diseases including fruit and seed rot in T. brownie [7].

Conclusion

Investigating pathogens affecting T. brownii, Alternaria spp, Cladosporium spp, Fusarium spp, Botryospaheria spp, and Pestalotia spp were isolated from flowers and fruits. Notably, Pestalotia spp and Fusarium spp pathogens were most evident than Rhizopus spp and Cladosporium spp. Furthermore, Fusarium spp and Bostryosphaeria spp affected seed germination; whereas Cladosporium spp are reported to affect leaves, branches, stems and fruit. Fungi pathogens infested T. brownii flowers and fruits during the growing period. Infected flowers, fruits, stems, and leaves exhibit localised symptoms such as rusts, anthracnose, powdery mildew, blights, spots and short holes associated with leaf fungal diseases [20-34].

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