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Full Length Research Paper

Protocol optimization for *in vitro* propagation of Kulfo, orange flesh sweet potato (*Ipomoea batatas*) variety using shoot tip culture

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Conventional propagation methods of sweet potato (*Ipomoea batatas* L. var *Kulfo*) through stem cutting require large amount of propagules and large space for preparation. It has high risk of disease transmission to the next generation. *In vitro* propagation is the best alternative to overcome such limitations. This study was conducted to optimize protocol for *in vitro* propagation of Kulfo sweet potato variety using 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) phytohormones for shoot multiplication and rooting, respectively. The result revealed that the highest shoot initiation (77.78%) and shoot length (4.40 cm) was observed in MS media supplemented with 0.5 mg/l BAP. Best shoot multiplication (5.33 shoots per explants) was obtained in MS medium supplemented with 1 mg/l BAP. MS medium supplemented with 0.5 mg/l IBA showed 100% rooting and average root length of 7.44 cm *in vitro*. In *ex vitro* conditions, 93.33% rooting was recorded. During acclimatization, 84 and 93% survival of *in vitro* and *ex vitro* rooted plantlets, respectively, were recorded. It could be concluded that MS media without BAP provides optimal condition for shoot initiation. MS supplemented with 1 mg/l BAP provides optimum condition for shoot multiplication. The *ex vitro* rooting could be better option to reduce *in vitro* rooting cost and for increased plantlet survival during acclimatization. Based on the result, 0.5 mg/l BAP for shoot initiation, 1 mg/l BAP of shoot multiplication and directly transferring to soil for rooting was recommended for micropropagation of Kulfo sweet potato variety. However, further studies will be needed in *ex vitro* root induction.

Key words: Sweet potato, micropropagation, shoot tip culture.

INTRODUCTION

In developing countries, sweet potato is an important part of food security packages (Dagne et al., 2014). It is

mainly cultivated for its expanded edible roots which contain high carbohydrate, minerals and vitamins like

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vitamin A (beta carotene) and C to a large sector of the global population (Tumwegamire et al., 2011; Shonga et al., 2013).

Sweet potato is traditionally multiplied mainly by stem cuttings which are a slow process, and diseases may accumulate in the vine cuttings from generation to generation. This could result in declining of root yield and loss of superior genotypes. This method requires large area, incurs high cost and consumes time. Despite many efforts, the underlying problem in this procedure is low frequency of regeneration, long periods of culture and frequent media changes (Gosukonda et al., 1995).

Micropropagation of sweet potato offers significant advantages in the production of large number of disease free clonal propagules within a short time, with the possibility of eliminating viral, bacterial and fungal infection and the production of high quality and uniform plantlets (Neja, 2009; Tekalign et al., 2012). *Kulfo* variety is high yielding, orange fleshed sweet potato with high quality in terms of vitamins A and C (Laban et al., 2015). There is high demand of orange fleshed sweet potato in Ethiopia but shortage of planting material is a critical problem. Therefore, the aim of this study was to optimize protocol for rapid micropropagation of *Kulfo* sweet potato variety in *in vitro* condition using shoot tip culture.

MATERIALS AND METHODS

Plant material

The study was conducted using orange fleshed sweet potato variety, *Kulfo*. This variety was obtained from Hawassa Agricultural Research Centre, Southern Nations Nationalities and Peoples' Region, Ethiopia. Vine cuttings of about 25 cm long were planted and grown in greenhouse at the College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia. The mother plants were irrigated twice per day and allowed to grow for a month after which actively growing shoot tips were collected and used as source of explants.

Stock solution and medium preparation

MS media (Murashige and Skoog, 1962) supplemented with various plant growth regulators were used. Stock solutions of the macronutrients, micronutrients, vitamins, iron source and plant growth regulators (1 mg: 1 ml) were prepared and stored at +4°C in refrigerator for immediate use. For dissolving the plant growth regulators, drop of ethanol was used for IBA and drop of NaOH for cytokinins (BAP) before making up the final volume with distilled water. The dissolved solution was poured into labeled volumetric flask to be fully dissolved and finally stored in refrigerator for later use.

The culture medium containing 30 g/l sucrose was prepared from their respective stock solutions. The plant growth regulators (BAP and IBA) were added to the medium as required with various concentrations. The mixture was stirred using magnetic stirrer and the volume was adjusted using double distilled water. Then, pH was adjusted in all cases to 5.8 using 1 M NaOH or 1 M HCl. Finally, 8.0 g/l agar was added and heated to melt throughout the experiment. Before autoclaving, the media were dispensed into sterilized culture jars. The media were steam sterilized using autoclave at a temperature of 121°C with a pressure of 0.15 Kpa for 15 min and

transferred to the culture room and stored under aseptic conditions for later use.

Sterilization and initiation of the cultures

Healthy vines shoot tip of *Kulfo* sweet potato variety was collected as explants. The explants were then washed with distilled water and sterilized by dipping in 70% ethanol for 1 min in a sterilized jar and washed using sterile distilled water three times for 5 min. They were then sterilized with 1% (v/v) commercial bleach (NaOCl) solution containing 3–4 drops of Tween-20 for 15 min and rinsed 4 times with sterile double distilled water each for 5 min with gentle shaking to remove the chemical residue. The damaged parts were excised off using a sterile scalpel and about 1 cm long explants were cultured into the nutrient media. The cultures were maintained at room temperature in the growth room with white florescent lamps of 16/8h light and dark photoperiod, respectively.

Effect of different concentration of BAP on shoot initiation

The sterilized explants were cultured on basal MS medium supplemented with various concentrations of BAP (0, 0.5, 1.0, 1.5, and 2.0 mg/l). The experiment was laid down in completely randomized design in factorial with three replications with five shoot tips per jar. After 3 weeks, percent shoot initiation and shoot length were recorded.

Effect of different concentrations of BAP on shoot multiplication

For shoot multiplication experiment, the initiated shoots were taken and cultured on hormone free MS medium for two weeks to avoid carry over effects in the next experiment. MS medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) were used for shoot multiplication. The experiment was arranged in completely randomized design with three replications with five shoots per jar. The cultures were placed in white florescent light room adjusted at 16/8 h light/dark at room temperature. Shoot number, shoot length and leave number were recorded after 5 weeks of culturing.

Effect of IBA for root formation

For *in vitro* rooting experiment, well developed shoots were cultured on hormone free MS medium for avoiding carry over effect. The shoots were transferred to half strength MS medium supplemented with 0.0, 0.1, 0.5, 0.75 and 1.0 mg/l IBA. The experiment was laid down as completely randomized design (CRD) with three replications with five shoots per jar were used. For *ex vitro* rooting experiment, 15 shoots were directly transferred to greenhouse from shoot multiplication media by carefully excising *in vitro* multiplied micro-shoots for rooting and hardening, simultaneously. After a month, number of roots, root length and percentage of rooted plantlets were recorded for both *in vitro* and *ex vitro* treatments.

Acclimatization

Plantlets with well-developed root and leaves were washed with tap water to remove adhering media and sucrose attached on the roots of plantlets. Fifteen plantlets were transferred to plastic pots in greenhouse containing hardening medium composed of soil, compost and sand (1:1:2) ratio, respectively. The plants were placed in pots covered with transparent plastic bags and irrigated

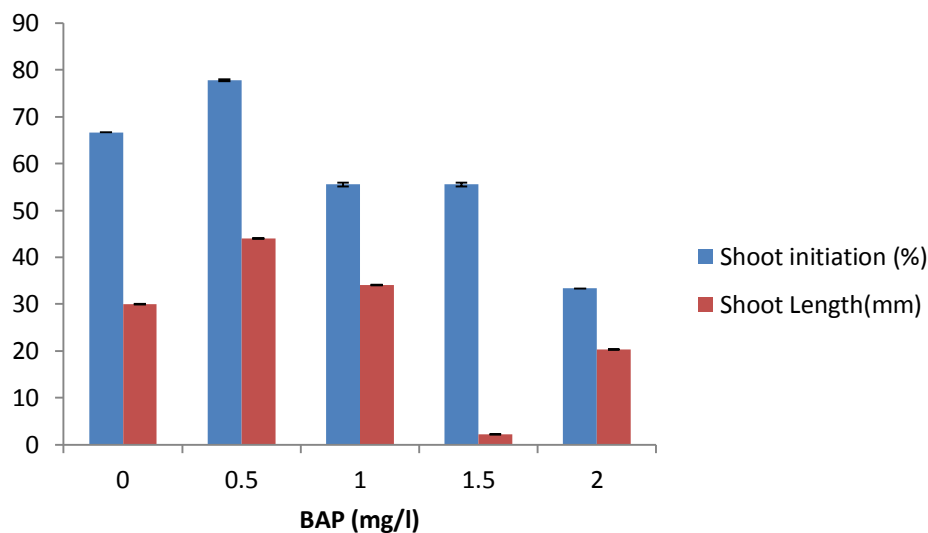


Figure 1. Effect of BAP on shoot Initiation of *Kulfo* sweet potato variety.

using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks. Finally, after 30 days, the survival rates of the plantlets were evaluated by counting the number of successfully acclimatized plantlets.

Data analysis

SAS software v9.2 (SAS Institute, 2008) was used for data analysis of variance and significance difference between treatments. Means separation was done with least significance difference (LSD) at 0.01 probability level.

RESULTS AND DISCUSSION

Shoot induction

MS medium supplemented with 0.5 mg/l BAP showed the maximum initiated shoots (77.78 ± 0.23) and shoot length (4.40 ± 0.11 cm) per explants (Figure 1).

A decrease in percent shoot initiation was observed for increasing BAP concentrations from 0.5 to 2 mg/l. Low concentrations of BAP were effective in rapid shoot initiation due to activation of cellular process. High concentrations (2 mg/l) of BAP resulted to significantly lower shoot initiation due to the inhibitory effect on metabolism and shoot elongation. This is due to high concentration of BAP that leads to metabolic inhibition. Gosukonda et al. (1995) found that different sweet potato varieties respond differently to *in vitro* shoot initiation media. However, in many cases higher concentrations of BAP were found to inhibit shoot elongation (George et al., 2008). The result was also in conformity with the finding of Sowal et al. (2002) who reported the effectiveness of low concentration of BAP to result in rapid shoot initiation due to the activation of tRNA cytokinins resulting in rapid proliferation of shoot primordial. In a similar study,

Khadiga et al. (2009) also reported that BAP at the concentration of 5 mg/l gives low number of regenerated shoots of sweet potato.

Effects of BAP on shoot multiplication

Maximum shoots per explant (5.33 ± 0.34) and shoot length (7.82 ± 0.02 cm) were observed on MS media supplemented with 1.0 mg/l BAP (Table 1 and Figure 2). Minimum shoots number (1.00 ± 0.00), shoot length (2.11 ± 0.12 cm) and leaves number (2.78 ± 0.19) were observed on MS media supplemented with the highest concentration of BAP (3.0 mg/l). At concentration of BAP below 1 mg/l, mean number of shoots and shoot length show significant difference while the leaf number did not show significant difference in their means. Consistent with the earlier report that the number of shoots, shoot length and leaf number starts declining as the concentration of BAP increased from 0.5-3.0 mg/l. It was noticed that at the BAP concentrations higher than 2.5 mg/l, shoots showed bushy feature and distorted growth due to inhibitory effect on shoot elongation and multiplication. In related studies, decline in shoot number, shoot length and leaf number was reported on Awassa-83, Beletech, Adu and Barkumie and other sweet potato genotypes (Addis, 2013, Neja, 2009; Geleta and Tileye, 2011).

A different approach by Tassew (2012) has been reported for the applications of combination of the two growth regulators (BAP and GA3) instead of BAP alone that has resulted to less number of shoot per node.

Effect of IBA in *in vitro* rooting

The highest *in vitro* rooting percentage (100 ± 0.00) was

Table 1. Effect of BAP on shoot multiplication.

BAP concentration (mg/l)	Shoot number	Shoot length	Leaf number
0	2.00 ^{ef} ± 0.00	5.10 ^g ± 0.10	3.22 ^{gh} ± 0.19
0.5	3.67 ^c ± 0.33	6.51 ^e ± 0.04	3.00 ^{gh} ± 0.33
1	5.33 ^b ± 0.34	7.82 ^b ± 0.02	6.33 ^d ± 0.34
1.5	2.67 ^{de} ± 0.33	6.44 ^e ± 0.03	5.11 ^e ± 0.01
2	4.33 ^c ± 0.00	7.20 ^d ± 0.10	8.22 ^b ± 0.19
2.5	2.33 ^{de} ± 0.34	5.20 ^g ± 0.10	5.44 ^e ± 0.20
3	1.00 ^g ± 0.00	2.11 ^j ± 0.12	2.78 ^h ± 0.19
CV	8.11	0.93	4.73

BAP=Benzyl Amino Purine, Means with the different letter in the same column is significant different at 0.01 probability level. Values are mean ±SD.



Figure 2. *In vitro* shoot multiplication of Kulfo genotype on MS medium containing 1.0 mg/l BAP after 5 weeks of culturing.

observed on ½ MS media supplemented with 0.5 mg/l IBA and IBA free media. The minimum *in vitro* rooting percentage (33.33±0.01) was observed on MS medium supplemented with 1 mg/l IBA. However, 93.33% root formation was obtained from *ex vitro* rooting. The maximum *ex vitro* rooting response was not significantly different from maximum *in vitro* rooting responses (Table 2). The maximum number of roots per shoot (7.44±0.38) with average root length (6.22±0.11 cm) was found on ½ MS medium supplemented with 0.5 mg/l IBA. A minimum of 3.33±0.33 and 4.09±0.11 cm number of roots and root lengths, respectively, were observed for ½ MS medium supplemented with 1.0 mg/l IBA (Table 2). As the concentration of IBA increased, number of root and length of roots were significantly reduced. This indicates that rooting was highly influenced by the concentrations of IBA used. The ease of rooting in sweet potato is due to the presence of high endogenous auxin concentration in the explanted organ. Hence, appropriate amounts of auxin in the rooting medium are crucial for root induction.

Similar result was reported by Geleta and Tileye (2011) on Awassa-83, Guntute and Awassa local sweet potato varieties.

From the rooting experiment, *ex vitro* could be taken as the best option that replaces *in vitro* root formation. It reduces the cost of the growth regulator. The ease of rooting in sweet potato is due to the presence of high endogenous auxin concentration in the explanted organ.

Roots developed through *ex vitro* rooting were significantly longer compared to those developed *in vitro* (Table 2). The highest root length (9.80±0.00 cm) was observed on *ex vitro* rooted shoots. The *ex vitro* system provides better rooting system during simultaneous rooting and acclimatization period and reduce the cost associated with growth regulators during *in vitro* rooting. There are a number of reports on *ex vitro* rooting in different crops such as Rotula (Martin, 2003), pistachio (Benmahioul et al., 2012), Agapanthus (Ponnusamy and Vanstaden, 2013), tea (Ranaweera et al., 2013), *Malus zumi* (Jin et al., 2008), *Hagenia abyssinica* (Tileye et al.,

Table 2. Effect of IBA on rooting of Kulfo sweet potato variety.

IBA (mg/l)	Rooting %	Number of roots	Root length
0	100 ^a ±0.00	1.67 ⁱ ±0.00	8.27 ^b ±0.05
0.1	88.89 ^{ab} ±0.00	4.22 ^d ±0.19	7.19 ^c ±0.03
0.5	100 ^a ±0.23	7.44 ^a ±0.38	6.22 ^e ±0.11
0.75	66.67 ^{abc} ±0.00	5.22 ^c ±0.19	4.99 ^f ±0.01
1	33.33 ^c ±0.01	3.33 ^{ef} ±0.33	4.09 ^g ±0.11
Ex	93.33 ^a ±0.00	3.85 ^{de} ±0.00	9.80 ^a ±0.00
CV	4.05	5.90	1.29

Ex=Ex *vitro* rooting; IBA=indole-3-butyric acid; CV=coefficient of variation. Means with the same letter in the same column are not significantly different at 0.01 probability level.



Figure 3. Rooting of Kulfo sweet potato variety. A) In vitro rooting at hormone free ½ MS medium. B) Roots of ex vitro rooted plantlets.

2007) and *Passiflora* (Mahipal et al., 2015). This is the first report on *ex vitro* rooting of shoots of sweet potato.

The *ex vitro* developed roots were non-fragile during handling (Figure 3). However, *in vitro* developed roots have been found to be thick, fragile and easily breakable during handling. This is due to the development of structural abnormalities in *in vitro* roots (Kataoka, 1994). This makes the *ex vitro* rooting method cost effective, time saving and more suitable for transferring into natural environment compared with the *in vitro* development of roots. Absences of root hairs in *in vitro* developed roots are also reported to affect their establishment in field soil under commercial-scale cultivation (Debergh and Maene, 1981). This makes the *ex vitro* rooting method cost effective, time saving and more suitable for transferring into natural environment.

Acclimatization of plantlets

The *in vitro* as well as *ex vitro* rooted plantlets were hardened in the greenhouse. After a month of acclimatization, 84% of plantlets survived and successfully established from *in vitro* rooted plantlets.

From the plantlets rooted in *ex vitro* system, 93% successfully survived and acclimatized (Figure 4). Better plantlets survivals were observed on *ex vitro* rooted plantlets than *in vitro* rooted plantlets. This is due to higher root length which playing crucial role in nutrient absorption (Kumar et al., 2014). The current results is in harmony with the finding of Berihu (2014) who reported 81.25% and 70.59 % plantlet survival, using the mixture of moist red soil, sand soil, and compost in the ratio of 1:2:1. Tassew (2012) also obtained 80% - 90% of survived plantlets after one month acclimatization.

Conclusion

Among BAP concentrations used for shoot initiation, 0.5 mg/l BAP was found to be the optimum concentration. The effects of BAP concentrations on shoot multiplications were also highly significant. The maximum shoot/explant (5.33 ±0.00) and shoot length (7.82±0.02 cm) were observed on MS medium supplemented with 1.0 mg/l BAP. For *in vitro* rooting, half strength MS medium with 0.5 mg/l IBA showed the highest value for percentages of rooting and number of root/shoot.



Figure 4. Acclimatized plantlets of Kulfo variety in the greenhouse. A) Plantlets covered with plastic bags (1st week of acclimatization). B) Acclimatized plantlets after one month in greenhouse.

Although, statically no significant difference was observed among 0.5 mg/l BAP and *ex vitro* rooting, but higher root length response were recorded at *ex vitro* rooted plantlets. About 84% of the *in vitro* rooted plantlets were acclimatized successfully, whereas, 93% of the *ex vitro* rooted plantlets survived and acclimatized successfully. Therefore, 0.5 mg/l BAP for shoot initiation, 1 mg/l BAP of shoot multiplication and directly transferring to soil for rooting was recommended for micropropagation of Kulfo sweet potato variety.

CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

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Full Length Research Paper

Abundance and impact of *Parthenium hysterophorus* L., an alien invasive herb on plant species diversity in invaded areas of Queen Elizabeth National Park, Uganda

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***Parthenium hysterophorus* is an alien annual herb that aggressively threatens biodiversity of Queen Elizabeth National Park (QENP) in Uganda. Occurrence, abundance and impact of *P. hysterophorus* on plant species diversity were examined. An observational inspection survey assessed the occurrence of *P. hysterophorus* while Quadrats sampled vegetation in *P. hysterophorus* invaded and uninvaded sites of Mweya Peninsula and along Kazinga Channel Track. Plant species richness, dominance, evenness and diversity of invaded and uninvaded sites were statistically different at $P < 0.05$. Species richness (R) and dominance (D) were higher in invaded sites (R = 58, D = 0.62) than uninvaded sites ($P = 0.043$; R = 39, $P = 0.04$; D = 0.46). Consequently, species diversity of other plants became less ($1-D = 0.38$) in invaded than uninvaded ($P = 0.039$; $1-D = 0.55$). Also, *P. hysterophorus* significantly starts to reduce species diversity and richness at very low levels of abundances, as low as (4.6%) and (7.7%), respectively, and dominates at a relatively higher level (40.2%). It was concluded that *P. hysterophorus* in QENP, negatively affects the plant species diversity at low levels of abundances through dominance.**

Key words: *Parthenium hysterophorus*, environmental impact, species abundance, species richness, weed spread.

INTRODUCTION

Parthenium hysterophorus is an alien annual herb that belongs to the Asteraceae family (Meena et al., 2017). Currently, it is aggressively threatening the biodiversity of Queen Elizabeth National Park (QENP) in Uganda. It originated from Southern United States of America (Thapa et al., 2018) and due to its high colonization vigour (Steven and Shabbir, 2014), it has reached as far

as East Africa in general and Uganda, in particular (Wabuye et al., 2014). In Uganda, *P. hysterophorus* has been recorded in the Eastern districts of Jinja and Busia, Central districts of Kampala and Masaka, Western districts of Mbarara and Kasese and the Northern district of Pader (Wabuye et al., 2014).

P. hysterophorus is an erect plant that attains a mean

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height of 2 m (Sahrawat et al., 2018). Its life cycle exhibits rosette and reproductive distinct growth stage. In rosette stage, the plant develops large simple leaves that create a wide cover which prevents undergrowth (Kaur et al., 2014). They later become multi-branched, forming bipinnate leaves at reproductive stage (Sahrawat et al., 2018). A single mature *Parthenium* plant produces a minimum of 25000 (Kaur et al., 2014) and a maximum of 100,000 seeds (Bobo and Abdeta, 2016). The seeds are then dispersed by vehicles, wind, water, machinery, animals, and along with fodders and grains (Meena et al., 2017; Birhanu and Khan, 2018). Seed germination takes place throughout the year since it is not limited by moisture availability (Kaur et al., 2014). Soon after the seedlings' emergence, the plant forms a dense canopy and then flowers within a month (Bobo and Abdeta, 2016). *P. hysterophorus* requires an average of 180-240 days to complete its life cycle (Kaur et al., 2014; Meena et al., 2017).

The negative effects of *P. hysterophorus* are not limited to plant species diversity but also impacts on animals (Bobo and Abdeta, 2016; Meena et al., 2017) that inhabit that same locality. Direct contact with the dry parts of *P. hysterophorus*, causes asthma and skin irritations (Kumar et al., 2012). Body dehydration, inflammation of soft body membranes, miscarriage and headache have also been reported (Shrestha et al., 2019). An amount of 10-50% of *P. hysterophorus* in the animals' fodder kills livestock and buffaloes (Sahrawat et al., 2018). *P. hysterophorus*' invasion reduces plant species diversity within national parks (Etana et al., 2015) which propels game animals to search for food beyond park boundaries, creating food insecurity in neighbouring communities (Abdulkerim-Ute and Legesse, 2016; Horo et al., 2020).

Although, *P. hysterophorus* is spreading rapidly (Thapa et al., 2018) and its occurrence impacting negatively on plant composition of rangelands (Khaket et al., 2015; Hassan et al., 2018), no documentation exists on how it has influenced the vegetation composition in Queen Elizabeth National Park (QENP). Thus, the examination of abundance for *P. hysterophorus* and how it impacts on plant species diversity, can help in monitoring of the habitat of QENP as an important aspect of wildlife and biological conservation (Pilliod and Arkle, 2013). It will also inform on detection of vegetation changes (Phillippoff and Cox, 2017), especially when such changes become detrimental to species (Nkoa et al., 2015). In this study, therefore, the occurrence and abundance of *P. hysterophorus* and its impact on plant species diversity of QENP were documented.

MATERIALS AND METHODS

Study area

The study was carried out between June 2014 and December 2015 in Mweya peninsula and along the Kazinga channel track located

in QENP. The Park is the largest protected area that lies within the Albertine rift valley in Western Uganda (Figure 1). QENP directly spans the equator line (00° 15'S, 30° 00'E) between lakes Edward and George. These two lakes are connected by a 35 km Kazinga channel at an oval land mass, the Mweya peninsula (4.22 km²), located at (0° 11' 40"S 29° 53'57" E and 890 meters above sea level. The park which is under the management of Uganda Wild Authority (UWA), covers an area of 1978 km². QENP receives mean maximum precipitation of 1390 mm and minimum of 750 mm annually. The mean annual maximum temperature is 28°C and minimum is 18°C (UWA, 2012). The original vegetation of the study area is typically savanna grassland, predominated by herbs, grasses, shrubs and trees. However, UWA (2012) reported that the park also inhabited other invasive species such as *Lantana camara*, *Dichrostachys cinerea*, *Imperata cylindrica*, *Opuntia vulgaris*. Thus, in QENP, adverse impacts of *P. hysterophorus* are aggravated by the existence of other invasive plant species.

QENP is Uganda's most popular tourist destination centre, which provides a rich habitat for 95 mammalian and 619 avian species. It is a haven for antelope species like Uganda kobs, duikers, Topis and sitatunga, as well as big mammals like lions, elephants, buffaloes and hippopotamuses. According to UWA (2012), an estimated human population of 30,000 people lives within the park in 11 fishing villages; while 50,000 people live in 52 parishes bordering it. This human population together with the UWA staff and tourists crisscross the park on a daily basis.

Study sites selection

Study sites were selected using two methods. These were based on preliminary information provided by QENP-invasive species management team on *P. hysterophorus* invasion, and guidelines given by Nkoa et al. (2015). Using the QENP-invasive species management team, hot spots of *P. hysterophorus* occurrence were located, and the clumpy spatial distribution pattern exhibited by *P. hysterophorus* within the located study sites necessitated stratification of the hot spots (herein termed as sampling sites), according to Nkoa et al., (2015).

Data collection methods

Occurrence, hotspots and spread of *P. hysterophorus* in QENP

An observational inspection survey was conducted to assess the occurrence of *P. hysterophorus* as an indicator of its hotspots and spread in the park. Guided by the presence of at least a single *Parthenium* plant, occurrence of *P. hysterophorus* was measured in a 1-m² randomly thrown quadrat within (50 x 50) m plots of each hot spot following survey procedure by Maszura et al. (2018). The number of spots for a specific hotspot inhabited by *P. hysterophorus* were recorded, and *P. hysterophorus* occurrence computed as percentage presence in each hot spot for comparisons (Bhusal et al., 2014).

Plant species abundance in *P. hysterophorus* invaded and uninvaded sites of QENP

A quadrat method was employed to generate the plant species abundances, for examination of *P. hysterophorus*' impact on species diversity (Arne et al., 2018; Maszura et al., 2018; Zereen et al., 2018). Three spots per hotspot with > 50% *P. hysterophorus* area cover, were considered for sampling (Bhusal et al., 2014).

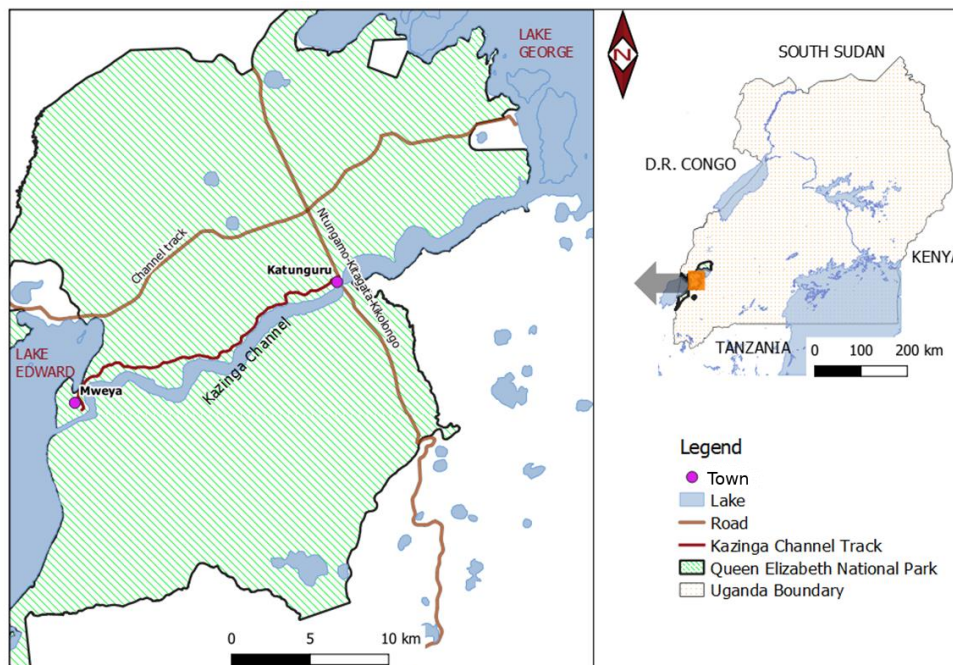


Figure 1. A map of Uganda showing the Location of the study areas in Queen Elizabeth National Park, Uganda. (Developed in QGIS 3.4.13).

Nine pairs of (10 m X 10 m) plots at 2 meters apart were demarcated for each hotspot; in a manner that nine plots were demarcated in *P. hysterothorus* invaded and other nine in uninvaded sites. A 1-m² quadrat was then randomly thrown in triplicate in each plot. Thus, a total of fifty-four (54) quadrats were placed in invaded and an equal number placed in uninvaded plots. All plant species within each quadrat were counted, recorded, collected and taken to Makerere University herbarium for identification. Plant species abundance was calculated as per a formula by Mahajan and Fatima (2017):

$$\text{Percentage abundance} = \frac{\text{Total count of an individual species}}{\text{Total number of all species recorded}} \times 100$$

Plant species diversity indices in *P. hysterothorus* invaded and uninvaded sites of QENP

Determination of plant species diversity indices; Species Simpson's index of diversity, dominance, and evenness for *P. hysterothorus* invaded and uninvaded sites were computed from plant species abundance using PAST computer software version 4.03, 2020 while species richness was compiled based on the number of plant species collected.

Data analyses

We analyzed data using Student's t-test for independent samples which compared the mean differences of Simpson's index of diversity, dominance, evenness, and richness between *P. hysterothorus* invaded and uninvaded sites while One Way ANOVA compared the computed indices between sampling sites at 0.05 level of significance using IBM SPSS Statistics 21, 2020.

A Principal Component Analysis (PCA) was run to correlate the plant species with *P. hysterothorus* abundance. Effects of *P. hysterothorus* abundance on species diversity indices were correlated by linear regression models.

RESULTS AND DISCUSSION

Occurrence, spread and hotspots of *P. hysterothorus* in QENP

From the survey with the invasive species management committee, the hot spots for *P. hysterothorus* were found to be motor-mechanical workshops, water drainage trenches, campsites, homesteads, trash burning sites located in Mweya Peninsula and spots along Kazinga channel track. *P. hysterothorus* percentage occurrences were generally high and ranged from 59.1 to 100%. Occurrence was highest in motor-mechanical workshops (100%), followed by burnt sites (85.7%), homesteads (77.1%), campsites (75.0%) then water drainage trenches (60.0%), and lastly, spots along Kazinga channel track (59.1%) as presented in Figure 2. It is evident that incoming vehicles were the most probable means of introduction of *P. hysterothorus* in these areas and its spread was further enhanced by crisscrossing of more vehicles. Mweya Peninsula once hosted the management headquarters for QENP and it is where vehicle washing and mechanical repairing of the vehicles took place. Thus, water from vehicle washing could have

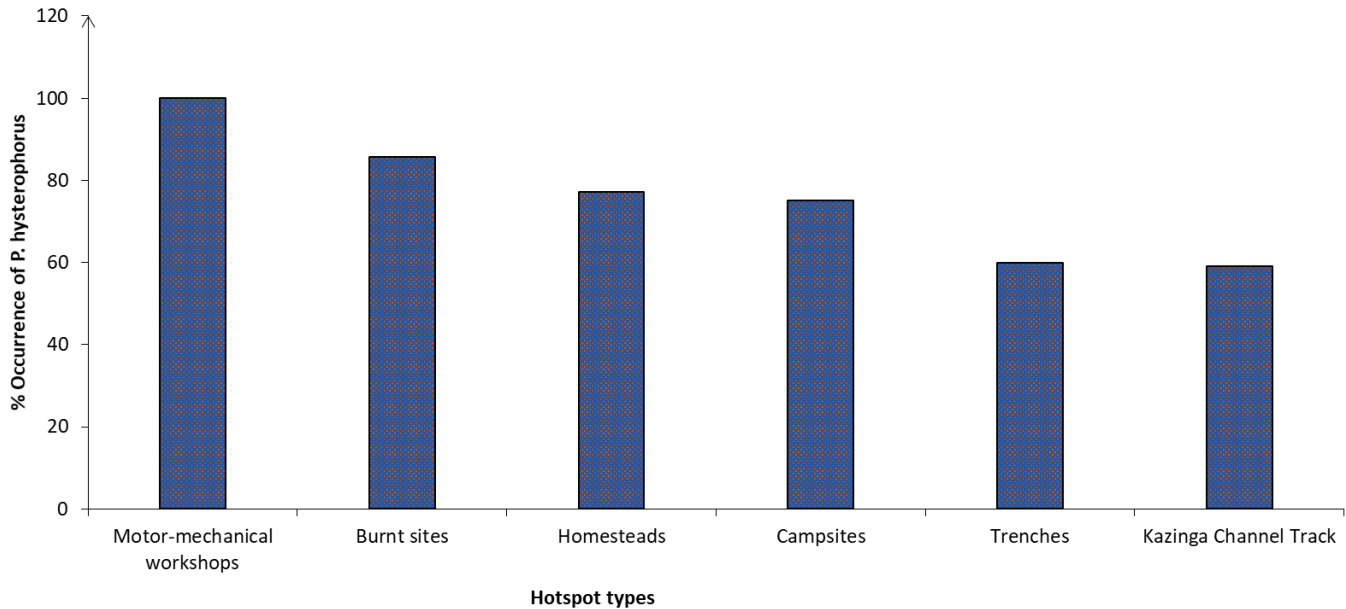


Figure 2. *P. hysterophorus* percentage occurrence for different hot spots.

spread and facilitated the seed germination of this noxious weed. The study findings are in line with CLIMEX simulation results that recorded *P. hysterophorus* in drainage trenches, dumpsites, abandoned buildings, construction sites, residential areas, rangelands and crop fields (Wabuye et al., 2014 ; Horo et al., 2020). The results also agree with a report from the distribution survey made in Nepal, where *P. hysterophorus* dispersal was directly associated with vehicle movements (Shrestha et al., 2019). Water channels were highly infested with Parthenium weed in Awash National Park in Ethiopia; thus, water was one of the dispersing agents of this weed (Etana et al., 2015). Tracks regularly used by animals to the peninsula were also observed to be lined by *P. hysterophorus*. These tracks included the visible ones made by hippopotamuses and elephants as they leave the waters of Kazinga channel. Therefore, animals' movements especially the big mammals such as elephants, buffaloes and hippopotamus are likely to be playing a big role in spreading the weed by carrying its seeds in their hooves, which can cause rapid spread of the weed and difficulty in its management.

Consistent with the findings of this study, Kuldip et al. (2011) noted that increased tourism and transportation intensified the spread of *P. hysterophorus* in North-Western Indian Himalaya. *P. hysterophorus* invasion was found in internally displaced people's camps in Pader district of Uganda, and near residential and worshipping places in Kampala (Wabuye et al., 2014) and in Malaysia (Maszura et al., 2018). It is highly likely that the spread of this weed is facilitated by human movements accidentally or due to its ornamental value such as

wreath making and wedding decorations in churches. It is possible that *P. hysterophorus* has spread in the same ways into the neighbouring communities of the QENP.

Plant abundance in *P. hysterophorus* invaded and uninvaded sites

In this study, plants species collected belonged to 27 genera and 16 families. Members of family Asteraceae were the most abundant (83.2%) in invaded sites, followed by Poaceae (11.6%) while members of other families that included Verbenaceae, Tiliaceae, Solanaceae composed of 5.4% are shown in Table 1. The table shows only plant species with percentage abundance ≥ 0.01 . Conversely, members of other plant families (48.8%) dominated the uninvaded sites followed by Poaceae (46.6%), whereas those of Asteraceae (4.2%) were the least abundant. In other studies, Poaceae (14.28%) and Asteraceae (9.52%) were among top three rich families in *P. hysterophorus* invaded sites (Gebrehiwot and Berhanu, 2015; Gadisa et al., 2019). Annual herbs of Asteraceae family have a high fecundity (Chauhan et al., 2019), that could have enabled them to compete favourably with members of other families for growth requirements. Moreover, invaded sites were characterized with bare soil patches due to disturbance, that could have allowed sunlight for secondary colonizers of Asteraceae family to grow.

We found that *P. hysterophorus* was the most abundant (79.0 %) among the flora in invaded sites followed by *Cyperus rotundus* (7.2%). *Cynodon dactylon* was

Table 1. Plant species abundances and their ranks in *P. hysterothorus* invaded and uninvaded sites.

Plant family/species	Plant origin	Counts		% Abundance		Rank	
		Invaded	Uninvaded	Invaded	Uninvaded	Invaded	Uninvaded
Acanthaceae							
<i>Hypoestes trifora</i>	IS	41	-	0.63	-	19	-
Amaranthaceae							
<i>Amaranthus spinosus</i> (L.)	IS	420	-	1.35	-	4	-
Asteraceae							
<i>Parthenium hysterophorus</i> (L.)	AN	24440	-	79.01	-	1	-
<i>Bidens pilosa</i> (L.)	IS	712	1	2.3	-	3	32
<i>Tagetes erecta</i> (L.)	IS	289	-	0.93	-	8	-
<i>Conyza bonariensis</i> (L.)	IS	235	-	0.76	-	9	-
<i>Conyza stricta</i> (Willd.var.)	IS	27	-	0.09	-	24	-
<i>Gnaphalium luteo-album</i> (L.)	IS	21	39	0.07	-	25	12
<i>Argeratum conyzoides</i> (L.)	IS	13	66	0.04	-	29	66
Capparaceae							
<i>Cleome monophylla</i> (L.)	IS	51	-	0.16	-	18	-
Convolvulaceae							
<i>Dichondra repens</i> (L.)	IS	10	43	0.03	1.69	30	10
Cyperaceae							
<i>Cyperus rotundus</i> (L.)	IS	2234	656	7.22	25.05	2	1
Malvaceae							
<i>Sida ovata</i> (Forssk.)	IS	219	24	0.71	0.94	10	15
Oxalidaceae							
<i>Oxalis corniculata</i> (L.)	IS	66	-	0.21	-	17	-
Fabaceae							
<i>Indigofera spicata</i> (Forssk.)	IS	35	339	0.11	13.28	22	3
Poaceae							
<i>Digitaria scalarum</i> (Schweint.) Chiov.	IS	395	0	1.28	-	5	-
<i>Cynodon dactylon</i> (Pers.)	IS	296	260	0.96	10.29	7	4
<i>Datylactenium aegyptium</i> (L.) Willd.	AN	219	547	0.71	21.4	10	2
<i>Eleusine indica</i> (L.) Gaertn	AN	149	13	0.48	0.48	13	16
<i>Chloris gayana</i> (Kunth.)	IS	104	188	0.34	7.37	14	5
<i>Eleusine jaegeri</i> (Var.)	IS	103	41	0.33	1.61	14	11
<i>Sporobolus africanus</i> (Poir.)	IS	20	26	0.06	1.02	26	14
<i>Hyparrhenia rufa</i> (Nees.) Stapf.	IS	10	82	0.03	3.21	30	7
<i>Cynodon nlemfuensis</i> (Vanderyst.)	IS	2	28	0.01	1.1	42	13
Polygonaceae							
<i>Oxygonum sinuatum</i> (Meisn.) Dammer	IS	213	140	0.69	5.48	12	6
Primulaceae							
<i>Anagallis arvensis</i> (L.)	IS	41	-	0.13	-	19	-
Solanaceae							
<i>Datura stramonium</i> (L.)	IS	33	-	0.11	-	22	-
Tiliaceae							
<i>Triumfetta rhomboidea</i>	AN	393	53	1.27	2.07	6	9
Verbenaceae							
<i>Lantana camara</i> (L.)	AN	102	-	0.33	-	14	-
Zygophyllaceae							
<i>Tribulus terrestris</i> (L.)	IS	38	6	0.12	0.23	21	22
Total counts		30931	2552				

Bolded, Family names; IS, Indigenous; AN, Alien.

recorded in all *P. hysterophorus* invaded sites, but with a low abundance (0.96%). Similarly, a report on weed survey done in Khyber Pakhtunkhwa in Pakistan, revealed that *P. hysterophorus* dominated the study sites with the highest abundance (63.4%) among the weeds and was followed by *C. dactylon* (11.37%) at most locations (Ali and Khan, 2017). *P. hysterophorus* abundance was also the highest (85.2%) at Kuala Muda in Malaysia (Maszura et al., 2018). In another related study, highest mean important value index (IVI) of (76.15%) was recorded for *P. hysterophorus* (Gebrehiwot and Berhanu, 2015) in Ethiopia. Musese et al. (2020) recorded the highest abundance (43.0%) of the same alien invasive species in Tanzania. High abundances of *P. hysterophorus* have been attributed to its allelopathic effects on native species (Meena et al., 2017; Birhanu and Khan, 2018), short life span and high fecundity (Bobo and Abdeta, 2016; Meena et al., 2017), vegetative regeneration (Rwomushana et al., 2019), absence of natural enemies (Meena et al., 2017) and diverse means of dispersal; which could have enhanced its rapid spread and fast colonization (Abdulkerim-Ute and Legesse, 2016).

Co-existence of plant species with *P. hysterophorus* at different sampling sites

Analysis of plant species that co-existed with *P. hysterophorus* at different sampling sites revealed a strong association of *P. hysterophorus* abundance with *Cyperus rotundus* and *Tribulus terrestris*. They clustered more closely with *P. hysterophorus* on negative axis of PC1 in Trenches (T). Other plant species such as *Bidens pilosa*, *Indigofera spicata*, *Cynodon dactylon*, *Oxalis corniculata*, *Portulaca oleracea*, and *Triumfetta rhomboidea* also showed a strong association and clustered close to negative axis of PC1 in motor-mechanical workshops (MW). *Digitaria scalarum*, *Cida ovata*, *Oxygonum sinuatum*, *Eleusine indica*, *Chloris gayana* clustered close to negative axis of PC2 around homesteads (HS), showing a weak association with *P. hysterophorus* abundance. *Datyloctenium aegyptium* and *Eleusine jaegeri* clustered on positive axis of PC2 at burnt sites (BA), also showing a weak association with abundance of the invading weed. *Galinsoga parviflora*, *Dichondra repens* clustered close to negative axis of PC2 showing a weak relationship (Figure 3). *C. rotundus*, which inhabits wet environments, co-existed in trenches where the Parthenium seeds could have dispersed by water currents. Secondary colonizers such as *B. pilosa* could have existed in motor-mechanical workshops following soil disturbance. *T. terrestris* was also recorded in high and low *P. hysterophorus* infested clusters of Simanjaro Rangeland in Tanzania (Musese et al., 2020). The co-existence can also be explained from

morphological perspective. For example, *C. dactylon* develops stolons and rhizomes which enhance its rapid vegetative growth that enables it to compete favourably with *P. hysterophorus*. Also, the stolons and rhizomes already in the soil could have been spread into fresh areas by a grader while making drainage systems. Furthermore, a study by Kruk and Satorre. (2008), reported that *C. dactylon* reproduces by seeds that have a low primary seed dormancy. Thus, seeds emerge from the soil faster than seedlings of other plants, which makes them strong competitors (Donato et al., 2019). Therefore, *C. dactylon* is a potential candidate for suppressing *P. hysterophorus* and is recommended for vegetation restoration in *P. hysterophorus* invaded sites. In other studies, *C. dactylon* was also found in *P. hysterophorus* invaded sites (Kumari et al., 2014) in India and Woreda in Ethiopia (Gadisa et al., 2019).

Plant species diversity indices in *P. hysterophorus* invaded and uninvaded sites in QENP

Generally, the study results showed a significant difference in species richness, dominance, evenness and diversity between *P. hysterophorus* invaded and uninvaded sites with $P < 0.05$ (Table 2). Although invaded sites were richer ($P = 0.043$, $R = 58$) in plant species than uninvaded ($R = 39$), uninvaded sites had more evenly distributed plant species ($P = 0.04$, $E = 0.63 \pm 0.02$) than invaded ($E = 0.23 \pm 0.08$). Species dominance was also found to be higher in invaded ($P = 0.04$, $D = 0.62 \pm 0.19$) than uninvaded sites ($D = 0.46 \pm 0.25$). Consequently, species diversity became significantly less ($P = 0.039$, $1-D = 0.38 \pm 0.19$) in invaded than uninvaded ($1-D = 0.55 \pm 0.25$). Reviewed impacts of *P. hysterophorus* on species diversity in India revealed a decrease (46.1%) in grass cover and a loss (398.1) g m^{-2} in dry biomass of Poaceae family (Birhanu and Khan, 2018). Assessment of *P. hysterophorus* impact on herbaceous plant diversity of Awash National Park in Ethiopia (Etana et al., 2015), attributed the difference in species richness to high regeneration of plant species that follow soil disturbance.

Effect of *P. hysterophorus* abundance on plant species diversity, dominance, richness and evenness in invaded sites of QENP

It was found that increasing relative abundance of *P. hysterophorus* causes a significant decline in plant species diversity ($R^2 = 0.9972$; $P < 0.001$), and plant species richness ($R^2 = 0.7377$; $P < 0.001$), but increases dominance in invaded sites ($R^2 = 0.9962$; $P < 0.001$). However, it does not contribute to species evenness in invaded sites ($R^2 = 0.0081$; $P = 0.643$) as indicated in

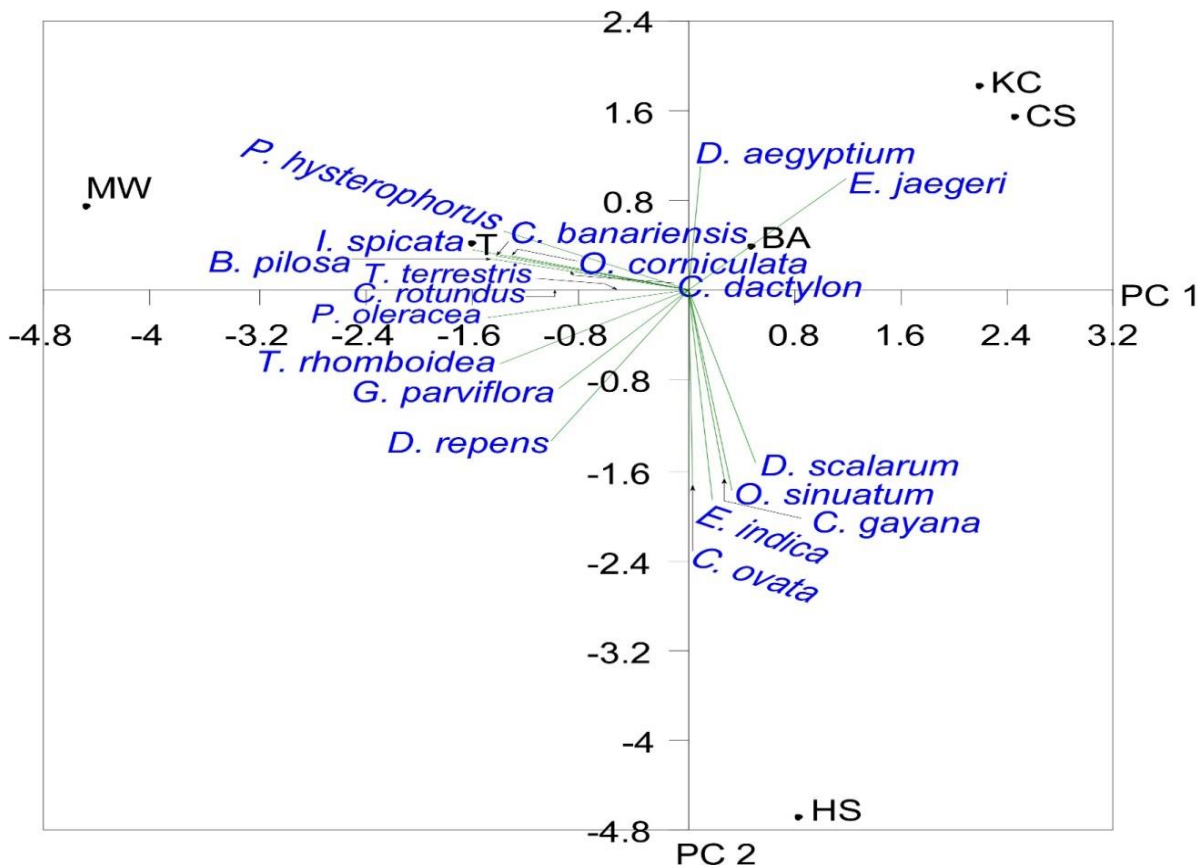


Figure 3. Scatter diagram showing plant species that co-exist with *P. hysterothorus* at Queen Elizabeth National Park.

Table 2. Means of species richness, dominance, evenness and Simpson’s diversity in *P. hysterothorus* invaded and uninvaded sites.

<i>P. hysterothorus</i> invasion status	No. of sampling sites	No. of quadrats	Diversity Indices			
			Species richness (R)	Dominance (D)	Evenness (E)	Simpsons iversity (1-D)
Invaded sites	6	54	*58	(Mean±SD) *0.62±0.19	(Mean±SD) *0.23±0.08	(Mean±SD) *0.38±0.19
Uninvaded sites	6	54	*39	*0.46±0.25	*0.63±0.21	*0.55±0.25

*Mean value is significant at P < 0.05.

Figure 4A to D. Furthermore, extrapolation of the generated linear regression models, based on linear equations ($y = -0.014x + 1.465$) in 4A, and ($y = -0.8896x + 106.87$) in 4B, the abundance at which *P. hysterothorus* starts to significantly reduce species diversity and richness is at very low percentage of 4.6 and 7.7% respectively. However, its effect on dominance according to ($y = 0.014x - 0.4631$) in 4C, was exerted at relatively higher percentage abundance (40.2%).

This confirms that *P. hysterothorus* reduced the

species diversity and richness of plant species and agrees with the documented impact of *P. hysterothorus* that also revealed a reduction of 80-90% carrying capacity of pasture in Central West Asia (Rwomushana et al., 2019). In addition, *P. hysterothorus* frequency was inversely proportional to species diversity and a significant shoot growth reduction (47.9%) and root growth inhibition (59.3%) was recorded on grassland species of Australia (Birhanu and Khan, 2018). The dominating effect of *P. hysterothorus* was attributed to

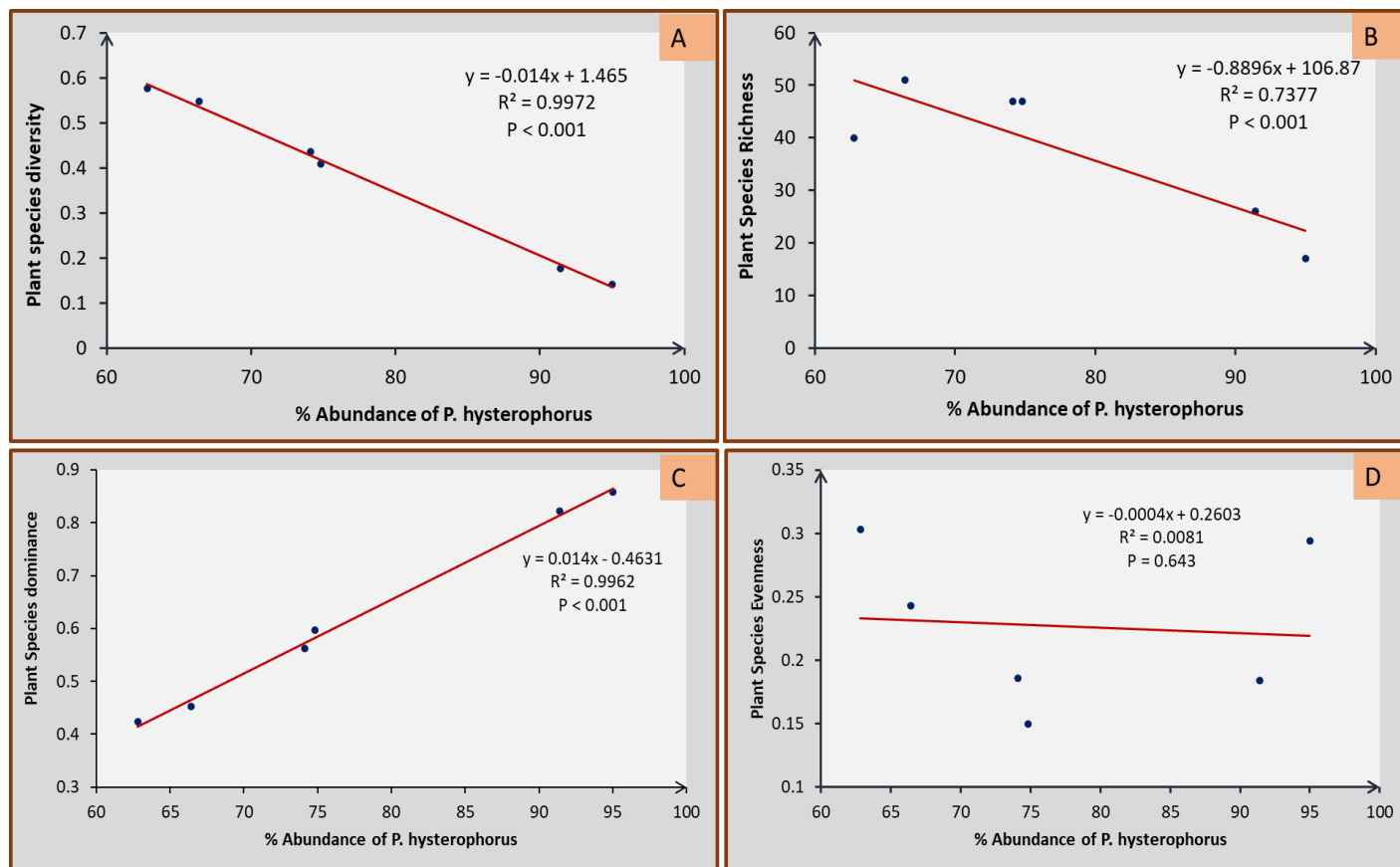


Figure 4. Effect of *P. hysterophorus* relative abundance on plant species diversity (A), richness (B), dominance (C) and evenness (D) at invaded sites in Queen Elizabeth National Park.

allelopathic nature of monoterpenes it contains (Belgeri and Adkins, 2015). Over 73.7% reduction in species richness in invaded areas as shown in Figure 4B, was accounted to *P. hysterophorus*. The same trend was reported by Gadisa et al. (2019), further confirming the detrimental effect of the weed especially in protected areas like QENP. Similarly, a decline (90%) in forage production was attributed to inhibitory potentials of *P. hysterophorus* on plant germination (Birhanu and Khan, 2018).

Conclusion

P. hysterophorus invasions in QENP, significantly reduces species diversity and richness, even at very low levels of abundance through its dominance at a relatively higher level.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Botryosphaeriaceae associated with baobab (*Adansonia digitata* L.) and marula (*Sclerocarya birrea* A. Rich.) in agroforestry systems in Kenya

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Indigenous fruit trees such as baobab and marula provide key nutrients and income for smallholders and enhance diversification of agroforestry systems in the drylands of Sub Saharan Africa. Cankers and diebacks are increasingly observed impacting baobab and marula in domestication trials and farms in Kenya, but little is known on disease occurrence and associated pathogens. Field disease incidence and severity was assessed. Fungal isolation and molecular identification was performed and pathogenicity of isolates was evaluated on baobab, marula and additional agroforestry trees. Nine taxa morphotypes belonging to genera *Lasiodiplodia*, *Neofusicoccum* and *Dothiorella* were identified co-occurring in both symptomatic and asymptomatic plant material. Seedlings inoculated with isolates of *L. pseudotheobromae*, *L. theobromae* and *N. parvum* showed similar symptoms with various degree of virulence. These findings suggest that species of Botryosphaeriaceae may occur as endophytes and also act as a disease complex, with the potential of infecting a wide range of trees in Eastern Kenya. Further investigation of ecology and impact of this potential threat to agroforestry systems in the African drylands, need to be performed in order to develop mitigation strategies.

Key words: *Adansonia digitata*, agroforestry, Botryosphaeriaceae, *Sclerocarya birrea*, tree cankers.

INTRODUCTION

Domestication of *Adansonia digitata* and *Sclerocarya birrea* within agroforestry systems in drylands of Kenya has contributed to nutritional security and source of livelihoods (Waldron et al., 2019). However, canker and dieback diseases associated with Botryosphaeriaceae fungi has greatly impacted trees healths in Africa, potentially frustrating the benefits of agroforestry for

smallholder farmers (Graziosi et al., 2019).

Species of Botryosphaeriaceae have been reported to cause serious disease on woody plants worldwide (Jami et al., 2014). Reports of dying baobab in South Africa were associated with *Lasiodiplodia theobromae* and *Neofusicoccum parvum* (Roux, 2002). Health assessment of Australian baobab (*Adansonia gregorii*) trees revealed

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eleven Botryosphaeriaceae species including *Lasiodiplodia theobromae* (Sakalidis et al., 2011), *Lasiodiplodia crassispora*, *L. pseudotheobromae*, *Neofusicoccum ribis*, *Pseudofusicoccum adansoniae* and *Lucania parva*. *Graphium* species was also identified associated with baobab in South Africa (Cruywagen et al., 2010; Farr and Rossman, 2016)

Botryosphaeriaceae causing canker and dieback in Kenya has been reported on *Grevillea robusta* in Eastern Kenya (Njuguna et al., 2011), on Meliaceae (Muthama et al., 2017) and on Eucalyptus (Machua et al., 2016). However, no isolation attempts have been done on indigenous baobab and marula in Kenya. Severe cankers associated with Botryosphaeriaceae have been observed in Kenya on domestication trials of baobab and marula causing growth loss, fruit rot and tree mortality; additionally due to their domestication trials, the biotic interaction in agroforestry systems provide an intriguing situation to study. Hence, this is the first detailed study of Botryosphaeriaceae attacking *A. digitata* and *S. birrea* in East Africa. The role of these fungi in the ecology of the trees from which they were collected should be considered in future studies.

The objective of this study was to characterize the diversity of Botryosphaeriaceae associated with cankers of native trees *A. digitata* and *S. birrea* in Kenya and to assess their pathogenicity on these hosts.

MATERIALS AND METHODS

Field survey and sampling

Survey was conducted in Eastern Kenya in Makueni and Kitui County in 2018. Three sites were selected across the two Agro-ecological zones of Mukange, Tiva and Ikanga.

Makueni country is hot and dry receiving mean annual rainfall of 231 and 361 mm during long and short rains respectively. The mean maximum temperature of the area is 25°C and the mean minimum temperature is 13°C (Jaetzold et al., 2010). Kitui is hot and dry with high temperature throughout the year ranging from 16 to 34°C (Jaetzold et al., 2012).

Samples were collected from symptomatic and asymptomatic material from across five farms in Kitui and Makueni County. Symptomatic trees were sampled based on occurrence of various disease symptoms; such as dieback of shoots and branches, cankers on trunk with resin flow and diseased leaves, leaf spots or blights.

Fungal isolation, characterization and growth rate studies

A total of 102 symptomatic and 18 healthy trees were sampled. Pieces were cut from disease growing edge and also from healthy samples; surface were sterilized and blotted dry with sterile filter papers. Pieces were plated on petri dishes containing 2% malt extract agar (MEA) amended with streptomycin sulfate (100 mg/l) (Merck, Germany) and incubated at 25°C. The isolates were replicated three times. The cultures were monitored daily for two weeks and colonies resembling Botryosphaeriaceae were sub cultured to fresh 2% (MEA) plates until purification.

After two weeks of incubation, nine morphotypes were distinguished by conidial characteristics aided by relevant keys,

publications and books on Botryosphaeriaceae fungi (Burgess et al., 2019). Ten morphological groupings of Botryosphaeriaceae isolated from healthy and asymptomatic tissues of baobab and marula in the three sites were selected for molecular studies. Ten isolates of each Botryosphaeriaceae occurring in each site and tree species were selected for molecular studies.

Growth rates were assessed between 15 and 35°C at 5°C intervals in culture growth. Three replicates of each isolate were used. Mycelial plugs of 6 mm diameter were taken from actively growing edges of week-old single mycelia cultures and transferred to the center of MEA 90 mm diameter petri-dishes. Three perpendicular measurements were taken of the colony diameter daily until mycelium of the fastest growing isolates had covered the plates. Macromorphological changes in the growing colonies (upper and lower sides) were studied. The experiment was monitored for color changes using color charts of Rayner (1970), for two weeks. Pycnidia were mounted in 85% lactic acid on microscopic slides and examined using a microscope.

Ten isolates from each morphotype were chosen for DNA extraction. Genomic DNA was extracted using CTAB (3%) and phenol-chloroform DNA extraction method as described by Gardes and Bruns (1993) with modifications according to Ihrmark et al. (2002). Part of ITS r DNA region was amplified and sequenced using fungal specific primer ITS1F (5'-CTTGGTCATTTAGAGGAGTAA-3') (Gardes and Bruns, 1993) and ITS4 (5'TCCTCCGCTTATTGATATGC-3). The amplified PCR products were purified AMPURE PCR purification kit (Beckman Coulter, USA) following manufacturer's instructions. The samples were sequenced in both directions at least twice using the PCR Big Dye © terminator cycle sequencing kit. PCR reaction mix was prepared as described by the manufacturer's instruction. The samples were sequenced in both directions twice using ITS1F and ITS 4 primers.

Edited nucleotide sequences were submitted to NCBI database sequences and identified using BLASTN (Ying et al., 2015) www.ncbi.nlm.nih.gov/BLAST/blast.cgi Published sequences from GenBank were used to identify sequences obtained from this study.

Phylogenetic analyses were done for ITS sequenced data. The edited nucleotide data and those from GenBank were aligned using MUSCLE and phylogenetic analysis was done in MEGA 7. The evolutionary history was inferred using Neighbor-joining method (Saitou and Nei, 1987). The tree was drawn to scale. The evolutionary distances were computed using Maximum Composite Likelihood method of Kumar et al. (2016). All positions containing gaps and missing data were eliminated from dataset.

Pathogenicity trial

Three potential canker and dieback fungi obtained diseased and healthy tissues were selected for the pathogenicity trials: *N. parvum*, *L. theobromae* and *L. pseudotheobromae*. The species were selected on the basis that they were the species isolated most frequently from diseased and symptomless samples of *A. digitata* and *S. birrea* trees. Healthy 8-month-old seedlings of marula, baobab, *Acacia xanthophloea* and *Calodendrum capense* were chosen for the pathogenicity assay. The part to be inoculated was sterilized with 70% ethanol and a vertical incision of approximately 1 cm was made using sterile blade and bark carefully lifted up. Mycelial plug 5 mm² were excised from four day old cultures using cork borer and placed at the centre of the incision and covered with parafilm. After inoculation, the seedlings were assessed regularly for canker symptoms development for 6 months. All the seedlings were selected, slit longitudinally and the total length of the internal lesion was recorded.

To complete Koch's postulates, three inoculated stems per isolates were randomly selected for re-isolation of inoculated fungus.

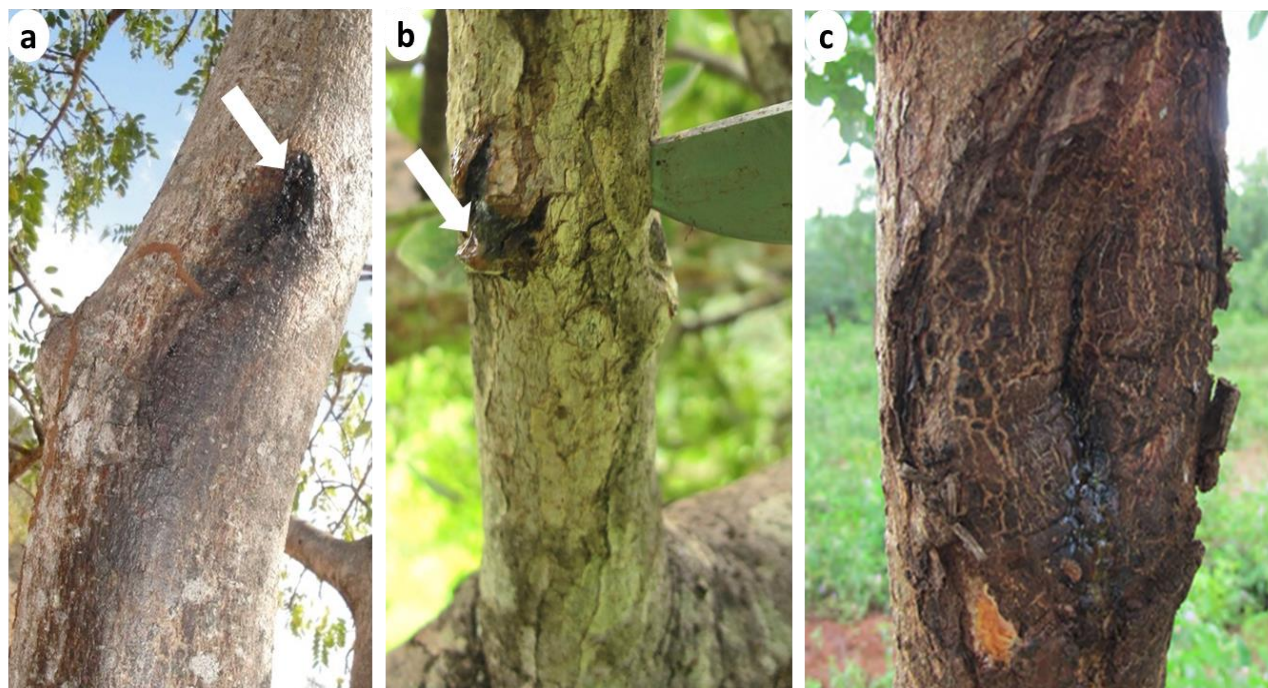


Figure 1. Disease symptoms observed in *Adansonia digitata* and *Sclerocarya birrea*.

Data analysis

GenStat Version 19.1 were used to analyze the data as needed and Minitab Version 15 was used to analyze field data collected. Pathogenicity trial was conducted using a randomized complete block design with four blocks comprising of 80 treatments including controls. Data was log transformed to satisfy the assumptions of ANOVA. One-way analysis of variance was used to assess difference in lesion lengths among fungal species with means separated using Turkey's test ($p=0.05$).

RESULTS

Field symptoms and abundance of fungal species on parts of *A. digitata* and *S. birrea* in Eastern Kenya

The main disease symptoms observed in the field were stem with resin flow (gummosis) (Figure 1). Cankers varied in size from small lesions to large open wounds.

The disease in the field was characterized by stem cankers with resin, dieback of branches and some leaf spots and blights. Disease incidence increased during dry season. About 87% of the diseased trees and 70% of healthy trees sampled yielded Botryosphaeriaceae fungi. Analysis of fungi occurring on different plant parts showed that most of the fungi were isolated from diseased stems (50.1%) followed by branches (33.5%), leaves (9.7%) and healthy plant parts (6.7%) (Table 1). *L. theobromae* and *L. pseudotheobromae* were the most frequently isolated species occurring on both symptomatic and asymptomatic tissues, with highest occurrence in

dieback and canker symptoms.

Morphological and molecular characterization

All the isolates produced aerial mycelium that was initially white turning greyish white, dark green or blackish grey after two weeks. About 450 isolates were morphologically characterized and 38 molecularly identified. The morphotypes corresponded to three main genera; *Lasiodiplodia*, *Neofusicoccum* and *Dothiorella*.

In MEA culture *Lasiodiplodia* initially had dense whitish mycelium turning smokey grey, and olivaceous grey color on the reverse (Figure 2b, d, f and g).

Dothiorella colonies were initially white to smokey grey with woolly aerial mycelia, becoming pale olivaceous grey within 5 to 7 days (Figure 2c). In culture, aerial mycelia of *Neofusicoccum* were fluffy and white becoming grayish and pale olivaceous gray and bluish black on the reverse (Figure 2a).

Growth rate studies

The fungi differed in their growth rates at five temperatures ($P<0.001$) (Table 2). *N. parvum* was the fastest species and colonized the plate within 24 h, achieving maximum growth at 30 to 35°C. *Lasiodiplodia mahajangana* obtained maximum growth at 30°C. All fungi grew favorably between 25 and 35°C and poorly at 15°C.

Table 1. Significance of occurrence of Botryosphaeriaceae fungi species on healthy and diseased parts of *A. digitata* and *S. birrea* in Eastern Kenya.

Tree species	Fungal species	Healthy			Diseased			% occurrence
		Leaves	Branch	Bark	Leaf spots and blight	dieback	Stem canker	
<i>A. digitata</i>	<i>L. theobromae</i>	-	++	+++	++	++++	+++++	18.2
	<i>L. pseudotheobromae</i>	-	-	+	-	+++	++++	15.2
	<i>L. parva</i>	-	+	+	-	++	+++	1.5
	<i>N. parvum</i>	+	++	++	++	++++	+++++	5.3
	<i>L. crassispora</i>	+	+	+	++	+++	+++	0.7
	<i>D. longicollis</i>	-	-	-	+	++	++	0.9
	<i>D. sarmentororum</i>	-	-	+	++	++	+++	0.8
	<i>Lasiodiplodia</i> sp.	-	++	++	++	++	+	1.3
<i>S. birrea</i>	<i>L. theobromae</i>	-	+	+	+	+++	++++	17.6
	<i>L. pseudotheobromae</i>	-	+	+	+	++	++	14.5
	<i>L. parva</i>	-	-	+	+	+	++	0.8
	<i>L. crassispora</i>	+	++	++	+	+++	+++	1.5
	<i>L. mahajangana</i>	-	+	-	+	++	++	0.1
	<i>Lasiodiplodia</i> sp.	-	-	+	++	+++	++++	9.5

- = Pathogen not detected in the tissue; + = occurrence not significant; ++ = 1-10% occurrence in the disease symptoms; +++ = 10-20% occurrence; ++++ = 20-30% occurrence; +++++ = >30%. Species with an occurrence of >++ were considered potentially important pathogen in the disease type.

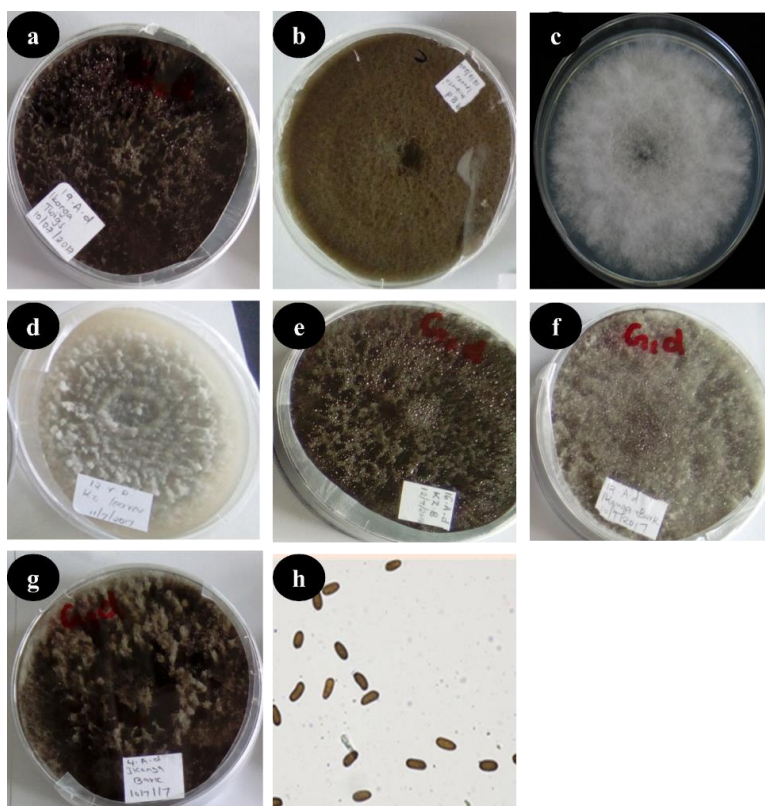
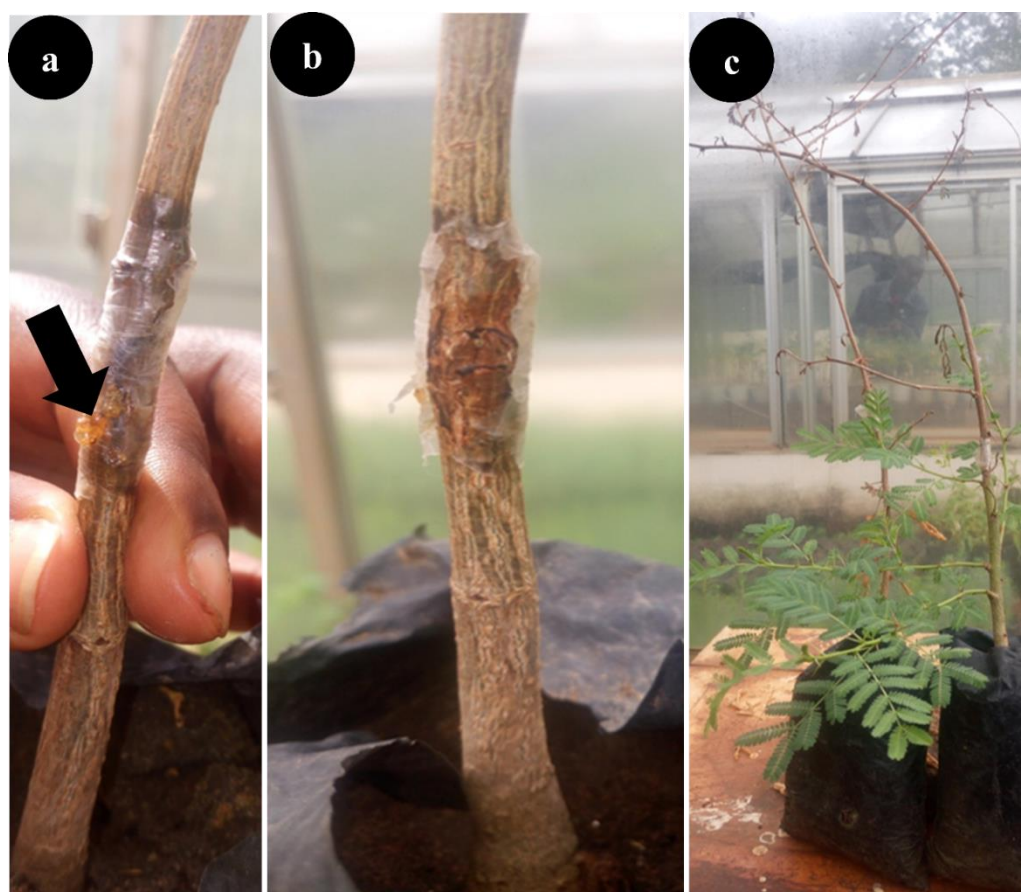


Figure 2. Macro and microscopic cultural and conidial characteristics of morphotypes. (a) *Neofusicoccum parvum*, (b) *Lasiodiplodia* sp., (c) *Dothiorella* sp., (d) *L. pseudotheobromae*, (e) *L. crassispora*, (f) *L. parva*, (g) *L. theobromae* and (h) spores of *Lasiodiplodia theobromae*.

Table 2. Growth rate of five Botryosphaeriaceae at five temperatures.

Temperature (°C)	Growth rate (mm/day)				
	Mean growth rates (mm day ⁻¹) ± SE				
	<i>N. parvum</i>	<i>L. theobromae</i>	<i>L. pseudotheobromae</i>	<i>Lasiodiplodia</i> sp.	<i>L. mahajangana</i>
15	3.3±0.2 ^{aa*}	3.7±0.2 ^{aa}	4.0±0.2 ^{ad}	4.5±0.2 ^{ab}	6.3±0.3 ^{da}
20	8.8±0.3 ^{ab}	10.0±0.1 ^{ba}	5.2±0.6 ^{bd}	8.3±0.4 ^{aac}	10.0±0.4 ^{abe}
25	13.2±0.1 ^{ac}	14.9±0.1 ^{bb}	6.5±0.4 ^{cc}	14.8±0.2 ^{bb}	14.6±0.3 ^{bc}
30	15.0±0.0 ^{ad}	14.1±0.1 ^{ba}	9.6±1.9 ^{cd}	12.8±0.4 ^{abc}	15.3±0.2 ^{cd}
35	15.6±0.1 ^{ad}	10.8±0.3 ^{ab}	7.5±0.8 ^{ea}	7.9±0.3 ^{ad}	5.4±0.3 ^{ac}

*Mean colony diameter LSD at 95% confidence interval. *Letters followed by the same superscript across the column (different temperatures) were not significant at 95% confidence interval.

**Figure 3.** Symptoms developed on inoculated seedlings.

Pathogenicity of three Botryosphaeriaceae species

Seedlings of the entire four tree species inoculated with Botryosphaeriaceae showed canker and dieback disease symptoms as observed for these four tree species in the field. The main symptoms caused by the *L. pseudotheobromae*, *N. parvum* and *L. theobromae* on inoculated seedlings were cankers, dieback and wound healing (Figure 3). The earliest symptom observed on

inoculated seedlings was resin production (gummosis), which occurred on approximately 90% of seedlings inoculated with Botryosphaeriaceae within 14 days. The seedlings developed canker symptoms characterized by necrosis of the inner bark and woody tissues, stem swelling and bending. Incidence of wound healing was highest on baobab and marula. Healed tissues were surrounded by layers of fleshy callous tissues around discoloured tissues where the fungus had been inoculated

Table 3. Summary of analysis of variance (ANOVA).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungal pathogen	3	16.62418	5.541392	999.71	<.001
Tree species	4	0.732276	0.183069	33.03	<.001
Fungal pathogen. Tree sps	5	0.422677	0.084535	15.25	<.001
Residual	67	0.371383	0.005543		
Total	79	18.15051			

Variate: Number of days.

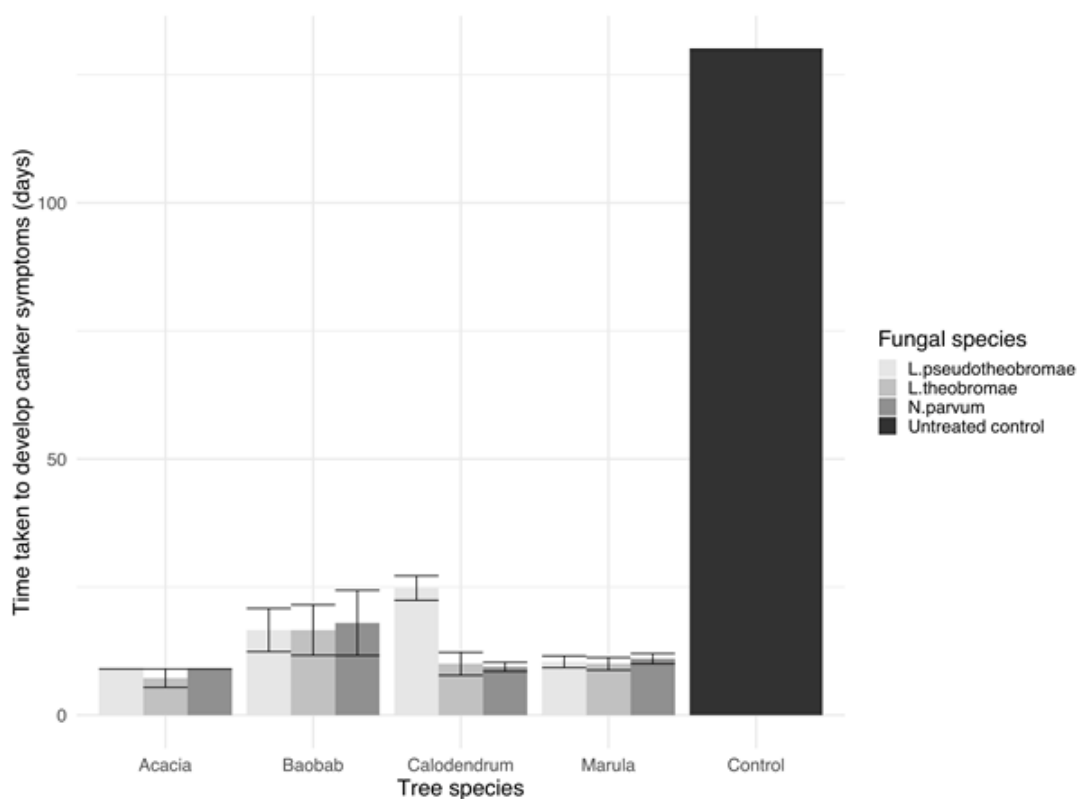


Figure 4. Mean days to show early canker and dieback symptoms in *Adansonia digitata*, *Sclerocarya birrea*, *Calodendrum capense* and *Acacia xanthophloea* seedlings inoculated with *L. pseudotheobromae*, *L. theobromae* and *N. parvum* under glass house conditions.

(Figure 3b). The symptoms caused by the three Botryosphaeriaceae species were generally indistinguishable among the four plant species.

There was significant difference in the mean number of days taken by each of the three inoculated fungal species to cause canker and dieback symptoms on the four tree species ($p \leq 0.001$; Table 3). Seedlings inoculated with *L. pseudotheobromae* were the first to develop canker symptoms on *Acacia* followed by *Calodendrum* (Figure 4) *L. theobromae* came second in developing the symptoms and *N. parvum* was little slower. *Acacia* and *Calodendrum* were highly susceptible to Botryosphaeriaceae but *S. birrea* and *A. digitata* were

less susceptible. Baobab and marula showed fastest wound healing after inoculation of Botryosphaeriaceae than other tree species inoculated with the same fungal species.

Internal lesions were identified by extensive discoloration of phloem and rotting of inner tissues (Figure 3). The size of the internal lesions in the four tree species were significantly different from the un-inoculated control seedlings and between fungal species ($p < 0.001$) (Table 4). All Botryosphaeriaceae species caused the longest lesions in *A. xanthophloea* and *C. capense*, moderate lesion on Marula (*S. birrea*) and shortest lesion on Baobab (*A. digitata*) (Figure 5).

Table 4. Summary of analysis of variance (ANOVA) at 95% confidence interval.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
fungal_sps	3	189.222	63.074	7.56	<.001
tree_species	4	584.554	146.138	17.52	<.001
fungal_sps. tree_species	5	393.818	78.764	9.44	<.001
Residual	67	558.988	8.343		
Total	79	1726.582			

Variate: lesion size (cm).

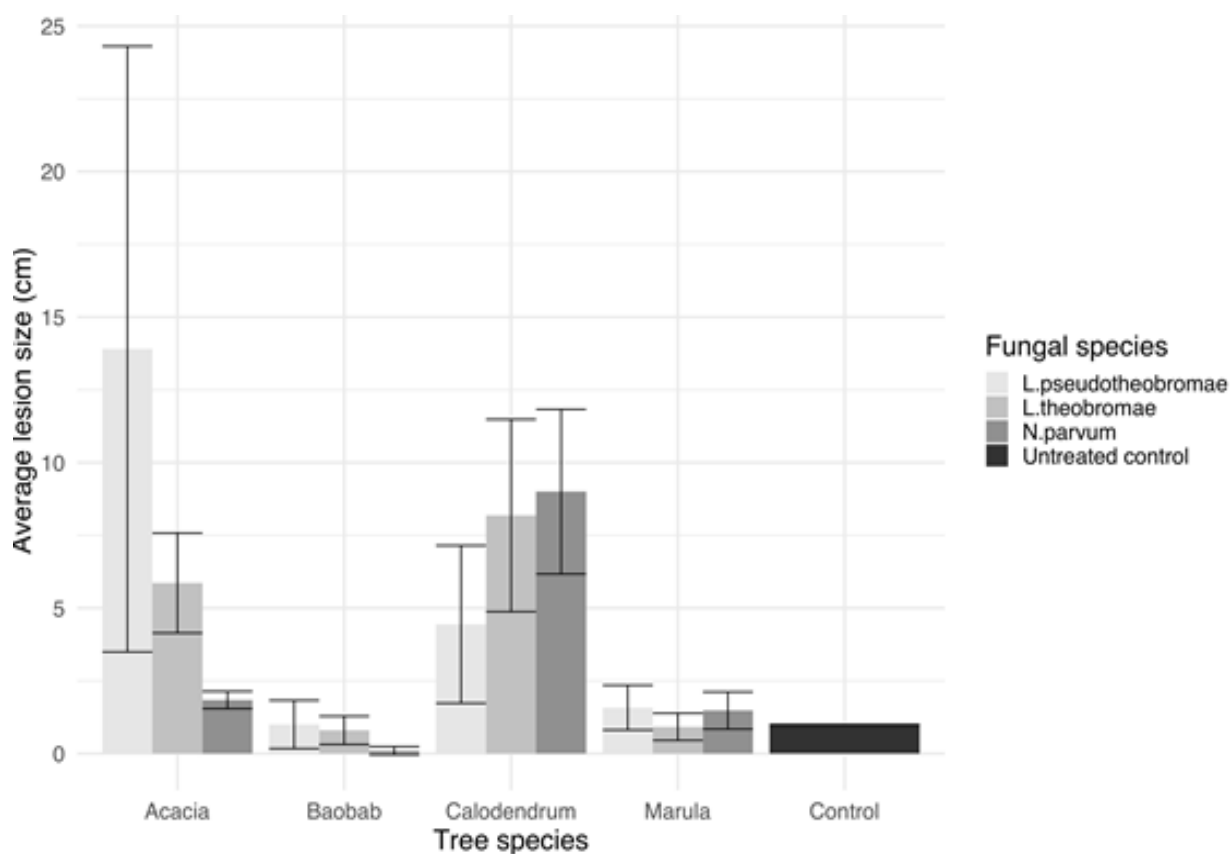


Figure 5. Mean internal lesion lengths (cm) after six months in *Adansonia digitata*, *Sclerocarrya birrea*, *Acacia xanthophloea* and *Calodendrum capense* seedlings inoculated with *Lasiodiplodia pseudotheobromae*, *L. theobromae* and *Neofusicoccum parvum* under glass house conditions in Kenya.

The longest lesion occurred on Acacia inoculated with *L. pseudotheobromae*, followed by *N. parvum* and *L. theobromae* showed slightly lower virulence.

Ranking of the scores obtained from the two variables (occurrence of early canker symptoms and size of internal lesions caused by each fungal species under glass house conditions) using Kruskal Wallis one-way analysis of variance showed the average rank for *L. pseudotheobromae*=50.67, *N. parvum*=49.67, *L. theobromae*=46.15 and Control=15.5 ($p < 0.001$) as shown in Table 5. Therefore, *L. pseudotheobromae* was the most

virulent species on all the tree species.

DISCUSSION

Canker and dieback disease was identified as a threat to cultivation of baobab and marula in Kenya and potential canker and dieback pathogens were also identified. This study described most comprehensive. Botryosphaeriaceae species associated with diseased and healthy samples of *A. digitata* and *S. birrea* in Eastern Kenya using

Table 5. One-way analysis of variance.

Sample	Size	Mean rank
<i>L. pseudotheobromae</i>	20	50.67
<i>N. parvum</i>	20	46.15
<i>L. theobromae</i>	20	49.67
Control (pure agar inoculation)	20	15.5
P value (Chi-square probability)	< 0.001	

morphological and molecular data on ITS region. Three fungal genera were identified by means of phenotypic characters and DNA sequence analyses. Majority of the isolates represented *L. pseudotheobromae*, *L. theobromae* and *N. parvum* forming more than 50% of the Botryosphaeriaceae associated with both *A. digitata* and *S. birrea*. The three fungal species showed a strong endophytic association with asymptomatic tissues of baobab and marula (Begoude et al., 2010). The endophytic nature of Botryosphaeriaceae could be triggered to pathogenic phase by unfavourable climatic conditions (Osoro et al., 2017). The abundance and isolation frequency of *L. theobromae*, *L. pseudotheobromae* and *N. parvum* on diseased plant parts indicated they could play a role in the disease. *L. theobromae*, *L. pseudotheobromae* and *N. parvum* are serious pathogens of woody tree species in Africa (Slippers et al., 2017). *L. theobromae* has been referred to as a widely distributed fungi in tropical and sub-tropical regions and is reported to infect more than 500 plant species, the fungi has been associated with shoot blight, dieback and stem cankers in a diverse group of hosts (Adesemoye et al., 2014). The wide range of temperatures in which the species of Botryosphaeriaceae described here can grow (with optimum ranging from 25 to 30°C) make it hypothesized that high temperatures favors the pathogenic phase of this pathogens. *L. mahajangana*, *L. theobromae*, *L. pseudotheobromae*, *L. iraniensis* and *L. crassispora* had previously been isolated from *S. birrea* (Mehl et al., 2017), while *L. crassispora*, *L. pseudotheobromae*, *L. parva* and *L. mahajangana* have been associated with *A. digitata* (Cruywagen et al., 2017). Reports of dying Baobab in South Africa were associated to *L. theobromae* and *N. parvum*. *L. Pseudotheobromae* appears to have a wide host range and geographic distribution (Rodriguez-Galvez et al., 2017). It has been associated with cankers, dieback and stem rot in mangoes (Ismail et al., 2012), trunk canker in *Acacia* (Castro Medina et al., 2013). *L. pseudotheobromae* is the most isolated species from baobabs in Africa with isolations from both asymptomatic and symptomatic tissues. It caused lesion on Australian *A. gregorii* but few isolates were observed in Kenya Baobab.

The ability of these pathogens, *L. pseudotheobromae*, *L. theobromae* and *N. parvum* to cause disease on four tree species were tested. The focus species were *A.*

digitata and *S. birrea*, the other two species, *C. capense* and *A. xanthophloea* were included because they are agroforestry trees and the disease seemed to be widespread on several hosts on farms. Early canker development gave a rapid indication of virulence on all tree species and ranking analysis showed *L. pseudotheobromae* to be the most virulent on all the four tree species. The three fungal species isolated from baobab and marula could cause the disease not only on same species but also on *C. capense* and *A. xanthophloea*.

Therefore, the susceptibility of the four tree species to attack by all the fungi tested indicated the plurivorous nature of Botryosphaeriaceae (Jeff-ego and Akinsanmi, 2018). Emergence of pathogens with a wide host range pose serious health risk to other crops within Agroforestry and reduce productivity of the system. Trees adapted to dry areas should be restricted to their climatic conditions; emphasis on site specificity for trees should be encouraged as a disease management strategy to reduce destruction by pathogens.

Wound healing characterized by formation of callous tissues around infected parts was an indication of a host response to limit the spread of infection from the point of inoculation. No wound healing occurred in *Acacia* and *Calodendrum*, which indicated that high relative susceptibility to infection was connected to low wound healing. Baobab and marula were the least susceptible to infection among the four tree species tested, which are indigenous to semi-arid areas of Kenya and seemed to be better adapted to semi-arid conditions. The three fungi produce indistinguishable symptoms and it was not possible to isolate the primary cause of the disease supporting previous observation by Njuguna et al. (2011). It is therefore concluded that canker and dieback disease was a disease complex. Co-occurrence of Botryosphaeriaceae fungi as observed may increase the ability of the pathogens to overcome the host's resistance especially under unfavorable environmental conditions, although its benefits are not yet clear. *Acacia xanthophloea* and *Calodendrum capense* were highly susceptible to the disease whereas *A. digitata* and *S. birrea*, the native species showed least susceptibility making it suitable for agroforestry systems in semi-arid areas. This study concluded that unspecific plurivorous nature of pathogens with stressful environment in

agroforestry system will increase the virulence of the fungi and susceptibility of the host.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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