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Assessment of different kenaf (*Hibiscus cannabinus* L.) accessions in hormone-free plant growth medium and identification of the associated fungal contaminants

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Abstract
Kenaf is an industrial crop, which serves as income to small-holder farmers in Sub-Saharan Africa. Unfortunately, the oily and recalcitrant nature of its seeds makes seed germination during conventional propagation almost impossible. Plant tissue culture is a viable alternative to conventional propagation of kenaf seeds, however, it requires the use of expensive plant growth hormones and it is prone to fungal contaminations. This study therefore aimed at comparing the seedling growth of four different accessions of kenaf seeds (Tianung 2, Ifeken 100, Ifeken 400, and Ifeken D1 400) in half-strength growth hormone-free Murashige and Skoog (MS) medium as well as to identify the associated fungal contaminants in this medium. Data were obtained on plant length (cm), plant width (mm), number of leaves, leaf length (cm), number of roots, and root length (cm) at 14 days after culture (DAC). There were significant differences in all the growth parameters observed ($p<0.05$) except the plant width (mm) [$p>0.05$] at 14 DAC. Ifeken 400 had the highest average plant length (14.5 cm) followed by Ifeken 100 (10.5 cm) at $p<0.05$. On the other hand, the least average plant length (4.16 cm) was observed from both Tianung 2 and Ifeken D1 400 at $p<0.05$. The highest average root length (5.3 cm) was produced by Ifeken 400 while Ifeken DI 400 had the least average root length (2.6 cm) [$p<0.05$]. The fungal contaminants associated with the kenaf cultures in the half-strength growth hormone-free MS media were *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. Therefore, half-strength growth hormone-free MS medium could be considered as a low cost alternative method of propagating kenaf seeds in order to prevent poor seed germination and allow for improvement of kenaf as an industrial crop.
Keywords: *Aspergillus* spp., Fungal contamination, *Hibiscus cannabinus* L., Plant growth hormones, Plant tissue culture
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1. Introduction

Kenaf (*Hibiscus cannabinus* L.) is an herbaceous plant belonging to the family Malvacea (Satya *et al*., 2013; Devadas *et al*., 2018; Odahara *et al*., 2020; Hidayat *et al*., 2022). Its center of origin is Sub-Saharan Africa but it is now grown mainly in Asia and Latin America (Xia *et al*., 2017). It is a fast-growing annual plant with high potential for fibre production (Hossain *et al*., 2011). It also has multipurpose uses which include paper pulp, fabrics, textile, building materials, bio-composites, bedding material and oil absorbents (Monti and Alexopoulou, 2013; Mahmood *et al*., 2018).

In spite of the benefits of kenaf, its production is faced with various biotic and abiotic stresses. Factors of climate change such as drought, poor soil fertility and loss of viability are among the abiotic stresses affecting kenaf production (Adebisi *et al*., 2013; Falasca *et al*., 2014; Afzal *et al*., 2020). Germination problem in kenaf is attributed to the fact that kenaf is an oil seed, hence, it loses its viability easily during storage. Therefore, there is need to use alternative means of propagation which evade the problems of germination. Plant tissue culture is an important technology in bioscience and over hundreds of plant species have been regenerated from this technique

(Jiao, 2001). The technique involves rapid multiplication and production of plant materials under controlled environment for commercial and industrial purposes (Cruz-Cruz *et al*., 2013; Tegen and Mohammed, 2016; Yadav *et al*., 2019).

However, propagation of plants through tissue culture technique is faced with microbial contaminations (Odutayo *et al*., 2007; Akinyemi and Esuola, 2012; Varghese and Joy, 2016). Presence of microbes in tissue culture is the major cause of mortality in culture, tissue necrosis, shoot proliferation reduction and reduced rooting system (Rajput *et al*., 2005; Niaz and Dawar, 2009; Oriowo *et al*., 2019). Of all the pathogens infecting plants in the world, fungi are the most important pathogens affecting seed production due to their ability to cause grain pre and post-emergence death, reduce seedling vigor leading to decrease in germination and variation in plant morphology (Rajput *et al*., 2005; Niaz and Dawar, 2009).

Therefore, we aimed to compare the seedling growth of different accessions of kenaf in hormone-free plant growth medium and report the associated fungal contaminants for future improvement of kenaf as an important industrial crop.

2. Materials and Methods

2.1 Plant materials

The following accessions of kenaf seeds; Tianung 2, Ifeken 100, Ifeken 400, and Ifeken D1 400 were collected from Kenaf and Jute Improvement Programme, Institute of Agricultural Research and Training, Ibadan, Nigeria.

2.2 Media composition and preparation

The plant medium used in this study is composed of half-strength growth hormone-free Murashige and Skoog (1962) [MS], 0.1 g L^{-1} Myo-inositol, 3% table sugar as carbohydrate and energy source and 10 mg L^{-1} Ascorbic Acid. The pH of the medium was adjusted to 5.8 with few drops of NaOH and HCl. Media were solidified with 0.7% purified agar (tissue culture grade) and melted in a microwave oven. Media were dispensed into 25 ml test tubes and sterilized using an autoclave at 121^{o}C for 15 min before use.

2.3 Explants surface disinfection

Twenty seeds (explants) each of the four accessions of kenaf were subjected to surface disinfection steps by first washing under running tap water with few drops of detergent for 1 h. The explants were transferred to the laminar flow chamber and following aseptic conditions they were washed with 70% Ethanol for 5 min, followed by washing with 3% of sodium hypochlorite (3.5 v/v) for 10 min with few drops of detergent. Finally, the explants were rinsed thoroughly three times with sterile double distilled water.

2.4 Growth culture conditions

The disinfected explants were inoculated into the sterilized medium in the test tubes. The culture tubes were sealed using parafilms and incubated in the growth room at the temperature of 25 ^{o}C $\pm$ 2 ^{o}C with fluorescent tubes providing 16 h light/8 h darkness. Growth parameters including plant length (cm), plant width (mm), number of leaves, leaf length (cm), root number, and root length (cm) were collected at 14 days after culture (DAC).

2.5 Microbial contaminants isolated from the micropropagated kenaf seeds

The microbial contaminants associated with the *in vitro* germinated seedlings of the different accessions of kenaf seeds were studied morphologically. A small portion of the fungal colony was picked with a sterile inoculating loop and placed on a clean slide. The slide was stained with lactophenol cotton blue and it was covered with a cover slip. The slide was viewed under the x40 objective lens of a compound microscope for the type of hyphae, conidia and spores that were present.

2.6 Statistical Analysis

The experiment was laid out in a Completely Randomized Design (CRD) in triplicates. Data collected were subjected to One way Analysis of Variance (ANOVA) and means were separated using Duncan's Multiple Range Test at $p < 0.05$. All analysis were done using SAS version 9.1.

3. Results

3.1 Average plant length (cm) and plant width (mm)

The result of the average plant length (cm) is highly significant among the accessions with Ifeken 400 having the highest average plant length of 14.5 cm followed by Ifeken 100 (10.5 cm), whereas both Tianung 2 and Ifeken D1 400 had the least average plant length of 4.16 cm at $p<0.05$ (Figure 1A-D and Figure 2A). Although, there was no significant difference in the average plant width (mm) in all of the accessions at $p>0.05$, Ifeken 100 and Ifeken D1

400 had the highest average plant width of 0.26 mm, Tianung 2 had 0.23 mm, and Ifeken 400 had the least of 0.2 mm (Figure 1A-D and Figure 2B).

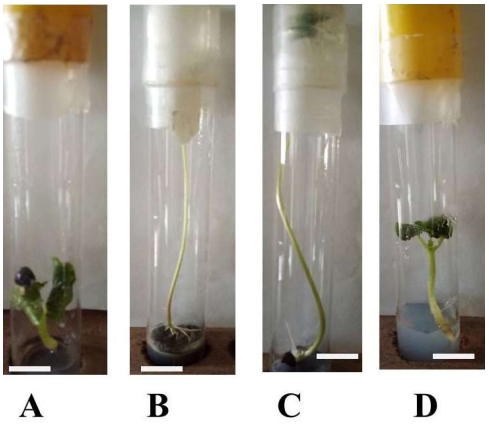

Figure 1. *In vitro* seed germination of four different accessions of kenaf, 14 days after culturing (DAC) (A) Tianung 2 (B) Ifeken 100 (C) Ifeken 400 (D) Ifeken D1 400. All scale bars = 1 cm.

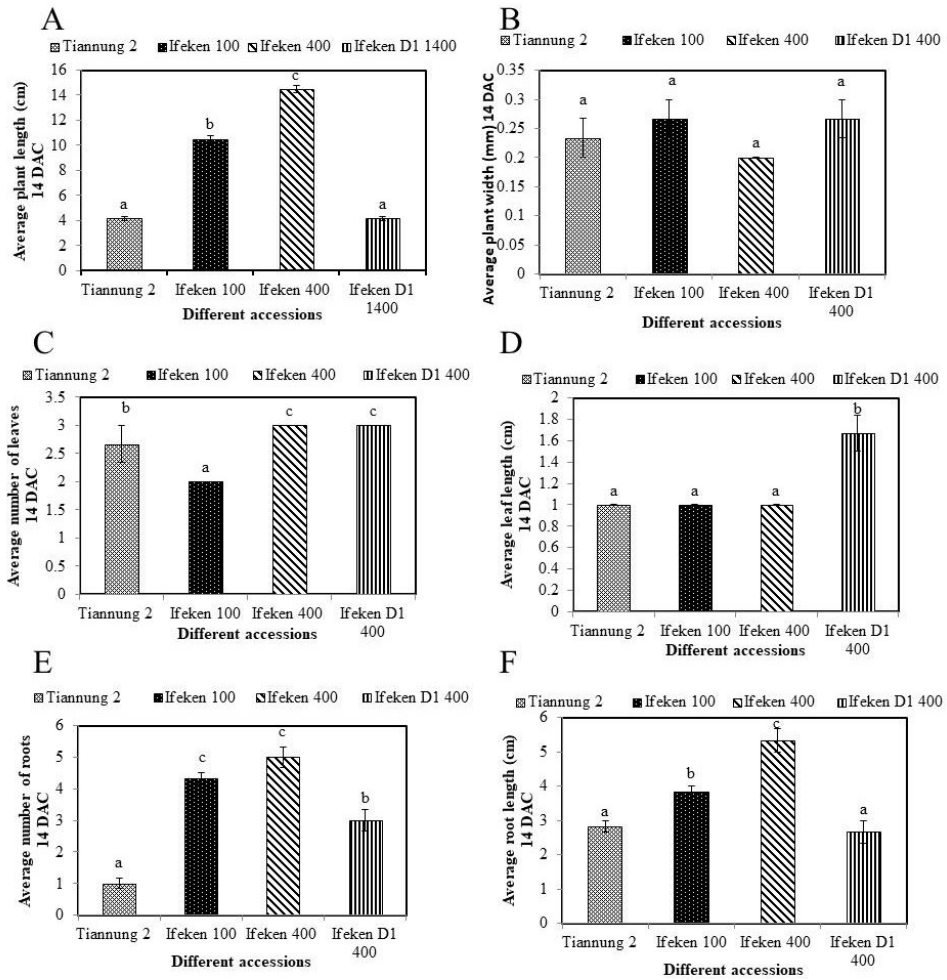

Figure 2. Different growth parameters of the four different accessions of kenaf during *in vitro* seed germination 14 days after culturing (DAC). (A) average plant length (cm) (B) average plant width (mm) (C) average number of leaves (D) average leaf length (cm) (E) average number of roots (F) average root length (cm). Letters with the same alphabets are not significantly different at p>0.05.

3.2 Average number of leaves and leaf length (cm)

The result of the average number of leaves is highly significant among the accessions with Ifeken 400 and Ifeken D1 400 having the highest average number of leaves of 3 each whereas Tianung 2 had 2.66 and Ifeken 100 had the least (2) at $p<0.05$ (Figure 2C). The average leaf length (cm) is also highly significant ($p<0.05$) among the accessions with Ifeken D1 400 having the highest average leaf length of 1.66 cm whereas Tianung 2, Ifeken 100, and Ifeken 400 all had 1 cm average leaf length (Figure 2D).

3.3 Average number of roots and root length (cm)

The result of the average number of plant roots is highly significant ($p<0.05$) among the accessions with Ifeken 400 having the highest average root number of 5 followed by Ifeken 100 (4.3), Ifeken D1 400 had 3, and Tianung 2 had the least average root number of 1 (Figure 2E). There is also significant difference ($p<0.05$) in the average root length in all of the accessions. Ifeken 400 had the highest average root length of 5.3 cm, followed by Ifeken 100 (3.8 cm), Tianung 2 had average root length of 2.8 cm, whereas Ifeken D1 400 had the least average root length of 2.6 cm (Figure 2F).

3.4 Microbial contaminants

The morphologically observed microbes related to the *in vitro* cultured seeds of the different accessions of kenaf were fungal isolates of *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., and mixed contaminants, containing *Aspergillus* spp., *Fusarium* spp. and *Rhizopus* spp. (Figure 3A-C and Figure 4A-D).

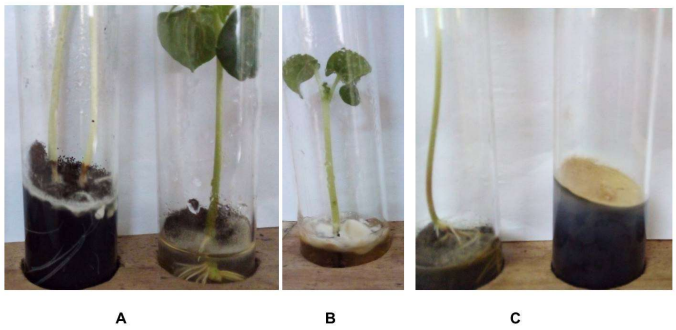

Figure 3. Microbial contaminants associated with the four different accessions of Kenaf during *in vitro* seed germination 14 days after culturing (DAC). (A). *Aspergillus* spp. (B). *Fusarium* spp. (C). Mixed contaminants.

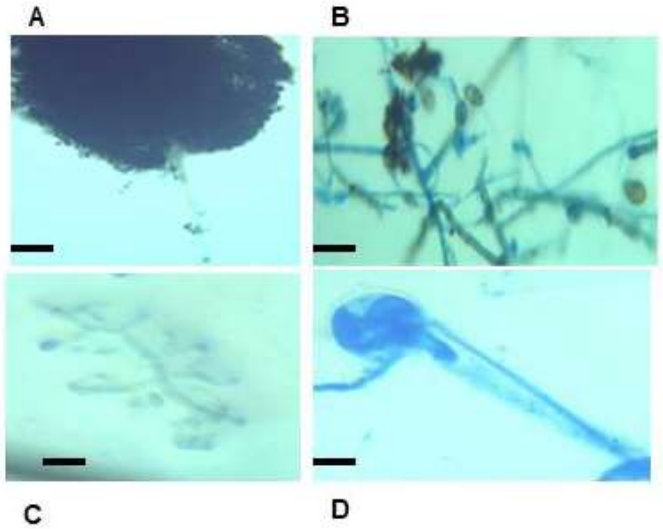

Figure 4. Micrograph of contaminated cultures of Kenaf accessions using a light microscope (A) *Aspergillus* spp. (B) *Fusarium oxysporum* showing the spores (C) *Penicillium* spp. (D) *Rhizopus* spp. All scale bars = 10 µm.

4. Discussion

This study revealed that using tissue culture technique for propagating kenaf seeds is a low cost alternative for evading the problem of poor seed germination. Akinyemi and Esuola (2012) reported tissue culture technique as the most practicable option for the conservation of fluted pumpkin which results from the recalcitrant nature of the seeds.

Results obtained from micropropagation of kenaf plants revealed that half-strength hormone-free MS medium supported the germination of kenaf seeds to generate shoots and roots. Several studies have reported the *in vitro* propagation of kenaf and other plants on MS medium using different plant organs (Samanthi *et al*., 2013; Xia *et al*., 2017; Galan-Avila *et al*., 2020).

Also, in spite of the growth of fungi on the half-strength hormone-free MS medium, the plants produce appreciable shoot and roots on the medium even without addition of plant growth regulators (PGRs). The study of Hlatshwayo *et al*. (2020) similarly reported the efficient micropropagation of endangered *Aloe peglerae* on half-strength MS medium in the absence of plant growth hormone.

The present study showed the presence of fungal isolates in the culture of kenaf grown on half-strength hormone-free MS medium despite the surface disinfection of the seeds with ethanol and hypochlorite solution. This could be attributed to the fact that some pathogens are seed-borne and are transmitted from the seeds into the culture, as well as possibility of contamination during the culturing process. These suggest that use of anti-fungal agents on kenaf seeds after disinfection process is necessary. The presence of fungal isolates in kenaf grown *in vitro* was likewise reported by Oriowo *et al*. (2019). Odutayo *et al*. (2007) also reported the presence of *Aspergillus* spp., *Fusarium* spp., and *Rhizopus* spp. in tissue culture of kenaf.

Conclusion

Plant tissue culture is an important technique used for the rapid multiplication of crops especially crops that are recalcitrant in nature (Akinyemi and Esuola, 2012, Odahara *et al*., 2020). In this study, we found that *in vitro* culturing of four different accessions of kenaf seeds in half-strength growth hormone-free MS medium resulted in appreciable plant and root lengths. This is an indication that tissue culture of kenaf seeds in low cost growth hormone-free MS medium could serve as an alternative technique for solving the problems of seed germination in kenaf propagated conventionally. The germination and the growth of kenaf *in vitro* despite the presence of fungal contaminations further buttress the importance of tissue culture in micropropagation of plants with possible seed-borne infections. Further studies should look into methods that can inhibit the growth of these fungal contaminations in the tissue culture for future improvement of kenaf as an industrial crop.

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Conflict of interest

We hereby declare that there are no conflicts of interest.

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