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OPTIMISED GERMINATION PROTOCOL FOR JACKFRUIT SEEDS AND EVALUATION OF METHODS FOR EXTRACTION OF DNA SUITABLE FOR GENETIC ANALYSIS

R.A. OJWANG, A.A. ADAN, E.N. NYABOGA, E.K. MUGE, B.N. MBATIA¹, G.O. OBIERO²
and D.O. OGOYF³

Department of Biochemistry, University of Nairobi, P. O. Box 30197, 00100, Nairobi, Kenya

¹School of Pharmacy and Health Sciences, United States International University, P. O. Box 14634, 00800, Nairobi, Kenya

²Centre for Biotechnology and Bioinformatics, University of Nairobi, P. O. Box 30197, 00100, Nairobi, Kenya

³Department of Biochemistry and Biotechnology, Technical University of Kenya, P. O. Box 52482, 00200, Nairobi, Kenya

Corresponding author: redemtorawuor@gmail.com

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ABSTRACT

Jackfruit (*Artocarpus heterophyllus*) is an underutilised plant that is promising in curbing food and nutritional security in sub-Saharan Africa. However, high level of secondary metabolites in its tissues significantly hampers its genetic characterisation for breeding purposes. Primarily, the compounds react with DNA during the extraction process, thus reducing its yield and quality. The utilisation of leaves from jackfruit seedlings is a potentially effective approach of addressing the challenge, however, limited information is available on efficient jackfruit seed germination procedures. Elucidating effective methods of jackfruit seed germination, and optimising protocols for DNA extraction is crucial in promoting its genetic characterisation studies for identification of superior varieties for propagation. The objective of this study was to evaluate methods of jackfruit seed germination, and DNA extraction procedures using jackfruit leaves. Pre-treatment of seeds with 3% hydrogen peroxide was effective in enhancing seed germination within a short time, compared to distilled water and 3% hydrochloric acid. We optimised a DNA extraction technique by combining CTAB-SDS based approach with an enhanced solvent extraction method. The technique yielded high quantity and quality of DNA from jackfruit leaves, which was appropriate for downstream polymerase chain reaction analysis. The sequence-related amplified polymorphism (SRAP) and simple sequence repeat (SSR) amplifications confirmed the effectiveness of the optimised CTAB-SDS based protocol for extraction of high quality DNA.

Key Words: *Artocarpus heterophyllus*, DNA isolation

RÉSUMÉ

Le jacquier (*Artocarpus heterophyllus*) est une plante sous-utilisée qui est prometteuse pour freiner la sécurité alimentaire et nutritionnelle en Afrique subsaharienne. Cependant, le niveau élevé de métabolites secondaires dans ses tissus entrave considérablement sa caractérisation génétique à des fins de sélection. Principalement, les composés réagissent avec l'ADN pendant le processus d'extraction, réduisant ainsi son rendement et sa qualité. L'utilisation de feuilles de semis de jacquier est une approche potentiellement efficace pour relever le défi, cependant, des informations limitées sont disponibles sur les procédures efficaces de germination des graines de jacquier. Éclaircir des méthodes efficaces de germination des graines de jacquier et optimiser les protocoles d'extraction d'ADN est crucial pour promouvoir ses études de caractérisation génétique pour l'identification de variétés supérieures pour la propagation. L'objectif de cette étude était d'évaluer les méthodes de germination des graines de jacquier et les procédures d'extraction d'ADN à l'aide de feuilles de jacquier. Le prétraitement des graines avec du peroxyde d'hydrogène à 3 % a été efficace pour améliorer la germination des graines en peu de temps, par rapport à l'eau distillée et à l'acide chlorhydrique à 3%. Nous avons optimisé une technique d'extraction d'ADN en combinant une approche basée sur CTAB-SDS avec une méthode d'extraction par solvant améliorée. La technique a donné une quantité et une qualité élevées d'ADN à partir de feuilles de jacquier, ce qui était approprié pour l'analyse en aval de la réaction en chaîne par polymérase. Les amplifications de polymorphisme amplifié lié à la séquence (SRAP) et de répétition de séquence simple (SSR) ont confirmé l'efficacité du protocole optimisé basé sur CTAB-SDS pour l'extraction d'ADN de haute qualité.

Mots Clés : *Artocarpus heterophyllus*, isolement de l'ADN

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus*) is a nutritious fruit with high medicinal value. It has been listed by the Food and Agriculture Organization (FAO) as a 'neglected' plant that can potentially reduce overreliance on a few fruit crops and promote biodiversity (FAO, 2017). Limited information is available on the genetic characteristics of jackfruits in sub-Saharan Africa to spur its development. The knowledge is particularly crucial in identifying superior jackfruit varieties for propagation in the region (Ojwang *et al.*, 2021).

Primarily, efficient seed germination and breaking of seed dormancy are considered a significant challenge in cultivation of most fruit trees from tropical areas. A major factor that is attributable to seed dormancy is the thick seed coatings. Notably, they prevent water and oxygen from entering into the seeds (Luna *et al.*, 2009).

Multiple pre-treatment techniques of breaking seed dormancy have been reported

(Travlos and Economou, 2006; Imani *et al.*, 2011; Abubakar and Maimuna, 2013). However, the efficacy of the pre-treatment approaches differs depending on the plant species. Hence, optimising a pre-treatment protocol for individual species is crucial for propagation of trees such as jackfruit.

Travlos and Economou (2006) revealed that sulphuric acid optimises seed germination in *Medicago arborea*. Abubakar and Maimuna (2013) reported that 50% hydrochloric acid (HCl) optimised seed germination in *Parkia biglobosa* species. Moreover, hydrogen peroxide solution was highly effective in the optimising seed germination of *Prunus* species (Imani *et al.*, 2011). However, no study has been reported for optimisation of jackfruit seed germination utilising these or other techniques. This knowledge is essential for developing jackfruit seedlings for genetic studies and propagation of the ones with superior traits in tropical regions (Sudan *et al.*, 2017).

One of the significant challenges that impedes genetic characterisation studies for

breeding purposes, is the high concentrations of secondary metabolites (Ojwang *et al.*, 2017; Sudan *et al.*, 2017). The metabolites cause shearing of the DNA or bind to taq polymerase; hence, inhibiting the polymerase chain reaction process (Sudan *et al.*, 2017). Standard techniques of DNA extraction in plants are ineffective in isolating DNA from species with high content of secondary metabolites (Doosty *et al.*, 2012). Therefore, optimisation of DNA protocols for individual plant species is crucial for plants with high phytochemical contents. Besides, existing DNA extraction protocols employ liquid nitrogen and β -mecarptoethanol, which are volatile, costly and hazardous (Sika *et al.*, 2015). Hence, developing a protocol that does not require these components is critical in enhancing genetic characterisation studies of tropical trees including jackfruit.

The efficiency of DNA extraction protocols for plants with high contents of secondary metabolites varies from one species to another. Doosty *et al.* (2012) examined the efficiency of four published protocols of DNA isolation from a medicinal plant *Satureja khuzistanica*. These included Murry and Thompson (1980), Dellaporta (1983), Doyle and Doyle (1990) and Kang and Yang (2004). Their findings revealed that modified Murry and Thompson (1980) procedure yielded good quality DNA; while the rest produced degraded DNA. The modifications included increasing the extraction buffer incubation period (one hour), reducing the centrifugation temperature (4 °C), increasing the concentration of NaCl (1.4M) and utilising leaves before flowering. Arruda *et al.* (2017) optimised the extraction of DNA from *Mimosa tenuiflora* plants, which are high in phytochemical metabolic products. The study tested eight different protocols, including Murray and Thompson (1980), Doyle and Doyle (1987), Roy *et al.* (1992), Haberer *et al.* (1996), Faleiro *et al.* (2003), Bonato *et al.* (2004), and Promega kit, but none of them had good quality DNA; and after modifications of CTAB based protocol, an

optimised one was developed. It involved addition of phenol, use of higher concentrations of CTAB (3%) and NaCl (2.5 M). Additionally, shorter duration of incubation (20 minutes) and a lower temperatures (60 °C) were used. The objective of this study was to optimise a protocol for jackfruit seed germination, as well as obtain quality DNA for molecular analysis.

MATERIALS AND METHODS

Sampling of jackfruit leaves. Young leaves from lower branches of mature Jackfruit trees (approximately 10-100 years) were collected from selected regions of Kenya and Uganda. Notably, the two countries were selected based on availability of the trees and mature fruits. A total of 41 and 79 leaf samples were obtained from Kenya (Kwale, Mombasa, Siaya and Kilifi Counties) and Uganda (Kampala, Wakiso, Mukono and Jinja districts), respectively.

The young leaves were quickly placed in airtight zip-lock polythene bags containing silica gel, and stored at -80 °C until use. The leaves were used for DNA extraction using four extraction techniques; namely Primeprep kit, CTAB-based extraction method (Dellaporta *et al.* 1983; Doyle and Doyle (1990), SDS-LiCl-based (Arif *et al.*, 2010), and Sucrose-based (Sudan *et al.* (2017). DNA obtained from all the four techniques was degraded. Hence, optimisation of jackfruit seed germination was performed to obtain young leaves from the seedlings for DNA extraction.

Optimisation of germination protocols. Mature fruits (30 brownish green) from Kenya and Uganda were cut and the seeds extracted for the germination study. The seeds were first planted directly in autoclaved soil, without pre-treatment, however; no germination was realised, thus the germination process was optimised through pre-treatment of the seeds with several chemicals to break their dormancy.

The seeds from individual fruits were divided into four groups and tested for

germination with different pre-treatment chemicals. The seeds were first wrapped in paper towels and placed in zip lock bags to preserve the moisture. The paper towels that contained seeds (three per fruit) were moistened with either 3% sulphuric acid (1st group), 3% hydrochloric acid (2nd group), hydrogen peroxide (3rd group) and distilled water (4th group). The zip lock bags containing seeds, with the different treatments, were placed in a growth chamber for 28 - 90 days at 26 ± 2 °C and allowed to germinate. The seeds were inspected twice a week and the germinated seeds were transferred to autoclaved soil in plastic pots, in the glasshouse. The germination percentage for each group was determined using Equation 1. The healthy seedlings were regarded as those that flourished after transplantation in the glass house.

$$\text{Germination(\%)} = \frac{\text{No. of germinated seeds}}{\text{Total number of sown seeds}} \times 100$$

..... Equation 1

DNA extraction methods. Young leaves from 4 week-old plants were used for optimisation of DNA extraction. Protocols of DNA extraction previously used in plants were tested. Leaf samples were ground using a pre-chilled mortar and pestle, and approximately 100 mg of each sample was subjected to the following procedure:

DNA extraction kit method. This method was based on Primeprep genomic DNA extraction kit for plants (GeNet Bio, Korea), according to the manual provided by the manufacturer (GeNet Bio, Korea).

CTAB based extraction. This method was based on work by Dellaporta *et al.* (1983) and Doyle and Doyle (1990). Briefly, the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer was prepared and incubated in a water bath at 60 °C for 20 minutes. Then, the ground leaves were mixed with 650 µl of

CTAB buffer and an equal volume of chloroform-isoamyl alcohol (24:1) was used for removal of secondary metabolites two times. The precipitation of DNA was performed using ammonium acetate and ice-cold absolute ethanol. Subsequently, the samples were incubated at -20 °C for 24 hours and washing of DNA was conducted using 70% ethanol. The RNA was removed from the sample using RNase and the extracted DNA was dissolved in distilled water.

SDS- LiCl based method. This method was adopted from Arif *et al.* (2010), and the extraction buffer consisted of Tris-HCl, EDTA, SDS, proteinase K and LiCl. Briefly, 100 mg of crushed leaves were mixed with the extraction buffer and vortexed for 5 minutes. The samples were then treated with RNase and extracted using phenol chloroform 1:1 v/v. The precipitation was done using ammonium acetate and ice-cold absolute ethanol. The extracted DNA was washed two times in aqueous ethanol (70%), and the dry DNA was dissolved in distilled water.

Sucrose based protocol for plants with high secondary metabolites. This method was adopted from Sudan *et al.* (2017). The extraction buffer contained Tris HCl, EDTA, NaCl, glycerol, and SDS. Briefly, the extraction buffer was warmed at 65 °C for 20 minutes and mixed with 100 mg of crushed leaf sample. The samples were treated twice using chloroform isoamyl alcohol and RNase was then added. The DNA was precipitated using ice-cold absolute ethanol and incubated at -20 °C for 10 minutes. The DNA pellets were washed twice in 75% ethanol and air dried before dissolving in TE buffer.

Modified CTAB-SDS based protocol for plants with high composition of phytochemicals. This is a modification of the method based on work by Dellaporta *et al.* (1983) and Doyle and Doyle (1990). The modifications were the addition of 20% (w/v)

SDS to the CTAB extraction buffer, a three-time phenol-chloroform (1:1 v/v) and chloroform isoamyl alcohol (24:1) steps and use of cold isopropanol for DNA precipitation instead of ammonium acetate and absolute ethanol. Briefly, the CTAB extraction buffer consisted of 2% (w/v) CTAB, 4% (w/v) PVP, 100 mM Tris-HCl (pH 8), 1.4 M NaCl, and 0.2 M EDTA (pH 8). Firstly, it was incubated in a water bath for 20 minutes at 60°C. Next, 650 µl of the buffer and 150 µl of 20% (w/v) SDS were mixed with 100 mg of crushed leaf sample in a microfuge tubes. Then, the samples were heated at 60 °C for 20 minutes and permitted to stand at room temperature for 10 minutes. Phenol chloroform volume of approximately 1.5 folds of the sample volume was added and mixed by inverting the tubes gently. Next, centrifugation (16060 ×g) was conducted for a duration of 10 minutes. Then, the supernatant was pipetted to a new micro-centrifuge tube and the step was repeated twice. Subsequently, 1.5 folds of chloroform-isoamyl alcohol (24:1) was added and the step was performed twice. Eventually, the extraction step was conducted using plain chloroform. The aqueous part was transferred into a new tube and mixed with 1.5 volumes of isopropanol, inverted repeatedly to mix, and then stored at -20 °C for 24 hours. Then, the samples were centrifuged for 5 minutes and air dried for 30 minutes at room temperature.

A volume of 500 µl of NaCl and 1 ml of RNase were then added to the sample and incubated at 37 °C for 30 minutes. Isopropanol was again added to the sample and DNA was precipitated for 30 minutes. The mixture was centrifuged, isopropanol removed, and sample was washed twice with 70% ethanol and dried. The extracted DNA was dissolved in 50 µl of double distilled water.

Agarose gel electrophoresis. The extracted DNA samples were run on 1% (w/v) agarose gel electrophoresis to determine its presence, quantity, quality and integrity.

Polymerase chain reaction (PCR) amplification using SSR and SRAP markers. The SSR (AH14 and AH31) and SRAP (ME5-EM7) primers utilised in this study were obtained from Liu *et al* (2016) and Li and Quiros (2001), respectively. The SSR primers were F-GCTTGTGGGTTCTGGGATCTAT and R-CAGACACTAGTTTGGATGTACT for AH14 and F-TCCTCTAACGTGCGCCCCTAAG and R-AAACCCAGCGTGCCACCATTG for AH31.

The PCR was undertaken using a thermocycler (Veriti Applied Biosystems, United States). Each reaction consisted of 4 µl of 5x PCR reaction buffer, 1 µl of MgCl₂, 0.5 µl of 10 mM dNTPs, 1 µl primers, 0.2 µl Taq polymerase (Biolabs, England), 2 µl of 10 ng DNA and sterile water was added to a final reaction volume of 20 µl. The nucleotide sequences for SRAP primers, ME5 and EM7 were TGAGTCCAAACCGGAAG and GAC TCGTACGAATTCAA, respectively. The PCR products for both SSR and SRAP markers were analysed through electrophoresis and viewed under UV transilluminator in a gel doc XR (Bio-Rad, USA).

RESULTS

Germination of pre-treated seeds. All the seeds treated with hydrogen peroxide germinated and were successfully transplanted into soil and acclimatised in the glasshouse (Fig. 1). Similarly, the distilled water samples also germinated but when transplanted, a few seedlings (4) did not flourish. Moreover, the HCl samples exhibited a slower pace of germination and when transplanted they all exhibited a very slow growth rate compared to the rest. Approximately, 9 of the replicates failed to acclimatise in the glasshouse and did not flourish like the rest.

Conversely, all the H₂SO₄ treated seed samples did not germinate. Overall, all samples treated with hydrogen peroxide, hydrochloric



Figure 1. Germination of chemical pre-treated seeds of jackfruit and acclimatisation of seedlings. (A) Seeds and the fleshy pulp region of jackfruit; (B) Seeds pre-treated with hydrogen peroxide germinating in zip lock bags; (C) Germinated seeds ready for transplanting to sterile soil in pots and (D) Seedlings acclimatising in the glasshouse.

acid and distilled water exhibited a high germination rates. The hydrogen peroxide treated seed samples had a higher germination rate (100%) compared to distilled water (95.56%) and HCl (90%) treated seeds. Furthermore, hydrogen peroxide treatment exhibited a faster rate of germination (4 - 6 weeks) compared to 3% distilled water (6 - 8 weeks) and 3% HCl (12 weeks). The seed samples treated with 3% sulphuric acid (H_2SO_4) did not germinate even after 12 weeks post-treatment.

Analysis of DNA using agarose gel electrophoresis. Extraction of DNA utilising a modified CTAB-SDS method yielded DNA with higher quantity and integrity compared to the rest. The PrimePrep kit, CTAB, SDS-

LiCl, and Sucrose based techniques yielded degraded DNA. Moreover, the extracted DNA pellets had brownish pigmentation and the solutions were not clear. Conversely, the modified method yielded clear and discrete bands with no visible contamination (Fig. 2).

Suitability test of the modified CTAB-SDS DNA extraction method. PCR analysis using SSR and SRAP markers on DNA from Primeprep Genomic DNA extraction Kit, CTAB, SDS-LiCl and Sucrose methods did not yield any amplification products on a 1 % gel electrophoresis (Fig. 3). However, the PCR amplification of DNA from modified CTAB-SDS method yielded amplicons. The modified CTAB-SDS extraction method was selected for subsequent validation for efficacy using

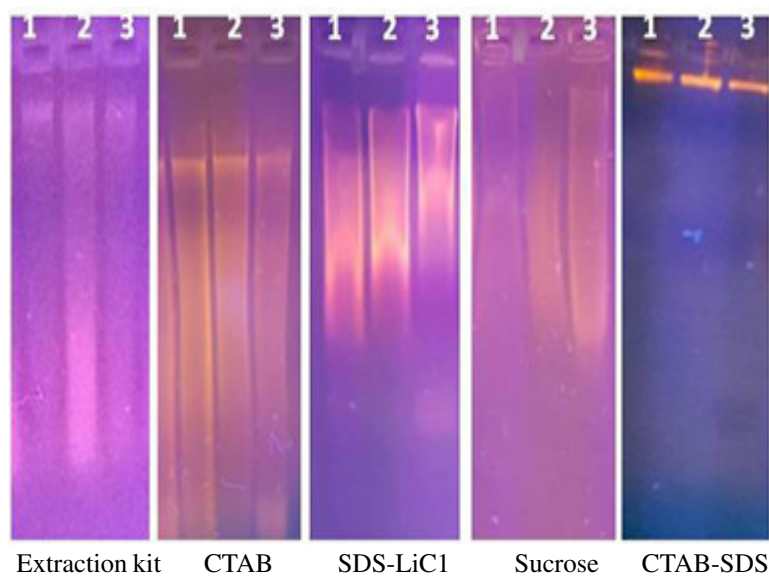


Figure 2. Comparison of five different DNA extraction methods. Extracted genomic DNA were analysed by 1% agarose gel electrophoresis and visualised under UV light.

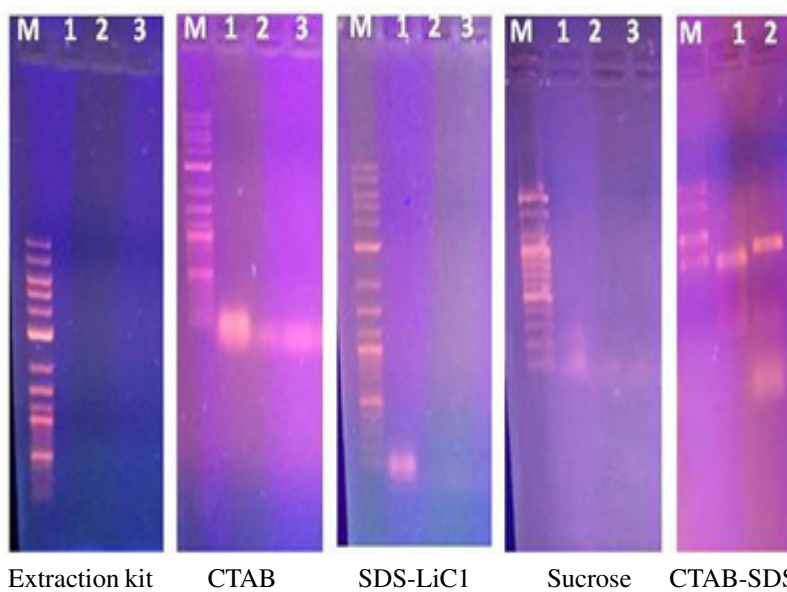


Figure 3. Electrophoretic profile of SSR amplification using primer AH14 on 1.5% agarose gel. Lane M - 1 kb molecular weight marker (Thermoscientific); and 1 - 3 represents samples isolated using the different DNA extraction methods.

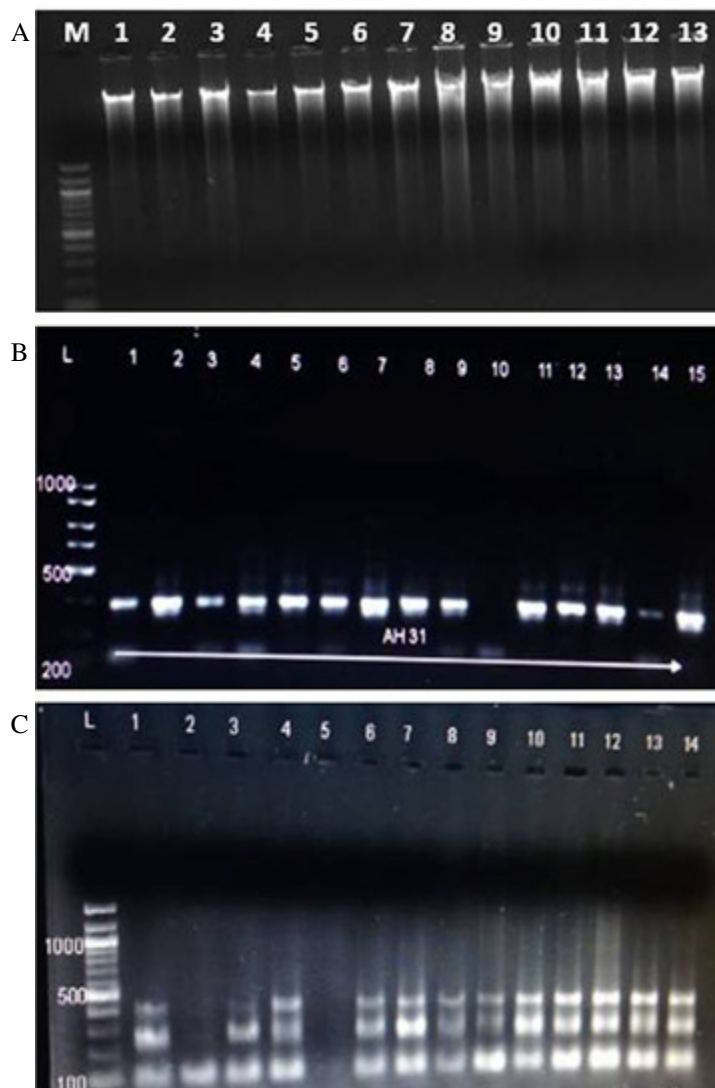


Figure 4. Performance of optimized CTAB-SDS method for DNA extraction from jackfruit leaves preserved in silica gel. (A) Electrophoresis of total DNA extracted from leaves of different jackfruit varieties using CTAB-SDS method; (B) Electrophoretic profile of SSR amplification using primer AH31 on 1.5% agarose gel; and (C) Electrophoretic profile of SRAP amplification using primer ME5-EM7 on 1.5% agarose gel. Lane L and M – 1 kb molecular weight marker (Fisher ThermoScientific); Lanes 1-3 represent samples from Muranga; 4-6, samples from Ukunda; 7-9, samples from Kampala; 10-12, samples from Mbale; and 13-15, samples from Ugenya.

jackfruit tree leaf samples from different regions in Kenya and Uganda. The modified (CTAB-SDS) yielded good quality bands (Fig. 4). All the DNA samples were successfully amplified using SSR and SRAP markers.

DISCUSSION

Seed germination and dominance. Pre-treatment of seeds with 3% hydrogen peroxide was the most effective in enhancing

germination within a short time compared to use of distilled water, 3% HCl or 3% H₂SO₄ (Fig. 1). Possibly, hydrogen peroxide was efficient in breaking the seed coat and making the seed more permeable to water and oxygen, thus facilitating cell multiplication and germination (Imani *et al.*, 2011). Primarily, hydrogen peroxide is naturally produced by plants during distress, which makes it ideal compared to other reagents used to stimulate germination. Hydrogen peroxide also has antimicrobial properties (Szopinska, 2014) and is ideal in preventing microbial pathogenic growth. There are limited published protocols on jackfruit seed germination and the findings of this study unveil an effective approach for faster and large scale propagation of Jackfruit seedlings. The technique can be used by researchers on jackfruit genetic studies, seedling vendors or farmers who intend to plant the fruit in large scale as the germination rate was 100%. Moreover, the small-scale farmers can use distilled water for faster propagation of the fruit as well.

Pretreatment of seeds with 3% HCl induced germination, but after a longer period (12 weeks). This implies that it is less effective in stimulating germination of jackfruit seeds. Seeds pre-treated with 3% H₂SO₄ did not germinate because the acid may have caused seed death (Imani *et al.*, 2011). Sulphuric acid has been effectively used in optimisation of seed germination of other plant species such as *Medicago arborea* (Travlos and Economou, 2006), and *Parkia biglobosa* (Abubakar and Maimuna, 2013). It implies that the optimisation protocols for seed germination vary from one plant species to another.

DNA extraction methods. The extraction kit, CTAB, SDS-LiCl and Sucrose based techniques yielded degraded DNA (Fig. 1); indicating that the techniques were not effective in removing secondary metabolites and essential oils, which are abundant in jackfruit leaves (Ojwang *et al.*, 2015; Ojwang *et al.*, 2017) and are known to interfere with

the quality of the extracted DNA. The extracts from these methods were mostly characterised by brown pigmentation during precipitation, implying a co-extraction of DNA with phytochemicals (Sudan *et al.*, 2017).

The modified CTAB-SDS based method was found to produce good quality DNA that yielded clear and distinct PCR amplicons on SSR and SRAP marker analysis (Fig. 4). This is an indication that the DNA was free of contaminants such as polysaccharides, polyphenols and other phytochemicals present in jackfruit leaves. The modified CTAB-SDS method included the use of additional steps of sample extraction in phenol-chloroform and chloroform-isoamyl alcohol. These repeated steps facilitated the removal of the secondary metabolites and essential oils (Doosty *et al.*, 2012) hence, leading to the extraction of high-quality DNA for genetic studies.

The modified protocol unlike those previously described, does not require the use of liquid nitrogen and β -mercaptoethanol. Few genetic characterisation studies on jackfruit have been conducted using DNA protocols that required liquid nitrogen and β -mercaptoethanol (Schnel *et al.*, 2001; Shyamamma *et al.*, 2008; Gopalsamy *et al.*, 2012; Arruda *et al.*, 2017). Liquid nitrogen is highly volatile and requires special handling equipment that may be unavailable in laboratories with low funding. Furthermore, β -mercaptoethanol is hazardous and has a strong pungent odour. The optimised method therefore, is suitable for laboratories with limited funding for example in sub-Saharan Africa.

CONCLUSION

This study has found out that administration of 3% hydrogen peroxide or distilled water pre-treatments is effective in breaking jackfruit seed dormancy. The 3% hydrogen peroxide technique can be potentially utilised in enhancing the propagation of jackfruit seedlings for large scale production. However, small-scale farmers can use water pre-treatment

option since it is more readily available and also has an equally higher germination rate. Moreover, the two techniques can be used to produce jackfruit seedlings for genetic characterisation studies. The optimised CTAB-SDS based method is suitable for extraction of high-quality DNA from leaves of jackfruit for genetic studies as well as in marker-assisted selection of superior varieties. This method can be adopted for related fruit tree species with high concentrations of secondary metabolic compounds such as polyphenols.

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