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Full Length Research Paper

Inclusion of African winged termites (*Macrotermes nigeriensis)* improves the nutrients and quality of fermented cassava mahewu

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Cassava mahewu is a non-alcoholic fermented beverage used by school children, but it has low contents of protein and micronutrients. This study examined the effect of inclusion of African winged-termite (AWT) on the nutritional quality and acceptability of cassava mahewu. Cassava flour was replaced with AWT at varying proportions of 100:0 (as control), 90:10, 80:20, 70:30 and 60:40% and fermented to obtain cassava mahewu. After freeze drying, proximate and micronutrients were determined using standard methods. Sensory attributes were assessed using 9-point Hedonic scale. The proximate compositions showed an improvement ($p<0.05$) in crude protein (1.35 to 32.65), fibre (1.26 to 4.0), fat (1.56 to 19.15) and ash (1.47 to 4.04 g/100 g) following the addition of AWT at the highest ratio compared with the control. A decrease ($P<0.05$) in carbohydrate (84.90 to 15.65 g/100 g) was recorded. The iron (1.53-31.65), zinc (0.70-4.60), vitamin C (4.90-13.90) and riboflavin (0.40- 2.09 mg/100 g) increased with the addition of AWT. In terms of overall acceptability, inclusion at 70:30 was significantly ($P<0.05$) rated higher than other ratios. Inclusion of termite flour prior to fermentation, improved the nutritional and sensorial qualities of cassava mahewu and hence could be utilized to manage the widespread nutritional deficiency in developing countries.

Key words: Cassava mahewu, edible insects, termites, enrichment, fermentation, nutrient, sensory quality.

INTRODUCTION

Protein-energy malnutrition is still highly prevalent in sub-Saharan Africa including Nigeria (Okwu et al., 2010; FAO,

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2021) due to high cost of nutritious and healthy foods (Bai et al., 2021) and overdependence on starch based food as the main staple at the expense of nutrient-rich foods. Currently, one out of five children under the age of six suffers from protein energy malnutrition and nearly half of all deaths in children under the age of five have been attributed to undernutrition (FAO, 2015). The world population is estimated to reach 10 billion people in 2050 (UN, 2019), consequently, conventional protein sources such as meat and fish may be insufficient and thus may not be within the reach of low-income households (Godfray et al., 2010; Agrodok, 2015). There is therefore need for alternative and cheaper sources of protein to meet the rising nutrient demands. One of the non-conventional nutrient-rich sources that have recently gained attention is edible insects.

As the world's population continues to grow rapidly, there is a renewed interest in the use of insects as human food. This is so because, edible insects are rich in protein with good amino acid, fatty acid profiles and high contents of a variety of micronutrients including vitamins, minerals and useful bioactive substances as compared to traditional sources of protein such as meat (Finke and Oonincx, 2014; Inje et al., 2018). The nutritional value of edible insects is largely variable due to thousands of species, the stage of metamorphosis and methods of processing (Finke and Oonincx, 2014; Kourimska and Adamkova, 2016; Mutungi et al., 2017). In Nigeria, over 30 species of insects are commonly used as food amongst which are locust, winged termites, grasshoppers, weevils and beetles (Ekop et al., 2010; Adeoye et al., 2014). While every measure is currently being taken to improve food production through conventional agriculture to meet the global food demands, almost zero interest has been shown to the consumption of edible insects and studies on the same is still very low as consumption is sometimes characterized by repulsive feelings and regarded as primitive behavior.

Winged termite (*Macrotermes natalensis*) is one of the most common species of insects belonging to the family, *termitidae.* It is the most represented insect in Nigeria in terms of acceptability and consumption (Adeoye et al., 2014). Winged termite is known locally in various parts of Nigeria by different names such as '*aku*' in Ibo, '*chinge*' in Hausa and '*Esusu*' in Yoruba and present every year at the onset of the rainy season. The termites contain high-quality nutrients including highly digestible proteins (Kinyoru et al., 2010) as well as minerals which are more bioavailable than minerals from plant foods (Ojha et al., 2021) with good flavor and texture, which makes it suitable to be added to a specific staple food to improve its nutrient quality. Though the nutrient composition of various insects species in Nigeria have been studied by several researchers (Ekop et al., 2010; Igwe et al., 2011a; Solomon and Prisca, 2012; Oibiokpa et al., 2017), very few studies are available on the possibility of enriching local staple foods such as cassava with nutrient-dense edible insect.

Cassava (*Manihot esculenta*) is a perennial woody shrub that belongs to the family Euphoriaceae. It is cultivated as an annual crop in tropical and subtropical areas of the world (FAO, 2015). Cassava is the cheapest food and one of the most utilized staple food crops in developing countries in Africa after maize, with Nigeria being the largest producer of cassava in the world (FAO, 2021). Cassava is widely consumed in diverse ways in the country as excellent source of carbohydrate. However, its protein and micronutrient quality is relatively poor (Montagnac et al., 2009). As a result, micronutrient deficiency diseases and lack of dietary diversity becomes a particularly severe problem in various communities where diets are based predominantly on cassava as main staple. Owing to the food importance of cassava, the improvement of nutritional quality of cassava-based food products has received considerable attention and significant efforts have been made to improve its nutritional value (Mesfin and Shimelis, 2013; Salvador et al., 2016; Boyiako et al., 2020), so that consumers can still derive essential nutrients other than carbohydrate from cassava.

Fermented foods, which form a considerable part of diet in Nigeria, are products obtained from the enzymatic modification of food by microorganisms to bring about desired biochemical changes, improving the nutritional value of foods, extending shelf-life and serves as potential sources of probiotics (Fadahunsi and Soremekun, 2017; Anyiam et al., 2020). Cassava mahewu is a novel cassava-based non-alcoholic fermented beverage widely used as complementary drink for school children in Mozambique and other parts of Southern African countries (Salvador et al., 2016). However, being a direct product of cassava, it is low in proteins and micronutrients (Boyiako et al., 2020). Hence, cassava mahewu drink alone cannot cater for the daily nutrients requirement of the school children. Therefore, this study was aimed to determine the suitability of blending African winged termite flour with cassava flour for cassava mahewu production and to evaluate the sensory and the nutritional contents of the final product. The findings from this work may encourage the use of edible insects in addressing the problem of protein-energy malnutrition and food insecurity especially in poor rural settings.

MATERIALS AND METHODS

Sample collection

Selection of edible winged termites (*Macrotermes nigeriensis*) for this study was based on the perceived nutritional value of the specie, its availability and local preference in the community. The adult winged termites were freshly collected in early morning hours during raining season (May to June) from residential buildings in Umudike community using traditional methods of attraction to light at night and handpicking. Harvested insects were transported under ice blocks to the laboratory at the Department of Biochemistry, Michael Okpara University of Agriculture, Umudike for processing.

Table 1. Ratio of composite blend formulation.

Sample	Cassava flour (%)	Termite flour (%)	Label
A	100	0	Negative control
B	90	10	Test diet 1
C	80	20	Test diet 2
D	70	30	Test diet 3
E	60	40	Test diet 4

Cassava roots used for mahewu preparation were sourced from the Natural Root Crops Research Institute, Umudike, Nigeria and transported in a container to the laboratory for processing into flour. The ethical guidelines provided by the ethical committee of College of Natural Science, Micheal Okpara University of Agriculture Umudike were strictly followed. All chemicals used in the study were of analytical grade.

Sample processing

The winged termites were washed three times with clean tap water to remove soil and dirt, de-winged, de-legged and blanched by soaking in boiled water for 2 min and drained before subjecting to oven drying at 45°C to constant weight for 12 h. The dried insects were milled into fine powder using an electric mill (BN-2001-62WC-Germany). The processed termite was sieved using a 250 µm mesh sieve to fine brown flour, packaged in labeled dry glass jar and stored in an airtight container until used for mahewu preparation. The collected cassava roots were cleaned, peeled and washed again with tap water to remove sand particles. They were cut into smaller pieces and oven dried at 50°C to constant weight before subjecting to milling. The milled white powder was sieved to produce smooth fine dried flour which was stored in an airtight container until used.

Composite blend formulation

Five experimental diets were formulated (Table 1). The milled cassava flour and winged termite flour were thoroughly mixed together with the aid of a laboratory sized mixer to make 100 g at different ratios and stored in well labeled plastic containers prior to fermentation. The formulation was designed in different proportions so as to obtain the most acceptable product that has the highest nutritional value.

Production of enriched cassava mahewu

Each formula in Table 1 was fermented to produce mahewu under controlled conditions using the method of Salvador et al. (2016) with little modifications. 20 g of each mixture was dissolved with 49 mL of distilled water and added to 150 ml of boiling water. The mixture was boiled for 5 min to gelatinize the starch and then cooled to a temperature of 25°C. The porridge was transferred to a 250 ml Erlenmeyer flask and 1.25 g of freeze dried starter culture was added as a source of inoculum, mixed thoroughly and allowed to ferment for 36 h at 30°C while monitoring the pH and titratable acidity (TTA). The fermented gruels were freeze dried at -20°C for 24 h to obtain the enriched samples in powdered form prior to biochemical analysis. All the solvents and chemicals used for the biochemical assay were of analytical grade.

Changes in pH and titratable acidity during fermentation

Titratable acidity (TTA) and pH were measured at the beginning and after every 12 h during fermentation according to the AOAC (2000) and Salvador et al. (2016). The pH values of the complementary beverage were determined in triplicate by a pH meter. For TTA, 5 g of dried sample was mixed with 25 ml distilled water, the mixture was allowed to stand for 15 min, shaken at 5 min intervals and centrifuged at 3000 rpm for 15 min. 10 ml of the filtrate were titrated against 0.1 M NaOH using 1% phenolphthalein (3 drops) as indicator. The acidity was calculated as % (W/V) lactic acid equivalent using the formula described by Salvador et al. (2016).

$$\% \text{ lactic acid} = \frac{ml\ of\ 0.1M\ NaOH\ (end\ point)}{ml\ of\ sample\ taken}\ x100$$

Proximate analysis

Proximate parameters such as % crude protein, crude fibre, ash, fat, carbohydrate and moisture contents were determined on each sample using the standard procedures described by AOAC (2000). Percentage moisture was calculated by drying the sample in an oven at 105°C for 3 h. The dried sample was put into a desiccator, allowed to cool and the reweighed. Crude protein was determined by Kjeldahl method using a nitrogen-protein conversion factor of 6.25, crude fibre by acid and alkaline hydrolysis, fat was determined by Soxhlet extraction method with petroleum ether. Percentage ash was evaluated by combusting the samples in silica crucible placed in a muffle furnace at a high temperature of 600°C. The percentage of carbohydrate present was determined by subtracting all of the components assayed (crude protein, crude fat, moisture, fibre and ash) from 100 (AOAC, 2000; Kambabazi et al., 2021).

Analysis for minerals

Samples were analyzed for selected minerals following the method described by Okalebo et al. (2002). A portion (2 g) of the ash obtained was digested with nitric and hydrochloric acid (1:3) and allowed to stand for 30 min. It was later filtered using Whatman filter paper (0.45 µm). The resulting extract was used for the determination of calcium, iron, manganese, zinc, and copper by the use of an atomic absorption spectrophotometer (ICP-OES 720 series Agilent). Standard solutions (Sigma Chemicals, USA) of each of the minerals were also prepared and co-analyzed with the sample extract preparations.

Analysis for vitamins

The B-complex vitamins (Thiamine, Riboflavin and Niacin) were determined following the methods described by Okwu and Josiah

Table 2. Changes in pH and TTA of *M. nigeriensis* (WT)-enriched cassava mahewu.

Sample	pH				Titratable acidity (%)			
Time (h)	0 h	12 h	24 h	36 h	0 h	12 h	24 h	36 h
Control	6.55±0.7	5.72±0.5±	4.79±0.2	4.53 ±0.2	0.07 ±0.01	0.12± 0.02	0.23±0.07	1.33±0.04
10% WT	6.35±0.0	5.66±0.4	4.75±0.1	4.49±0.6	0.08±0.01	0.16±0.04	0.29±0.06	1.39±0.02
20% WT	6.10±0.8	5.35±0.2	4.67±0.7	4.41±0.7	0.09±0.02	0.15±0.01	0.33±0.01	1.49±0.06
30% WT	5.95±0.6	5.35±0.4	4.63±0.4	4.53±0.4	0.09±0.01	0.17±0.03	0.55±0.07	1.48±0.01
40% WT	6.04±0.2	5.41±0.2	4.47±0.4	4.30±0.8	0.08±0.07	0.19±0.02	0.68±0.03	1.46±0.07

(2006), while vitamin C content of the enriched fermented cassava mahewu was determined by the methods described by Desai and Desai (2019) using the spectrophotometric methods.

Sensory evaluation

The sensorial analysis was carried out following the methods described by Boyiako et al. (2020). A semi-trained panel of thirty members who were familiar with the standard product evaluated the sensory properties of the enriched product. The panelists were asked to rate each sensory attribute of all samples including the standard cassava mahewu. The fermented samples were coded with letters and served to the panelists at random to guard against any bias. The basis for evaluation included appearance/thickness, taste, flavour, colour and overall acceptability. The rating was done on a 9-point hedonic scale (9. Like extremely; 8. Like very much; 7. Like moderately; 6. Like slightly; 5. Neither like nor dislike; 4. Dislike slightly; 3. Dislike moderately; 2. Dislike very much; 1. Dislike extremely). Water was provided to rinse the mouth after each testing.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data generated using the Statistical Package for Social Sciences (SPSS) version 17.0. Least significant difference (LSD) test ($P<0.05$) for means separation was done using Statistix 10. Results were expressed as mean ± standard deviation of triplicate determinations

RESULTS

Changes in pH and titratable acidity during fermentation

The result of changes in pH and titratable acidity (TTA) during the production of the improved cassava mahewu is shown in Table 2. It was observed that the highest pH (6.55) was recorded at 0 h which decreased to the lowest pH of 4.30 for 40% at 36 h. Titratable acidity (TTA) increased from minimum of 0.07% at 0 h to a maximum of 1.56% at the end of the fermentation in all the enriched samples.

Proximate composition of improved cassava mahewu

The quantitative estimations (in percentages) of the proximate compositions of *M. nigeriensis*-enriched mahewu are shown in Table 3. A significant ($p<0.05$) improvement in % protein (from 2.87 to 32.65), fat (0.65 to 29.15), fibre (1.26 to 4.04) and ash (1.55 to 4.14) was observed with the inclusion of *M. nigeriensis* at different blend ratios. Cassava mahewu with 40% *M. nigeriensis* inclusion had the highest amount of protein, fat and ash content when compared with the control mahewu. No significant difference ($p>0.05$) was recorded in water content of the mahewu with the inclusion of *M. nigeriensis* flour. However, a significant decrease ($p<0.05$) in carbohydrate content was recorded in the mahewu following the inclusion of *M. nigeriensis* flour at all levels.

Mineral composition of termite-improved cassava mahewu

Table 4 shows the selected mineral composition of termite flour incorporated cassava mahewu at different blend levels. The mineral contents of all the composite blends increased significantly compared with the control mahewu ($p<0.05$). It was observed that the sample having 30% termite flour inclusion had a significant ($p<0.05$) higher improvement in calcium, zinc and manganese. However, iron content was higher in 40% termite flour inclusion when compared with the control mahewu which recorded the least in all minerals assayed.

Effect of *M. nigeriensis* flour inclusion on vitamin content of cassava mahewu

Results of the vitamins assay of *M. nigeriensis*-improved cassava mahewu are shown in Table 5. It was observed that inclusion of termite flour at different proportions significantly improved the amount of vitamin C (from 42.4 to 55.8 mg/100 g), riboflavin (0.15 to 1.80 mg/100 g), thiamine (0.67 to 2.30 mg/100 g) and niacin (0.85 to 1.98 mg/100 g). Inclusion at 30% had higher vitamin C and niacin content than other proportions and the control. Riboflavin and thiamine were higher in 40% inclusion.

Table 3. Effect of inclusion of *M. nigeriensis* (WT) on proximate composition (%) of cassava mahewu.

Components	Protein	Fat	Fibre	Ash	Water	Carbohydrate
Control	2.87±0.07^{a}	0.65±0.19^{a}	1.2±0.04^{a}	1.65±0.06^{a}	8.75±0.20^{a}	84.08±2.1^{a}
10% WT	11.09±0.8^{b}	8.10±1.3^{b}	2.20±0.9^{b}	1.88±0.9^{ab}	8.20±1.04^{a}	68.58±1.2^{b}
20% WT	14.1±1.7^{c}	15.0±1.13^{c}	3.47±0.8^{c}	2.48±0.5^{b}	9.80±0.84^{ab}	56.18±4.1^{c}
30% WT	22.96±2.6^{d}	21.85±1.63^{d}	4.04±1.2^{d}	3.71±0.8^{c}	9.75±0.17^{ab}	38.68±3.3^{d}
40% WT	32.65±1.74^{e}	29.15±1.35^{e}	3.84±0.6^{cd}	4.14±0.3^{c}	10.15±0.28^{b}	21.17±2.4^{e}

Mean with different superscript (a-b-c) are significantly different at (P<0.05) along the columns.

Table 4. Effect of inclusion of winged termite on mineral composition (mg/100 g) of cassava mahewu.

Component	Calcium	Iron	Zinc	Copper	Manganese
Control	32.12±2.4^{a}	1.84±0.2^{a}	0.46±0.0^{a}	0.65±0.01^{a}	0.55±0.05^{a}
10% WT	48.20±3.8^{b}	15.7±1.5^{b}	2.96±0.23^{b}	1.84±0.12^{b}	0.76±0.04^{ab}
20% WT	55.65±0.22^{c}	26.70±1.8^{c}	4.60±0.28^{c}	2.05±0.35^{b}	1.12±0.28^{b}
30% WT	65.37±2.87^{d}	31.65±2.19^{d}	4.65±1.07^{c}	3.85±0.35^{c}	3.70±0.14^{c}
40% WT	62.40±4.24^{cd}	58.84±3.48^{e}	3.70±1.14^{c}	4.12±2.90^{c}	2.01±0.11^{d}

Mean with different superscript (a-b-c) are significantly different at (P<0.05) along the columns.

Effects of winged termite inclusion on the sensory attributes of cassava mahewu

The sensory evaluation of the fermented beverage prepared from various percentage inclusion of termite flour (Table 6) showed that the sample with 30% termite inclusion was rated significantly higher (P<0.05) for taste (7.56) than other samples. Sample with 10% termite inclusion had the least score for taste (5.80). Cassava mahewu with 40% termite flour was rated significantly higher (p<0.05) for flavour (7.80) than other samples. The control sample was more preferred in terms of appearance/thickness (7.90) and colour (7.20) followed by 10% inclusion. Inclusion at 40% recorded the least (5.60) in terms of colour.

In terms of the overall acceptability (Figure 1) of the product, termite flour inclusion at 30% was significantly (P<0.05) rated higher than other samples. Inclusion at 10 and 20% showed no significant difference (P<0.05) in terms of acceptability when compared with the standard cassava mahewu. Cassava mahewu with 40% winged termite inclusion had the least score for overall acceptability.

DISCUSSION

Providing information about the benefits of entomophagy and incorporating same in traditional foods can increase the willingness to eat this kind of food due to their high nutritional profile (Finke and Oonincx, 2014). In the present study, the fermentation of winged termite-incorporated cassava mahewu was characterized by a decrease in pH and a corresponding increase in TTA. This observed inverse proportional trend between pH and TTA was previously reported by Salvador et al. (2016) and Fadahunsi and Soremekun (2017). This might probably be caused by the microbial activities during fermentation which degraded some of the carbohydrates content into lactic acids and other organic acids, consequently causing the fall in pH and increase in TTA. According to Agarry et al. (2010), a decrease in pH and corresponding increase in TTA is a key factor for growth of microorganisms during fermentation which signified a good rate of fermentation. Moreover, cassava flour had been reported as being a good substrate for lactic acid production through fermentation (Salvador et al., 2016). In addition, the occurrence of un-dissociated forms of organic acids at low pH could inhibit a broad spectrum of pathogens thus improving the microbiological stability of the final product (Blandino et al., 2003). This is because most food pathogens are not able to survive at low pH. Therefore, the decrease in pH observed in this study is advantageous to the final product in terms of prolonging its shelf life and microbial stability.

The need for improving the nutritional quality of cassava mahewu with edible insect becomes evident with the observed lower values of protein, ash, and crude fibre contents of standard cassava mahewu used as control which could be due to losses during cassava processing (Montagnac et al., 2009) or complete absence of the nutrient. Following the inclusion of winged termite flour at

Table 5. Effect of inclusion of winged termite on vitamin composition (mg/100 g) of cassava mahewu.

Component	Vitamin C	Thiamine	Riboflavin	Niacin
Control mahewu	42.4±1.07^{a}	0.67±0.04^{a}	0.15±0.03^{a}	0.85±0.02^{a}
10% Inclusion	42.8±2.14^{a}	0.75±0.07^{ab}	0.89±0.20^{b}	0.88±0.14^{a}
20% Inclusion	53.4±3.2^{b}	1.30±0.14^{b}	1.22±0.07^{bc}	1.60±0.08^{ac}
30% Inclusion	55.8±5.2^{b}	1.56±0.07^{bc}	1.64±0.14^{c}	2.08±0.12^{c}
40% Inclusion	52.6±2.3^{b}	2.30±0.14^{c}	1.80±0.12^{c}	1.98±0.28^{c}

Mean with different superscript (a-b-c) are significantly different at (P<0.05) along the columns.

Table 6. Sensory properties of cassava mahewu improved with winged termite.

Sample	Flavour	Taste	Appearance/thickness	Colour
Control	6.11±1.2^{a}	6.80±0.4^{ac}	7.90±0.4^{a}	7.20±1.2^{a}
10% inclusion	6.12±0.8^{a}	5.80±0.3^{a}	7.10±1.0^{a}	6.82±0.8^{ab}
20% inclusion	5.80±1.0^{a}	6.30±0.5^{a}	6.56±2.1^{ab}	5.80±0.0^{4b}
30% inclusion	6.22±0.6^{a}	7.56±1.7^{b}	6.22±0.3^{b}	5.78±0.9^{b}
40% inclusion	7.80±0.6^{b}	6.50±0.7^{c}	5.78±1.6^{b}	5.60±1.0^{b}

Mean with different superscript (a-b-c) are significantly different at (P<0.05) along the columns.

different proportions, a significant increase ($p<0.05$) in protein content was observed when compared with the control standard mahewu. The result indicated that the winged-termite is a good source of protein for man and animals which is an agreement with the report of Kinyuru et al. (2010) and Igwe et al. (2011a). Since an adult male with 70 kg average body weight requires 35 g of protein daily, therefore only about 164 g (on dry weight) of the termite-enriched cassava mahewu beverage would be required to provide an average adult minimum daily protein requirement, with an allowance of 25% made for protein indigestibility and the limiting sulphur amino acid content (Igwe et al., 2011b; Inje et al., 2018). That means a lower amount (<164 g on dry weight) of the fermented beverage needs to be consumed per day by children in order to meet their daily protein requirement.

The carbohydrate content of the fermented cassava mahewu decreased significantly ($p<0.05$) with the addition of edible winged-termite flour compared with the control. This could be attributed to the effect of fermentation on the carbohydrate content. The indigenous microflora associated with the natural fermentation of cassava mahewu has been reported to be amylolytic (Omenu et al., 2007) thereby causing high hydrolysis of carbohydrate to simple molecules. The fermenters utilize carbohydrates as an energy source during fermentation and produce carbon dioxide as a by-product. This is in agreement with the reports of Fadahunsi and Soremekun (2017) and Boyiako et al. (2020) who revealed that fermentation of starchy foods generally leads to a decrease in the level of carbohydrates content.

A significant improvement ($p<0.05$) in fat content of the cassava mahewu was observed following the inclusion of African winged-termite flour at different proportions. This indicates that the termite used for enrichment is an important source of fat and oil. Winged-termites have been reported to contain appreciable higher amounts of good fats which are clearly superior when compared with conventional foods of animal origin (Igwe et al., 2011b). Over 60% of the fatty acids present in edible insects generally are in the form of highly desirable mono- and polyunsaturated fatty acids (MUFA and PUFA) (Igwe et al., 2011a; Rumpold and Schluter, 2013). Saturated fatty acids are not good for human consumption because they have been implicated in certain cardiovascular disorders such as atherosclerosis, cancer and aging due to their greatest negative effect on LDL cholesterol. In contrast, MUFA and PUFA present in insects have been shown to decrease plasma cholesterol concentrations (Igwe et al., 2011b). Therefore, the increase in fat content observed may not necessarily portend risk for its consumers. Moreover, the termite is prepared by the use of de-fating process that reduces the fat content before consumption.

The crude fibre of the enriched fermented cassava mahewu also increased significantly compared with the 100% cassava mahewu. This could be accredited to the addition of the edible termite flour which has been reported to be high in dietary fibre (Igwe et al., 2011; Akullo et al., 2018). Fiber in the diet is desirable because

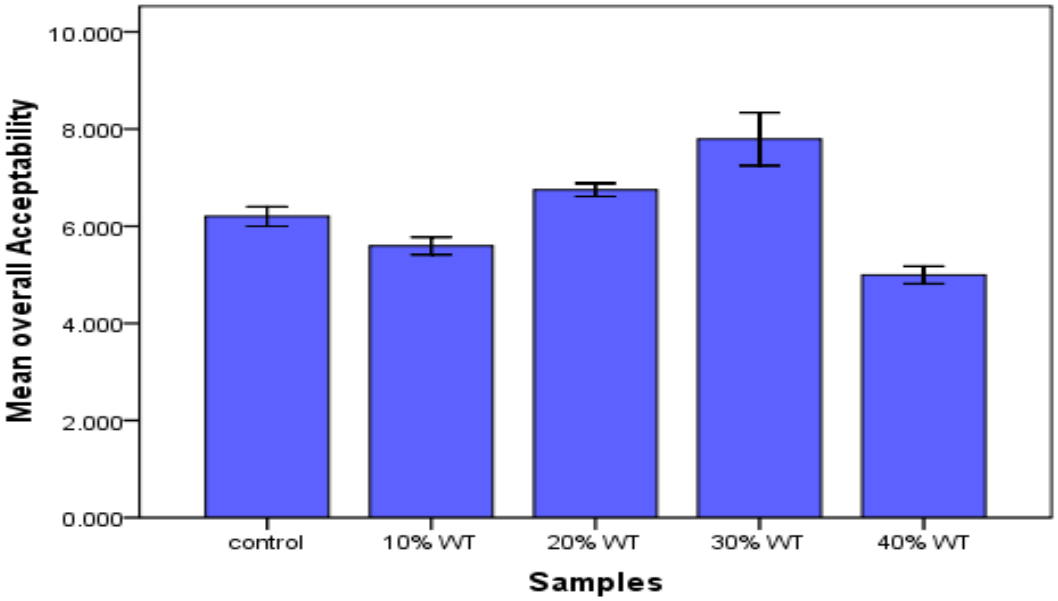

Figure 1. Consumer's acceptability of termite flour-improved cassava mahewu.

it lowers the serum cholesterol and enhances the excretion of fecal steroids thereby reducing the risk of atherosclerosis. Significant increase ($p<0.05$) of the ash content seen in this study implied that the improved product had considerable amount of minerals because the level of ash content is a reflection of the total available minerals in any food sample. As observed, the termite-enriched cassava beverage has a fair content of iron, zinc, manganese and copper. These minerals are known to play important metabolic and physiologic roles in the living system. Iron is an indispensable nutrient that is involved in oxygen transport and plays a vital role in brain and cognitive development (Akeredolu et al., 2011). It is well known that young children are vulnerable to the effects of iron deficiency because of rapid growth and development of their organs. Iron deficiency is the most common cause of anemia (Miller, 2013) and the most prevalent important nutritional problem in children. It threatens over 60% of women and children in most developing countries (WHO, 2010). Low intake of iron especially in children, can contribute to anemia which indicates the vulnerability of this age group to the iron deficiencies.

Zinc is another essential element and it is apparently deficient in the diets of many people in developing countries. Zinc and manganese for example, strengthen the immune system and act as cofactors for many enzymes participating in the metabolism of carbohydrates, proteins, fats and nucleic acids. Low zinc status in children has been associated with retarded growth, poor appetite and impaired sense of taste (WHO, 2010). Assuming good bioavailability of the iron contents, the improved fermented cassava mahewu can contribute significant amount of Iron to children and women especially during pregnancy in attempts to mitigate the risk of iron deficient anemia and undernutrition of micronutrient that is wide spread in developing countries. Similarly, calcium is an essential mineral which plays vital roles by virtue of its phosphate salts in neuromuscular function in many enzyme-mediated processes like blood clotting, tooth and bone formation (Igwe et al., 2011a). Children need calcium for strong bone formation. Apart from the nutritional point of view, calcium has also received substantial interest in the medical field for its role in osteoporosis and several other chronic diseases including hypertension and colon cancer (Thacher et al., 2012). An increase in the amount of calcium was observed in the present study following the addition of termite flour. The present findings is in agreement with Boyiako et al. (2020) who reported increase in calcium and other mineral content of cassava mahewu when enriched with beetroot juice.

The vitamin contents of *winged termite*-improved cassava mahewu showed that it contained appreciably high amounts of vitamins C, riboflavin (B2), niacin (B3) and thiamine (B1) which increased appreciably with the inclusion of termite flour at different levels. Vitamin C maintains blood vessels flexibility and improves blood circulation in the arteries (Miller, 2013; Desai and Desai, 2019). One of most important benefit derivable from vitamins C from food is their role as antioxidants while that of the B-vitamins is their role as co-enzymes in several enzyme systems of the body. The high vitamins and minerals contents present the developed product enriched as a highly potential good source of vitamins for malnourished children especially in poor-resource

settings.

The sensory evaluation of the samples showed that the enriched cassava product with 30% termite inclusion was rated highest for taste and overall acceptability. Previous report has shown that the taste and flavor of food have the most pronounced effect on consumer acceptance of the food (Kourimska and Adamkova, 2016). Dietary fat in food increases the palatability of food by absorbing and retaining the flavor. Hence, the increased fat content of the winged-termite could have contributed to the taste, flavor and acceptability of the termite-improved cassava mahewu by the consumers. Nevertheless, the sensory evaluation proved that our product gained a level of consumer acceptance comparable with the standard.

Conclusion

The study has revealed African winged termite (*M. nigeriensis*) as a very good source of proteins and micronutrients necessary for combating protein-energy malnutrition and micronutrient deficiencies rampant in various developing world today. The inclusion of edible *M. nigeriensis* flour at different levels improved the nutritional quality of fermented cassava mahewu. With regards to the sensory properties and consumer acceptability, cassava mahewu having 70:30% termite flours recorded the highest preference. Therefore, the enriched cassava mahewu could be utilized to manage the widespread nutrient deficiency in developing countries where cassava is regarded as the main staple. However, more research is needed on the technological and safety aspects of such product in order to promote the use of insects as food and mainstream acceptance of such products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Cryopreservation of plant genetic resources: A legacy for humanity

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Plant genetic resources are essential for agri-food security in the world and conservation of genetic diversity. Cryopreservation is an *in vitro* culture technique used for long-term plant conservation, by freezing the tissue at low temperatures usually with liquid nitrogen (-196ºC). During cryopreservation, cell division and metabolic activity of the explants are quiescent. There are different cryopreservation techniques used for many species and recently it has been observed that the use of aluminum cryoplates or foils increase plant survival and regeneration. The explants are exposed to a lot of stress in the different stages of cryopreservation, especially during chemical or physical dehydration and during thawing. Cryopreserved plants are exposed to physical, chemical and physiological cell damage and oxidative stress. The principal cause of plant cell mortality is membrane rupture due to ice crystal formation. The cryoprotective substances prevent ice formation and optimal dehydration is necessary for plant survival and regeneration. Different cryopreservation stages could alter genetic stability, especially during plant regeneration by the use of plant growth regulators. DNA alteration during *in vitro* culture depends on different factors, mainly cryopreservation technique and plant species. Molecular markers are used to detect variations in the DNA of cryopreserved plants. A successful cryopreservation protocol depends on survival, regeneration and genetic stability of plant materials.

Key words: Cryopreservation, plant genetic resources, cryogenic damage, plant regeneration, genetic stability.

INTRODUCTION

Plant genetic resources (PGR) are any vegetal genetic materials with real or potential value for agri-food security (Sonnino, 2017). Only 30 crops cover 95% of global agri-food needs and these have been used to produce new varieties, which have important characteristics such as higher production and tolerance to biotic and abiotic stresses (Kaviani, 2011; Shiferaw et al., 2011; FAO, 2021). Therefore, it is essential to preserve PGR because they represent a great part of plant biodiversity.

The preservation of plant species depends greatly on whether their seeds are of orthodox or recalcitrant nature and the latter species are often conserved in field

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collections (Guzmán et al., 2017). Nevertheless, PGR maintained in field are affected by biotic and abiotic factors (Niino and Arizaga, 2015). Thus, an alternative is *in vitro* medium or long-term conservation. *In vitro* plant culture requires multiple techniques that enable the growth of cells, tissues and organs in aseptic culture media (Oseni et al., 2018).

Cryopreservation consists of the interruption of metabolic functions of biological materials by decreasing the temperature with liquid nitrogen (LN) (-196°C), while maintaining viability (Niino and Arizaga, 2015). Cryopreservation in liquid nitrogen is an alternative for long-term conservation of PGR (Panis, 2019). During cryopreservation, the cell cycle and metabolic and biochemical activity are detained, therefore, the biological material can be safeguarded for practically indefinite periods (Benson et al., 2006). After cryopreservation, plant materials will recover and regenerate into a plant, depending on treatments before and after exposure to LN and there is a minimal risk of DNA alterations (Adu-Gyamfi et al., 2016; Nuc et al., 2016; Gross et al., 2017). The genetic stability of cryopreserved plant material is assessed with morphological, cytological, biochemical and, for the most part, molecular markers (Harding, 2004).

Different cryopreservation protocols have been developed for various species using different types of explants such as seeds (Schofield et al., 2018), synthetic seeds (Petrus et al., 2019), shoots (Bruňáková and Čellárová, 2016), apexes (Liu et al., 2017), pollen (Souza et al., 2018), embryogenic cultures (Varis et al. 2017), zygotic embryos (le Roux et al. 2016) and cell suspensions (Titova et al., 2021). The choice of explant used for cryopreservation is dependent on the objective; and while seeds and embryos are the main source of genetic diversity, shoot tips and dormant buds are clonal materials (Reed, 2017).

The response of plant material depends on the species and its genetic variability, explant type, cryopreservation technique as well as the stressful environment of tissue culture (Kaya and Souza, 2017; Popova and Kim, 2019; Bednarek and Orłowska, 2020). Therefore, plant cryopreservation protocols developed, are specific for each plant material and focus on achieving the maximum post-cryogenic survival and regeneration (Harding, 2004).

TECHNIQUES USED IN CRYOPRESERVATION

Cryopreservation techniques are classified into conventional and new techniques. The first ones are based on the partial chemical dehydration of the explants with osmoprotectors followed by gradual freezing with automatic freezers (Engelmann, 2011). The new techniques are based on vitrification and the use of aluminum cryoplates that facilitate the manipulation of the explants as well as faster cooling and heating (Matsumoto, 2017).

Plant vitrification requires a highly concentrated solution that sufficiently dehydrates tissues and solidifies intracellular water into metastable glass to avoid crystallization in the cell (Matsumoto, 2017). On the other hand, the aluminum cryoplates have been successful to obtain high shoot regeneration, greater than 90% in various species (Yamamoto et al., 2015; Rafique et al., 2016; Dhungana et al., 2017; Thammasiri et al., 2019; Pettinelli et al., 2020; Zhang et al., 2020; Benelli et al., 2021; Tanaka et al., 2021). Table 1 shows the main plant cryopreservation methods developed in different species. There are many combinations of procedures carried out in cryopreservation techniques like encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pre-growth, pre-growth-drying and droplet-vitrification (Engelmann, 2004).

Stages of cryopreservation

Cryopreservation includes different stages depending on the selected technique, involving preparation and explant excision, preculture, cryoprotection, vitrification/dehydration, fast cooling in LN, rewarming, cryoprotector elimination, regeneration and plant culture (Volk et al., 2004; Liu et al., 2017; Streczynski et al., 2019). These stages are schematized in Figure 1. The critical point is an optimum dehydration to avoid lethal ice crystals formation in plant cells (Pence et al., 2020). To reduce explant water content, air-drying in laminar flow hood, silica gel, cryoprotectants, slow cooling and pre-culture are used (O'Brien et al., 2021). The selection depends on explant tolerance to stress, and these can be used singly or combined.

MAIN COMPLICATIONS DURING CRYOPRESERVATION

Cryopreservation causes stress in plant cells that are subjected to excision, osmotic dehydration and sudden changes in temperature (Uchendu et al., 2013). This stress reduces plants survival and regeneration (Lynch et al., 2011). Freezing injuries affect cell membranes that lose the fluidity of their lipid components (Centinari et al., 2016). Biological membranes define cell limits and internal organelles; they are highly dynamic which allows the maintenance of integrity, resistance to stress, flexibility, fluidity and electrical insulation (Gould, 2018). Plant membranes are constituted mainly of galactolipids, phospholipids and lysophospholipids. Some classes with large polar head groups are phosphatidylcholine and digalactosil diacylglycerol, while others have small head groups such as phosphatidylethanolamine, monogalactosyldiacylglycerol, and phosphatidic acid (Lin et al., 2021).

Table 1. Main plant cryopreservation methods developed in different species.

Cryopreservation methods	Species	Preculture	Osmoprotection	Vitrification	Rewarming	Plant regeneration	Reference
Vitrification	Apical meristems of *Chlorophytum borivilianum*	MS medium with 0.5 M glycerol for 48 h at 25°C	18.4% glycerol and 13.7% sucrose for 20 min	PVS2 for 30 min at 0°C	40% sucrose for 10 min	33%	Chauhan et al. (2021)
Pregrowth-dehydration	Embryogenic tissue of *Picea abies* L.	0.25-1 M sucrose and 10 µM ABA for 7 days	N/A	Dehydration with silica gel for 2 h at 25°C	Water bath at 42°C for 3 min, after on ice for 2-3 min and 1-0 M sucrose for 1.5 h	276 embryos g^{-1}	Hazubska-Przybył et al. (2013)
D-Crio-plate (vitrification by air current on aluminium cryo-plates)	Buds of *Juncus decipiens* Nakai	MS medium with 0.3 M sucrose overnight at 25°C	2 M glycerol and 1.0 M sucrose solution for 30 min	Air current of a laminar flow cabinet for 3 h at 25°C	1 M sucrose for 15 min	90%	Niino et al. (2013)
Air drying (flash drying, normal drying)	Zygotic embryos of *Seemannaralia gerrardii*	Electrolysed water of CaMg solution (0.5 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$) for 30 min in the dark	N/A	Flash-dried for 50 min at 25°C	Electrolysed water of CaMg solution (0.5 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$) for 30 min in the dark	70%	Berjak et al. (2011)
V-Crio-plate (vitrification and aluminium cryo-plates)	Shoot tips of *Tanacetum cinerariifolium*	MS medium with 0.5 M sucrose for 2 days at 5°C	2 M glycerol and 1.4 M sucrose solution for 30-60 min	PVS 7M for 40 min at 25°C	1 M sucrose for 15 min	90%	Yamamoto et al. (2011)
Droplet vitrification (droplets of cryoprotectant on an aluminium foil strip)	Meristems of *Musa* spp.	MS medium with 3, 6 y 9% sucrose for 1-2 days at 25°C	2 M glycerol and 0.4 M sucrose solution for 20 min	PVS2 for 30 min at 0°C	1.2 M sucrose for 15 min	52.9%	Panis et al. (2005)
Pregrowth	Meristems of *Musa* spp.	MS medium with 0.4 M sucrose and 1 µM IAA for 2 weeks at	N/A	N/A	Water bath at 40°C for 1.5 min.	66%	Panis et al. (2002)
Encapsulation-dehydration	Apices of *Wasabia japonica*	½ MS medium with 0.3 M sucrose for 1 day at 20°C	Encapsulation into 2% Na-alginate beads containing 0.4 M sucrose and treated with MS medium and 0.8 M sucrose solution for 16 h at 25°C	Dehydration with silica for 4 h at 25°C	Water bath at 35°C for 3 min	75%	Sakai et al.,(2000)
Encapsulation-vitrification	Apices of *Mentha spicata*	MS medium with 30 g L^{-1} sucrose for 3 weeks at 4°C	Encapsulation into 2% Na-alginate beads containing 0.4 M sucrose and treated with 2 M glycerol and 0.4 M sucrose solution for 1 h	PVS2 for 3 h at 0°C	Water bath at 38°C for 1 min and MS medium with 1.2 sucrose for 10 min	87%	Sakai et al. (2000)

ABA: Abscisic acid. IAA: Indole-3-acetic acid. MS: Murashige and Skoog (1962). N/A: Does not apply. PVS: Plant Vitrification Solution (30% glycerol, 19.5% ethylene glycol and 0.6 M sucrose). PVS2: 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide and 0.4 M sucrose.

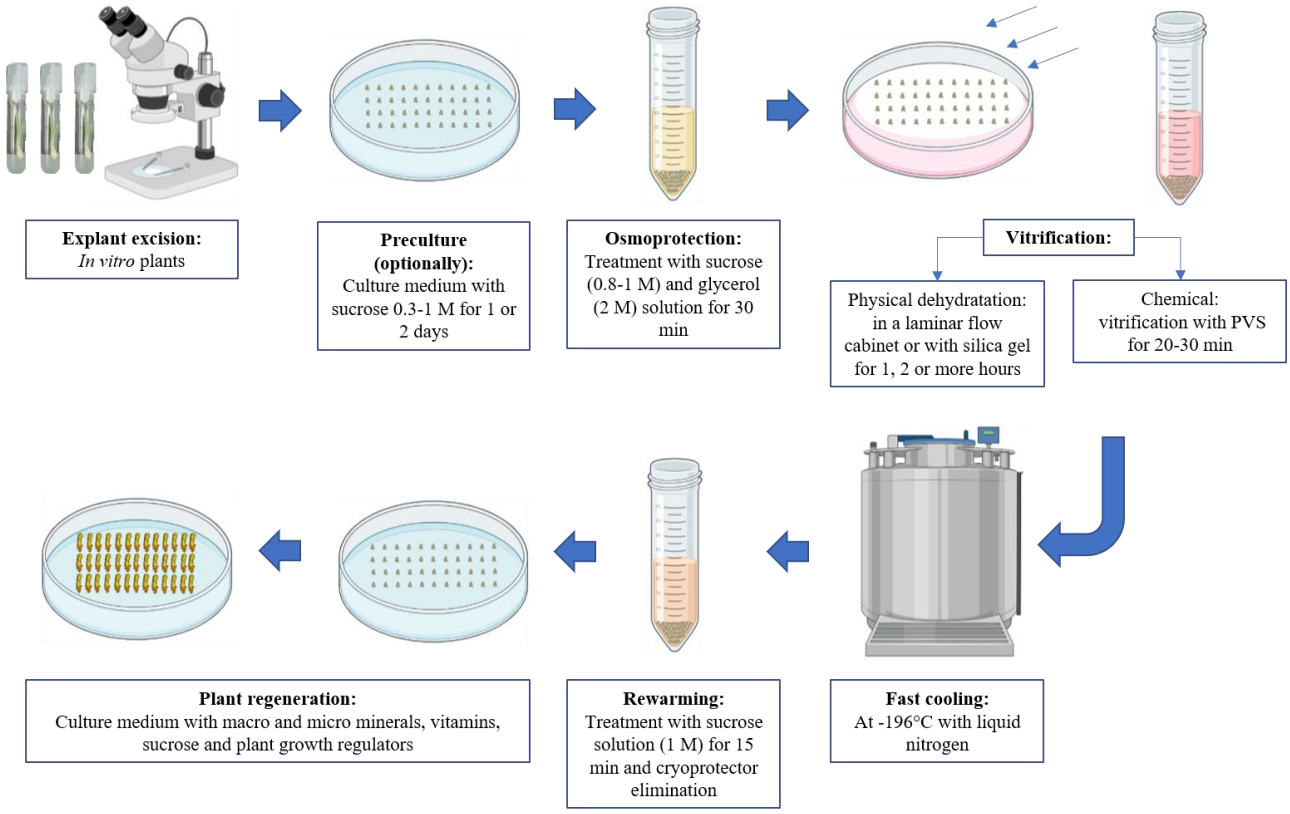

Figure 1. Stages of cryopreservation. PVS: Plant vitrification solution.

Cryogenic damage

Temperature variations affect the lipid composition of membranes and therefore their biophysical properties (Cook et al., 2021). The integrity of the plasma membrane is a factor on which cell viability depends because it is considered a primary site of cryogenic injury. The mortality of plant explants is mainly due to improper thawing which causes cryogenic damage on cell membrane (Yang et al., 2017). In cryopreserved maize zygotic embryos, different degrees of cell lesions have been observed. The main symptoms include plasmolysis, mitochondrial condensation, increased heterochromatin, nuclear contraction and chromatin condensation, as well as rupture of the cell wall, cell membrane and nuclear envelope (Wen et al., 2010).

Oxidative stress

Cryopreservation procedures can cause physical, chemical and physiological cell damage in addition to oxidative stress (Martín and González-Benito, 2005). The first immediate response to biotic and abiotic stress in plant cells is formation of reactive oxygen species (ROS) and these tend to decline when the stress disappears (Huang et al., 2019). ROS are free radicals like superoxide anion (O_2^{2-}), hydroperoxyl radical ($OH_2^{\bullet}$), alkoxy radical ($RO^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$), also nonradical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Mehla et al., 2017; Hasanuzzaman et al., 2019). These signaling molecules also regulate plant metabolism and are produced in organelles like chloroplast, mitochondria, peroxisome and cytoplasm (Das et al., 2015; Huang et al., 2019). However, an excessive concentration of intracellular ROS leads to oxidative stress and damage to lipids, proteins and DNA; therefore, it alters the plasma membrane and metabolic pathways, ending in cell death (Nita and Grzybowski, 2016).

Favorably, plant cells have enzymatic and non-enzymatic antioxidant systems that maintain ROS homeostasis (You and Chan, 2015). The enzymatic antioxidant systems are superoxide dismutase, catalase and peroxides involved in the reduction process of O^{2-} to H_2O_2. The non-enzymatic components are generally ascorbic acid, α-tocopherol, flavonoids, glutathione, carotenoids, lipids, and phenolic compounds, which are efficient antioxidants (Nadarajah, 2020).

However, even when antioxidant substances are used during cryopreservation, these systems are often impaired during freezing and thawing (Kaczmarczyk et

al., 2012). The factors associated with oxidative stress during cryopreservation are diverse, including disinfection agents, explant cutting, osmotic agents, freezing and thawing (Chen et al., 2014). Particularly, plant tissues are exposed to cell lysis during aseptic establishment process and this causes the synthesis and release of phenolic compounds, whose high accumulation is toxic and causes cell death (Jones and Saxena, 2013).

The most common disinfection agents used are sodium hypochlorite (Bello et al., 2018), mercuric chloride (Haider et al., 2015), ethanol (Maina et al., 2010), ozone (Cabrera Jova and González, 2014), antibiotics and commercial fungicides (Ray and Ali, 2016). Surface disinfectants cause oxidative stress and cell death depending on the concentration used, contact time and type of plant explant (Cuba-Díaz et al., 2020).

During cryopreservation, direct mechanical damage causes cell wall injury and produces an explosion of ROS composed primarily of hydrogen peroxide (Whitaker et al., 2010; Skyba et al., 2012). Excess of hydrogen peroxide triggers autophagy by chloroplasts and peroxisomes as well as programmed cell death (Smirnoff and Arnaud, 2019).

On the other hand, oxidative stress caused by cryoprotectants induces lipidic peroxidation and oxidative phosphorylation damage (Ren et al., 2013). Lipid peroxidation is the decomposition of lipids into aldehydes such as 4-hydroxynonenal and malondialdehyde under the action of ROS (Liu et al., 2021). Cryoprotectants like polyethylene glycol, mannitol and sucrose are osmotic stress agents under *in vitro* culture conditions (Şen, 2012). In addition, sorbitol pretreatments increase intracellular hydrogen peroxide concentrations (Lynch et al., 2011).

Plant cells are exposed to osmotic stress during the osmoprotection and dehydration process, which can lead to excessive ROS release (Whitaker et al., 2010; Rahmah et al., 2015). Osmotic dehydration involves treating tissues with concentrated sugar solutions or other osmotically active substances, which allows water to flow through membranes from a lower solute concentration to a higher concentration (Volk and Walters, 2006).

Therefore, plants need to increase the cryostability capacity of the plasma membrane to tolerate various stresses caused by freezing and thus accelerate the recovery process after thawing (Uemura et al., 2009).

CRYOPROTECTIVE AGENTS

Cryoprotective agents (CPAs) are high or low molecular weight water-soluble substances that facilitate dehydration and decrease the osmotic gradient of cells through vitrification during cryopreservation and long-term storage in liquid nitrogen (Yang et al., 2017).

The function of CPAs is to prevent ice formation and cryogenic damage through interference of hydrogen bonds in water molecules (Towey and Dougan, 2012). Most of the time, plant survival depends on optimal dehydration with CPAs (Elliot et al., 2017).

Permeable CPAs to plasma membrane are low molecular weight and the most widely used are methanol, dimethyl sulfoxide, glycerol, propylene glycol, ethylene glycol and formamide. Non-permeable CPAs are of high molecular weight, among which are polyvinylpyrrolidone, hydroxyethyl starch and some sugars (Gurruchaga et al., 2018).

However, the toxicity of penetrating CPAs is an obstacle to successful cryopreservation (Streczynski et al., 2019). The CPAs toxicity is associated with temperature, CPAs concentration, time of exposure, plant species and tissue development (Rahmah et al., 2015). CPAs are toxic when they break membranes, alter enzyme function or if cell viability is decreased (Best, 2015).

PLANT REGENERATION

Plant regeneration after a cryopreservation process will depend mainly on the species, explant type and the cryopreservation technique used (Mathew et al., 2018). The time required for plant regeneration is variable; in some cases, it takes a few months and, in others, it takes up to a year or more to obtain fully acclimatized plants (Kaczmarczyk et al., 2012).

Regeneration rates are also influenced by the sensitivity of plant tissue to various types of cryopreservation stress (Uchendu et al., 2013). Therefore, before and after freezing, specific solutions are used to cryoprotect cells and prevent or reduce recrystallization (Yang et al., 2017) which reduces negative impact of cryopreservation stress in cells.

Another important aspect during plant regeneration after cryopreservation is the genetic stability, because the aim of plant genetic resources conservation is to ensure the true-to-type status of the regenerants (Wang et al., 2021).

Genetic stability of cryopreserved plants

During plant cryopreservation, genetic stability evaluation helps to verify if the DNA from cryopreserved plants remains intact (Dar et al., 2019). In general, nulls or minimal differences have been observed in plant materials before and after cryopreservation (Matsumoto et al., 2013).

However, freezing and thawing could alter lipids and proteins, which can cause chromosomal damage and induce genetic and epigenetic changes (Chatterjee et al., 2016). Other factors that could alter DNA are plant growth regulators used for plant regeneration after

Table 2. Evaluation of genetic stability in plant cryopreservation.

Molecular marker	Species	Cryopreservation technique	Genetic stability	Reference
MSAP, RAPD and AFLP markers	*Mentha x piperita* L.	Encapsulation-dehydratation	MSAP results revealed variation in the DNA methylation pattern of mint apices after cryopreservation. RAPD and AFLP markers showed a complete genetic stability.	Ibáñez et al., 2019)
Transcript expression patterns and qPCR methods	*Arabidopsis thaliana* (cv. Columbia)	Vitrification (PVS2 and PVS3)	Changes in transcript expression in shoot tips.	Gross et al. (2017)
Microsatellites	*Picea abies* L. Karst	Slow-cooling	Identical prior to and following cryopreservation of somatic embryos.	Varis et al. (2017)
ISSR markers	*Malus domestica*	Two-step freezing	No polymorphism found between the mother plant and regenerants before and after cryopreservation of apple dormant buds.	Yi et al. (2015)
RAPD markers	*Eutrema japonicum Matsum.*	*Vitrification (PVS2)*	No significant differences were observed using RAPD PCR in wasabi plants regenerated from shoot tips after cryopreservation.	Matsumoto et al. (2013)
RAPD markers	*Prunus armeniaca L.*	*Encapsulation-dehydration*	No changes in genetic stability in apricot shoot tips after cryopreservation.	Soliman (2013)

AFLP: Amplified Fragment Length Polymorphism. ISSR: Inter Simple Sequence Repeat. MSAP: Methylation Sensitive Amplification Polymorphism. qPCR: Real-Time Polymerase Chain Reaction. PVS: Plant Vitrification Solution. RAPD: Random Amplified Polymorphic DNA.

cryopreservation; these could induce somaclonal variation in the regenerated plants (Bairu et al., 2011; Sales and Butardo, 2014; Butiuc-Keul et al., 2016).

Furthermore, different stages of cryopreservation affect cell functionality, protein expression and DNA stability in different ways (Chatterjee et al., 2016). In general, substances used in pretreatment, preculture and cryoprotection stages could affect genetic stability of explants (Martín et al., 2011). Specifically, histone methylation and modification have been observed during preculture and vitrification (Heringer et al., 2013). Likewise, increased methylation has been reported in tissue culture associated with the use of CPAs and stress conditions (Smulders and de Klerk, 2011; Orlowska et al., 2016; Gross et al., 2017; Ibáñez et al., 2019).

Molecular markers are used to detect variations or polymorphisms between individuals for specific regions of DNA. These polymorphisms can be used to construct genetic maps and evaluate differences between markers in the expression of traits in a population (Marwal et al., 2014).

Some molecular markers that have been widely used in plant cryopreservation are presented in Table 2. Nevertheless, DNA alteration during *in vitro* culture depends on the species, genotypes and culture conditions (Surenciski et al., 2007).

CONCLUSION

The bases of food security are PGR because they provide a source of energy, fiber and metabolites of industrial and pharmaceutical interest. Cryopreservation is a viable alternative to safeguard these valuable resources. Plant cryopreservation is a multiple stage process and there are different plant cryopreservation techniques that have been used for different explants. Nevertheless, different responses have been observed because there are many factors that influence the development of plants after cryopreservation protocols in such a way that the existing protocols are specific for each species and genotypes. ROS production is the primary explant response after cryopreservation, and it has been associated with low plant survival and regeneration. In this sense, avoiding ice crystal formation through CPAs is crucial during osmoprotection.

The success of a cryopreservation protocol depends on survival, regeneration and genetic stability of the plant material. These are

fundamental variables for the development of suitable cryopreservation protocols for plant germplasm.

It is necessary to develop cryopreservation protocols for species of agri-food interest that are not yet cryopreserved, wild relatives and for those that are at risk of loss. In addition, it is necessary to optimize existing protocols for their application in different genotypes.

The use of cryopreservation for long-term PGR conservation contributes to ensure agricultural and food security for many generations to come, either through the use of these resources to repopulate or to carry out genetic improvement and generate varieties better adapted to the challenges ahead.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

PGR, Plant genetic resources; NL, liquid nitrogen; ROS, reactive oxygen species; **CPAs,** cryoprotective agents.

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Biogenic synthesis of silver nanoparticles using *Azadirachta indica* methanolic bark extract and their anti-proliferative activities against DU-145 human prostate cancer cells

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Phytomedicine coupled with nanotechnology has shown possibilities in treating different ailments including cancer. In the current study, silver nanoparticles (AgNPs) were biogenically synthesized using methanolic extracts of *Azadirachta indica* (neem) barks and tested for cytotoxic and anti-proliferative activities on normal cells (Vero E6) and human prostate cancer cells (DU-145), respectively by resazurin assay. Characterization of the AgNPs using UV-Vis, Fourier transmission infrared spectroscopy (FTIR) and Zetasizer Nano were performed. The UV-Vis spectroscopy showed surface plasmon resonance of 428 nm indicating the successful formation of silver nanoparticles. The FTIR spectra indicated the presence of ketones, carboxylic acid, amines, aldehydes, secondary alcohols, alkenes, and carbohydrates. Zetasizer revealed the average particle size and zeta potential of 58.8nm and -33Mv respectively for the biogenic AgNPs. IC_{50} values were found to be 41.78±0.82, 8.02±0.18 and 6.37±0.34 µg/ml for crude extract, biogenic AgNPs and control drug (doxorubicin), respectively. While there was no significant difference ($p>0.05$) in anti-proliferative activities between biogenic AgNPs and control drug (doxorubicin), results showed a significant difference ($p<0.05$) in their cytotoxic activities. Selectivity index of the AgNPs was recorded to be 2.1 while that of control drug was 1.4; showing the potential of AgNPs to select cancerous cells over normal cells. We deduce that, the biogenic AgNPs has good anti-proliferative activities against human prostate cancer cells (DU 145) in a selective manner.

Key words: Silver nanoparticles, *Azadiracta indica*, biogenic synthesis, cancer, prostate cancer.

INTRODUCTION

Cancer burden in developing countries has increased to 80% and estimated to increase to 85% by 2030 in sub-Saharan Africa (Morhason-Bello et al., 2013). However, the region receives only 5% of the global resources

dedicated to cancer (Morhason-Bello et al., 2013) prompting for an urgent need for novel therapeutics to combat the situation. Currently, cancer is managed through different ways such as surgery, chemotherapy, radiation therapy and immunotherapy (Suh et al., 2020). Regarding chemotherapy, there are several issues reported such as toxicity, resistance of cancer cells, high cost and poor delivery systems (Senapati et al., 2018) and therefore there is a need for effective and less toxic drugs for cancer. To date, there is a global resurgence in interest and use of plant-based therapies due to the fact that plant derived products have shown promise in treating different cancer types such as colorectal, breast, prostate, stomach, esophageal and liver cancer among others (Roy and Bharadvaja, 2017).

Despite of this pike in the use of plant derived materials for cancer treatment, there are issues reported on limited bioavailability of active phytochemicals to the target cells (Siddiqui and Sanna, 2016). Such, the limitation entails poor stability due to gastric and colonic acidity, poor solubility of the ingredient, poor metabolism by the effect of gut microflora, poor absorption across the intestinal wall, poor active efflux mechanism and first-pass metabolic effects hampering the success of these products through pre- and clinical trials (Siddiqui and Sanna, 2016). In that view, developed novel drug delivery system and carriers for herbal drugs should preferably attain some prerequisites including proper delivering of the drug at a rate oriented by the needs of the body, over a given period of treatment and ultimately present the active ingredients of the drug to the site of action (Martínez-Ballesta et al., 2018). Several tactics have been explored to increase drug solubility, sustainability, bioavailability and gastrointestinal permeability including nanotechnology that has tremendously gained attention in the development of new pharmaceutical carrier and delivery systems. Encapsulation of plant phytochemicals into a biodegradable and biocompatible nanoparticle remains a solution to this problem. Nanomaterials can overcome limitations associated with conventional crude extract delivery, increase the action of plant extracts, reduce the required dose and side effects, and eventually improve the activity (Gudise et al., 2021).

Among other plants, *Azadadiracta indica* extracts have been encapsulated in various metallic nanoparticles (NPs) such as silver and gold (Hareesh et al., 2016) among others. The silver nanoparticles (AgNPs) is one of the most studied and utilized nanoparticles in drug formulation due to its potent broad spectrum of activities (for instance inhibitory activity towards nearly 650 microbes), extremely large surface area, strong permeability and little drug resistance (Ivanova et al., 2018).

Silver nanoparticles biogenically synthesized using methanolic bark extracts of *A. indica* has shown antiproliferative activity against CT26 colorectal cancer cell lines (Sokei, 2018). In the study, results showed that the nanoformulation had significantly higher ($p>0.05$) activity (IC_{50} 15.13 µg/ml) compared to the methanolic extract (IC_{50} 87.46 µg/ml) with a higher selectivity index (2.03) compared to the methanolic crude extract (1.08) and that of doxorubicin (1.56). Furthermore, the NPs increased *in vivo* tumor growth inhibitory activities in Swiss albino rats at an inhibitory capacity of 71.96% at 30 mg/kg body weight compared to the crude extracts (Sokei, 2018). Given good activities of the NPs on colorectal cancer, it is of great interest therefore to explore their potential in treating other adenornocarcinoma (cancer developing from secretory cells). In this study, we studied cytotocity and antiproliferative activities of the silver nanoparticles biosynthesized using methanolic barks extracts of *A. indica* on normal cells (Vero E6) and human prostate cancer cell lines (DU 145), respectively.

MATERIALS AND METHODS

Collection of plant

About 7 kg of barks of the neem plant (*A. indica*) was harvested from a farm sustainably (by cutting branches and later pealing them) at farm in Juja town, Kiambu County, Kenya Republic 10 5' S, 370 10' E, 1520 m alt. in September, 2020. After proper authentication by a botanist, the materials were harvested and a voucher specimen deposited at Jomo Kenyatta University of Agriculture and Technology (JKUAT) botany herbarium and assigned voucher specimen number SK-001.

Methanolic extraction

The method as described by Sokei (2018) was adopted. Briefly, the barks of *A. indica* were washed with distilled water, air-dried and crushed into coarse powder. The powdered bark (100 g) was soaked in 400 ml, 70% methanol. The mixture was allowed to stand for 72 h with vigorous shaking using a shaker (GYROMAX^{TM} 727, Amerex Instruments, Inc.). After incubation, the methanolic extract was then filtered and the filtrate was concentrated under pressure at 60°C in a rotary evaporator (LabTech®, Daihan Labtech Co. Ltd.) to remove methanol. The resulting extract broth was kept at 4°C for downstream use. In case of crude extract preparation, the methanolic extract was dried in the water bath for 96 h at 60°C to obtain the dry powder of the crude extracts.

Biogenic synthesis of AgNPs using *A. indica* methanolic barks extracts

The AgNPs were formulated using the extract broth with 1 mM of

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silver nitrate ($AgNO_3$) solution following the protocol followed by Nghilokwa et al. (2020). Primary characterization was done using UV spectrophotometer at wavelength between 300 and 800 nm and later washed three times with deionized water for complete purification and later frozen at -80°C for 48 h before drying in lyophilizer (freeze dryer, FDL-10N-50-BA, MRC, Laboratory equipment manufacturer, UK) and stored at 4°C.

Classification and characterization of *A. indica* silver nanoparticles

The synthesized nanoparticles were subjected to UV-Vis Spectroscopy (JENWAY, Bibby Scientific Ltd, UK) and Fourier-transform infrared spectroscopy (Perkin-Elmer FTIR spectrophotometer Norwalk, CT, USA) as in Sokei (2018). Also, Zetasizer Nano (Malvern Instruments Ltd., United Kingdom) was used to uncover the size and zeta potential.

Cell lines

DU 145 (human prostate cancer) and Vero E6 (normal) cells obtained from ATCC (Manassas, VA, USA) were used. As in Sokei (2018), the cells were grown in MEM medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% antibiotic (Penicillin/Streptomycin) in a T75 culture flask and incubated at 37°C and 5% CO_2 to attain confluence of 80%.

***In vitro* anti-proliferative activity of *A. indica* extract and *A. indica* AgNPs on human prostate cancer cell line (DU145)**

DU145 prostate cancer cells were prepared for anti-proliferative assay in conditions previously reported by Sokei (2018) and later by Gavamukulya et al. (2021). In this case, 20 µl of resazurin dye (0.15 mg/ml) was used and optical density (OD) was read using the MULTISKAN GO plate reader (Thermo Scientific) at 562 nm and a reference wavelength of 690 nm. From the readings, IC_{50s} were established.

***In vitro* cytotoxic activity of *A. indica* extract and *A. indica* AgNPs on Vero E6 (Normal) cell line**

Exponentially growing Vero E6 cells were washed and prepared for cytotoxicity assay in conditions previously reported by Sokei (2018). Subsequently, 20 µl of resazurin dye (0.15 mg/ml) was added and optical density (OD) read using the MULTISKAN GO plate reader (Thermo Scientific) at 562 nm. From the readings, CC_{50s} were established.

Tumor selectivity index (TSI)

Tumor selectivity index (TSI) indicates the ability of the extract/drug to exert selective toxicity to cancer cells while sparing the normal ones. It was calculated by the following equation (Indrayanto et al., 2020):

TSI = mean CC_{50} against normal cells/mean IC_{50} against tumor cells,

where CC_{50} is the concentration of extract/product that exerted cytotoxic effect to 50% of the normal cells (Vero E6) while IC_{50} stands for the concentration of the products that inhibited the growth of cancer cells by 50%.

Statistical analysis

Data from UV-Vis spectrophotometry and FTIR were handled with origin software for plotting graphs while absorbance data from Resazurin assay were handled in GraphPad Prism 9.2.0. First, the drug concentration and absorbance readings were log transformed and normalized, respectively. Secondly, nonlinear regression was used to compute the IC_{50} and CC_{50} for each treatment. Thirdly, differences in groups were compared using One-way ANOVA followed by post hoc analysis using Tukey's test. The IC_{50} and CC_{50} values were expressed as mean ± standard deviation (sd) and a significance level was set at $p \leq 0.05$.

Ethical approval

Approval for experimentations was granted by the University of Nairobi Animal Ethics Committee at department of veterinary physiology and anatomy and issued in a letter with reference number: FVM BAUEC/2021/308.

RESULTS AND DISCUSSION

Biogenic synthesis and characterization of silver nanoparticles

UV-Vis spectroscopy

The UV-Vis spectra illustrated in Figure 1, depicted absorption peaks for the particle sizes at different time intervals. Color change from rusty orange at time 0 to dark brown at 72 h and peak formation at about 380 to 480 nm confirmed bio-reduction of silver ions by the extract and the successful nanoformulation. In this case, the highest peak corresponds to 428 nm surface plasmon resonance (SPR). The similar results were reported earlier on silver nanoparticles synthesized by leaf extract of *Amorphophallus paeoniifolius* (Gomathi et al., 2019). This optical properties is due to collective electron oscillations induced by electromagnetic radiation which depends on the shape, geometrical arrangement and degree of interaction between particles (Chen and Jensen, 2016). The correlation between the SPR and geometrical properties therefore qualifies the SPR to be used as fast and simple morphological characterization tool for particles.

The SPR obtained in this study therefore indicates the synthesis of spherical molecules. When the amount of the particles was small, the absorption band was broad with a low peak intensity while as the amount of the particles increased with time, the absorption spectrum became narrower with an increase in the intensity of the absorption band (Jain and Mehata, 2017).

Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR was done in order to study different functional

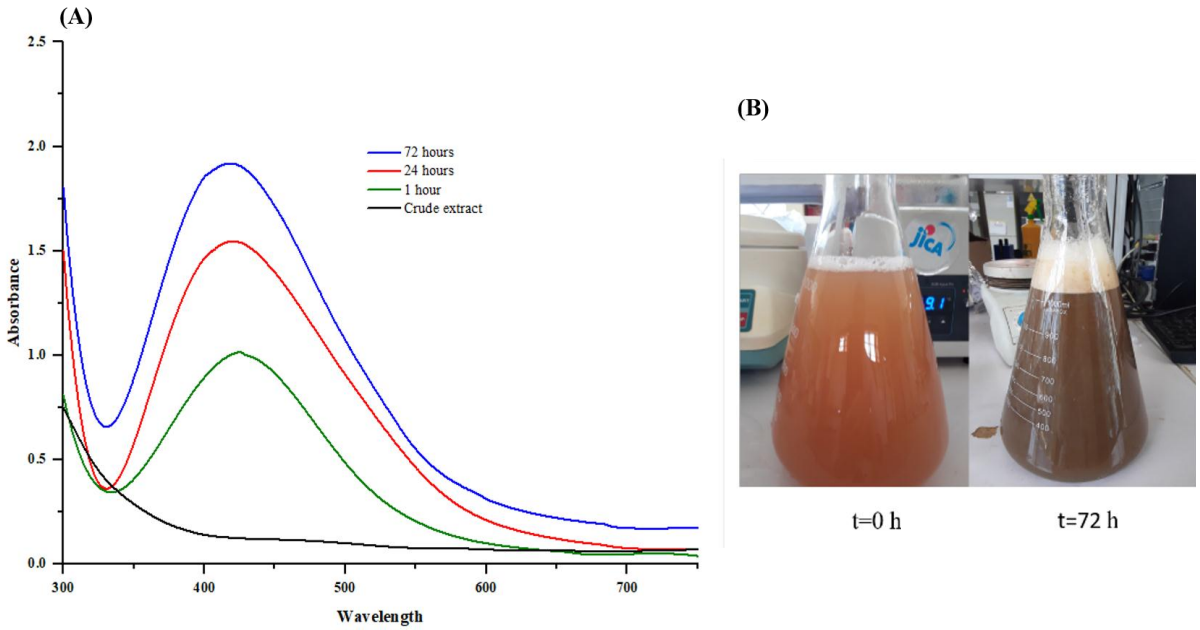

Figure 1. (A) UV-Vis spectra bands measured from 300 to 800 nm showing surface plasmon resonance (SPR) of biogenic silver nanoparticles at different time intervals of synthesis. (B) Reacting components at time=0 with rusty yellow color and time=72 h with dark brown color. The spectra bands and color change in the solution indicates successful formation of the nanoparticles.

groups present in the products and more importantly those involved in reduction and capping processes during synthesis of the NPs. Results showed the presence of different functional groups such as the polyenes, ketones, phenols, alcohol, alkane, alkene, amines, carbohydrates, amino acids and ethers, as reported by other authors (Nghilokwa et al., 2020; Sokei, 2018). Biomolecules such as phenolics, flavonoids, sesquiterpenes, and terpenoids found in the crude extract is implicated in the conversion of the ionic form of silver (Ag^{+}) to the metallic nanoform (Ag^{0}).

In the results presented in Figure 2, the wider spectral peak between 3369.5 and 2959 cm^{-1} are assigned to –OH and –CH stretching, respectively as it was also earlier reported (Velmurugan et al., 2015). Other peaks at 1633 cm^{-1} for C=O showing ketones and carboxylic acid, 1396.4 cm^{-1} for N-CH_3 of amines and aldehydes, 1211.2 cm^{-1} for –CHOH indicating secondary alcohols and 1110.9 cm^{-1} for In-CH^3 indicating alkenes. Also, 1072.2 cm^{-1} for C-N stretching indicates aliphatic amines and C-O stretching for aliphatic aryl, unsaturated diacylperoxides and carbohydrates, 702 cm^{-1} for phenols and 617.2 cm^{-1} for vinyl hydrocarbons. Shifts in the peaks where O-H (for phenols) and C-H (for alkane) functional groups resides, is an indication of involvements of such groups in bio-reduction and capping of ionic silver to its metallic nanoform. Other studies indicated that the reducing phytochemicals in the neem (*A. indica*) leaf consisted mainly of terpenoids, nimbin and quercetin which served as capping and stabilizing agents in addition to reduction (Sironmani, 2016). This concludes that, plants and particularly neem contains phytochemicals that can synthesize metallic nanoparticles in an ecofriendly, less toxic and cost effective manner.

Particle size determination and zeta potential measurements

Particle size determination of the biogenic AgNPs were performed by zeta sizer (Malvern, Zetasizer Nano, ZSP) and presented as percentage intensity. Laser diffraction revealed that particles obtained are polydisperse mixture with the size ranging from 24.4 to 141 nm (Figure 3). The average diameter of the particles was found to be 58.8 nm.

The size of the nanoscale materials provides significant control over physical and chemical properties including their interaction with biomolecules and cell organelles (Tavakol et al., 2017). Nanoparticles with smaller size conferring higher surface area can easily penetrate into the cells and mitochondria. However, NPs with higher surface area can play an important damaging role in cell through interaction with proteins due to more exposed atoms and active sites on the surface of small particles than the larger ones (Tavakol et al., 2017). In general, the size of NPs affects their toxicity levels whereby the smaller sized NPs becoming more toxic that large sized

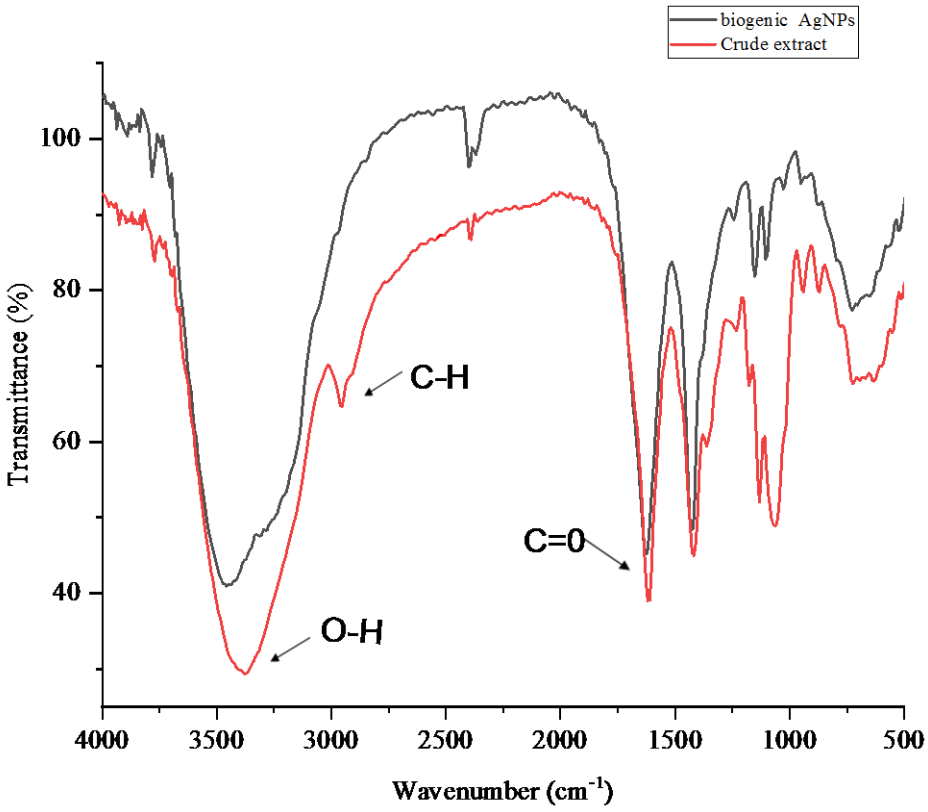

Figure 2. An overlay plot showing FTIR absorbance spectra of the crude extract of *A. indica* and its AgNPs. There is a slight shift in the peaks at -OH and -CH stretchings, indicating involvement of the groups in AgNPs synthesis.

ones exhibiting the highest activity in cell morphological changes, cell membrane damage, ROS generation, cell cycle arrest and cell apoptosis among others (Zhang et al., 2016). Silver nanoparticles of average size above 50 nm show to have less toxicity compared to those of smaller size below 50 nm (Zhang et al., 2016) and that entails that the biogenic AgNPs used in this study of average size 58.8 nm can be less toxic.

On the other hand, zeta potential measurement of the AgNPs was determined in water as solvent and found to be -33 mV (Figure 4) confirming the repulsion among the particles and thus increase in stability of the formulation. Zeta potential being the electrical charge on the surface of materials surrounded in the medium, is another important factor affecting different cell responses as compared to particle size (Tavakol et al., 2017). Overall, with higher zeta potential, the stability and assimilation of the materials to the body is guaranteed since the adherence and clumping is minimized (Santana et al., 2019). While nanoparticles with a zeta potential between -10 and +10 mV are considered approximately neutral, the nanoparticles with zeta potentials of greater than +30 mV or less than -30 mV are considered strongly cationic and strongly anionic, respectively (Santana et al., 2019). Results from this report concurs with silver nanoparticles (AgNPs) bio-stabilized using aqueous callus extract of *Gymnema sylvestre* and showed to have a zeta potential of -36.1 mV (Netala et al., 2016).

Resazurin assays

Table 1 shows results for anti-proliferative activities and cytotoxicity of crude extracts of *A. indica* and its silver nanoparticles. Antiproliferative effects are as shown in Figure 5 with dose-response curve for cell viability on DU154 prostate cancer cells in which the increase in drug concentration decreases the cell viability. The study on human prostate cancer DU145 cells showed that doxorubicin had a 6-fold higher activity (6.37 µg/ml) than the *A. indica* extract (IC_{50} 41.78 µg/ml). Also, from this study, biogenic AgNPs showed higher activity (8.02 µg/ml) compared to their crude counterpart (IC_{50} 41.78 µg/ml), a 5-fold increase with no significant difference to doxorubicin ($p>0.05$). This concurs with results by Sokei (2018) where AgNPs increased activity on CT26 colorectal cancer cell lines at ~6-folds compared to bark methanolic crude extracts. Different studies show varying activities of *A. indica* crude extracts for instance, Kashif et al. (2018) reported on the activity of methanolic oil extract

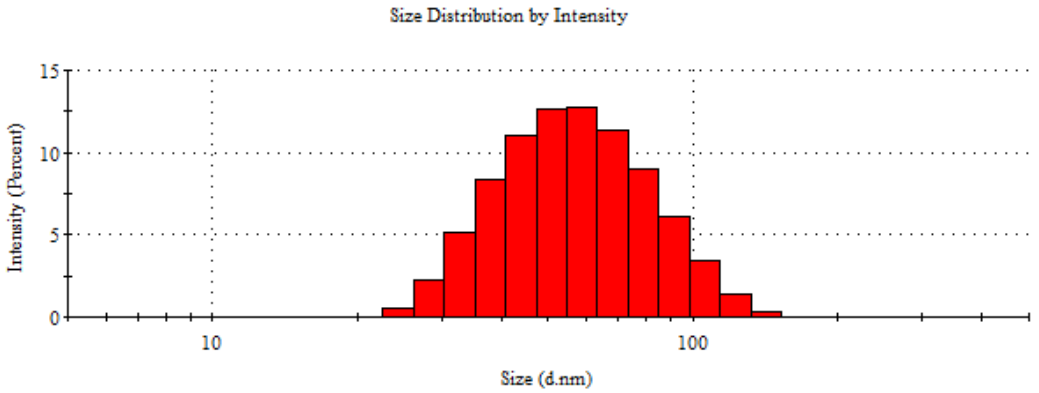

Figure 3. Size distribution by intensity of biogenic AgNPs. The sizes depicted by Zetasizer spanned from 24.4 nm to 141 nm where the average size was found to be 58.8 nm.

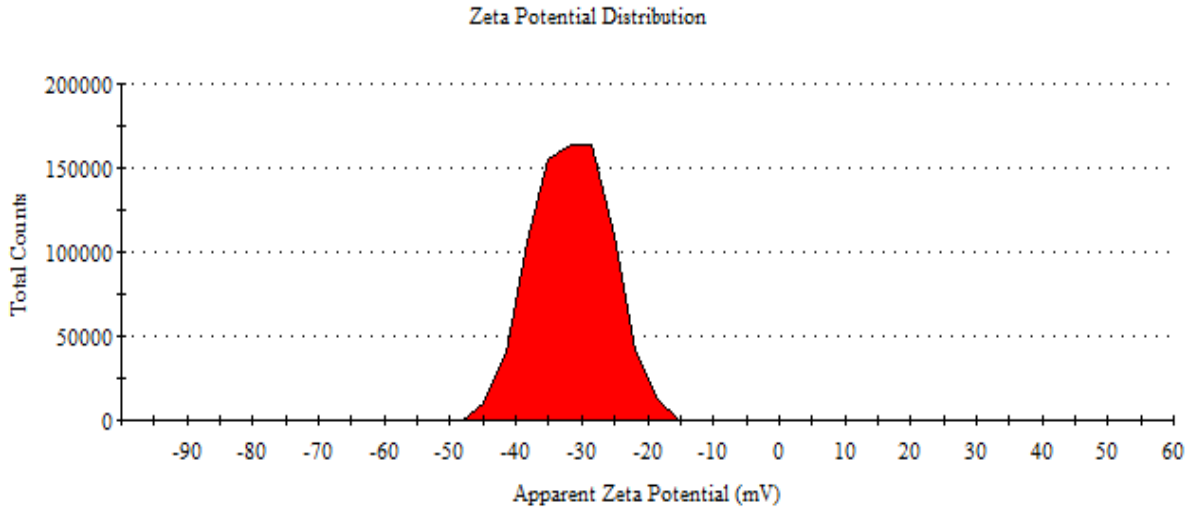

Figure 4. Zeta potential of biogenic AgNPs as depicted by Zetasizer (Malvern, ZSP). The strong negative charge of -33mV was realized.

of *A indica* (157.15 µg/mL) against DU-145 human prostate cancer cells after 24 h exposure. The reason behind these discrepancies could be attributed to differences in cell types, extracts and assays used.

Generally, the findings suggest that nanoformulating the *A. indica* extract significantly improved its anti-proliferative activity. Many studies reported the enhancement of activities given to crude extracts of plants through nanoformulation including antidiabetic and antihyperlipidemic effects (Gudise et al., 2021), antitumor activities against colorectal cancer (Sokei, 2018) among others. This is the reason behind an extensive proposal to combine herbal medicine with nanoscience since nanomaterials can overcome limitations associated with conventional crude extract delivery leading to improvement of the activity (Gudise et al., 2021).

On the other hand, resazurin assay revealed the control drug (doxorubicin) to be cytotoxic to Vero E6 cells (8.97 µg/ml) with significant difference ($p<0.05$) from the biogenic AgNPs (17.01 µg/ml). On the other hand, the crude extract showed less cytotoxicity (67.25 µg/ml) with a significant difference ($p<0.05$) to both control and biogenic AgNPs. Figure 6 presents the cytotoxicity results with dose-response curve for cell viability on Vero E6 cells in which the increase in drug concentration also decreased the cell viability. Cytotoxicity being the measure of the ability of a test substance to kill normal cells is used as one of the screening tests to observe the cell growth, reproduction and morphological effects by medical devices. In this study, *A. indica* extract was nearly 7-times less cytotoxic than the doxorubicin but 3 times less toxic compared to biogenic AgNPs, results correspond with Sokei (2018).

Also, the results are consistent with findings where *A. indica* was reported to contain phytoconstituents that can combat oxidative damage, enhance the immunity, reduce

Table 1. IC_{50} and CC_{50} values and their respective selectivity index of *A. indica* bark methanolic extract, its silver nanoparticles and control (doxorubicin).

Treatment	IC_{50} (µg/ml)	CC_{50} (µg/ml)	Selectivity index (CC_{50}/IC_{50})
Neem bark crude extract	41.78±0.82[a]	67.25±12.29[a]	1.6
Biogenic AgNPs	8.02±0.18[b]	17.01±1.75[b]	2.1
Control (Doxorubicin)	6.37±0.34[b]	8.97±0.59[c]	1.4

There is significant difference among means (p>0.05) following One-way ANOVA. Also, values in the same columns with the different superscripts are significantly different at (P <0.05) following Tukey's multiple comparison test (Post hoc analysis).

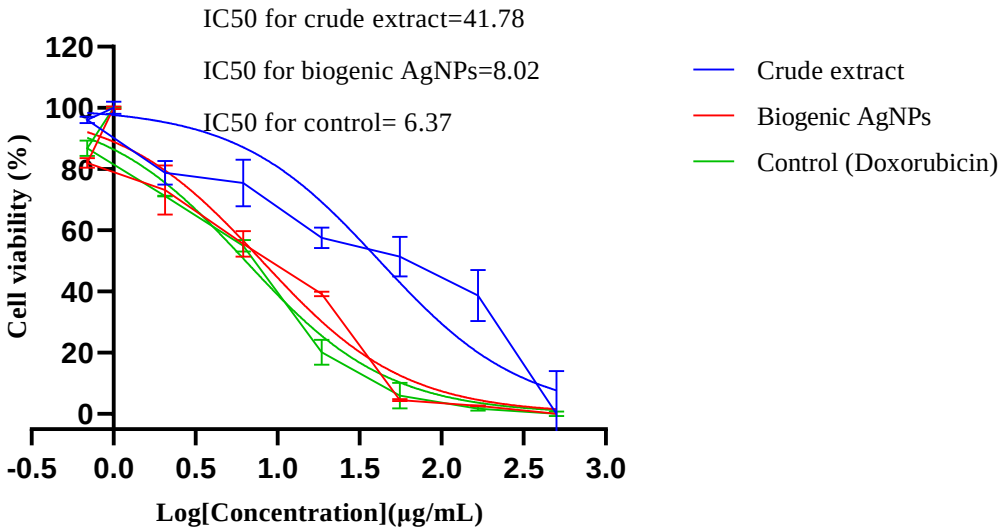

Figure 5. Dose response curve for cell viability of DU145 Human Prostate cancer cell lines. The IC50s for the crude extract, biogenic AgNPs and control (doxorubicin) were found to be 41.78, 8.02 and 6.37 µg/ml respectively.

inflammation, and interfere with the growth of cancer cells thereby making it non-toxic to normal cells (Sironmani, 2016). However, doxorubicin was 2-times more cytotoxic than the AgNPs which agrees with the findings by Buttacavoli et al. (2018), where the AgNPs were found to be 2.8 fold less cytotoxic than doxorubicin. In this study, the nanoparticles showed a 4-fold increase in cytotoxicity compared to that of crude extract, implying that nanoformulating plant phytochemicals increase cytotoxicity. Explanation to this is backed up by other reports which implicate both the AgNPs and Ag^+ released by AgNPs involvement in the mechanism of cytotoxicity in different forms. First, AgNPs perhaps provide a perfect surface outside the mitochondria for the univalent reduction of oxygen to superoxide from electron through the electron transport chain (Buttacavoli et al., 2018). Secondly, Ag^+ may interfere with the functioning of proteins and DNA as it binds to them (Abdelsalam et al., 2018). Thirdly, oxidative stress coupled with generation of reactive oxygen species (ROS) occurring as an early event, leads to NP-induced toxicity (Mao et al., 2016).

Nevertheless, from the results in Table 1, it was revealed that biogenic AgNPs had the highest selectivity index (SI) of 2.1, while the control (doxorubicin) had the lowest selectivity index of 1.4, and thus the higher toxicity of the later to non-target cells. Selectivity index (SI) can be defined as the ratio of the toxic concentration of a sample against its effective bioactive concentration, thus the ideal drug should have a relatively high toxic concentration but with a very low active concentration. A selectivity index greater than 2 is appropriate for a good anticancer drug candidate (Koch et al., 2005). From the current study, *A. indica* extract and doxorubicin had poor selectivity (SI < 2). As the SI demonstrates the differential activity of a drug, the greater the SI value, the more selective it is. Therefore, SI value less than 2 indicates general toxicity of the pure compound (Koch et al., 2005). Results from the ongoing report synchronizes with other findings where biogenic AgNPs acted more selectively on human lung cancer cells and caused less toxicity on normal cells (S.I = 2.2) (Gurunathan et al., 2015). Also AgNPs biosynthesized by *Annona muricata* fruits acted

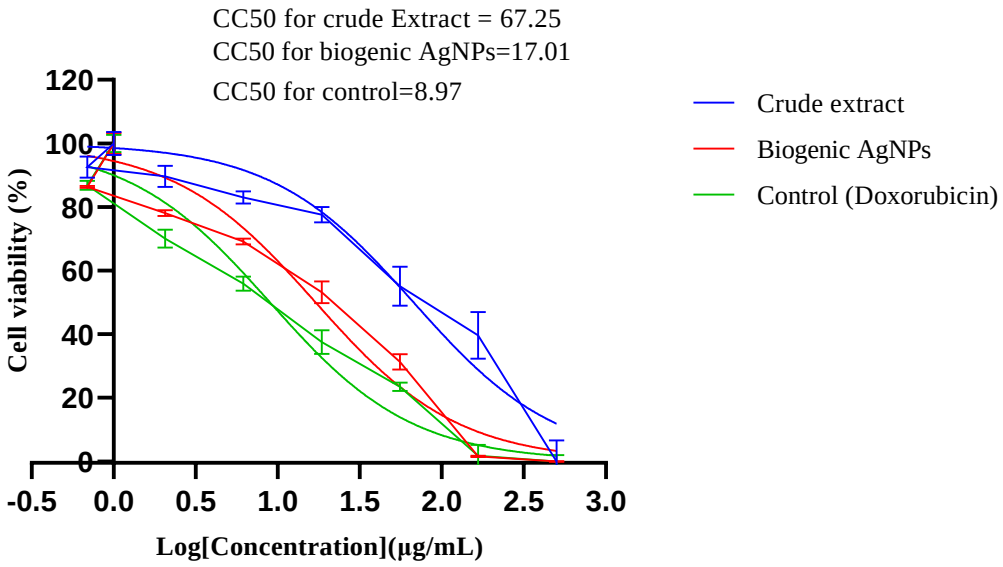

Figure 6. Dose-response curve for cell viability of Vero E6 cells. The CC50s for the crude extract, biogenic AgNPs and control (doxorubicin) were found to be 67.25, 17.01 and 8.97 µg/ml respectively.

selectively against cervical and prostate cancer cells sparing normal cells with a selectivity index above 7 (Gavamukulya et al., 2021) and neem synthesized AgNPs induced cytotoxicity selectively in human gastric cancer cells indicating induction of apoptosis (Sironmani, 2016). The improved selectivity can be attributed to the nature of the size and surface modifications of the nanoparticles (Sokei, 2018). Since the biogenic AgNPs can kill cancer cells more selectively, they can therefore be an effective approach for controlling and ultimately eradicate cancer. Consequently, they can serve as drug delivery strategies, allowing the use of lower doses of drugs to reduce cytotoxic effects and therefore increase therapeutic efficacy. The biogenic AgNPs synthesized and tested in this report can act as a good candidate for cancer management due to its good selectivity index, size and stability. Therefore, preclinical trials can be set using experimental animals to test its efficacy and safety *in vivo.*

Conclusion

The present study confirms that phytochemicals present in bark methanolic extracts of *A. indica* can synthesize AgNPs in an ecofriendly manner. Also, our data suggest that the biogenic AgNPs synthesized hereby has good anti-proliferative activities against DU145 prostate cancer *in vitro*. The results further provide promising evidence that the biogenic AgNPs can selectively kill cancer cells while sparing normal cells with a selectivity index above 2 which is indicative of a potential anticancer candidate. Therefore, we explore the relevance of these findings for further safety and efficacy assessment *in vivo* against human prostate cancer or any other adenocarcinoma.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Polymorphism of the heat shock protein 70 gene in indigenous chickens from different agro-climatic zones in Kenya

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Climate change leads to heat stress, which is one of the most challenging environmental conditions affecting poultry. Studying thermotolerance in poultry is crucial because it may identify genetic lines that can withstand heat stress. This study aimed to investigate polymorphisms in the heat shock protein 70 (*HSP70*) gene in indigenous chicken. In Kenya, we collected 277 blood samples from selected chicken ecotypes from Turkana, Western Highlands, Lake Victoria, and Lamu and extracted their genomic DNA. The first 360 bp region of *HSP70* was amplified using conventional PCR and then sequenced using the Sanger ABI 3730 method. The detailed analysis of the *HSP70* gene fragment revealed three *HSP70* haplotypes (GC, AC, and AG). Phylogenetic analysis of *HSP70* revealed the presence of the ancestral haplotype GC, which dominated in Turkana ecotype. High *HSP70* nucleotide diversity indices were recorded in the Turkana ecotype, while low values were recorded in the Lamu ecotype. In addition, most of the *HSP70* gene variations occurred within populations. Our study provides the first account of the *HSP70* polymorphisms in indigenous chickens in Kenya. The results obtained should pave the way for further in-depth studies on genes involved in heat stress regulation.

Key words: Climate change, family-poultry, heat stress, single nucleotide polymorphisms, thermotolerance.

INTRODUCTION

Climate change is one of the notable challenges affecting Africa today (Haile et al., 2020). The African continent suffers from climatic variation leading to multiple stresses like heat stress, adversely affecting the agricultural sector since it gives rise to low productivity and diseases, and therefore threatens food security. Feather coverage and lack of sweat glands make heat dissipation to the environment by chickens difficult, thus making them susceptible to heat stress (Tamzil, 2014). The adverse effects of heat stress in poultry includes a reduction in the following: egg production (4.99-57%), egg weight (2.78-14.3%), body weight (3.74-32.6%), body weight gain (11-

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50%), daily feed intake (16.09-46.33%), increased water consumption and feed conversion ratio and sometimes mortality (Abdel-Moneim et al., 2021; Barrett et al., 2019; Habashy et al., 2017; Hirakawa et al., 2020; Kumar et al., 2021; Saleh et al., 2020).

The ever-increasing human population has increased the demand for chicken products like eggs and meat, with a corresponding erosion of chicken genetic resources owing to crossbreeding, particularly in emerging countries like Kenya, where there is abundant diversity. Although many crossbreeding programs are geared towards improving chicken production, other factors like the heat tolerance ability of improved chickens are not taken into consideration. To come up with highly productive chickens, it is necessary to consider heat tolerance since climate change may lead to heat stress, reduced chickens' productivity and increased mortality. Climate change adaptation has been adopted by many existing technologies in poultry breeding programs; and has fostered the breeding of heat-tolerant indigenous chickens with exotic breeds to promote heat tolerance (Aengwanich, 2008; Hoffmann, 2010; Lu et al., 2007; Renaudeau et al., 2012).

Specific genes are beneficial in heat stress tolerance, including the heat shock protein family, like the *HSP70* gene (Fujimoto and Nakai, 2010; Kregel, 2002; Takii et al., 2017). *HSP70* is a molecular chaperone that is required for the correct folding of newly synthesized proteins and maintaining protein homeostasis (Feder and Hofmann, 1999; Fujimoto and Nakai, 2010; Hartl et al., 1992; Mayer and Bukau, 2005; Rosenzweig et al., 2019; Zeng et al., 2013). It also has a role in apoptosis (Kregel, 2002), but most importantly in regulating heat shock response and acquiring thermotolerance (Hartl et al., 1992; Mayer and Bukau, 2005; Rosenzweig et al., 2019).

Certain genes, like the frizzled (F), dwarf (*dw*), naked-neck gene (*Na*), have been linked with heat tolerance in chickens (Cahaner et al., 1993; Deeb and Cahaner, 2001; Magothe et al., 2010). The scaleless mutation (*sc/sc*) causes a lack of almost all the body feathers, spurs, and foot scales in chicken, which happens during embryogenesis due to the failure of skin patterning (Wells et al., 2012). This mutation has enabled the chicken to tolerate heat in hot climates (Cahaner et al., 2008; Renaudeau et al., 2012; Wells et al., 2012). Different types of combs have also been attributed to heat regulation in indigenous chickens (Moraa et al., 2015). Several countries have introduced some of these genes in poultry breeding programs to improve the performance and adaptability of chickens to hot ambient temperatures (Carabaño et al., 2019; Fathi et al., 2013, 2014).

Various studies have shown that *HSP70* expression in many tissues is highly activated under heat stress in chicken (Amrutkar et al., 2014; Galal and Radwan, 2020; Givisiez et al., 1999), in turkeys (Wang and Edens, 1998), and quails (Atkinson and Dean, 1985). Mahmoud (2000) identified polymorphic sites on the *HSP70* gene upstream from the coding region in birds subjected to heat stress. Their study also found that birds more resistant to heat had only the P*st*I *HSP70* allele, while two different alleles for the same gene were found in the other breeds (Mahmoud and Edens, 2005). They, therefore, concluded that polymorphisms in the *HSP70* gene might be helpful to the breeding companies for the production of heat-tolerant chickens. Polymorphisms have been detected in the regulatory and coding regions of *HSP70* in heat-tolerant chickens (Zhang et al., 2002). The beginning of the promoter and coding regions of *HSP70* have been analyzed in chickens with various heat tolerance abilities. Two single nucleotide polymorphisms (SNPs), which were silent mutations, were identified: A+258G and C+276G (Mazzi et al., 2003). The different genotypes at polymorphism sites A+258G and C+276G were associated with the mRNA expression level of *HSP70* in the leg muscle and liver, with the heterozygotes having higher expression levels compared to the other genotypes (Zhen et al., 2006).

This study aimed to evaluate the presence of polymorphism in the promoter and the beginning of coding regions of the *HSP70* gene in chickens adapted to divergent climatic conditions in Kenya. Chickens in heat-stressed environment in Turkana and Lamu were compared to chickens in the cold environment from the Western Highlands. In contrast, chickens from moderate environment, Lake Victoria, acted as the controls. The presence of polymorphism is an indication that they could be associated with the phenotypes that are heat tolerant. Therefore, our research will play a significant role in future molecular breeding programs, primarily in exploiting useful genes during heat stress exposure in poultry.

MATERIALS AND METHODS

Study clearance

The study was authorized by the Director of Veterinary Services of the State Department for Livestock, Ministry of Agriculture, Livestock, Fisheries and Irrigation of Kenya. Authorization number: RES/POL/VOL.XXVII/162.

Study areas

Four chicken ecotypes were sampled from Turkana, Western Highlands, Lake Victoria, and Lamu in Kenya (Figure 1). Seven agro-climatic zones characterize Kenya. Turkana is found in Zones VI and VII, which form the driest parts in Kenya and are considered deserts. Annual rainfall of 200 to 600 mm describes these zones, and the temperature can reach a high of 42°C. This weather condition is quite unreliable for crop and livestock production. The Western Highlands occupy zones I and II, which are considered wet. The zones experience low temperatures of below 10°C and receive an annual rainfall of about 1000 mm. Lake Victoria lies in Zone III, which is the most significant zone for agricultural activities. It receives an annual rainfall of 500 to 1000 mm and a temperature range of between 17.1 - 29.4°C. Lamu is found in Zone IV, which is

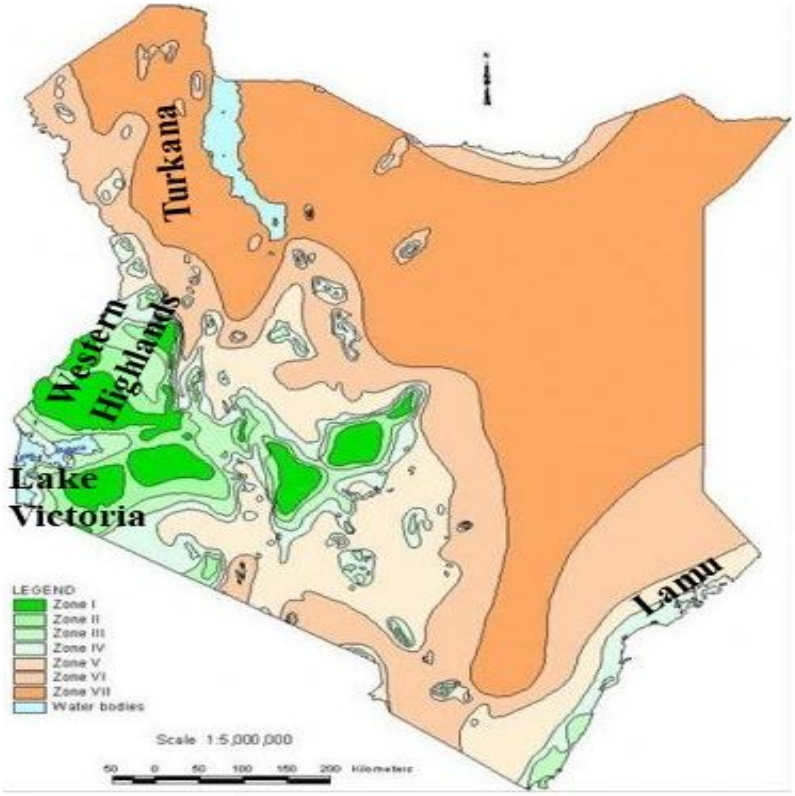

Figure 1. Map of Kenya showing the study areas within the seven agro-climatic zones in Kenya.
Source: Infonet Biovision (2008).

dry and humid with an annual rainfall of 800 mm and a temperature range of 24.1 - 29.2°C. These zones have previously been described (https://infonet-biovision.org/EnvironmentalHealth/AEZs-Kenya-System, Accessed 2021-11-06; Sombroek et al., 1982).

Sample collection and DNA extraction

We employed a cross-sectional purposive sampling design. Samples were collected using a rural participatory approach method, where we explained to the farmers as regards our research and sought their consent before taking samples. Questionnaires were administered to the farmers and essential information was recorded about their chickens. Blood samples were collected from 277 genetically unrelated indigenous chickens from Turkana (89), Western Highlands (73), Lake Victoria (23), and Lamu (92), as shown in Figure 1. In each farm, two mature birds, a cock and a hen (above six months) were chosen from a flock as previously described (Mwacharo et al., 2011). The blood samples were spotted on Whatman® FTA® Classic Cards (Whatman Biosciences) and stored at room temperature before DNA extraction. The genomic DNA was extracted from the air-dried blood preserved on Whatman® FTA® Classic Cards (Whatman Biosciences) following the manufacturers' instructions.

PCR amplification and sequencing

The first 360-bp of the *HSP70* gene promoter and the beginning of the coding region was amplified via PCR using forward primer HSPF (5'AACCGCACCACACCCAGCTATG-3') and reverse primer HSPR (5'CTGGGAGTCGTTGAAGTAAGCG-3') (Akaboot et al., 2012). The amplified region corresponded to position 52,784,305-52,784,621 of the galGal4 Chromosome 5 of chicken *HSP70* (NP_001006686) (www.ensembl.org).

PCR amplifications were performed in 25µl reaction volumes containing 1 X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 0.1% Triton X-100), 2.5 mM of each dNTP, 10 pM of the reverse and forward primers, and 1 unit of Taq DNA polymerase (Promega, Madison WI, USA) and 20 ng template genomic DNA. Thermocycling conditions were as follows: 94°C (3 min), 35 cycles of 94°C (1 min), 58°C (1 min) and 72°C (2 min) and a final extension step at 72°C (10 min).

A 1% agarose gel was used to run the PCR products using 1X TBE buffer (89 mM Tris, 2 mM Na2EDTA), 89 mM Boric acid at a voltage of 100 V for 25 min. The gels were stained using GelRed™ Nucleic acid gel stain and visualized under UV light (BTS-20 model, UVLtec Ltd., UK). Further, a 1 kb DNA ladder was used in identifying the approximate size of the molecule run on the gel.

Upon visualization on agarose gel, the positive PCR products were sequenced at Macrogen in Europe. The products were sequenced in both the reverse and forward directions using the Sanger ABI 3730 method.

Data analysis

The chromatograms were visualized and manually edited using Chromas Lite v.2.1.1 (Technelysium Pty Ltd, Australia). The primers were thereafter trimmed out and a consensus nucleotide sequence

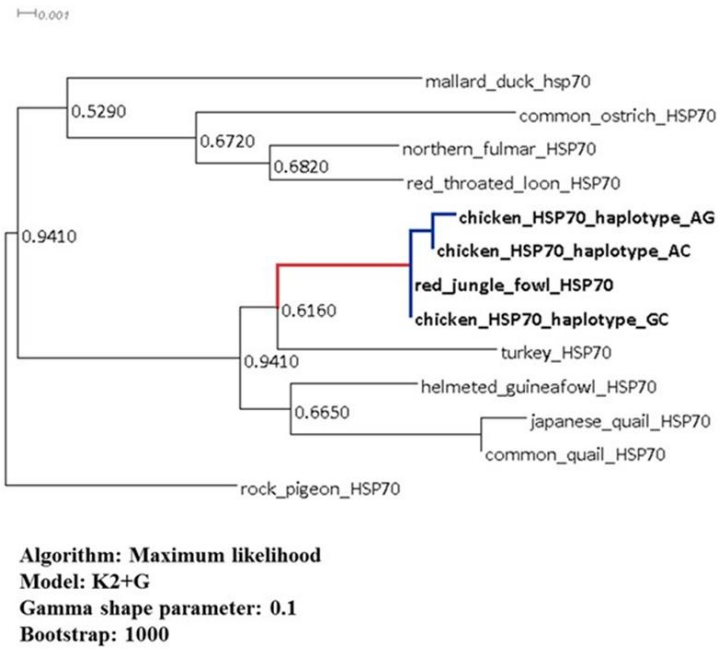

Figure 2. A rooted maximum likelihood tree of the three *HSP70* haplotypes and other avian *HSP70*.

was created. The consensus was aligned against reference sequences from GenBank using Clustal X v.2.1 (Larkin et al., 2007).

We constructed the haplotypes manually and using DnaSP v.5.10 (Librado and Rozas, 2009). Genetic diversity indices on *HSP70* for the ecotypes were determined using DnaSP v.5.10 and ARLEQUIN v.3.5.1.2 (Excoffier and Lischer, 2010). We constructed a phylogenetic tree using the observed *HSP70* haplotypes and the reference sequences downloaded from GenBank using the maximum likelihood algorithm implemented in MEGA v.6.06. The representation of the evolutionary history of the analyzed taxa was inferred with a bootstrap consensus tree constructed with 1,000 replicates (Tamura et al., 2013). A split decomposition network was constructed on *HSP70* haplotypes using splits tree v.4.14.2 (Bandelt and Dress, 1992) and the Analysis of Molecular Variance (AMOVA) was used in assessing the population genetic structure (Excoffier and Lischer, 2010).

Ethics approval and consent to participate

The study received permission from the Director of Veterinary Services, under the State Department for Livestock, Ministry of Agriculture, Livestock, Fisheries and Irrigation in Kenya under permit number RES/POL/VOL.XXVII/162.

We also obtained clearance from the County Directors of Agriculture, Livestock, and Fisheries in the sampled regions. Consent was sought from the farmers before recording information and taking samples from their chicken.

RESULTS

Genetic polymorphisms in *HSP70* gene

Three *HSP70* haplotypes were identified after comparing the 277 sequences of the study with other avian *HSP70* sequences downloaded from GenBank. We renamed the haplotypes to haplotype AC, haplotype GC, and haplotype AG. The haplotype sequences were submitted to the GenBank under accession numbers KT948639, KT948640, and KT948641.

When we aligned the three haplotypes generated with the ancestral red jungle fowl (NC_006092.3) using Muscle v.3 (Edgar, 2004), two polymorphic sites were observed at positions 153 and 171, corresponding to position 52,784,398 and 52,784,416, respectively in the Gallus_gallus-4.0 chromosome 5 from UCSC genome browser.

Phylogenetic analyses

Phylogenetic analysis of our *HSP70* haplotypes with other Avian *HSP70* sequences downloaded from GenBank (common ostrich; XM_009675580.1, mallard duck; XM_005022658.1, Japanese quail; AB259847.1, common quail; EU622852.1, northern fulmar; XM_009576438.1, rock pigeon; XM_005506375.1, helmeted guinea fowl; AB096696, and the red-throated loon; JJRM01051595) showed clustering of haplotype GC to the ancestral red jungle fowl (Figure 2), while haplotype AG and AC clustered together. The tree was rooted using the rock pigeon *HSP70* sequence.

Further analysis with splits decomposition showed the red jungle fowl clustering with haplotype GC. These were genetically distant from chicken haplotypes AG and AC,

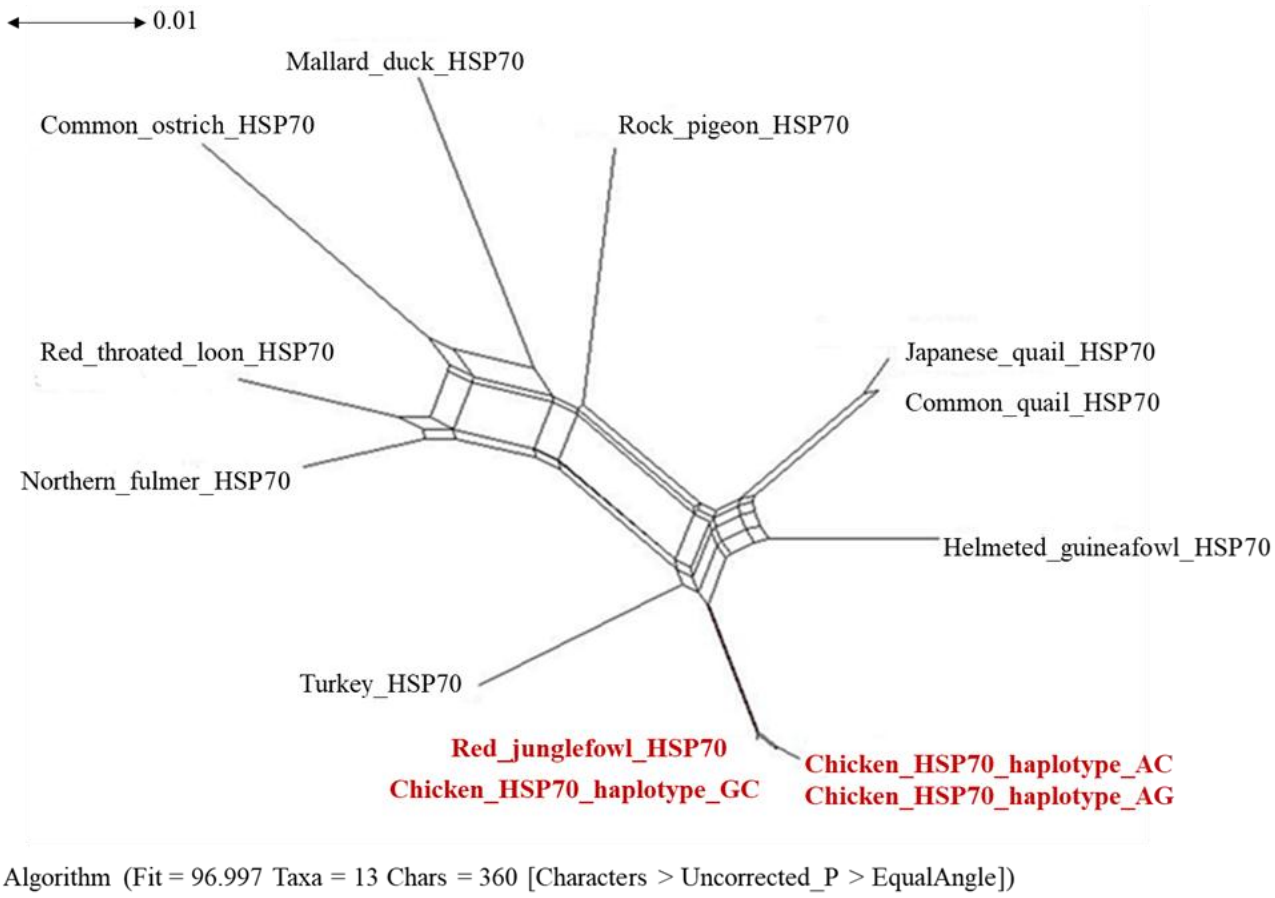

Figure 3. Splits decomposition network of the three *HSP70* haplotypes with other avian *HSP70* sequences.

which clustered together. The other avian species were genetically distant from the chicken haplotypes (Figure 3).

Distribution of the *HSP70* haplotypes in the sampled chicken ecotypes

The relative frequencies of observed haplotypes in the sampled chicken ecotypes are shown in Figure 4. Haplotype GC was frequently distributed in all the chicken ecotypes but dominated in the Turkana chicken ecotype. Haplotype AG was equitably distributed in all the ecotypes but had the highest frequency in the Lamu chicken ecotype. Haplotype AC had the lowest frequencies in all the chicken ecotypes (Figure 4).

Diversity indices

We calculated the number of homozygotes and heterozygotes, observed and expected heterozygosity, and nucleotide diversity indices for the indigenous chicken ecotypes (Table 1). Lower estimates of expected heterozygosity (H_E) were calculated for the Lamu ecotype compared to the other ecotypes. Turkana populations had the highest estimates of H_E. Western Highlands ecotype displayed the lowest values for observed heterozygosity (H_O), while the Lake Victoria ecotype displayed the highest values of H_O. Turkana ecotype recorded the highest nucleotide diversity (P_i) values, while the Lamu ecotype had the lowest values for the P_i (Table 1).

AMOVA

To infer the *HSP70* haplotypes population genetic structure of indigenous chicken ecotypes in Kenya, AMOVA was analyzed. The variations were assessed using 1000 replicates in *HSP70* haplotypes among the ecotypes, among individuals within the ecotypes, and within individuals using AMOVA of pair-wise differences as implemented in Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). Based on pair-wise differences in AMOVA, variance among the ecotypes accounted for 7.99% of the total variance in the chicken *HSP70* gene. Still, the variance among individuals within the ecotypes accounted for 2.39% of the total variance. Conversely, the variance within individuals was high, and it accounted

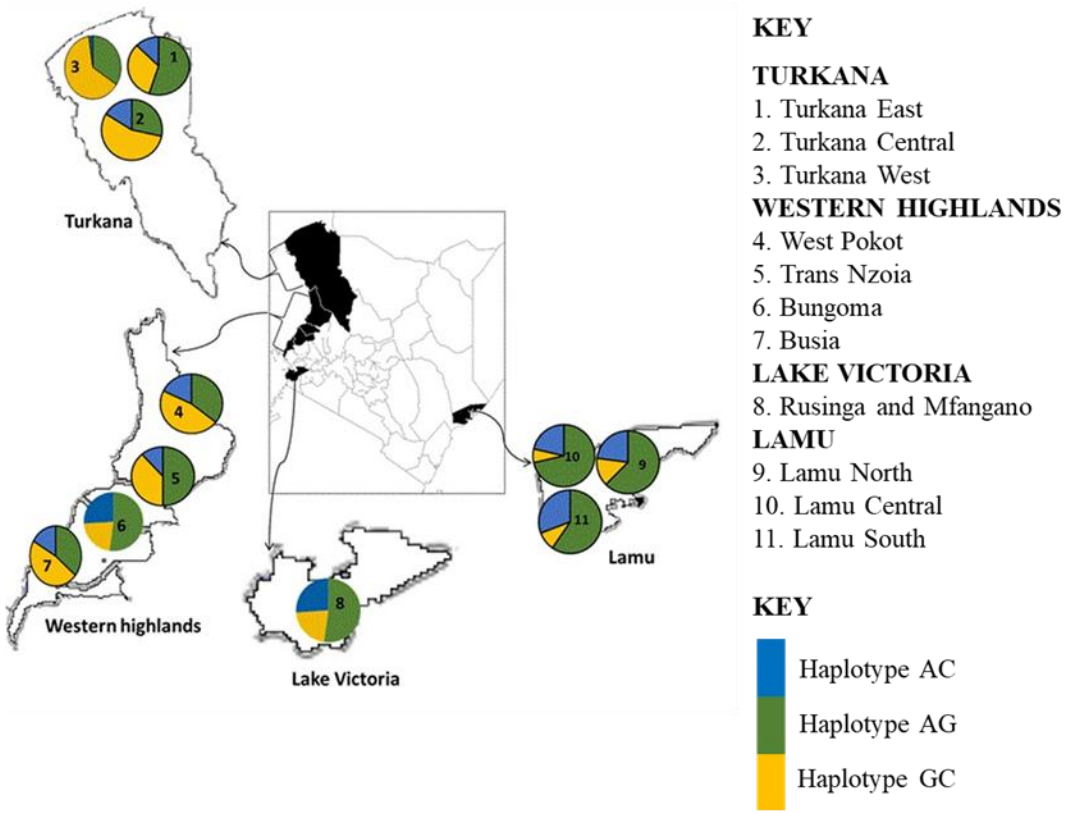

Figure 4. Chicken *HSP70* haplotype distribution in the different agro-climatic zones in Kenya.

Table 1. Diversity indices of *HSP70* gene in indigenous chicken ecotypes from different agro-climatic zones in Kenya.

Ecotype	Number of homozygotes	Number of heterozygotes	H_O	H_E	P_i
Turkana	96	118	0.551	0.629	0.003±0.002
Western Highlands	50	60	0.545	0.628	0.002±0.002
Lake Victoria	12	34	0.739	0.619	0.002±0.002
Lamu	80	104	0.565	0.499	0.001±0.001

H_E = Expected heterozygosity; H_O = observed heterozygosity; P_i=Nucleotide diversity.

for 89.62% of the total variance (Table 2).

DISCUSSION

Heat tolerance in livestock production, especially poultry, has interested many researchers in the recent past due to the need for genetic lines that can withstand the harsh effects of climate change (Duangjinda et al., 2017). Indigenous poultry is diverse and displays phenotypic differences among the various breeds (Moraa et al., 2015). The phenotypic differences observed between various poultry species can be due to the modification of gene products and their expression levels due to genetic changes, and these affect the efficiency of birds to heat tolerance (Hoffmann and Willi, 2008; McManus et al., 2010; Walser et al., 2006; Wray, 2007). Several researchers have used the *HSP70* gene as a model phenotype to study regulatory mutations in response to environmental changes (Chen et al., 2007; Hoffmann and Willi, 2008; Kang et al., 2009; Walser et al., 2006; Wray, 2007). Structural polymorphism of chicken *HSP70* genes has been shown to play a vital role in chicken heat tolerance (Aryani et al., 2019; Galal and Radwan, 2020; Mahmoud, 2000; Mazzi et al., 2003; Wang et al., 2014). Therefore, in this study, we examined the polymorphism of the *HSP70* gene via DNA sequencing of chicken ecotypes from Turkana, Lamu, Western Highlands, and Lake Victoria. Three *HSP70* haplotypes, viz: haplotype GC, AC, and AG were identified. These haplotypes were

Table 2. AMOVA of the *HSP70* gene in indigenous chicken ecotypes from different agro-climatic zones in Kenya.

Variance components	Hierarchy	d.f.	variation	Percentage of variation	*p*-value
Among ecotypes	1	4	22.4	7.9	0.000±0.000
Among individuals within ecotypes	2	549	164.9	2.3	0.219±0.004
Within individuals	3	554	158	89.6	0.003±0.001

d.f. = degrees of freedom; Significance (P<0.01).

shared within and between the chicken ecotypes. The extent of *HSP70* haplotype sharing among indigenous chicken ecotypes, which can be due to extensive inbreeding practices, shows a lack of population structure (Chen et al., 2016). Most farmers practice unsupervised crossbreeding between indigenous and commercial chickens to obtain performing hybrid that can produce more eggs and meat, well adapted to local climate. In the long run, this practice leads to genetic erosion of genes that could be advantageous for adaptation to harsh environmental conditions and in disease resistance (Aengwanich, 2008; Bekele et al., 2010; Kingori et al., 2010; Mohammed et al., 2005). In order to maintain the unique attributes of indigenous chicken which are valued by producers and to avoid genetic erosion and dilution, breeding programs targeting improvement of indigenous chickens should focus on within breed selection rather than crossbreeding with commercial chicken breeds (Okeno et al., 2012). There should also be capacity building among farmers and local communities through training, education, raising awareness and sharing information (Yonas, 2020).

In all the ecotypes, the most frequent haplotype was chicken *HSP70* haplotype AG with high frequencies observed in the Lamu chicken ecotype. Contrastingly, in all the ecotypes, haplotype AC had the lowest frequencies. The phylogenetic analysis of the *HSP70* haplotypes indicated that chickens share homology with the turkey. They are relatively distant from *HSP70* of other related poultry (helmeted guinea fowls, Japanese quail, and common quails) but distantly related to mallard duck, common ostrich, northern fulmar, and red-throated loon. A closer comparison with other avian species, including the ancestral red jungle fowl sequences, indicated that haplotype GC is the ancestral haplotype since it clustered with the ancestral red jungle fowl, and this haplotype dominated in Turkana chicken ecotype.

Polymorphism in the chicken *HSP70* gene is related to heat stress resistance (Zhang et al., 2002). It is clear from this study that haplotype AG and GC could be advantageous to heat tolerance in indigenous chickens since haplotype GC dominated in Turkana chicken ecotype. In contrast, haplotype AG was dominant in the Lamu chicken ecotype. Turkana and Lamu are arid and semi-arid lands characterized by high ambient temperatures of up to 42ºC, and we hypothesize that the two haplotypes have a crucial role in heat tolerance. This result is in concordance with what was reported by Mazzi et al. (2003). Results from their study on different breeds of broiler chickens identified polymorphism in the coding region of the *HSP70* genes at positions g.370A>G and g.388C>G. Three of the detected alleles (*hsp70-1*, *hsp70-2*, and *hsp70-3*) were associated with the nature of resistance to heat stress. They concluded that these alleles could be used as a candidate gene for heat stress (Mazzi et al., 2003). Another study by Aryani et al. (2019) reported four *HSP70* haplotypes in their chicken populations: H1, H2, H3, and H4. The H4 haplotype was only found in chickens that were resistant to heat stress. A study by Mack et al. (2013) indicated that the C-69A>G SNP in the 5′-flanking region of the *HSP70* gene affects chicken thermotolerance traits in white recessive rock chickens exposed to thermoneutral temperatures. The authors also found that the GG genotype might be advantageous for the prevention of heat stress. Thus, this SNP may be a potential molecular marker for further genetic improvement of thermotolerance in chicken (Mack et al., 2013).

Additional research on the role of *HSP70* on heat stress has been done on indigenous chickens from Indonesia (Tamzil et al., 2013), Taiwan (Liang et al., 2016), and Thailand (Duangjinda et al., 2017). Polymorphisms in the *HSP70* gene have also been reported in ducks (Xia et al., 2013), pigs (Chen et al., 2003; Jin et al., 2005; Ruohonen-Lehto et al., 1993), and cattle (Basiricò et al., 2011).

Low P_i values were recorded for chickens in this study, and these results were similar to those of Gan et al. (2015). These results showed that the genetic diversity of chickens has declined over time. This phenomenon could be attributed to the breeding practices, genetic drift, and a small base population that led to beneficial allele fixation.
The chicken *HSP70* AMOVA test results indicated significant genetic differentiation of the *HSP70* gene within individuals. On the other hand, the genetic difference among the chicken populations and individuals within the population was not considerable. These results revealed a lack of population structure within indigenous chicken ecotypes in Kenya. Lack of population structure in chicken has also been reported previously by Chen et al. (2016). We, therefore, hypothesized that the significant genetic differentiation within individuals in the *HSP70* gene in chickens and the change in *HSP70* gene sequence may be due to the natural and artificial selection as well as the long history of domestication and evolution, and could play a significant role in heat stress.

It has been shown that individual variations in heat shock responses may be related to DNA polymorphisms in the *HSP70* gene in birds (Maak et al., 2003). Genetic changes bring about variations in gene products and their expression levels. These genetic changes could be one factor contributing to phenotypic variations between various species (De La Rosa et al., 1998). Several studies have indicated that mutations in gene sequencing alter gene expression, morphology, and physiology (Deeb and Cahaner, 2001; Iwamoto et al., 2008). For instance, changes in the AT content of the promoter affect the expression of *HSP70*, which plays a significant role in regulatory evolution (Chen et al., 2011). Therefore, this indicates that other changes in the *HSP70* gene sequence could contribute to the evolution of this gene.

The different heat-tolerant traits in the chicken are brought about by the coding regions and polymorphism regulation of *HSP70* (Zhang et al., 2002). Therefore, the use of modern molecular breeding technology to identify genetic markers related to thermotolerance could allow for the possibility of direct gene selection for heat stress.

CONCLUSION AND RECOMMENDATIONS

Results obtained from this study have demonstrated the structural polymorphism in the *HSP70* gene in the Kenyan chicken ecotypes, as shown by the variations in the partial *HSP70* gene. It is also evident that indigenous chicken ecotypes in Kenya may genetically harbor different levels of thermotolerance. Also, results from this study have formed an informational database for chickens in response to adaptation to various agro-climatic zones. This information can be implemented by breeding programs to develop heat-resistant chicken that can produce maximum yields, thus improving food security in the face of climate change.

In line with the present study, future research areas should include samples of other chicken ecotypes from other agro-climatic zones to study the *HSP70* gene polymorphisms that exist.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In vitro propagation of two commercially important bamboo species (*Bambusa tulda* Roxb. and *Dendrocalamus stocksii* Munro.)

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Bamboo plant is an important biodegradable raw material which can play important role in rejuvenation of Indian rural economy through positively impacting agricultural, industrial, energy and in environmental sectors. Traditional methods of vegetative propagation of this plant have insufficient multiplication rate. In the present investigation, *in vitro* bud break and aseptic culture establishment in relation to different seasons of Bihar were evaluated for large scale clonal propagation of two commercially important bamboo species, *Bambusa tulda* Roxb. and *Dendrocalamus stocksii* Munro. The experiment was conducted thrice using a completely randomized design with 10 replicate per treatment. Mean calculation was performed using Duncan's Multiple Range Test (DMRT) at P<0.05. Summer climate (April-June) was the most congenial season for initiation and establishment of aseptic cultures. 6 Benzylaminopurine (BAP) without supplementation of any additive resulted in significant bud breakage in *B. tulda*, however, in case of *D. stocksii,* Thidiazurone (TDZ) with the additives resulted in comparatively better response. For multiplication, BAP (2.5 mg/l) showed maximum number of shoots (24±6.2) in *D. stocksii*. However, in *B. tulda* high multiplication rate with adequate shoot length was observed in semi solid media supplemented with BAP (1 mg/l). For both the species, the survival rate during hardening (primary and secondary) was maximum during monsoon season. The refined *in vitro* regeneration system of two commercially important bamboo species developed is efficient and will be an impetus to raising bamboo nurseries of elite germplasm for bamboo growing areas of Bihar.

Key words: MS media, clonal propagation, *in vitro* regeneration, phytohormones, 6benzylaminopurine, thidiazurone.

INTRODUCTION

Bamboo, belongs to the highest evolved flowering family, Poaceae. It is a natural biodegradable raw material, which can play important role in rejuvenation of Indian rural economy through impacting in agricultural, industrial, energy and environmental sectors. Among the flowering plants, it is one of the highest carbon sequesters, environment friendly and suited to adapt and grow even on degraded lands (Hossain et al., 2015; Singh et al., 2020; Waghmare et al., 2021). This plant can grow very fast (0.9 to 1.2 m/day) within four to five years and can regrow after harvesting, without the need of replanting and thereby, making it a perennial renewable bio-resource. Due to its versatility, this plant is also known as green gold, poor man's timber, friends of the people and also cradle to coffin timber (Sawarkar et al., 2020; Singh et al., 2021).

The green cover in Bihar had reduced to 9% after the bifurcation of Jharkhand from Bihar state in 2000. The population density of this state is quite high (1106 people/km^2) and the average land holdings is quite low, 0.24 ha (2011 census) (India, 2021). In addition, farmers of this state also face extreme causalities both with floods and droughts every year. It was thereby, decided by the state Department of Environment, Forest and Climate Change (DEFCC) to increase the green cover from 15 to 17% and subsequently to 19% through massive development of plantations under ' Haryali Mission'. Bamboo is among one of such plants for integrating it into agriculture by planting on farm boundaries, farm-land (Agro forestry) and non-agricultural land (waste land, degraded lands and in homesteads). Bamboos can also provide farmers a perennial source of income (Hoogendoorn and Benton, 2014; Liese and Kohl, 2015).

Since time immemorial, bamboos have grown naturally but they are now also being cultivated. However, this plant has also witnessed serious depletion as is the case with other plants in the natural habitats. In order to conserve the depleting bamboo resource, the commercially important species need to be selected and propagated. However, the propagation of this plant is impeded by long flowering cycles that can last 40 to120 years, lack of seed availability and also short viability of seeds. Traditional methods of vegetative propagations (rhizome splitting, offset cuttings, branch and branch cuttings) have low multiplication rate. Also, these methods are season specific, being bulky and so inconvenient in handling and transport (Waghmare et al., 2021; Mustafa et al., 2021).

These gaps can be filled by micropropagation. Through tissue culture techniques, large scale mass clonal propagation of high quality germplasms can be made (Goyal and Sen, 2016). India possesses world's largest bamboo resources, next to China, with 136 species and 23 genera spread over sixteen millions hectares of land (Forest Survey of India, 2019).

In this study, two economically important bamboo species (*Bambusa tulda* Roxb. and *Dendrocalamus stocksii Munro.)* were selected due to their adaptability to the local climatic conditions. D. stocksii is thorn less, solid stemmed, loosely spaced, erect bamboo growing upto 9m height with the diameter ranging from 30 to 50 mm. The leaves are lanceolate (10-20 cm long) and are most suited for agroforestry as it has low canopy coverage. It is utilized for furniture, basket making, poles, stakes, crafts and also in construction. *B. tulda* is another commercially important bamboo species, with the characteristics of being tall, quick growing and with the ability to grow up to 13 to 26 m in height. It also prevents soil erosion. This bamboo has high demand as it being one of the fastest growing plants and culms are used to manufacture many handicrafts, furniture, house construction, paper, and pulp production.

Earlier work on the development of protocols for micro clonal propagation of these two species of bamboos showed challenges with rhizogenesis and hardening of the seedlings (Waikhom and Louis, 2014; Sharma and Sharma, 2013; Somashekar et al., 2018; Rajput et al., 2019).

The main objective of the study was to evaluate *in vitro* bud break and aseptic culture establishment in relation to different seasons of Bihar for above two commercially important bamboo species. Effects of different PGRs and additives in various concentrations and combinations to initiate healthy multiple shoots under *in vitro* conditions were monitored. Influence of seasons on hardening (primary and secondary) of tissue culture raised seedlings with survival efficiencies was also observed.

MATERIALS AND METHODS

Source of explants

Explants were collected from actively growing shoots of four years old healthy culms of *D. stocksii* and *B. tulda* which were brought from Institute of Wood Science and Technology (IWST) Bengaluru and were planted and maintained at Plant Tissue Culture (PTC) Lab, Tej Narayan Banaili (TNB) College campus, TilkaManjhi Bhagalpur University (TMBU) Bhagalpur (Figures 3a and 4a). The explants were collected in different seasons (winter, spring, summer and monsoons) from April 2018 to March 2019 following the methods of Saxena (1990) and Choudhary et al. (2016) and were brought to the PTC Lab, TNB College campus Bhagalpur for further processing. The study site, Bhagalpur district is located in the south of Bihar (at the 25.24 latitude and 86.98 longitude) on the bank of the river Ganga. The area is characterized by hot humid summer (36 to 18.7°C) and cool winter (23.4 to 5.2°C).

Surface sterilization

At first the shoots were surface sterilized with the help of cotton swab dipped in 70% ethanol in order to remove the dust and microorganisms. Shoots were then washed in running tap water Explants of the size of 1.5 to 3.0 cm in length were cut into pieces containing one node axillary buds. Surface sterilization procedures. of explants were also observed in order to monitor the bud breakage response and the levels of contamination. In the present investigation, four different procedures were applied for treatment of explants. Those were named as T1, T2, T3, and T4 **(Table 1).**

After each treatment, explants were rinsed with autoclaved distilled water for three to four times. Then the nodal segments were brought to the laminar flow chamber under aseptic conditions and were sterilized by treating buds with 70% ethanol (for 30 to 35 sec) followed by three rinses with autoclaved distilled water. Explants were then treated with 0.1% $HgCl_2$ for 5 min and were subsequently washed with distilled water three to four times.

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Table 1. Steps of different surface sterilization procedures of explants.

Steps	T1	T2	T3	T4
Outside Laminar	Tween2.0+ streptomycin + 0.025% $HgCl_2$ for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0 + Dettol + 0.025% $HgCl_2$ for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0+ Dettol for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0+ Dettol for 10 min followed by treatment with1% bavistin for 10 min
Inside Laminar	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$ for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$ for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 1% NaOCl for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$for 5 min

Culture conditions

The modified MS media was used for shoot initiation of *D. stocksii* and *B. tulda*. The MS media (consisting of salt, vitamins and 3% sucrose) was prepared using 100 mg/l myoinositol. Different plant growth regulators (PGRs)-BAP, TDZ and NAA were added at various concentrations to the MS media, before adjustment of pH at 6.0. Then the media was autoclaved at 121°C at 6.80 kg (15 lb) for 20 min. The cultures of all growth stages were incubated under artificial physical conditions 22±2°C, 65 to 70% humidity and 16 h of photoperiod (using white fluorescent tube).

Axillary bud initiation

Following different sterilization treatments (T1, T2, T3 & T4), per cent bud breakage and contamination level in both genus (*B. tulda* and *D. stocksii*) were monitored in different seasons (winter, spring, summer and monsoon) (Table 2). Experiments were conducted thrice for each sterilization procedure for each season. 10 explants were used for one experiment and in each culture tube 15 ml nutrient media (MS+ NAA 0.1 mg/l+ BAP 1.0 mg/l) was employed. Contamination level and bud breakage per cent were calculated after 10 days of inoculation.

For monitoring the influence of various phytohormones on auxiliary bud initiation, explants were inoculated in test tubes containing 15 ml of initiation media supplemented with different concentrations of growth hormones (BAP, TDZ and NAA) alone or with additive. Three concentrations of BAP (1, 2.5 and 5 mg/l) and TDZ (0.1, 0.25 and 0.5 mg/l) were tested. Since TDZ being strong cytokinines and thereby, comparatively less concentration of this cytokinine was taken in our experiments. For observation, 10 tubes were taken for each treatment having one explant (observation unit) in each tube. The experiments were done in triplicate. For each genus a total of 360 experiment units, each time with 120 units were employed. Treatments were made in completely randomized design. After inoculation being made in nutrient media, explants were kept in culture room and observations were made after 21 days (3 weeks) onward till when the auxiliary bud broken and a number of shoots proliferated. Number of shoots was calculated by counting the shoot number in each explant. Shoot length was measured in cm by scale (Table 3).

***In vitro* shoot multiplication**

Initiated axillary buds were separated and subsequently cultured for multiple shoot proliferations. Established aseptic cultures of proliferated clumps of shoots were propagated in semi-solid (clerigel used as solidifying agent) and liquid MS media under different cytokinins concentrations BAP (1, 2.5, and 5 mg/l) and TDZ (0.1, 0.25 and 0.5 mg/l). The clumps of 5 to 10 shoots were used for *in vitro* shoots multiplication. The sub-culturing of *in vitro* raised shoots was carried out by transferring in fresh MS media at regular intervals. For liquid media, sub-culturing time intervals were kept 8 to 10 days, however, for solid media it was in between 15 and 21 days. During sub-culturing, dead parts of the plantlets were removed carefully. Also, periodical transfers were made in order to avoid browning and leaching and to maintain healthy cultures. The performance of multiplication in different concentrations of growth hormones was calculated by observing the number of shoots and shoot length.

The shoot length and shoot number were also observed when media were supplemented with different concentrations of additives, Ascorbic acid (AA), Cysteine (Cys) and Citric acid (CA) applied alone or in combinations (50 mg/l AA, 50 mg/l AA + 50 mg/l Cys, 50 mg/l AA + 25 mg/lCys. AA + 50 mg/l CA, 50 mg/l AA + 25 mg/l CA, 50 mg/l AA + 25 mg/l Cys + 25 mg/l CA).

In vitro rooting

After 5 to 6 successful passages of cycles, the well-developed clump with 3 to 4 shoots was inoculated into the MS rooting media. The *in vitro* raised shoots of adequate heights were transferred to full strength and half strength of MS media containing different concentrations of auxins such as IBA (1.0, 2.5 and 5.0 mg/l), NAA (1.0, 2.5 and 5.0 mg/l) in order to access their response on rooting. The performance of *in vitro* rooting was calculated after 4 weeks (30 days) of incubation by observing root per cent and the mean number of roots in each clump.

$$\text{Rooting (\%)} = \frac{\text{Number of clumps in which roots were present}}{\text{Number of clumps used in rooting experiment}} \times 100$$

Acclimatization

For acclimatization, *in vitro* raised bamboo plantlets seedlings were transferred into portrays containing different potting mixtures (**Figure** 1) and then they were subsequently allowed to grow inside the closed tunnel of green house for three weeks. Then the partially acclimatized plantlets were kept in green house (outside of closed tunnel) for 3 weeks. Primary hardened plantlets were subsequently transferred to the propagation bed of the Net/Shade house for a period of 45 days. The survival rate during hardening processes was observed in different seasons (winter, spring, summer and monsoons).

Experimental design and statistical analysis

The experiment was conducted thrice using a completely

Table 2. Initiation of *B. tulda* and *D. stocksii* in different seasons after following different sterilization procedures of explants.

Sterilization techniques	Contamination and bud breakage %	Winter		Spring		Summer		Monsoon	
		B. tulda	***D. stocksii***	***B. tulda***	***D. stocksii***	***B. tulda***	***D. stocksii***	***B. tulda***	***D. stocksii***
T1	Contamination%	40.3±.088	32.0±7.52	23.0±3.61	21.1±3.50	26.3±0.89	12.4±1.60	74.0±4.00	65.5±2.21
	Bud breakage%	44.3±5.17	62.7±7.48	72.3±0.88	64±0.71	76.0±1.15	82.3±1.86	45.3±1.86	72.3±3.18
T2	Contamination%	15.0±2.52	16.6±3.59	10.0±1.53	9.09±0.23	8.0±0.58	7.33±0.69	36.0±2.08	22.5±3.39
	Bud breakage%	33.3±7.54	66±7.47	80.7±2.85	70±2.00	91.7±0.88	87.33±0.98	59.3±2.73	72.6±3.17
T3	Contamination%	71.7±0.88	73.4±5.27	60.7±4.67	58.8±6.2	51.7±4.10	49.06±3.75	80.3±4.91	83.5±1.36
	Bud breakage%	19.7±0.89	44.0±2.16	45.7±2.87	63.5±4.5	53.3±7.26	62.3±5.04	30.0±4.16	54.3±6.36
T4	Contamination%	77.7±2.19	74.5±4.49	38.0±4.16	58.3±2.0	34.7±2.33	43.63±5.61	85.0±1.73	81.5±1.64
	Bud breakage%	16.3±3.28	41.2±1.88	58.7±2.03	61±8.00	62.7±5.59	56±4.72	50.0±3.06	37.3±1.86

T1, T2, T3 and T4 are the surface sterilization techniques adopted for different explants.

Table 3. Initiation in *B. tulda* and *D. stocksii*: Influence of different concentrations of cytokinins in relation to shoot number and shoot length.

Different combinations of growth hormones	***B. tulda***		***D. stocksii***	
	Number of shoots (Mean±SE)	**Shoot length (cm) (Mean±SE)**	**Number of shoots (Mean±SE)**	**Shoot length (cm) (Mean±SE)**
MS + BAP 1 mg/l + NAA 0.1 mg/l	$5.5±0.63^{ab}$	$7.16±0.27^{a}$	$2.0±0.30^{ghijk}$	$7.14±0.19^{bc}$
MS + BAP 2.5 mg/l + NAA 0.1 mg/l	$5.9±0.64^{a}$	$6.43±0.31^{ab}$	$3.8±0.25^{abcde}$	$5.56±0.26^{de}$
MS + BAP 5 mg/l + NAA 0.1 mg/l	$4.6±0.37^{abc}$	$4.1±0.30^{cde}$	$4.2±0.20^{abc}$	$4.48±0.16^{gh}$
MS + TDZ 0.1 mg/l + NAA 0.1 mg/l	$1.5±0.22^{efghi}$	$4.67±0.30^{c}$	$1.5±0.17^{jk}$	$7.19±0.29^{ab}$
MS + TDZ 0.25 mg/l + NAA 0.1 mg/l	$2.4±0.33^{def}$	$3.67±0.33^{def}$	$2.9±0.28^{cdefghi}$	$5.27±0.33^{ef}$
MS + TDZ 0.5 mg/l + NAA 0.1 mg/l	$2.1±0.59^{efg}$	$1.91±0.19^{ejk}$	$4.1±0.38^{abcd}$	$2.61±0.22^{ij}$
MS + BAP 1 mg/l + NAA 0.1 mg/l + Additives	$3.8±0.48^{cd}$	$4.53±0.36^{cd}$	$2.1±0.23^{ghij}$	$6.53±0.31^{bcd}$
MS + BAP 2.5 mg/l + NAA 0.1 mg/l + Additives	$2.9±0.62^{de}$	$3.36±0.37^{efgh}$	$4.9±0.66^{ab}$	$5.11±0.19^{efg}$
MS + BAP 5 mg/l + NAA 0.1 mg/l + Additives	$2±0.49^{efgh}$	$2.13±0.20^{ij}$	$3.3±0.54^{cdefg}$	$2.33±0.16^{ijk}$
MS + TDZ 0.1 mg/l + NAA 0.1 mg/l + Additives	$1.8±0.24^{efghi}$	$3.47±0.22^{efg}$	$3.7±0.53^{bcdef}$	$7.93±0.24^{a}$
MS + TDZ 0.25 mg/l + NAA 0.1 mg/l + Additives	$1.7±0.15^{efghij}$	$2.75±0.2^{ghi}$	$5.1±0.41^{a}$	$3.10±0.12^{l}$
MS + TDZ 5 mg/l + NAA 0.1 mg/l + Additives	$1.1±0.31^{efghijk}$	$1.95±0.13^{ijk}$	$3.1±0.55^{cdefgh}$	$2.40±0.21^{ijk}$

Values are given in mean (n =10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

randomized design with 10 replicates per treatment. The effects of PGRs and additives on initiation and multiplication of both the species were calculated and the level of significance was determined through analysis of variance (ANOVA). Mean calculations were performed using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

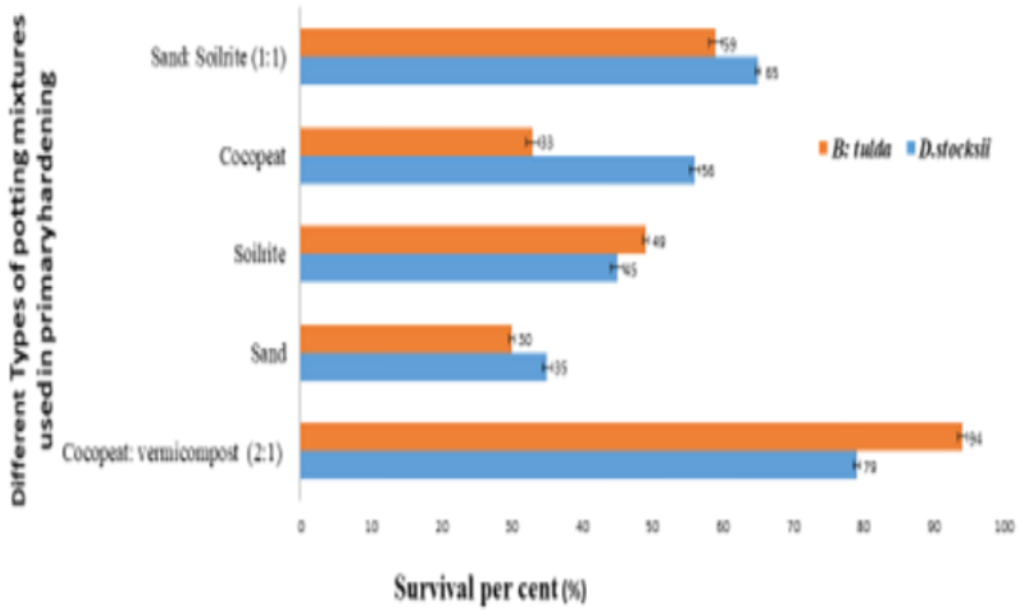

Figure 1. Primary hardening of *B. tulda* and *D. stocksii* in different potting mixture.

Evaluation of efficient sterilization techniques, axillary bud initiation and rooting per cent was made through the experimental designs as previously discussed.

RESULTS AND DISCUSSION

Initiation and establishment of *in vitro* aseptic culture

Initiation in different seasons

Seasons had marked impact on the establishment of aseptic cultures and on multiple bud breaks. In T2 treatment, there was maximum bud proliferation with least microbial contamination in all the seasons followed by treatment T1 (with the application of antibiotic streptomycin) (Tables 1 and 2). Comparatively less bud break was observed in explant treated with antibiotic as surface sterilants. Microbial contamination was the maximum during the monsoons in treatment T4 (85%) and T3 (80.3%). Earlier workers have also reported the retarded growth of explants with the applications of antibiotics (Yasodha et al., 2008; Negi and Saxena, 2011). Moreover, the treatment with antibiotics is not only cost effective but also time consuming. Negative effects of antibiotics at higher concentrations have been attributed to their toxic effects on morphogenesis (Mathias and Mukasa, 1987; Venkatasalam et al., 2013; Buckseth et al., 2017).

The rate of contamination and bud initiation percentage of both the species varied in different seasons. Summer climate (April-June) was the most congenial season for initiation and establishment of aseptic cultures. In case of *D. stocksii* the per cent bud break response was maximum (87.33%) during summer season followed by monsoons (72.6%), when they were treated as T2 sterilization technique. During the monsoons, the microbial contamination percent was comparatively high (22.5%). Microbial load was also maximum during rainy seasons (65.5, 83.5 and 81.5%) compared to other seasons. This might be due to prolonged high humidity coupled with high temperature prevailing in this locality during monsoons. In case of *B. tulda* also, the summer was the most suitable season for bud initiation due to high percent (91.7%) of bud breakage with minimal contamination level (8.0%), followed by spring season with 80.7% bud breakage and 10.0% microbial contamination (Table 2). Previous workers have also reported the influence of climatological factors on the levels of microbe contamination and establishment of aseptic cultures (Saxena and Dhawan, 1999; Kiran and Ansari, 2000; Choudhary et al., 2017; Sandhu et al., 2018). In an investigation, working on *Dendrocalamus asper*, Nadha et al. (2013) observed that the rainfall had the direct influence on contamination rate and survival per cent of explants. In *D. stocksii,* the contamination level was moderately low during winters and spring, 16.6 and 9.09%, respectively, when treated with T2 procedure. However, in the same sterilization procedure (T2), the bud proliferation percentage (66.1%) was comparatively lesser in winters compared to other seasons. It might be due to high phenol production during this season (Banerjee et al., 2011). In *B. tulda* also bud breakage percentage was less (33.3%) during winter, even after following the same sterilization technique. Browning and death are the common problems associated with the

culture of woody plants which might be attributed to the oxidation of phenolic compounds in explants tissues (Choudhary et al., 2004; Arya et al., 2008; Suwal et al., 2020).

Influence of PGR

The influence of PGRs combinations varied in both the species of bamboos. In case of *D. stocksii*, TDZ when applied along with additives resulted in high number of shoots and also high shoot length (Table 3). The maximum number (5.1±0.41) of shoots was observed in the combinations of MS + TDZ 0.25 mg/l+ NAA .1 mg/l+ additive, followed by the combinations of MS+ BAP 2.5 mg/l + NAA 0.1 mg/l + additives, with 4.9 ± 0.66 shoots. The shoot length was high (7.93±0.24) in TDZ concentration of 0.1 mg/l in combination with additives. However, *B. tulda*, TDZ as cytokinine was not very effective for bud proliferation. In this case, BAP alone at the concentration of 2.5 mg/l and 1 mg/l was found as the most suitable phytohormones for bud breakage (Table 3). The number of shoots was 5.9±0.64 at concentration of 2.5 mg/l, followed by 5.5 ± 0.63 at the concentration of 1.0 mg/l. The shoot length was adversely affected with the increase in the concentrations of the PGR. At low concentration of PGR, there was high shoot length; however, at considerably higher concentrations there was minimum length. Similar findings were also observed in case of *D. stocksii*. The high level of BAP or cytokinine has been reported to induce programmed cell death in cultures (Singh et al., 2012). Moreover, cytokinine at higher level is also known to promote ethylene biosynthesis and thereby, adversely affecting the growth in culture (Suwal et al., 2020).

Different cytokinines (BAP and TDZ) had varied responses towards both the species. The BAP without additive resulted in significant numbers of bud breakage in *B. tulda.* Contrary to this, TDZ with additives had better response in *D. stocksii*. This might be due to the endogenous level of growth hormones in the tissues of the culture as well as the extent of phenolic which might vary in different strains of the same species (Sandhu et al., 2017; Jiménez, 2005).

Previous researchers working on different bamboo species have also reported high percentage of bud breakage, when BAP was applied without additives as in *B. tulda* (Choudhary et al., 2020), *Dendrocalamus hamiltonii* (Agnihotri and Nandi, 2009; Sood et al., 2002), *Bambusa bambos* (Arya and Sharma, 1998) and *Guadua angustifolia* (Jiménez et al., 2006). However, Kabade (2009) and Beena et al. (2012) observed multiple shoot induction when TDZ was applied in combination with NAA. Somesekhar et al. (2008) working on *D. stocksii* reported the synergistic effect of BAP with NAA for high shoot induction with adequate shoot length. The endogenous auxins and the added auxins in the media might have resulted in increased total auxin levels of seedlings in cultures resulting thereby in high apical dominance and subsequent suppression of shoot proliferation. However, interaction with cytokinine might promote shoot proliferation (Rasool et al., 2009; Bin Azizan, 2017).

Shoot multiplication

Influence of PGR concentrations

After 45 days of inoculation, shoots developed well in a group of 5-20, then the clumps of 5-10 micro-shoots were transferred to different types of nutrient media (MS semi solid and liquid media) containing different concentrations of cytokinins (BAP and TDZ). The number of shoots as well as shoot lengths of *D. stocksii* and *B. tulda* cultures was evaluated subsequently after 15 to 21 days of sub culturing (Table 4). In case of *D. stocksii,* the number of shoots was maximum (24.87±0.62) in liquid media supplemented with BAP 2.5 mg/l, followed by 20.37±0.27 in the semisolid media; however, with same concentration (2.5 mg/l) of BAP. Shoot length was maximum (7.0±0.14) at the BAP concentration of 1.0 mg/l followed by 5.23±0.11 at the concentration of 2.5 mg/l of BAP. Moreover, for *D. stocksii* the liquid media supplemented with 2.5 mg/l BAP was most congenial as it resulted in maximum shoot number with adequate length (5.23±0.11). Another cytokinine (TDZ) was not very effective due to slow rate of multiplication along with dwarf shoots.

In *B. tulda,* the multiplication rate and shoot length were high in semi-solid media. The number of shoots was observed maximum (24.44±0.50) in semi solid media supplemented with BAP at 1.0 mg/l, followed by 13.44±83 shoots at BAP concentration of 2.5 mg/l.

Physical state of media (liquid/semisolid) requirements depends upon the nature of the explant used for the micropropagation. In case of *D. stocksii*, MS liquid media resulted in better response than semi solid media. Browning and leaching were comparatively lower in this media. This might be due to easier uptake of nutrients and regulators without any restrictions compared to gelling agents (Negi and Saxena, 2011). Earlier, Saxena (1990) reported multiplication of *B. tulda* in liquid media. However, in case of *B. tulda* we found semisolid media (with clerigel) as most suited for mass propagation. In liquid media, there was slower rate of multiplication after 2 to 3 cycles, which might be due to hyperhydricity (Saxena and Bhojwani, 1993; Arshad et al., 2005). Among the different cytokinines, BAP at the concentrations of 1 to 2.5 mg/l was found most suitable phytohormones for *in vitro* multiplication of bamboo shoots (Choudhary et al., 2016; Mane et al., 2020). However, concentrations above this range influenced multiplication but resulted in stunted shoot growth. Previous workers have also confirmed it while working on different species of bamboos (Arya and Arya, 1997; Bag et al., 2000; Bhadrawale et al., 2018).

Table 4. Multiplication of *B. tulda* and *D. stocksii* in different combinations of growth hormones.

Concentration of growth hormones	Types of media	*B. tulda*		*D. stocksii*	
		Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
BAP 1 mg/l	Semi solid	24.44±0.50^{a}	4.72±0.46^{b}	15.23±0.17^{c}	3.35±0.17^{def}
	Liquid	11.67±0.28^{bc}	5.66±0.51^{a}	12.56±0.11^{ef}	7.01±0.14^{a}
BAP 2.5 mg/l	Semi solid	11.33±0.70^{cd}	3.02±0.18^{cdef}	20.37±0.27^{b}	2.49±0.18^{ghi}
	Liquid	13.44±0.83^{b}	3.36±0.19^{cd}	24.87±0.62^{a}	5.23±0.11^{b}
BAP 5 mg/l	Semi solid	8.22±0.90^{ef}	2.52±0.17^{fghi}	12.8±0.79^{e}	1.79±0.07^{k}
	Liquid	6.33±0.33^{ghi}	2.33±0.12^{fgh}	14.7±0.52^{cd}	2.46±0.08^{ghij}
TDZ 0.1 mg/l	Semi solid	6.86±0.35^{fgh}	2.87±0.27^{cdefg}	7.61±0.25^{ij}	3.58±0.30^{de}
	Liquid	8.78±0.618^{e}	3.49±0.05^{c}	9.7±0.29^{h}	5.04±0.18^{bc}
TDZ 0.25 mg/l	Semi solid	7.89±0.26^{efg}	2.09±0.23^{ghij}	8.1±0.23^{i}	2.96±0.19^{fg}
	Liquid	5.89±0.63^{hij}	3.23±0.17^{cde}	11.88±0.42^{efg}	3.61±0.13^{ed}
TDZ 5 mg/l	Semi solid	5.22±0.27^{hijk}	1.47±0.13^{ijk}	5.771±0.20^{k}	1.82±0.14^{k}
	Liquid	4.33±0.23^{jk}	1.68±0.06^{hijk}	6.55±0.29^{jk}	2.59±0.08^{gh}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Influence of additives

Influence of different types of additives and their combined effects was also evaluated. After 1 to 2 sub-culturing, cultures of *D. stocksii* became brown and died due to secretion of phenolic compounds. In order to overcome the browning and leaching of those cultured plants, the effect of various combinations of additives in different concentrations was monitored. Additives in combination of 50 mg AA + 25 mg CA + 25 Cys. mg/l resulted in maximum (13.3±0.67) shoot multiplication with high (6.69±0.18 cm) shoot length (Table 5). Our findings are in conformity with the observations of previous workers (Rathore and Ravishankar Rai, 2005; Somashekar et al., 2008; Rajput et al., 2020). However, in case of *B. tulda,* MS basal media without the addition of any additives resulted in maximum number of shoots (21.44±7.15) and shoot length (4.64±1.55 cm) (Table 5). Earlier, Thorpe et al. (1991) and Prutpongse and Gavinlertvatana (1992) have also reported micropropagation of some of the bamboo species in MS media supplemented either with cytokinine or BAP.

Rooting

In vitro rooting was observed in both the species of bamboos by adding different concentrations of auxin either in full or half strength of MS basal media. In *D. stocksii*, the half strength of MS media was suffice for *in vitro* rooting, however, in case of *B. tulda* full strength resulted comparatively in better rooting percentage (Table 6). In *D. stocksii*, rooting percentage was maximum (81%) with 4.2±0.39 roots when half strength of MS media was supplemented with NAA 2.5 mg/l (Figure 4f and g). However, in *B. tulda*, full strength of MS media containing NAA 5 mg/l resulted in maximum percent (97%) of rooting with 6.44±2.15 roots in average (Figure 3f and g). Among different auxins, NAA was the most effective hormone for root induction and growth for both species. However, previous workers reported high efficiency of rooting in IBA alone in case of *Bomarea glaucescens* (Shirin and Rana, 2007) or by supplementing combinations of auxins in *Dendrocalamus strictus* (Chaturvedi et al., 1993). Responses of phytohormones on cultures might be influenced with the nature of strains of different species. There are several reports, suggesting half MS strength as most appropriate for rooting, however, in case of *B. tulda* we found MS full strength as most suitable for *in vitro* rooting. In half strength of MS, there was profuse rooting but the mortality rate was comparatively high due to dryness and browning. This might be due to nutrient deficiency. Root induction is the high energy demanding process and thereby, the endogenous requirements of metabolic substrates might vary in different species (Yasodha et al., 2008; Sandhu et al., 2018).

In conformity with the previous reports on different species of bamboos, we observed half strength of MS media as more effective in root induction than MS full strength for *D. stocksii.* Singh et al. (2012) on *D. asper* and *D. hamiltonii*, Ramanayake and Yakandwall (1997)

Table 5. Effect of additives on shoot multiplication of *B.tulda* and *D. stocksii*.

Different concentration and combination of additives	*B. tulda*		*D. stocksii*	
	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
Without Additives	21.44±7.15^{a}	4.64±1.55^{a}	11.8±1.01^{ab}	4.93±0.16^{b}
50 mg Ascorbic acid	9.56±3.19^{b}	2.91±0.97^{b}	4.4±0.27^{ef}	3.369±0.09^{de}
50 mg Ascorbic acid + 50mg Cysteine	4.33±1.44^{defg}	2.31±0.77^{bc}	5.2±0.42^{e}	2.627±0.08^{f}
50 mg Ascorbic acid + 25mg Cysteine	8.22±2.74^{bc}	2.26±0.75^{bcde}	9.5±0.43^{cd}	3.44±0.17^{d}
50 mg Ascorbic acid + 50 mg Citricacid	4.67±1.56^{def}	2.28±0.76^{bcd}	4.9±0.28^{ef}	2.6±0.10^{f}
50 mg ascorbic acid + 25 mg citric acid	5.78±1.93^{cde}	1.79±0.66^{cdef}	10.78±1.23^{bc}	4.4±0.09^{c}
50 mg Ascorbic acid + 25 mg Cysteine+ 25 mg Citric acid	6.33±2.11^{cd}	1.60±0.53^{f}	13.3±0.67^{a}	6.69±0.18^{a}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Table 6. Effect of different auxins in rooting (%) and numbers of roots of *B. tulda* and *D. stocksii*.

Auxins concentration	*B. tulda*		*D. stocksii*	
	Rooting (%)	Number of roots in clump (Mean±SE)	Rooting (%)	Number of roots in clump (Mean±SE)
MS + NAA (1.0 mg/l)	43	3±0.17^{d}	42	0.5±0.17^{efghij}
MS + NAA (2.5 mg/l)	75	4.11±0.20^{b}	76	1.5±0.27^{c}
MS + NAA (5.0 mg/l)	97	6.44±0.53^{c}	65	1.3±0.26^{cde}
MS + IBA (1.0 mg/l)	11	0.78±0.22^{ghijk}	17	0.4±0.16^{ghijk}
MS + IBA (2.5 mg/l)	41	1.56±0.24^{fg}	54	1.4±0.22^{cd}
MS + IBA (5.0 mg/l)	45	1.89 ±0.11^{f}	40	1±0.30^{cdefg}
MS/2 + NAA (1.0 mg/l)	33	1.44±0.18^{fgh}	72	0.9±0.10^{defgh}
MS/2 + NAA (2.5 mg/l)	41	2.67±0.25^{de}	81	4.2±0.39^{a}
MS/2 + NAA (5.0 mg/l)	68	3.56±0.24^{bc}	73	2.5±0.34^{b}
MS/2 + IBA (1.0 mg/l)	12	0.44±0.24^{jk}	23	0.3±0.15^{hijk}
MS/2+ IBA(2.5 mg/l)	24	1.11±0.35^{fghij}	60	1.2±0.20^{cdef}
MS/2+ IBA(5.0 mg/l)	30	1.33±0.17^{fghi}	40	0.8±0.36^{cdefghi}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

on *Dendrocalamu giganteus*, Rathore and Ravishankar Rai (2005) on *D. stocksii*. Somasheker et al. (2008) found rooting in one fourth strength of MS media. The low strength of MS media create partial nutrient stress (Singh et al., 2012) and also provide low osmotic potential resulting thereby, plantlets to produce more roots, early adaptation during acclimatization and to induce them to become autotrophic (Arab et al., 2018).

Acclimatization and field transfer

Primary hardening

Well developed *in vitro* rooted plantlets were transferred to greenhouse under closed tunnel. The development and growth of plants were monitored in five different potting mixtures (cocopeat + vermicompost; sand; soilrite; cocopeat; sand + soilrite) for primary hardening. The use of 2:1 ratio of cocopeat and vermicompost was found most suitable potting mixture with high survival rate (79% in *D. stocksii* and 94% in *B. tulda*) for both species (Figure 1). The vermicompost being used in potting mixture was effective in providing high porosity and better aeration for root growth (Singh et al., 2012).

Secondary hardening and field transfer

Influence of seasons on survival of seedlings in hardening

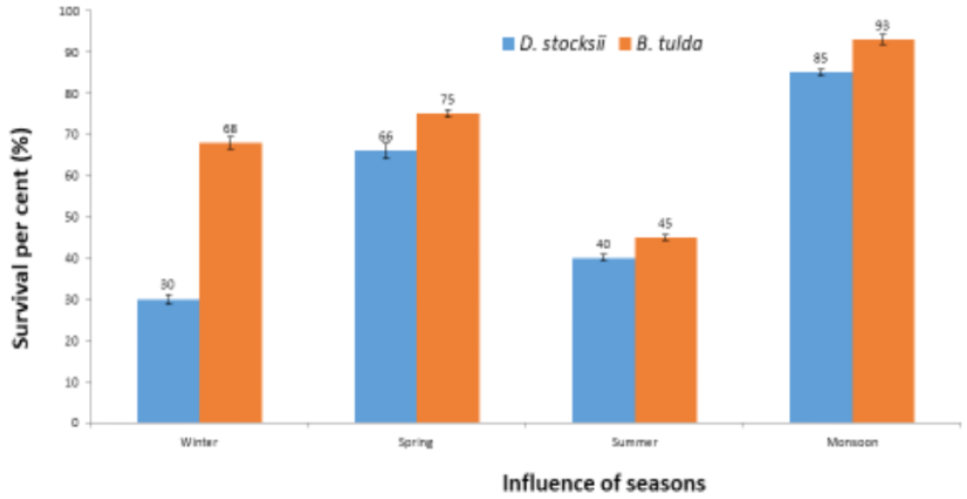

Figure 2. Secondary hardening of *B. tulda* and *D. stocksii* in different seasons.

Figure 3. *In vitro* propagation of *B. tulda* using axillary node explants: (a) Source of explants, (b) Explants used for initiation, (c) One explant inoculated in MS media (MS+NAA 0.1 mg/l+BAP 1.0 mg/l), (d) Bud proliferation, (e) Multiple shoot regeneration, (f &g) Regenerated plantlets with well-developed roots, (h) Partially acclimatized plants in green house, (i) Fully acclimatized plant in shade house, and (j) One years old tissue culture raised plants in field.

was also monitored. After 30 to 45 days of primary hardening, plants were transferred to the Net or Shade house on mother bed constituting of sand and cow dung (1:1). For both species of bamboos, survival rate was maximum during monsoons (93% in *B. tulda* and 85 % *D. stocksii*) (Figure 2). High survival rate in hardening during July and August has also been reported earlier on different bamboos (Mishra et al., 2008; Singh et al., 2012,

Figure 4. *In vitro* propagation of *D. stocksii* using axillary node explants: (a) Source of explants, (b) Explants used for initiation, (c) One explant inoculated in MS media (MS+NAA 0.1 mg/l+BAP 1.0 mg/l), (d) Bud proliferation, (e) Multiple shoot regeneration, (f & g) Regenerated plantlets with well-developed roots, (h) Partially acclimatized plants in green house, (i) Fully acclimatized plant in shade house, and (j) One years old tissue culture raised plants in field.

2021). With the important variables taken into account, the study reveals about the large scale mass clonal propagation of two important bamboo species (*B. tulda* and *D. stocksii*). These findings can be helpful for industrial adoption of *in vitro* propagation technology for large scale commercial production.

However, in tissue culture raised plants, heterogeneity to some extent limit the purpose of *in vitro* propagation system. Therefore, it is advisable to test clonal fidelity after 10 sub culturing cycles. In our investigations, we followed up to 9-10 cycles of sub-culturing during multiplication. Axillary branching is, however, least susceptible to soma clonal variations (Negi and Sexena, 2011).

Conclusion

Because of the long flowering cycles of *B. tulda* and *D. stocksii* and the limitations of vegetative propagation, the difficulties are confronted with regards to the supply and the growing demand of these two commercially important bamboo species. In the present investigation, an efficient refined protocol for large scale mass clonal propagation, multiplication with high rooting and acclimatization in the soil with abundant growth performance has been developed. These findings can be helpful for industrial adoption of *in vitro* propagation technology for large scale production of high quality planting materials (true to the type). These two bamboo species have tremendous potential to develop agro industrialization in rural areas and to bring marginal lands into use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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