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Low cost alternatives for micro propagation of Moringa (*Moringa stenopetala* L.)

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Abstract

Moringa (*Moringa stenopetala*) is widely cultivated in southern Ethiopia for its nutrition and medicinal value. Micro propagation to preserve the plant becomes costly due to the tissue culture media cost. The aim of the study was to evaluate low cost alternative media components for micro propagation of Moringa (*Moringa stenopetala*). Enset starch as gelling agent and table sugar as a carbon source were used as a low cost alternative media components for moringa culture initiation and multiplication using shoot tip. The highest mean numbers of shoots per explants (6.94±0.26), shoot length (4.88±0.16cm) and leaf number (17.07±0.86) were obtained in media containing 80g/l enset starch as an alternative gelling agent. The response of the explant for 30g/l sucrose and table sugar were not significantly different. Utilization of enset starch as gelling agent reduced the gelling agent cost by 70% while table sugar reduced sucrose cost by 95%. The micro propagation rates under the experimental conditions using the low cost media alternative were equal to or higher than the conventional media components. Therefore, the obtained result can be used in commercial micropropagation of moringa. However, studies on transparency of enset starch gel at higher concentration could be explored for wider application in tissue culture laboratories.

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Introduction

Moringa is a softwood perennial tree that belongs to the monogenetic family Moringaceae which is native to Asia and Africa. *Moringa stenopetala* is commonly cultivated in South Ethiopia, North Kenya and Eastern Somalia (Fahey, 2005). Its leaves are known for high nutritional value which is eaten as a fresh salad, cooked or dried in the form of powder. It is rich in vitamins, minerals and proteins. Moringa seeds are also rich in oils. In addition, its leaves have medicinal value such as antimicrobial and anti-diabetic effects (Fahey, 2005). Therefore, it has a potential to alleviate nutritional deficiency in developing countries.

Moringa is a cross pollinated plant and traditional propagation is by seeds and shoots cuttings. Sexual propagation needs presence of sufficient individual plants in the area and high seed germination efficiency (Nouman *et al*., 2012). Propagation using the cutting of the mother plant limb causes death of the mother plant. However, this may also be not possible in some cases and may even lead to loss from cultivation. Preservation of the species for ethno-botanical, dietary and pharmacological applications is a growing concern. Micro-propagation is an alternative procedure, but high cost associated with the culture media prevented Ethiopia from benefiting from tissue culture technology.

Tissue culture technology normally requires sophisticated equipment's and expensive chemicals which makes its operation costly (Chen, 2016). It offers an opportunity for producing healthy plantlet materials and seed system for vegetatively propagated plants (Twaij *et al*., 2020). However, adoption of the technology in Ethiopia is slow. This is mainly due to high cost of operation, requirement of sophisticated equipment's and expensive chemicals (Amare and Dugassa, 2022). There is high demand for tissue cultured products such as hybrid coffee plantlets by farmers, but little supply and high cost makes unaffordable for tissue culture products (Ogero *et al*., 2011; Abebaw *et al.,* 2021).

To alleviate such problems, low cost alternatives for chemicals of high purity, temperature and photoperiod controlled growth were reported in many literatures. To mention some, commercial grade sugar in India for banana micro propagation (Ganapathi *et al*., 1995), solar illuminated tissue culture rooms in Cuba, refined and unrefined white and brown sugar for coconut culture (Bonaobra *et al*., 1994; Silvosa-Millado *et al*., 2020) and sugarcane in Ethiopia (Getnet *et al*., 2016). In addition, gelling agents such as isubgol, sago, enset starch, corn starch for the culture of fruit crops were reported (Bhattacharyya *et al.,* 1994; Zimmerman *et al*., 1995; Mengesha *et al*., 2012; Amlesom *et al*., 2021; Dhawale *et al*., 2021). The aim of this study was to evaluate the possibility of using locally available gelling agents and carbon sources for moringa micro propagation.

Materials and methods

Explant and media preparations

Moringa stenopetala seeds were collected from Jimma agriculture research center, Ethiopia. The seed pods were removed, sterilized and allowed to germinate on petridish with cotton support moisten with distilled water until sprouted out its radicle. Then the germinated seeds were aseptically transferred to MS medium free of growth regulators (MS+3% sucrose + 0.8% agar). After emergence of the first pair of leaves, the shoot tip of the seedlings were taken and used as sources of the explant for tissue culture experiments.

MS media supplemented with BAP growth regulator was used (Murashige and Skoog, 1962). Stock solutions of the macronutrients, micronutrients, vitamins, iron source and plant growth regulators (1mg/ml) were prepared and stored at +4°C in refrigerator for immediate use. The culture medium containing 30g/l sucrose and 1mg/l BAP was prepared from their respective stock solutions. The mixture was stirred using magnetic stirrer and the volume was adjusted using double distilled water. Then, pH was adjusted in all cases to 5.8 using 1M NaOH or 1M HCl. Finally, 8.0g/l agar, 40g/l, 60g/l, 80g/l or 100g/l enset starch was added to the medium and heated to melt. Before autoclaving, the media were dispensed into sterilized culture jars. The media were steam sterilized using autoclave at a

temperature of 121°C with a pressure of 0.15 Kpa for 15m and transferred to the culture room and stored under aseptic conditions for later use (Kumari and Singh, 2012).

Culture initiation

Healthy shoot tips of Moringa were collected as explants. The explants were then washed with distilled water and sterilized by dipping in 70% ethanol for 1min in a sterilized jar and washed using sterile distilled water three times for 5 min. They were then sterilized with 1% (v/v) commercial bleach (NaOCl) solution containing 3-4 drops of Tween-20 for 15 minutes and rinsed 4 times with sterile double distilled water each for 5min with gentle shacking to remove the chemical residue. The damaged parts were excised off using a sterile scalpel and about 1cm long explants were cultured into the nutrient media. The cultures were maintained at room temperature in the growth room with white florescent lamps of 16/8h light and dark photoperiod, respectively (Vettorazzi *et al*., 2017).

Shoot multiplication

For shoot multiplication experiment, the initiated shoots (2-3cm long) were taken and cultured on fresh MS medium containing galling agent (8g/l agar, 40g/l, 60g/l, 80g/l, or 100g/l enset starch), 30g/l sucrose or table sugar and 1.0mg/l BAP. The treatments were replicated three times. The experiment was arranged in completely randomized design with three replications with five shoots per jar. The cultures were placed in white florescent light room adjusted at 16/8h light/dark at 25°C. Shoot number, shoot length and leave number were recorded after 5 weeks of culturing (Vettorazzi *et al*., 2017).

Rooting of generated shoots

For *in vitro* rooting experiment, well developed shoots were cultured on hormone free MS medium for avoiding carry over effect. The shoots were transferred on fresh MS medium containing galling agent (8g/l agar, 40g/l, 60g/l, 80g/l, or 100g/lenset starch), 30g/l sucrose or table sugar and 1.0mg/l IBA. The experiment was laid down in completely randomized designs (CRD) with three replications. After a month, number of roots, root length and percentage of rooted plantlets were recorded (Ayenew *et al.,* 2012; Ayelign *et al*., 2012).

Acclimatization

Plantlets with well-developed root and leaves were washed with tap water to remove adhering media and sucrose attached on the roots of plantlets. Thirty plantlets were transferred to plastic 30cm diameter pots in greenhouse containing hardening medium composed of soil, compost and sand at ratio of 1:1:2, respectively. The plants were placed in pots covered with transparent plastic bags and irrigated using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks besides reducing watering frequency. Finally, after 30 days, the survival rates of the plantlets were evaluated by counting the number of successfully acclimatized plantlets (Khadiga *et al.* 2009; Saini *et al.,* 2012).

Data Analysis

All the experiments were setup in completely randomized design with three replications. The results were expressed as mean ±SD of three experiments. SAS software v9.2 (SAS Institute, 2008) was used for analysis of variance and significance difference between treatments. Means separation was done with Least Significance Difference (LSD) at $p < 0.01$.

Result

Cost comparisons

The cost of moringa micropropagation was significantly reduced using low cost media substitutes for gelling agent and carbon source. Cost reduction of 70% and 95% for gelling agent and carbon source, respectively, were achieved (Table 1).

Table 1. Cost analysis between conventional media components and low cost alternatives.

Conventional MS medium(g/l)	Low cost substitute (g/l)	Cost in 1litre of the medium (ETB)		Cost reduction (%)
		Conventional	Low cost alternative	
8g agar	60gEnset starch	20	6	70%
30g Sucrose	30g table sugar	30	1.5	95%

1USD= 54.5ETB

Shoot initiation and multiplication

Shoot induction in conventional and alternative gelling agent is not significantly different. Hundred percent of initiation was observed from four different concentrations of gelling agents (8g/l of agar and 60g/l, 80g/l and 100g/l of enset starch). But the 40g/l enset starch resulted 86.68% shoot induction (Table 2). At enset starch concentration of 60g/l, reduced gel strength and anchoring capacity of *in vitro* explants were observed. The anchoring quality of enset starch gel was improved at concentrations above 60g/l and become stronger at 80g/l enset starch.

Table 2. Effect of bulla concentration for *in vitro* shoot initiation percentage.

Gelling agent(g/l)	% of initiation
Agar (8g)	100^{a}
Enset starch (40g)	86.68^{b}
Enset starch (60g)	100^{a}
Enset starch (80g)	100^{a}
Enset starch (100g)	100^{a}

The same letter indicates no significant difference in their mean value at $p<0.01$.

The number of shoots, shoot lengths and number of leaves produced on conventional media and media containing 80g/l enset starch as gelling agent did not show significant difference. An average of 6.94±0.26 shoots with 4.88±0.16cm shoot length and 17.07±0.86 leaves per plantlets were recorded at enset starch concentration of 80g/l. The 60g/l enset starch concentration also resulted relatively lower shoot multiplication response (4.55±0.2) compared with agar.

Enset starch effect on root development

The rooting experiment indicated that higher root lengths of $6.51±0.49^{cm}$, $6.41±0.54^{cm}$ was obtained on gelling agents of 8g/l agar and 60g/l enset starch, respectively (Table 3). However, 80g/l enset starch showed reduced root length (5.51±0.43cm) but with better gelling quality for the explants to anchor. The 100g/l gel is very hard, reduced root elongation and development. The times taken for rooting the plantlets were longer for the low cost gelling agents. In general, the response of the explants for shoot induction, multiplication and rooting in media containing the conventional agar and 80g/l enset starch as gelling agent were almost similar. From our observation, it is recognized that increasing the concentration of enset starch reduces gel transparency which makes detection of contamination very difficult in tissue culture jars (Fig. 1C).

Table 3. Different gelling agent concentration of multiplication media.

Gelling agents(g/l)	No of shoot	Shoot length(cm)	No of leaves	No.root	Root length(cm)
Gelling agent					
Agar(8g)	$7.01±0.3^{a}$	$4.90±0.18^{a}$	$16.81±1.05^{a}$	$9.09±0.73^{a}$	$6.51±0.49^{a}$
Enset starch (40g)	$1.76±0.134^{e}$	$2.08±0.13^{e}$	$6.07±0.26^{f}$	$1.51±0.21^{b}$	$2.60±0.44^{b}$
Enset starch (60g)	$4.55±0.2^{d}$	$3.11±0.25^{d}$	$6.82±0.16^{f}$	$8.51±0.21^{a}$	$6.41±0.54^{a}$
Enset starch (80g)	$6.94±0.26^{a}$	$4.88±0.16^{a}$	$17.07±0.86^{a}$	$8.41±0.41^{a}$	$5.51±0.43^{b}$
Enset starch (100g)	$6.412±0.29^{c}$	$3.43±0.22^{c}$	$8.01±0.0.74^{e}$	$2.16±0.19^{b}$	$3.21±0.28^{c}$
Carbon source					
Sucrose 30g	$6.41±0.44^{a}$	$4.61±0.20^{a}$	$15.14±28^{a}$	-	-
Table sugar 30g	$6.35±0.35^{a}$	$4.50±0.37^{a}$	$15.00±0.74^{a}$	-	-

The same letter indicates no significant difference in their means at $p<0.01$

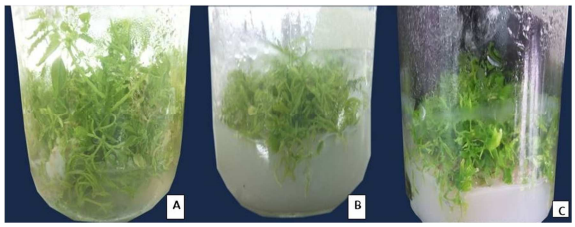

Fig. 1. Effect of gelling agent on multiplication of moringa, A) 8g/l agar, B) 60g/l enset starch, C) 80g/l enset starch (scale by bar 2cm).

Out of the sucrose and table sugar concentrations tested, 30g/l sucrose and table sugar resulted similar shoot multiplication response on moringa. Maximum shoot number of 6.41±0.44 and 6.35±0.35; shoot length of 4.61±0.20cm and 4.50±0.37cm and leaf number of 15.14±28 and 15.00±0.74 were recorded for 30g/l sucrose and table sugar, respectively (Table 3).

Any increase in concentration above 30g/l for both sucrose and table sugar did not show further increase in shoot multiplication response. However, higher rooting recorded at high sucrose concentrations (Data not presented).

For hardening experiment in the greenhouse, the rooted shoots were removed from the culture bottles, washed thoroughly to remove remnants of agar and transplanted to pot containing soil and compost. The plants were regularly watered and covered with plastic cover to ensure high humidity. After a month of acclimatization, 84% of plantlets were survived and successfully established from *in vitro* rooted plantlets (Fig. 2).

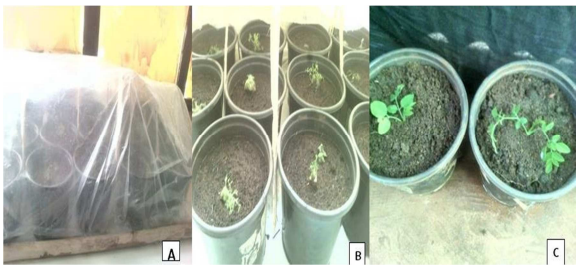

Fig. 2. Acclimatized plantlets of Moringa; A) plantlets covered with plastic cover (first week), B) after two weeks in green house, C) after four week in green house.

Discussion

Cost associated with nutrient media is one of the points that need intervention to advance tissue culture technologies in sub-Saharan Africa (Pant, 2016; Amare and Dugassa, 2022). There are no significant differences between conventional media components and low cost alternative media components such as enset starch as gelling agent and table sugar as carbon source suggesting the ability of enset starch to support and supply nutrients to the moringa explants. The explant response is as good as the conventional agar. Interestingly, growth of shoots and roots were not halt but enhanced due to some inherent nutritional composition of the enset starch. Different reports exist showing efforts to address media cost. For instance, table sugar has been utilized as an alternative carbon source ((Kaur *et al.*, 2005; Demo *et al.*, 2008). Molasses has been used as sources of vitamins and carbohydrates (Santana *et al.,* 2009). Furthermore, different kinds of fertilizers in form of spray have been used as alternative sources of mineral elements (Mahalakshmi *et al.,* 2018). Some of the mineral elements such as silver nitrate reported to function as hormone supplements during shoot multiplication phase (Cardoso, 2019).

In this experiment, substitution of carbon source with table sugar resulted highest cost reduction. Cost reduction of up to 70% was achieved using enset starch as gelling agent without its effect on shoot multiplication and rooting. Similar results with 66 to 90% gelling agent cost reduction were reported using potato starch, rice flour, cassava flour and corn flour (Daud *et al.,* 2011; Sharifi *et al.*, 2010; Ogero *et al.*, 2011; Amlesom *et al.*, 2021) and balanga seeds (Jabeen *et al.,* 2017). Therefore, use of low cost alternative media effectively reduced production cost for moringa micropropagation.

Well-developed leaves and stems were essential for photosynthesis and hence for good acclimatization in green house systems which further determines survival of the tissue culture plantlets. Gelling agents are known to affect leaf development, *in vitro* growth (Nery *et al.*, 2021) and callus development (Kumar *et al.*, 2021). The shoot development response of moringa explants on conventional and low cost alternative media did not show any significant difference. In both cases they have well developed leaves and shoots. Eight gram enset starch had superior performance in number of shoots and shoots length as well as number of leaves development then followed by 60g/l and 100g/l enset starch, respectively. A similar result was reported for micropropagation of sweet potato using alternative gelling agents (Ogero *et al.,* 2011) and pineapple using enset starch as alternative gelling agent (Ayenew *et al.*, 2012). In addition to gelling agent, applications of table sugar resulted similar shoot production response, which is in agreement with Getnet *et al.* (2016) report by using 40g/l table sugar.

Production of tissue culture plantlets with well-developed roots in *in vitro* or *ex vitro* conditions enhance plant survival during acclimatization (Jorge, 2002; Lebedev *et al.,* 2019). The rooting response of Moringa in conventional media components and low cost media alternatives such as 80g/l enset starch shown significant differences. They have relatively different performance with the conventional media component on rooting. The number of roots in conventional media is relatively higher than low cost media alternatives.

This is due to factors like availability of minerals and inhibitory hormones in enset starch that inhibit rooting.

Conclusion

In conclusion, the use of low cost media alternative for micropropagation of moringa is an indication for the possibility to use low cost alternatives for production tissue culture plantlets to improve productivity of farmers in sub-Saharan Africa. Use of these media alternatives to other crops will enhance establishment of affordable tissue culture systems in Ethiopia as well. In addition, improvement in the transparency of enset starch gel could be explored for wider applications. The described protocol can be successfully used for the low cost commercial propagation of moringa.

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