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ORIGINAL ARTICLE

In-vitro propagation of multi-use enset [*Ensete ventricosum* (Welw.) Cheesman] landraces using *bulla* as gelling agents

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Abstract

Enset is a perennial, multipurpose crop that is cultivated and consumed in Ethiopia. Nowadays, its traditional propagation systems face a challenge due to biotic and abiotic factors. Thus, shoot tip culture can be very advantageous for the quick multiplication of healthy plantlets to secure the conservation as well as propagation of the enset crop. Therefore, this study was designed to develop an efficient micro-propagation protocol for three popular multi-use enset genotypes by using locally available *bulla* and agar as gelling agents separately. The experiment was conducted in a completely randomized design with three replications in a factorial arrangement. About 1.0 cm long shoot tips were cultured on MS medium supplemented with 1 to 6 mg/l of BAP separately or in combination with IAA. It was found that the 8% (w/v) enset *bulla* was ideal and provided significant figures in the number and length of shoots and roots per shoot and also early initiation of shoots and roots when compared with 0.6% (w/v) agar-gelled MS media. MS medium containing 2.0–3.0 mg/l BAP was the appropriate concentration for in-vitro shoot induction and growth. The presence of 5.0 mg/l BAP alone, and 5.0 mg/l BAP in combination with 1.0 mg/l IAA was suitable for multiple shoot induction, whereas, 2.0 mg/l IBA and 1.0 mg/NAA separately were found to be the optimum concentration for root induction and development. Thus, *bulla* in addition to its alternative gelling potential with low cost has potential play an essential role in the rapid production and conservation of enset with desirable traits and disease-free plantlets for farmers.

Key message

Enset is an important food security and multipurpose crop. *Bulla*, which is extracted from enset, is an alternative gelling agent that is more efficient in the in vitro propagation of three different multi-use enset genotypes when compared to agar gelled medium.

Keywords Alternative solidifing agent · Conservation · Micro-propagation · Plant growth regulators · Shoot-tip · Tissue culture

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Abbreviations

AC	Activated charcoal
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BAP	6-Benzylaminopurine
CRD	Completely randomized design
DMRT	Duncan's multiple range test
IAA	Indole − 3-acetic acid
IBA	Indole-3-butric acid
MS	Murashige and Skoog
NAA	*α*-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
PGR	Plant growth regulator
SAS	Statistical analysis system

Introduction

Enset (*Ensete ventricosum* (Welw.) Cheesman) is a giant perennial herbaceous monocarpic multipurpose crop. It belongs to the family Musaceae in the order Zingiberales. The Musaceae family comprises the genera *Musa* and *Ensete*, which are found in both Africa and Asia (Borrell et al. 2019). *E. ventricosum* is likely the most widely distributed species within Musaceae, existing throughout much of central, southeast, and east Africa (Baker and Simmonds 1953). However, its domestication, cultivation, and human consumption as food are limited to Ethiopia (Brandt et al. 1997). Enset is a major food crop for more than 25 million people living in central, south, and southwest Ethiopia (Yemataw et al. 2016; Borrell et al. 2020). It is also used for animal feed, fuel wood, construction materials, and as a traditional herbal medicine for different human and livestock diseases (Brandt et al.1997).

Enset-based farming system is an indigenous and sustainable agricultural system that covers large areas of land in Ethiopia (Westphal 1975; Borrell et al. 2020). Farmers in the enset growing regions have a wealth of knowledge in tackling variety selection and cultivations, as well as pest and disease management which are well adapted to their socioeconomic and environmental conditions (Yemataw et al. 2016). Since enset is an indigenous crop, almost all production and processing practices are based on farmers' experiences (Bosha 2018).

Cultivated enset is most commonly propagated by vegetative means using the corm of a three- or four-year-old plant after cutting away the pseudostem at about 10 cm above the ground, then exposed to sunlight for two days, and subsequently buried again in the soil after removing the apical meristem area from the central part of the corm. After 2–3 months, new suckers will emerge (Tsegaye 2002; Blomme et al. 2018). This traditional method, however, is very tedious and laborious (Birmeta and Welander 2004) and also results in a poor propagation rate and diseased planting materials (Tripathi et al. 2017). Although enset can also be propagated by seeds, seed production is not a common practice, as farmers do not usually postpone harvesting until the maturity of the seeds (Negash 2001; Abebe 2005). During the maturation of the seeds, the enset becomes dried up, resulting in a total loss of its food value (Negash et al. 2000). Moreover, the hard, irregularly shaped seeds are very difficult to germinate. This results in a low germination rate (12%) and a long germination time (Karlsson et al. 2015).

According to Westwood et al. (2021), more than 20% of plant species are threatened with extinction. Therefore, to establish a sustainable food system, it is essential to conserve plant species, especially food crops, and their regionally well-adapted cultivars (Bhat et al. 2022). Plant tissue culture is a technique that involves the growth and multiplication of totipotent cells, tissues, and organs of plants on defined solid or liquid media comprising nutrients under an aseptic and controlled environment (Thorpe 2007; George et al. 2008) and will assist in conserving rare plants from extinction (Pegg 2002). It is also used for cryopreservation, conservation of rare and highly endangered plants, and the production of secondary metabolites (Kaczmarczyk et al. 2011; Coste et al. 2012; Chandana et al. 2018). Micropropagation is one of the tissue culture techniques used for the production of 'disease-free', high-quality, and uniform planting material within a relatively short time for commercial purposes, independent of the season (Feyissa et al. 2005; Garcia-Gonzales et al. 2010). However, the full application of these techniques depends on the cost of their ingredients. Of the different gelling agents, agar is the most commonly used and expensive gelling agent for the preparation of solid and semi-solid media for plant culture as compared to other ingredients (Babbar et al. 2005; Esekiel 2010; Jain-Raina and Babbar 2011), and it contributes about 70–75% of the total production cost (Deb and Pongener 2010; Ebile et al. 2022). Subsequently, efforts have been taken to identify less expensive alternatives to agar as gelling agents to reduce medium costs without compromising on the micropropagation rate or the quality of the plants produced. According to Nene and Sheila (1999), Raghu et al. (2007), and Daud et al. (2011), various alternative gelling agents such as potato, rice, barley, wheat, cassava, and corn starches have been used as sources, either singly or in combination, with varying degrees of success.

During traditional processing, enset crops produce three main starchy food products: *qocho*, *bulla*, and corm (*amicho*) (Tsegaye and Struik 2002). *Bulla* is one of the starchy products of enset, which is obtained by squeezing a mixture of the unfermented decorticated pseudostem and pulverized corm and decanting the liquid, followed by air drying (Daba and Shigeta 2016). It is considered the best quality enset food (Wolde-Gebriel et al. 2006) and is consumed mainly as porridge, in gruel, and crumbled form (Olango et al. 2014). According to ESTC (2003), starch produced from enset can be used for the paper, textile, and adhesive industries. Enset starch also has the potential to be used in binding and disintegrating compressed tablets (Gebre and Nikolayev 1993; Gebre-Mariam and Schmidt 1996). Moreover, enset derivative flour or *bulla* in in vitro propagation media of pineapple (Ayenew et al. 2012), vanilla (Mengesha et al. 2012), and cassava (Ayalew et al. 2017) has been used as a gelling agent by substituting expensive conventional agar and saving the production costs of the culture media. Hirose et al. (2010) verified that enset starch is used for both industrial and food purposes and found it to have high gelatinization properties.

The varietal diversity of the enset crop is relatively wide,and due to these features and others, the nutritional composition of the enset product *bulla* showed variability. The reports of Daba and Shigeta (2016) and Tuffa (2019) indicated that the proximate values ranged from 0.45 to 1.0% (for protein, fat, fiber, and ash) and minerals content in mg/100 g (calcium, potassium, magnesium, phosphorous, iron, and zinc were 11.4–58.7, 270–337, 5.2–11.9, 30.1–33.0, 2.5-7.0, and 0.2–4.5, respectively) for *bulla* from different enset landraces.

Despite the significance of enset as a food, feed, fiber, and medicinal crop, insufficient research has been conducted to improve its cultivation and production, and also to maintain its genetic resources. Moreover, nowadays many landraces are disappearing from farmers' home gardens due to biotic and abiotic factors, farmers' selection pressures, and changes in land use systems. Hence, micropropagation could be very advantageous for enset to produce healthy plantlets free from diseases that are easily transmitted when suckers are used as a source of new planting materials. Furthermore, micropropagation serves as a backup strategy for the conservation of the existing diversity of enset germplasm. Although there are a limited number of papers on the micropropagation of enset, as far as we know, no reports have been published in which *bulla* has been used as a gelling agent in in-vitro plant tissue culture on enset plants. Therefore, this study aims to develop and optimize an efficient protocol for the shoot tips of farmers' selected three multi-use enset landraces on *bulla* and agar-gelled media with different concentrations of cytokinin and auxin. The research aimed at testing the hypothesis of whether or not *bulla* is an efficient gelling agent and whether using it would be more economical than using agar.

Materials and methods

Plant materials

Four-month-old suckers of three enset landraces, namely *Astara*, *Gimbo*, and *Sisqella* were collected from a farmer's home garden in Hadiya zone, southern Ethiopia, and were planted subsequently in pots containing different soil mixtures in the greenhouse of Addis Ababa University until they resumed growth for three to five months before culturing, as shown in Fig. 1A. These landraces are highly recognized by local people for their medicinal and edible sweet corm (*Astara*), high yield and quality of *qocho* and *bulla* (*Gimbo*), strong and durable fiber, and early maturing ability (*Sisqella*) (unpublished data) and are also supported by previous works (Tsegaye and Struik 2002; Yemataw et al. 2014).

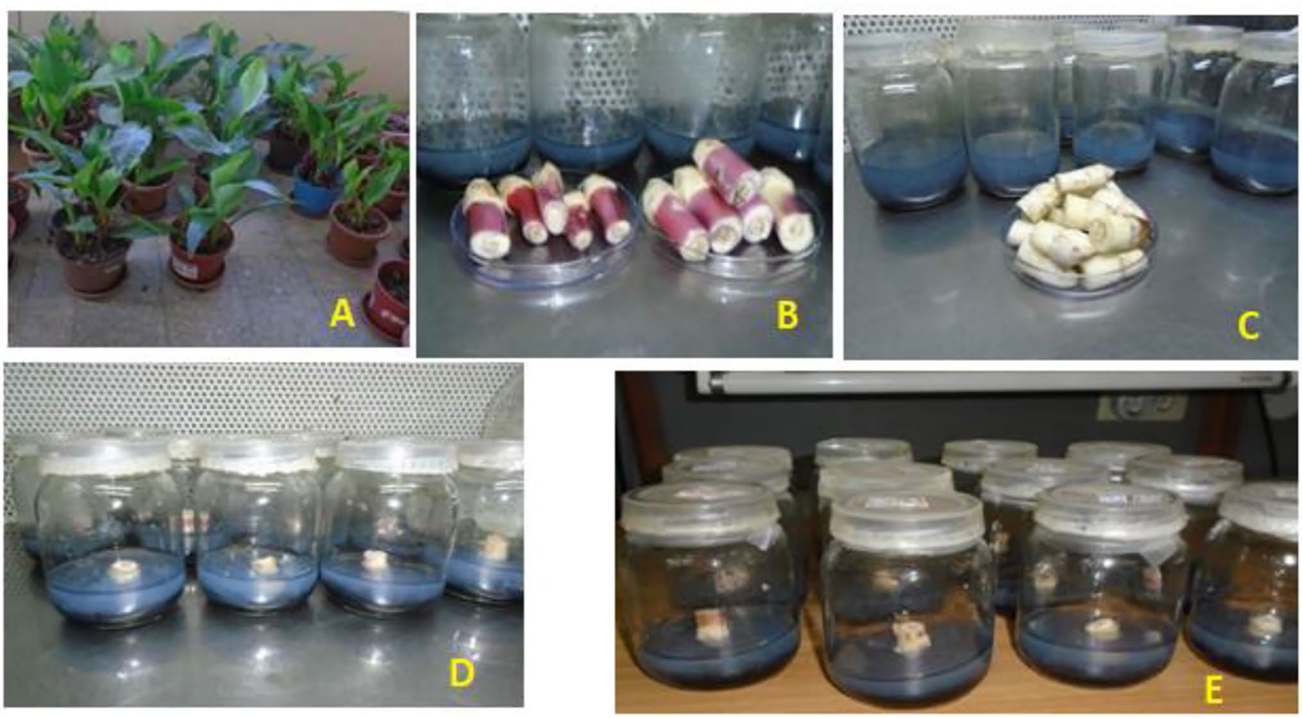

Fig. 1 Some mother enset suckers used as explant source, preparation for shoot initiation and shoot tip culture on different gelling agents: (**A**) Enset mother sucker used for explant source in the greenhouse; (**B**) Trimmed enset genotype *Astara* (left) and *Gimbo* (right); (**C**) Aseptically prepared genotype *Sisqella* sucker in laminar flow hood cabinet ready for shoot tip inoculation; (**D**) Shoot tip culture on 0.6% agar gelled MS medium; (**E**) Shoot tip culture on 8% *bulla* gelled MS medium

Extraction and analysis of some nutritional contents of *bulla*

The mass mixture from the scraped pseudostem and grated corms of two mature enset landraces of *Gimbo* were squeezed into the pit which was covered with enset leaves and a plastic sheet by using the bamboo-made sieve to collect the liquid. Then the resulting liquid was left overnight for sedimentation and the supernatant was discarded to obtain *bulla* (Fig. 2). Thereafter, its surface was rinsed with clean water and dried in a moisture extraction oven. The dried *bulla* was milled into powder using a milling machine and weighed, packaged in zipped polythene bags to prevent rehydration, and stored in cool dry cardboard until required.

The extracted *bulla* flour was characterized for moisture content, crude protein, crude fat, crude fiber, and total ash using the methods developed by the Association of Official Analytical Chemists (AOAC 2000). The procedures, respectively, were 925.09, 979.09, 920.39, 962.09, and 923.03 in which triplicate analysis was carried out in all cases. The total carbohydrate content was calculated by difference from other nutrients, using the formula as follows: Carbohydrate (%) = 100 – (% crude protein + % crude fiber + % total ash + % crude fat). The pH of *bulla* flour was determined from a 1/10 dilution of the sample. Minerals analysis were also determined according to the standard method of AOAC (2000) at the Center for Food Science and Nutrition, and in the Department of Chemistry Addis Ababa University, Ethiopia.

Gelling of culture medium using *bulla* flour

For this experiment, the MS culture medium was gelled with 5%, 6%, 7%, 8%, 9%, and 10% *bulla* flour, and 0.6% agar (Agar Plant Culture Test, Himedia Pvt. Ltd., Mumbai, India) was used as a control. The *bulla* flour was mixed with a portion of the cold culture medium in a 1-liter beaker separately, and then it was gently added to the remaining preheated medium at about 80^0 C while being continuously stirred vigorously; otherwise, it settles at the bottom of the MS media jar. The pH of *bulla* flour was acidic (based on this study and other previous reports). Hence, it was required to adjust the pH to 5.75 just after the addition of *bulla* flour in the growth medium. The gelled culture medium was cooled down to about 60^0 C, carefully dispensed into 50 ml glass jars or Magenta GA-7 culture vessels, and autoclaved at 121^0 C for 20 min. Then it was allowed to cool in a laminar airflow hood before culture.

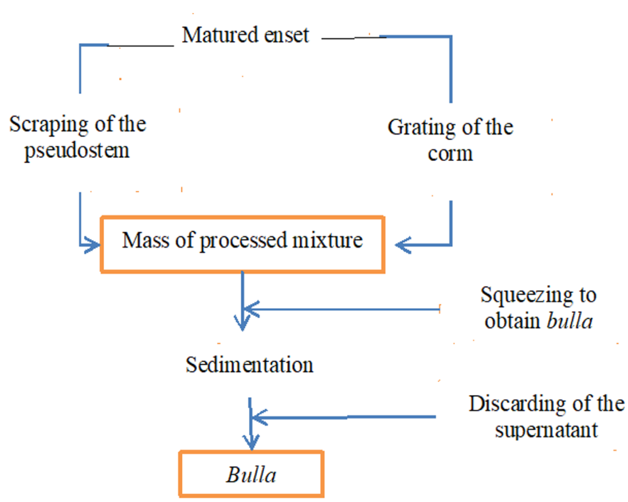

Fig. 2 Simplified flowchart that shows how *bulla* was produced

Preparation of stock solution and culture media

In this experiment, the MS (Murashige and Skoog 1962) basal medium was used throughout each activity. The full strength (for initiation and multiplication) and the half strength (for rooting) of each stock solution were individually prepared and then stored at -20 °C until the experiment was employed. In the same manner, the plant growth regulators (PGRs) used for this study were 6-benzyl aminopurine (BAP), α-naphthalene acetic acid (NAA), indol-3-butyric acid (IBA), and indol- acetic acid (IAA). Full-strength MS medium was prepared, and 3% sucrose was used as a carbon source, and 0.2% of activated charcoal (AC) was incorporated to prevent blackening due to polyphenol oxidation of explants (Diro et al. 2004). Different concentrations of PGR were supplemented as given later, followed by adjusting the pH to 5.75, and subsequently, 6.0 g/l agar was added and melted on a stirring hot plate.

Culture initiation

Healthy suckers of explants were uprooted, and the leaves, pseudostem, and roots were carefully removed without affecting the shoot tips and corms. The explants were washed with household detergent and running tap water for 5 to 10 min. The outer layers, or leaf sheaths, of the explants were detached, and the shoots were trimmed to 3.0 cm length and 3.0 cm width (Fig. 1B). Then, the explants were dipped in 70% (v/v) ethanol for 5 min followed by 4 times rinsing in sterile distilled water, and further sterilized with 20% sodium hypochlorite (NaOCl) for 20 and 10 min along with 3 drops of Tween-20, followed by 4 times rinsing with sterile distilled water. In each sterilization step, the outer tissue of the suckers that were exposed to disinfection solutions was removed, and the shoot tips were trimmed from all edges. Thereafter, the size of each explant shoot tip was further trimmed to 1.0 cm in length (Fig. 1C). The sterilized shoot tips of explants were cultured on a shoot initiation medium

(Figs. 1D and E) and maintained in a relatively controlled culture room at 16 h photoperiod and $40 \pm 5 \mu molm^{-2}s^{-1}$ with light intensity using cool white fluorescent light at 25 ± 2 °C until the end of experiments.

Shoot initiation

For shoot initiation, surface sterilized explants were cultured in culture jars containing 50 ml of MS medium with 3% sucrose, 5–10% *bulla* powder, 0.2% activated charcoal (AC) and supplemented with different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/l) in combination with IAA (1.0 and 2.0 mg/l). The experiment was carried out with three replications for each treatment level, with each Magenta GA-7 or culture jar containing one explant, for a total of 10 explants per treatment. Then, after culturing, the mouth of each culture vessel was appropriately closed with its cap and parafilm. The days for shoot initiation for each genotype and the shoot heights after a month were recorded.

Shoot multiplication

The initiated shoots were transferred to shoot multiplication MS medium supplemented with different concentrations of plant growth regulators as in shoot initiation. During transferring, the induced shoot tip was carefully removed near the corm without harming it by using a sterile surgical blade. After one month of incubation in the growth room, well-developed plantlets were carefully separated from the explants and transferred to root induction media. The number of days for micro-shoot initiation, the number of induced shoots, and shoot height per explant were recorded.

Root induction

After 30 days of multiplication on MS media, shoots were separated and transferred to a half-strength MS basal medium containing the same concentrations of IBA and NAA 0.0, 1.0, 2.0, and 3.0 mg/l each separately. Growth regulators free MS medium was used as a control. Three replications with one explant per culture vessel were used. Finally, similar to the initiation and multiplication stages, cultures were placed in a controlled growth room and the number and length of roots were recorded after one month of culture.

Acclimatization

After four weeks on rooting medium, healthy plantlets with long roots and elongated shoots were carefully removed from the culture vessel, and the roots were washed thoroughly with tap water. The plantlets were transferred to pots containing a sterile soil mixture of red, compost, and sand in their respective ratios of 1:2:1. The plantlets were covered with a white, transparent polythene bag for five days and then placed in the greenhouse for acclimatization.

Experimental design and data analysis

The experiment was designed in a Completely Randomized Design (CRD) with factorial arrangements of nineteen treatments. Each treatment comprised ten explants in three replications, with one explant per culture vessel. The data for explants` responses was recorded for the number of days to shoot and root initiation, the number of shoots and roots per plant, and shoot and root length (cm). All the recorded data were subjected to statistical analyses using SAS statistical software version 9.4, and an ANOVA was constructed, followed by Duncan's multiple range test (DMRT) at a 0.05% probability level, and the results were presented as means of the independent replications with standard error (SE±). The costs of *bulla* and plant propagation agar were computed using Microsoft Excel 2010. The photograph illustrations were taken with Sonny's 20.1 megapixel camera with 4X optical zoom.

Results

Applying bulla as gelling agent

It was observed that MS media supplemented with *bulla* at a concentration of 50 and 60 g/l didn't solidify. Our result also indicated that MS media containing 70 g/l *bulla* was semi-liquid. However, *bulla* at a concentration of 80 g/l was solidified. This exhibited that *bulla* at a concentration of 80 g/l has a similar nature of gelling and media stability to 6 g/l agar. However, 90 g/l and above became too hard to be used for culture. Based on our result, the mean values of the proximate compositions (moisture, protein, fiber, fat, and ash), total carbohydrate contents, pH, and some mineral values of *bulla* obtained from the enset *Gimbo* genotype are presented in Table 1.

Surface sterilization

In the present study, surface disinfection of shoot tip explants was effective by applying 70% v/v ethanol for 5 min followed by double sterilization with a 20% sodium hypochlorite solution containing 5% active chlorine first for 20 min and then for 10 min with three drops of Tween 20. Thus, this sterilization method for explants showed a considerable reduction in microbial contamination and a

Table 1 Proximate, pH, and minerial values for *bulla* flour of *Gimbo* genotype

Parameter	%	Parameter	mg/100 g
Moisture(wb)	49.4	Calcium	35.4
Crude protein (db)	0.7	Magnesium	14.7
Crude fiber (db)	0.86	Phosphorus	30.4
Crude fat (db)	0.52	Potassium	158.6
Total ash (db)	0.8	Sodium	3.2
Carbohydrate	47.72	Iron	2.8
pH	3.87	Nitrogen	0.11%

N.B: wb = wet basis, db = dry basis

better survival rate of the shoot tips of explants in culture media. However, after 17 days of culture, some microbial growth was observed around the explants on the MS media and destroyed about 53%, 60%, and 71% of explants in the culture for *Sisqella*, *Gimbo*, and *Astara* genotypes, respectively. Most probably, the major source of contamination in this case, was observed to be endophytes. Therefore, to reduce or destroy these endophytic contaminants, 500 mg/l cefotaxime was included in the culture medium. Thus, the application of cefotaxime in the culture medium significantly reduced the loss of explants during this experiment (data not shown here).

Shoot initiation

The shoot tips observed notable differences in days on shoot initiation among the three enset genotypes at 6 different levels of BAP with 2 levels of IAA combinations on the cultured media gelled with 80 g/l of the *bulla* (Tables 2 and 3). The results revealed that the relatively shortest period of days for shoot initiation was in genotype *Sisqella* at 2.0 mg/l BAP alone (4.0 days) and 3.0 mg/l BAP in combination with 1.0 mg/l IAA (4.25 days). In genotype *Gimbo*, single shoot initiation was shown after 5.0 days of culture on MS media with the same levels of BAP, and BAP in combination with 1.0 mg/l IAA as in *Sisqella*. On the contrary, a longer period (6.25 days) was recorded for *Astara*, with a similar concentration of BAP and IAA as in both *Sisqella* and *Gimbo*. However, the maximum period of days in shoot induction was recorded for all genotypes on MS media gelled with 6.0 g/l of agar as compared to 80 g/l of *bulla* with the same concentrations of BAP and IAA (Tables 2 and 3). Based on this result, BAP concentrations at 5.0 and 6.0 mg/l separately or in combination with 2.0 mg/l IAA caused a long period of days in shoot initiation in all explants studied on both gelled media types. This indicated the different responses of genotypes to the growth regulators and gelling agents.

In this experiment, after 30 days of culture on the initiation medium, the length of initiated shoots showed significant variations among genotypes in both gelling agents with different concentrations of BAP alone and combined with two levels of IAA. The highest shoot length was obtained on MS medium gelled with *bulla* and supplemented with 2.0 mg/l BAP alone for *Sisqella* (9.5 cm), and 3.0 mg/l BAP in combination with 1.0 mg/l IAA for *Gimbo* (8.5 cm) and *Astara* (6.55 cm) (Table 2). In contrast, the highest shoot length for *Sisqella* (6.0 cm), *Gimbo* (5.0 cm), and *Astara* (4.25 cm) was observed on MS medium gelled with agar (Table 3). These values were almost non-comparable with the maximum shoot length observed on MS media gelled with *bulla* and supplemented with the same levels of BAP and IAA (Tables 2 and 3). Media gelled with *bulla* and agar, and supplemented with 6 mg/l BAP and 2 mg/l IAA resulted in shorter shoot length followed by shoots from hormone-free MS medium (Tables 2 and 3).

Shoot multiplication

Results of this study indicated that different multiplication rates were observed for shoot tips to induce multiple shoots among the studied genotypes after culturing on MS media gelled with *bulla* and agar individually, and supplemened with BAP and IAA at different concentrations. Consequently, the results revealed that *Sisqella* produced multiple shoots in the fewest days, followed by *Gimbo* and *Astara* in the MS medium supplemented with 5.0 mg/l BAP and 1.0 mg/l IAA after the 13th, 19th, and 22nd days of culture in *bulla*-gelled media, respectively (Tables 2 and 3). However, on the same media with agar gelled more days to multiple shoot induction were recorded for *Sisqella* (22), *Gimbo* (25), and *Astara* (26) after culture. The second fewer number of days to multiple shoot induction was observed in the *bulla*-gelled media supplemented with 4.0 mg/l BAP alone for 17, 22, and 26 days to produce more normal shoots per shoot tip for *Sisqella*, *Gimbo*, and *Astara*, respectively. In this way, the *bulla*-gelled media used proved better for multiple shoot induction from the shoot tip of explants. Similarly, BAP without a combination with IAA is also suitable for multiple shoot induction.

The number of micro-shoots produced per explant also showed notable differences among genotypes, gelling agents, and growth regulators concentrations. The highest number of micro-shoots was recorded for *Gimbo* (18) on MS media supplemented with 5.0 mg/l BAP in combination with 1.0 mg/l IAA, followed by *Sisqella* (14) that contained 4.0 mg/l BAP on medium gelled with *bulla*. Whereas *Astara* resulted in less shoot proliferation (6) as compared to the other two genotypes on the same MS media (Table 2). However, on agar-gelled MS media *Gimbo*, *Sisqella*, and *Astara* produced 14, 11, and 4 shoots per explant, respectively, on the same concentrations of growth regulators. Explants cultured on growth regulators free MS medium produced the lowest number of shoots for all studied genotypes.

Table 2 In vitro shoot proliferation and growth of three enset genotypes on *bulla* -gelled MS media containing BAP (6-Benzylaminopurine) alone and BAP in combination with IAA (Indole − 3-acetic acid) at different concentrations

mg/l BAP	IAA	Enset Genotypes											
		Astara				*Gimbo*				*Sisqella*			
		DS	DM	NS	LS	DS	DM	NS	LS	DS	DM	NS	LS
0	0	9.55^{a}	37.25^{a}	1.75^{j}	1.82^{k}	8.35^{a}	32.17^{a}	3.37^{n}	1.75^{L}	7.33^{a}	27.50^{a}	2.50^{L}	2.00^{m}
1	0	7.25d^{e}	29.25^{b}	2.13^{i}	4.52^{e}	6.25^{gh}	25.05^{d}	4.50^{m}	5.33^{e}	5.25^{ef}	22.42^{b}	3.75^{k}	6.50^{d}
2	0	6.05^{j}	28.50^{c}	2.73^{g}	6.05^{b}	5.05^{j}	24.48^{f}	6.53^{k}	7.53^{b}	4.05^{j}	21.25^{c}	5.60^{ij}	9.50^{a}
3	0	6.50^{h}	27.50^{ef}	2.18^{i}	5.13^{c}	5.50^{i}	23.53^{i}	8.67^{i}	6.00^{d}	4.53^{h}	19.50^{e}	7.00^{h}	8.12^{c}
4	0	6.75^{f}	26.05^{j}	2.47^{h}	3.03^{h}	5.65^{i}	22.05^{L}	12.60^{e}	3.50^{h}	5.05^{f}	17.05^{i}	14.00^{a}	4.33^{gh}
5	0	7.06^{e}	26.53^{hi}	3.20^{f}	2.53^{i}	6.13^{h}	22.50^{k}	13.32^{d}	3.08^{i}	6.05^{d}	18.05^{h}	11.17^{cd}	3.65^{i}
6	0	7.25^{de}	27.75^{e}	3.67^{e}	2.12^{j}	7.18^{d}	23.75^{hi}	12.12^{f}	2.75^{j}	6.50^{b}	18.75^{fg}	12.11^{bc}	2.64^{kl}
1	1	7.05^{e}	28.75^{c}	1.35^{k}	3.50^{g}	6.50^{f}	24.13^{g}	3.18^{n}	4.20^{fg}	5.13^{f}	21.00^{c}	3.75^{k}	4.28^{h}
2	1	6.50^{h}	28.05^{d}	2.10^{i}	4.05^{f}	6.05^{h}	23.08^{j}	5.00^{L}	6.72^{c}	4.63^{gh}	20.08^{d}	8.25^{g}	6.53^{d}
3	1	6.25^{i}	27.42^{f}	3.52^{e}	6.55^{a}	5.05^{j}	23.50^{i}	11.18^{h}	8.50^{a}	4.25^{i}	19.07^{ef}	9.58^{ef}	8.57^{b}
4	1	7.42^{d}	26.70^{h}	4.50^{c}	3.30^{gh}	6.50^{f}	22.53^{k}	15.50^{b}	4.02^{g}	5.25^{ef}	19.50^{e}	10.50^{de}	4.52^{g}
5	1	8.05^{c}	21.87^{k}	6.12^{a}	2.52^{i}	6.75^{e}	19.05^{m}	18.02^{a}	2.72^{j}	5.42^{e}	13.05^{j}	12.00^{bc}	2.75^{k}
6	1	8.42^{b}	26.50^{hi}	5.23^{b}	2.03^{jk}	7.05^{d}	23.85^{h}	11.53^{g}	2.05^{k}	6.08^{d}	20.33^{d}	10.17^{de}	2.53^{kl}
1	2	7.25^{de}	26.82^{gh}	1.25^{k}	4.80^{d}	6.13^{h}	24.18^{g}	2.50^{o}	5.20^{e}	5.25^{ef}	21.50^{c}	3.50^{k}	5.43^{e}
2	2	6.53^{gh}	26.73^{h}	1.73^{j}	4.08^{f}	6.25^{gh}	24.50^{ef}	4.77^{lm}	4.43^{f}	4.75^{g}	21.08^{c}	5.00^{j}	5.03^{f}
3	2	6.72^{fg}	26.55^{hi}	2.10^{i}	3.15^{h}	6.35^{fg}	24.75^{e}	8.14^{j}	3.50^{h}	5.10^{f}	19.50^{e}	6.15^{hi}	3.77^{i}
4	2	7.13^{e}	26.27^{ij}	4.05^{d}	2.48^{i}	6.75^{e}	24.58^{ef}	13.34^{d}	2.60^{j}	5.25^{ef}	18.50^{gh}	9.17^{fg}	3.05^{j}
5	2	8.05^{c}	27.05^{g}	5.03^{b}	1.78^{k}	7.53^{c}	25.68^{c}	15.53^{b}	2.07^{k}	6.28^{c}	18.33^{gh}	11.13^{cd}	2.49^{L}
6	2	8.53^{b}	28.67^{c}	4.45^{c}	1.10^{L}	8.05^{b}	26.13^{b}	14.20^{c}	1.53^{L}	6.43^{bc}	22.50^{b}	12.25^{b}	1.53^{n}
SE±		0.11	0.18	0.14	0.16	0.12	0.15	0.20	0.16	0.11	0.29	0.56	0.14
CV		1.53	0.65	4.37	4.62	1.80	0.60	2.05	3.81	2.07	1.47	6.80	3.00

N.B: DS = No. of days for single shoot initiation, DM = No. of days for multiple shoot induction, NS = number shoot per explant, LS = Shoot length (cm), SE = standard error, and CV = coefficient of variation

Generally, this experiment revealed that medium gelled with *bulla* resulted in a higher number of micro-shoots per explant than medium gelled with agar (Tables 2 and 3).

Rooting

Well-developed shoots from multiplication media were transferred to a half-strength MS medium containing *bulla* or agar as a gelling agent and supplemented with IBA or NAA at a concentration of 1.0, 2.0, and 3.0 mg/l for root induction. Among all studied genotypes, the highest root number was observed in *bulla*-gelled medium supplemented with 2.0 mg/l IBA (5.5) for *Sisqella* followed by *Gimbo* (4.5) after 11.0 and 12.5 days of culture, respectively (Table 4). The same genotypes produced the second-highest number of roots on a medium containing 1.0 mg/l NAA gelled with *bulla.* The rooting medium supplemented with 3.0 mg/l IBA as well as 2.0 and 3.0 mg/l NAA produced the lowest root number (1.45) per shoot. These revealed that the rate of root formation was gradually reduced with increasing concentrations of IBA and NAA. Similarly, no root induction was observed on growth regulator-free medium gelled with agar in all examined enset genotypes until a month. However, rooted plantlets were observed on both hormone-free media gelled with *bulla*. Moreover, spontaneous root induction was also observed on multiplication media gelled with *bulla* in all genotypes.

The highest root length was recorded from shoots planted on *bulla*-gelled media containing 1.0 mg/l IBA for *Sisqella* (7.02 cm), followed by *Gimbo* (6.25 cm) and *Astara* (5.50 cm) (Table 4; Figs. 3 h to j). The MS media supplemented with 1.0 mg/l NAA also resulted in slightly comparable root length, of 6.5, 6.0, and 5.25 cm for *Sisqella*, *Gimbo*, and *Astara*, respectively. However, the lowest root length per shoot was observed for all the genotypes on agar-gelled media (Table 5) as compared to *bulla*-gelled media. According to this study, 2.0 and 3.0 mg/l NAA and 3.0 mg/l IBA showed the lowest root length followed by both auxins-free media (Table 4). In general, the examined genotypes showed a different response.

Acclimatization

After a month of acclimatization (Figs. 4), 92.50, 83.35, and 71.15% of *Sisqella*, *Gimbo*, and *Astara* plantlets that were taken from *bulla*-gelled media survived, respectively. While, the survival percentage of the same genotypes obtained from agar-gelled media was found to be 85.50% for *Sisqella*, 74.75% for *Gimbo*, and 65.50% for *Astara*.

Cost analysis

Based on this simple comparative cost analysis, prices for agar standard (plant propagation agar) and enset flour (*bulla*) were compared (Table 6). Considerable cost reduction was obtained by using *bulla* as a gelling agent instead of agar. Accordingly, using enset *bulla* for micro-propagation of enset shoot tips as an alternative source was efficient and resulted in a cost reduction of 73.3% (Table 6).

Discussion

Enset farmers in many enset growing regions have been cultivating the crop in the traditional method for many generations. However, nowadays its production is limited by various man-made and natural factors.Thus, in addition to on-farm maintenance appropriate tissue culture approaches were found indispensable for the rapid propagation, distribution, and conservation of high-quality planting material as well as for improving the production and productivity of the crop.

Bulla as a gelling agent

The result of this study revealed that *bulla* flour at a concentration of 80 g/l was the optimal amount to solidify and stabilize the MS medium in the in-vitro culture of enset shoot tips. Similar results have been reported by Ayenew et al. (2012), Mengesha et al. (2012), and Ayalew et al. (2017). They observed that a medium supplemented with 80 g/l of *bulla* powder was optimum and can stabilize the MS media in all phases of in vitro culture of pineapple, vanilla, and cassava, respectively. Hirose et al. (2017) confirmed the high gelatinization property of enset starch for food and industrial uses. Similarly, ESTC (2003) reported that starch produced from enset can be used for the paper, textile, and adhesive industries. Furthermore, Gebre and Nikolayev(1993) and Gebre-Mariam and Schmidt, (1996) observed that enset starch has a better potential to be used in binding and disintegrating compressed tablets. In most enset-growing regions of Ethiopia, farmers also consider *bulla* to be the best quality of enset food (Wolde-Gebriel et al. 2006; Daba and Shigeta 2016) and consume it mainly as porridge, gruel, and in crumbled form (Brandt et al. 1997; Olango et al. 2014). However, its proximate and mineral content were lower than those of corm and *qocho* (unpublished data).

Table 3 In vitro shoot proliferation and growth of three enset genotypes on agar- gelled MS media containing BAP (6-Benzylaminopurine) alone and BAP in combination with IAA (Indole − 3-acetic acid) at different concentrations

mg/l BAP	IAA	Enset Genotypes											
		Astara				*Gimbo*				*Sisqella*			
		DS	DM	NS	LS	DS	DM	NS	LS	DS	DM	NS	LS
0	0	11.22^{a}	37.00^{a}	1.25^{i}	1.08^{j}	9.25^{a}	34.08^{a}	2.52^{m}	1.53^{k}	8.50^{a}	32.00^{a}	2.10^{o}	1.53^{k}
1	0	10.13^{b}	32.00^{b}	1.80^{g}	2.00^{f}	8.05^{d}	29.22^{b}	4.00^{k}	3.53^{cd}	7.05^{d}	27.10^{b}	3.25^{L}	4.10^{e}
2	0	8.53^{fg}	30.91^{c}	1.83^{g}	4.25^{a}	7.08^{g}	27.50^{c}	4.53^{j}	5.00^{a}	6.05^{g}	26.03^{c}	3.50^{k}	6.02^{a}
3	0	8.50^{fg}	28.13^{h}	2.03^{f}	3.03^{c}	7.65^{ef}	27.05^{d}	5.05^{i}	4.00^{b}	6.48^{f}	24.53^{f}	4.03^{j}	4.50^{d}
4	0	8.75^{ef}	27.13^{j}	4.05^{a}	2.53^{d}	7.75^{e}	26.10^{f}	14.07^{a}	2.70^{g}	6.75^{e}	24.08^{g}	11.10^{a}	3.02^{g}
5	0	9.05^{d}	27.25^{j}	2.33^{e}	2.00^{f}	8.25^{cd}	25.10^{h}	11.10^{b}	2.50^{h}	7.12^{d}	23.50^{h}	9.03^{c}	2.53^{h}
6	0	9.50^{c}	26.50^{k}	2.50^{e}	1.80^{g}	8.45^{bc}	26.05^{f}	9.08^{d}	2.03^{i}	7.65^{bc}	23.05^{i}	6.25^{f}	2.32^{i}
1	1	8.25^{hi}	28.50^{g}	1.00^{j}	2.25^{e}	7.50^{ef}	26.5^{e}	1.50^{o}	3.50^{d}	7.12d	26.10^{c}	2.50^{n}	3.04^{g}
2	1	7.85^{j}	28.75^{f}	1.50^{h}	3.15^{c}	7.00^{g}	26.05^{f}	3.03^{L}	3.70^{c}	6.37^{f}	25.00^{e}	5.03^{h}	5.03^{b}
3	1	8.15^{hi}	28.53^{fg}	2.03^{f}	3.03^{c}	7.42^{f}	25.75^{g}	7.05^{f}	3.50d	6.18^{g}	24.50^{f}	6.03^{g}	4.57^{d}
4	1	8.50^{fg}	28.12^{h}	3.03^{d}	2.60^{d}	7.75^{e}	25.50^{g}	10.58^{c}	3.03f	6.48^{f}	24.10^{g}	8.07^{d}	3.05^{g}
5	1	8.75^{ef}	26.00^{L}	4.00^{a}	2.03^{f}	7.65^{ef}	25.12^{h}	14.05^{a}	2.05i	7.53^{c}	22.05^{k}	9.50^{b}	2.50^{h}
6	1	9.25^{d}	27.50^{i}	3.50^{c}	1.27^{i}	8.53^{b}	27.05^{d}	11.06^{b}	1.53k	7.65^{bc}	24.18^{g}	6.25^{f}	1.53^{k}
1	2	8.50^{fg}	29.08^{e}	1.25^{i}	3.60^{b}	7.75^{e}	26.75^{e}	1.75^{n}	4.03b	6.70^{e}	25.05^{e}	2.50^{n}	4.75^{c}
2	2	8.13^{i}	28.75^{f}	1.53^{h}	3.02^{c}	7.13^{g}	26.50^{e}	3.05^{L}	3.28^{e}	6.45^{f}	24.00^{g}	2.75^{m}	3.53^{f}
3	2	8.38^{gh}	28.25^{h}	1.75^{g}	2.25^{e}	7.63^{ef}	26.05^{f}	5.50^{h}	2.50^{h}	6.75^{e}	23.53^{h}	4.53^{i}	2.50^{h}
4	2	8.62^{efg}	28.50^{g}	2.50^{e}	2.00^{f}	7.65^{ef}	26.08^{f}	6.25^{g}	2.05^{i}	7.10^{d}	23.13^{i}	5.03^{h}	2.25^{i}
5	2	8.82^{e}	29.13^{e}	3.75^{b}	1.50^{h}	8.13^{d}	25.50^{g}	7.50^{e}	1.75^{j}	7.65^{bc}	22.50^{j}	7.00^{e}	1.73^{j}
6	2	9.28^{cd}	29.50^{d}	3.50^{c}	0.75^{k}	8.63^{b}	27.75^{c}	9.02^{d}	1.12^{L}	7.75^{b}	25.53^{d}	6.07^{fg}	0.80^{L}
SE±		0.14	0.13	0.11	0. 09	0.14	0.15	0.11	0.10	0.11	0.13	0.12	0.09
CV		1.54	0.44	4.55	3.96	1.74	0.56	1.68	3.69	1.55	0.52	2.10	2.88

N.B, DS = No. of days for single shoot initiation, DM = No. of days for multiple shoot induction, NS = number shoot per explant, LS = Shoot length (cm), SE = standard error, and CV = coefficient of variation

Table 4 In vitro root induction and growth of three enset genotypes on *bulla*- gelled MS media containing different concentrations of IBA (Indole-3-butric acid) and NAA (α-Naphthalene acetic acid)

mg/l IBA	NAA	Enset genotypes								
		Astara			*Gimbo*			*Sisqella*		
		DS	NR	RL	DS	NR	RL	DS	NR	RL
0	0	24.53^{a}	1.07^{g}	1.69^{g}	22.53^{a}	1.20^{f}	1.95^{g}	21.13^{a}	1.73^{f}	2.25^{f}
1	0	14.50^{e}	1.90^{e}	5.50^{a}	13.60^{e}	2.18^{d}	6.25^{a}	13.00^{e}	2.33^{e}	7.02^{a}
2	0	13.25^{g}	3.03^{a}	4.85^{c}	12.50^{g}	4.50^{a}	5.77^{c}	11.05^{g}	5.55a	6.05^{c}
3	0	15.53^{d}	2.52^{c}	4.50^{d}	14.50^{d}	2.50^{c}	5.02^{d}	13.57^{d}	2.75^{c}	5.88^{c}
0	1	14.00^{f}	2.88^{b}	5.25^{b}	13.05^{f}	3.60^{b}	6.02^{b}	12.53^{f}	5.03^{b}	6.50^{b}
0	2	17.50^{c}	2.20d	3.95^{e}	16.53^{c}	2.25^{d}	4.13^{e}	15.50^{c}	2.50^{d}	4.77^{d}
0	3	19.05^{b}	1.45^{f}	3.00^{f}	17.05^{b}	1.60^{e}	3.47^{f}	16.08^{b}	1.85^{f}	3.85^{e}
SE±		0.14	0.08	0.07	0.12	0.10	0.08	0.13	0.08	0.08
CV		0.84	3.76	1.73	0.77	3.73	1.70	0.90	2.58	1.62

N.B: DS = days for single root initiation, NR = mean number root /explant, RL = mean root length (cm), SE = standard error, and CV = coeffient of variation

Fig. 3 Shoot initiation, multiple bud proliferation and rooting of three enset genotypes: (**A**) Shoot tip initiated after five days of inoculation on the agar (two jars) and bulla (three jars) gelled MS media containing 2.0 mg/l BAP; (**B**-**D**) Multiple proliferated shoots from the enset genotype *Astara*, *Gimbo* and *Sisqella*, respectively, on the 8% *bulla* gelled MS medium containing 5.0 mg/l BAP with 1.0 mg/l IAA after two to three weeks; (**E**-**G**) In vitro elongated multiple micro-shoots of the genotype *Astara*, *Gimbo* and *Sisqella*, respectively, on the 8% of bulla gelled MS medium after eight weeks; (**H**-**J**) Roots of single plantlets developed from the genotype *Astara*, *Gimbo* and *Sisqella*, respectively, on the 8% of *bulla* gelled 1/2 MS medium containing 1.0 mg/l IBA

Surface sterilization

Initially, during this experiment, the presence of microbial contaminations was a major challenge, especially in the culture initiation steps. This could be attributed to the fact that explants may hold a wide range of microbial contaminants. Thus, to eliminate this source of contamination, in the present study, the explant tissues were sterilized carefully on their surfaces before culturing on the MS media. The results of the current study showed that surface disinfection experiments were effective when shoot tips of enset were sterilized with 70% ethanol for 5 min, followed by 20% NaOCl for 20 and 10 min. This is in line with the findings of Gezahegn and Mekbib (2016), who reported 95% clean explants resulted when shoot tip explants were treated with 70% ethanol for 5 min, followed by double sterilization with 20% chlorox for 10 min, and then 20 min for all the clones until 10 days of inoculation in MS medum. However, Zinabu et al.(2018) reported that enset shoot tip explants were disinfected with 20% NaOCl for 20 and 10 min after being treated with 70% ethanol for 10 min. But in the present study, during the process of surface sterilization, the treatment of enset shoot tips with 70% ethanol for 10 min resulted in the loss of explants from all three genotypes. Likewise, after three weeks of culture, endophyte contamination caused more than 50% of the explants in the culture

Table 5 In vitro root induction and growth of three enset genotypes on agar- gelled MS media containing different concentrations of IBA (Indole-3-butric acid) and NAA (α-Naphthalene acetic acid)

(mg/g) IBA	NAA	Enset genotypes								
		Astara			*Gimbo*			*Sisqella*		
		DS	NR	RL	DS	NR	RL	DS	NR	RL
0	0	33.50^{a}	0.87^{e}	1.22^{d}	32.30^{a}	1.05^{g}	1.42^{f}	31.25^{a}	1.10^{f}	1.57^{f}
1	0	17.05^{f}	1.52^{c}	3.15^{a}	16.10^{f}	1.65^{e}	3.93^{a}	15.13^{f}	2.15^{d}	4.82^{a}
2	0	16.10^{g}	2.78^{a}	2.90^{b}	15.08^{g}	3.20^{a}	3.89^{ab}	14.13^{g}	4.08^{a}	4.08^{c}
3	0	18.05^{e}	1.73^{b}	2.82^{b}	17.50^{d}	2.62^{c}	3.53^{c}	16.25^{c}	3.12^{c}	3.90^{d}
0	1	18.50^{d}	2.75^{a}	3.08^{a}	16.50^{e}	3.00^{b}	3.82^{b}	15.72^{e}	3.72^{b}	4.30^{b}
0	2	19.13^{c}	1.60^{bc}	2.80^{b}	18.10^{c}	2.07^{d}	3.15^{d}	16.05^{d}	2.10^{d}	3.83^{d}
0	3	21.05^{b}	1.15^{d}	2.52^{c}	19.00^{b}	1.24^{f}	2.80^{e}	18.05^{b}	1.60^{e}	3.25^{e}
SE±		0.10	0.08	0.08	0.16	0.09	0.05	0.10	0.08	0.09
CV		0.52	4.69	2.98	0.85	4.45	1.60	0.56	3.05	2.43

N.B: DS = days for single root initiation, NR = mean number root /explant, RL = mean root length (cm), SE = standard error, and CV = coeffient of variation

Fig. 4 Acclimatization of some in vitro grown plantlets of enset genotypes: (**A**) Plantlets in the pot of sterilized soil composted of sand: loam: red (1 : 2 : 1) covered with plastic bags; (**B**) Established plantlets after 8 days of plastic cover; (**C**) Acclimatized *Sisqella* plantlets in the greenhouse after 30 days; (**D**) *Astara* plantlets that were acclimatized in the greenhouse after 60 days

Table 6 Simple comparative cost analysis for agar standard and enset flour (*bulla*)

Gelling agents	Amount used per litre	Cost per Kg ETB	Cost per g ETB	Cost per L ETB	Cost saved per L in %
Agar	6 g/l	4000	4	24	
Bulla	80 g/l	80	0.08	6.4	73.3

Note: ETB = Ethiopian birr

to be destroyed for all genotypes examined in this study. Previous researchers also mentioned that besides surface contaminants of explants, endogenous contaminants were the key problem during in vitro propagation activity of enset (Negash 2001; Birmeta and Welander 2004; Gezahegn and Mekbib 2016; Zinabu et al. 2018). In this study, to avoid contamination caused by endogenous microbes, we applied 500 mg/l cefotaxime to the culture media. Similarly, Zinabu et al. (2018) reported that 99% clean explants were obtained when 500 mg/l cefotaxime was included in a medium on the *Bededet* cultivar of enset. According to Khan et al.(2018), endophytic microbes residing within the explants are recognized as a main constraint to the establishment, growth, and multiplication of tissue-cultured plants, as they are more challenging to eradicate by using standard surface sterilization procedures. However, the endophytic contamination could be removed by supplying different antibiotics to the culture media (Fang and Hsu 2012; El-Banna et al. 2021). In a similar study, numerous antimicrobial compounds have been thoroughly used to prevent the growth of endophytes in media during in vitro plant cultures of different plant species, such as *Bambusa nutans* (Ray et al. 2017), banana (El-Banna et al. 2021), *Solanum tuberosum* (Mora et al. 2022), and *Ipomoea batatas* (Pérez-Pazos et al. 2023). Generally, an effective sterilization procedure as well as using antimicrobial agents in some cases in media are important steps in the reduction of exophytic and endophytic contamination in in vitro culture. According to Yildiz (2012) and Khan et al.

(2018), success in tissue culture depends on the effectiveness of the sterilization methods used on the explants before culture initiation, and the confirmation of the endophytic nature of the contaminants.

Shoot initiation

We have been able to compare for the first time the use of locally available and low-cost gelling agent *bulla* flour with plant propagation agar for micropropagation of the three enset genotypes on MS media supplemented with different concentrations of BAP and IAA. The results of this study indicated significant variations were found in days to shoot initiation among the three enset genotypes. Gezahegn and Mekbib (2016) reported an almost similar period (5.25 to 9.0 days) for the single shoot initiation on MS media gelled with 6 g/l agar, but the medium was supplemented with 4.5-6.0 mg/l BAP and 1.0-1.5 mg/l NAA separately and in combinations. However, Diro et al. (2005) reported single shoot initiation after two weeks when enset shoot tip explants were cultured on MS medium gelled with 11 g/l of agar and supplemented with 2.5 mg/L BAP alone. The most likely reasons for this early induction in the present study were the types of *bulla* and the amount of solidifying agent (6 g/l of agar) used. A more concentrated gelling agent can reduce nutrient uptake by explants since the medium becomes very compact. Moreover, Matheka et al. (2019) reported higher levels of BAP to induce buds that grew slowly and were highly blackened. This is in line with the present study, were relatively low BAP concentrations (2.0 and 3.0 mg/l) with or without the combination of 1.0 mg/l of IAA supplemented the fewest number of days for shoot induction. The results of this study also revealed that significant variations existed among the tested genotypes in terms of the length in both gelling agents with different concentrations of BAP alone and combined with two levels of IAA. Similarly, Kahia et al. (2015) indicated that in vitro bud differentiation and development in bananas of the same family as enset were genotype-dependent.

Shoot multiplication

In this study, to obtain optimum conditions for multiple shoot formation from all explant types examined, after one month of shoot induction, the central part of the shoot tip at the base of corms was carefully removed to avoid apical dominance of enset shoot explant. Then, the excised explants were subcultured on MS media supplemented with BAP and IAA separately or in combination. This experiment was done after several preliminary trials in which different levels of BAP (from 1.0 mg/l to 6.0 mg/l) and IAA (1.0 mg/l and 2.0 mg/l) were compared with or without removing the central core of shoot tips. Thereafter, all non-excised explants of shoot tips gave rise to one complete normal shoot rather than multiple shoots until 45 days of sub-culturing on MS multiplication media (data not shown). But the explants that lack apical dominance produced multiple shoot buds at different levels based upon concentrations of growth hormones, genotypes, and gelling agents. A similar observation was also reported by Birmeta and Welander (2004) and Diro et al. (2005).

The results of this experiment showed the effects of the two gelling agents (*bulla* and agar) for multiple shoot induction on MS medium supplemented with BAP and IAA at different concentrations for the three enset genotypes. All the examined genotypes have shown differences in the period of multiple shoot induction, the number of shoots, and the length of shoots per shoot tip of the explants. For all tested enset genotypes, a better growth response was observed with *bulla* flour gelled media than agar. Almost similar results have been reported in pineapple, vallina, and cassava by Ayenew et al. (2012), Mengesha et al. (2012), and Ayalew et al. (2017), respectively, using *bulla* flour as an alternative gelling agent on MS media. However, their reports did not indicate the kinds of plant growth hormones or their concentration levels in the MS medium. In terms of the number of days for multiple shoot induction, the lowest number of days (13–22) were observed in the MS media supplemented with 5.0 mg/l BAP and 1.0 mg/l IAA on *bulla*-gelled media. However, on agar-gelled media with the same level of hormones and genotypes, it took from 22 to 26 days in the present study. However, Gezahegn and Mekbib (2016) reported slightly shorter durations (11.67 to 25.33 days) for Mazia, Arkiya, and Digomerza enset cultivars cultured on agar-gelled media having 4.5- 6.0 mg/l BAP and 2.0 mg/l NAA for multiple shoot induction. This variation could be a result of genotype differences, the types and amounts of macro- and mirco nutrients, and hormones that were used.

Successful multiplication is one of the most essential steps in micro-propagation. The current findings revealed that the highest and lowest number of shoots were 18 and 6 for *Gimbo* and *Astara* genotypes on *bulla*-gelled media containing 5.0 mg/l BAP and 1.0 mg/l IAA in one sub-culture after 30 days, but on agar-gelled media, the two genotypes produced 14 and 4 shoots per explant, respectively. Similarly, Gezahegn and Mekbib (2016) reported 23 shoots per explant. However, Negash et al. (2000) reported one to two shoots per shoot tip from the in vitro regeneration of the three enset clones on MS medium containing 2.25 mg/l BAP in combination with 0.2 mg/l IAA. In addition, Diro et al. (2005) obtained 3.7 shoots per shoot after splitting the explants into two and culturing them separately. Furthermore, Birmeta and Welander (2004) reported about 75 shoot buds that can potentially grow to shoots per explant in one subculture through meristem wounding of initiated explants. This variation may occur due to differences in genotypes, size of explants, media types and their gelling agents, the concentration of growth hormones, and light intensity in the growth room. Generally, our results show that *bulla*-gelled media produce better responses in all aspects for all the tested genotypes of enset.

Rooting

The formation of the root is also a crucial step in micro-propagation because its success could depend on the number and length of roots per plantlet. In the present study, well-developed shoots from multiplication media were transferred to half-strength MS medium gelled with *bulla* or agar supplemented with IBA or NAA at a concentrations of 1.0, 2.0, and 3.0 mg/l for root initiation. The result of this study shows that the fewest number of days (11.0–13.25) was observed on MS media gelled with *bulla* and supplemented with 2.0 mg/l IBA, but 14–16 days for root induction were recorded on the same media gelled with agar for all studied genotypes of enset. This result is slightly in agreement with Gezahegn and Mekbib (2016), who reported that 10.5 to 12.83 days are required for root induction on MS medium containing 1.0–2.0 mg/l IBA. In addition, Negash et al. (2000) mentioned that root formation occurred two weeks after transfer to root induction medium supplemented with 5 µM IBA, 1 µM IAA, and 1 µM BAP in combination for all three enset clones. However, Matheka et al. (2019) reported 3–5 days for root development after the culture of shoots on media gelled with 3 g/l gelrite. The most probable reasons for this early initiation are the type of gelling agent, and the concentration of chemicals and hormones in the media employed (Saraswathi et al. 2016; Bhat et al. 2022).

In terms of root number per shoot, our results are in agreement with the report of Gezahegn and Mekbib (2016). They reported the production of an average of 3.6 roots per explant after 12–14 days post-culture of explants on MS media with 1.0 mg/l IBA. On the other hand, Matheka et al. (2019) reported a maximum of 12 roots per shoot on a medium containing 1.0 mg/l IBA for *Bededet* enset genotypes. This is contrary to current findings of a maximum of 5.5 roots per plantlet. This might be due to genotype differences, the concentration of growth regulators and media, and the types of gelling agents. For example, genotype *Sisqella* responded better for root number and length, whereas poor response was observed for *Astara* culturing on both media types separately in the present study. Root induction and development are dependent on several factors, including media type and plant genotype (Kahia et al. 2015).

Acclimatization

The results of this study showed that the survival rate of the plantlets varied among the examined genotypes and gelling agents after 30 days in the greenhouse. Generally, all the studied genotypes of plantlets from a *bulla*-containing medium grew better than those from an agar-gelled medium. This is in agreement with the work of Ayenew et al. (2012) on pineapple, who reported 95% and 90% survival from *bulla* and agar medium, respectively. This was also supported by Mengesha et al. (2012), who reported that 90% of survival in greenhouse conditions for vanilla plantlets was derived from a *bulla* gelling agent. This might be attributed to the presence of different nutritional supplements in *bulla* compared to agar. The present study also indicated that a different survival rate was observed among genotypes, which agrees with the findings of Gezahegn and Mekbib (2016). They described that *Degomerza* performed better than *Mazia* and *Arkyia* enset genotypes. Similarly, Negash et al. (2000) reported that the *Nobo* enset genotype revealed a better survival rate than the *Choro* and *Ketano* genotypes. This might be due to genetic differences in the enset genotypes.

Simple cost analysis of *bulla* as a gelling agent

Few reports have mentioned the use of *bulla* as a potential gelling agent for micro-propagation of crops such as pineapple (Ayenew et al. 2012), vanilla (Mengesh et al. 2012), and cassava (Ayalew et al. 2017). However, this is the first report in which the use of *bulla* instead of agar as an alternative gelling agent has been reported on the in vitro growth of enset. The unit production cost of micro-propagation in most cases is limited to the full application of these techniques. According to Saraswathi et al. (2015) and Ebile et al. (2022), agar, which is an expensive gelling agent, has been widely used for solid media in tissue culture. Similarly, Kacar et al. (2010) stated that because of the high price of tissue culture- grade agar, attempts have been made to identify suitable alternatives. The result of the present study indicated that using enset *bulla* as an alternative source saves 73.3% in cost. In a similar study, Ayenew et al. (2012), Mengesh et al. (2012), and Ayalew et al. (2017) reported the use of *bulla* as an alternative source of agar for micro-propagation of pineapple, vanilla, and cassava, and they obtained 76%, 72%, and 65-86% of gelling cost reduction, respectively. Overall, the findings are in line with the original hypothesis that the gelling agent, enset product *bulla*, is locally available in farmers' homegardens and a less expensive alternative to agar that can be used in all stages of micro-propagation without compromising culture quality. Furthermore, the results of this study indicated that the supplement of *bulla* reduced the direct dependency on plant tissue culture agar. A similar observation was made by Daud et al. (2011), who studied alternative sources of agar such as potato starch, rice flour, cassava flour, and corn flour and obtained a 66-90% gelling cost reduction. Likewise, Kodym and Arias (2001) reported a 90% cost reduction by replacing sucrose and GelriteTM with locally available commercial sugar and starch-Gelrite™ mixtures, respectively. Ebile et al. (2022) demonstrated that there is a great opportunity to use some commonly available resources that are within the means of smallholder farmers in developing countries for media preparation in tissue culture technology to propagate indigenous as well as endangered crops.

Conclusion and recommendations

The present study showed a successful protocol for shoot tip in vitro culture studies of the multi-use plant *E. ventricosum* employing *bulla* as the gelling agent. Moreover, this research has for the first time compared and verified the effect of two gelling agents, *bulla* extracted from enset and agar, on in vitro shoot initiation, multiplication, and root development for three enset genotypes. The results showed that *bulla* can provide a significantly higher number and length of shoots and roots per shoot and also early initiation of shoots and roots for all the studied genotypes when compared with agar-gelled MS media. In addition, *bulla* is a less expensive and locally available resource, which could substitute conventional agar and result in an overall cost reduction for micro-propagation of enset. Thus, it provides additional supplements and the possibility of a backup for on-farm maintenance as well as mass in vitro propagation of enset genetic resources. It is accordingly imperative to make all feasible efforts to establish the *bulla* production firms and small-scale enset producers with cost-reducing harvesting and processing technologies that link sustainable enset farming systems with market opportunity. Furthermore, there is the need for domestication and expansion of enset plants to different regions and countries, as well as promoting the potential of enset *bulla* for micro-propagation and other industrial purposes.

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Declarations

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