

Micro-Propagation of *Vanilla planifolia* Using Enset (*Ensete ventricosum* (Welw, cheesman)) Starch as a Gelling Agent

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Abstract: *Vanilla planifolia* micro-propagation efficiency was evaluated using enset starch (bulla) as an alternative gelling agent. Particularly, optimization of bulla concentration levels and assessment of costs saved using bulla were carried out in this study since *in vitro* media ingredient, especially agar, comprises of the highest cost as compared to others ingredients. The results revealed that shoot numbers and fresh weight were significantly improved in multiplication media gelled with bulla (8%). Although all tested gelling agents apparent 100% rooting, increased newly growth of shoots was obtained on media supplemented with bulla (8%) followed by bulla (10%) and agar. Substitution of agar with bulla (8%) could save about 72.5% of agar cost per a litter media. Generally, replacing agar with 6-10% bulla could decline the cost of agar about 50-72.5% per a litter media. However, bulla resulted in poor media clarity that cause problem in contamination detection and enhanced root proliferation during multiplication phase. Hence, bulla seems to be more preferable in rooting phase that needs short growth period and improved the plantlets growth. In the future, detail studies would be needed on characterization of bulla starch to assure the principal active ingredients depending upon source of cultivars and locations as well as to assess effects on different crops.

Keywords: Alternative gelling agent, bulla starch, cost reduction, *in vitro* propagation, vanilla

INTRODUCTION

Vanilla planifolia Andr is commercially cultivated for its pods or beans. The pods (beans) of vanilla are the source of the popular flavoring substance called vanillin, which is mainly used in flavoring cakes, ice creams, sweets, chocolate, beverages and in cosmetics and perfume industry, condiments and oleoresin (Goodenough, 1982).

Vanilla is generally propagated by stem cutting of mature vine. This conventional propagation is slow, labor intensive and time consuming that impedes the growth and development of the mother plant when harvesting stems for propagation (Geetha and Shetty, 2000; Giridhar *et al.*, 2001). It is crucial to exploit the potential of tissue culture to effectively multiply and supply the required amount of planting materials for large-scale plantation (Abebe *et al.*, 2009). An efficient micro-propagation protocol of vanilla using agar gelling agent has been established previously (Kononowicz and Janick, 1984; George and Ravishankar, 1997; Geetha and Shetty, 2000; Abebe *et al.*, 2009). Large-scale mass propagation of vanilla demands economical, rapid multiplication and highly reproducible tissue culture protocol. The culture media ingredients, particularly the gelling agent called agar, comprises the highest cost as compared to others ingredients (Esekiel, 2010). Hence,

in order to reduce the total cost of *in vitro* multiplication of vanilla, identifying and optimizing alternatives cheap and locally accessible gelling agents by substituting with kitchen necessity starches is a decisive solution.

Plant starches are found to be suitable for gelling agents in tissue culture comparing with conventional gelling agent, agar. Some of the commonly used plant starches for plant tissue culture studies as alternative gelling agent include potato, rice, cassava and corn starches (Daud *et al.*, 2011). Of which, cassava flour is found to be a proper gelling agent (Gerbe and Sathyanarayana, 2001) as well as a potential cheap substitute for agar due to its local availability (Maliro and Lameck, 2004), particularly in Africa. Application of alternative gelling agents, therefore, is an appropriate option to achieve the optimum benefit and low cost for vanilla *in vitro* culture.

Enset (*Ensete ventricosum* (Welw.), Cheesman) is one of the widely cultivated Ethiopian indigenous crops producing different starchy food products such as kocho, bulla and amicho (Tsegaye and Struik, 2002). Bulla is a water-insoluble starchy product, which is processed by squeezing and decanting the liquid followed by drying. Bulla is considered as the best quality enset food obtained from fully matured enset

plant (Pijls *et al.*, 2006). Due to the quality and starchy nature of the product, bulla powder seems to be a critical alternative gelling agent for vanilla micro-propagation. Thus, this study aims:

- To evaluate the micro-propagation efficiency of vanilla using bulla gelling agent.
- To optimize the concentration levels of bulla.
- To assess the costs saved using bulla as a gelling agent instead of analytic grade agar.

MATERIALS AND METHODS

Experiments location and duration: The study was conducted at Plant Biotechnology Research Laboratory, Jimma Agricultural Research Center, Ethiopian Institute of Agricultural Research, Ethiopia. All experiments presented in this study and its preliminary evaluations were carried out between the beginning of 2011 and February 2012.

Source of explants: Plantlets were multiplied under *in vitro* condition as per the protocol established for *Vanilla prolifera* by Abebe *et al.* (2009) in plant biotechnology laboratory, Jimma Agricultural Research Center, Ethiopia. Healthy and uniform *in vitro* multiplied explants were selected and used for both multiplication and rooting phases' gelling agent experiments.

Enset starch called 'bulla' preparation: For this study, bulla powder was prepared and supplied by Tekabe small-scale food processor in Jimma, Ethiopia. The leaf sheaths and corm (underground stem base) of fully matured enset plant were collected, scraped and mixed together. The pulp was squeezed into large bowls to collect the liquid. Then, the liquid was decanted to collect the starch. The surface of starch was rinsed with clean water and scooped into trays for sun drying at ambient temperature. Finally, the dried starch was milled into powder using a household milling machine, packaged in sealed polyethylene bags and stored under room temperature (Pijls *et al.*, 2006).

Nutrient medium preparation: Two gelling agents such as agar (Sigma chemical company, Germany) and bulla powder (Tekabe small-scale food processor, Jimma, Ethiopia) extracted from *Ensete ventricosum* were used in all experiments. The nutrient media were prepared following protocols reported by Abebe *et al.* (2009). The experimental media were gelled with different gelling agents namely 0.8% agar, 8 and 10% bulla as well as a composite mixture consisting of 6%

bulla with 0.2% agar. The levels of bulla were formulated based on our prior preliminary investigations (data not published). The mixture of bulla with media was made carefully since it precipitates at the bottom and forms aggregates. So, bulla powder was first mixed into the cold media and heated on stove with continuous agitation followed by dispensing of the media. Dispensed media in Jam jars were autoclaved at 1.06 kg/cm² and 121⁰C for 20 min.

***In vitro* multiplication and rooting:** *In vitro* multiplication experiment using nodal explants was conducted on media gelled with two types of gelling agents and different concentrations. The nodal explants preparation for both multiplication and rooting were made according to Abebe *et al.* (2009) protocol. Young explants containing two nodes were cut off and transferred into both multiplication and rooting media gelled with different gelling agents and/or doses. Particularly, short and individual explants were excised and cultured into multiplication media, whereas long and strong explants were transferred into rooting media. Both multiplication and rooting' experiments were kept in growth rooms for 60 and 45 days after culturing, respectively. Three explants were cultured per a Jam jar for both multiplication and rooting experiments as well as replicated 10 times.

Cost of gelling agent calculation: A cost benefit analysis of two gelling agents using in vanilla micro-propagation was made. The cost of gelling agents was calculated per a litter of medium and per a kilogram of gelling agents. Then, the total cost saved using bulla per a litter of media was estimated using the following mathematical formula:

$$\text{Cost saved (\%)} = [100 - (\text{bulla cost/agar cost}) \times 100]$$

Plantlet acclimatization: Acclimatization of well elongated and rooted *in vitro* raised vanilla plantlets was made following the procedures reported by Abebe *et al.* (2009) and Mengesha *et al.* (accepted). The survival plantlets percentage was obtained by number of plantlets survive after a month divided by the total plantlets transferred to greenhouse.

Data collection: Quantitative and qualitative data were collected from multiplication and rooting phases after 60 and 45 days, respectively. The quantitative parameters collected from multiplied sample explants were number of nodes, rooted shoots (%), shoot height (cm), number of shoots and total fresh weight (g). While, in rooting phase, rooting ability (%), number of

roots, root length and newly emerged shoots' height (cm) were collected. Total fresh weight was measured immediately after removing the plantlets from the culture jar and washing the adhering gelling agents from the plantlets through running tap water. On the other hand, the qualitative data such as media clarity and stability as well as multiplied, rooted and acclimatized plantlets' images were collected. The images were taken using Sony Cyber-shot 14.1 Mega pixels digital camera. Then, the images were adjusted using differential interference brightness and contrast setting of Adobe photoshop CS.

Statistical analysis: The experiments were laid out with Complete Randomized Design (CRD). The quantitative data were subjected to analysis using SAS, statistical software package (Version 8.01) (SAS, 2001) according to Montgomery (2005). Significant mean values were compared using the procedure of REGWQ test (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*).

RESULTS AND DISCUSSION

Media clarity and stability: Media clarity is one of the characteristics of good gelling agent utilization since it is a critical point in tissue culture for frequent detection of microbial contamination. In the present study, the sole utilization of bulla powder as a gelling agent led to poor clarity of media as compared to agar and composite (agar + bulla) gelling agents (Fig. 1). This is in line with media clarity observed during supplement of cassava starch gelling agent in potato micro-propagation (Kurua *et al.*, 2008). Bulla resulted in a cloudy media that affected the detection of microbial contamination in multiplication phase since the culture

period is longer in this phase than rooting phase does. This clarity, however, was improved a little bit with supplement of 0.2% of agar mixing with bulla (6%), which also enhanced gel strength when using reduced bulla concentration.

The media supplemented with both composite and sole bulla (8 and 10%) gelling agents were stabilized in multiplication phase for more than 90 days. This indicated that gelled media with bulla starch may have an increased water holding capacity because of the ability of the starch to form hydrogen bond (Lupano and Gonzalez, 1999). Almost similar media stability duration was observed between agar and bulla. The longer gelled media stability using bulla starch is in contradiction to cassava starch that losses its stability due to dropping of the pH in the medium after certain period of time (Kurua *et al.*, 2008).

In vitro multiplication: Vanilla is micro-propagated efficiently using nodal explants (Geetha and Shetty, 2000; Abebe *et al.*, 2009). During the current study, *in vitro* multiplication of vanilla was conducted on media supplemented with different gelling agents. Here, bulla was considered as an alternative gelling agent that is needed to be optimized the dose and evaluated the effect on *in vitro* multiplication. Highly significance ($p < 0.01$) and significance ($p < 0.05$) differences for several growth parameters were observed among gelling agent types and/or doses. Higher significant differences for average shoot height and number of nodes among treatments were obtained (Table 1). The result revealed that bulla with increased dose (10%) showed a good average shoot height and number of nodes compared to the tested gelling agents and doses. This is most probably because bulla starch, which

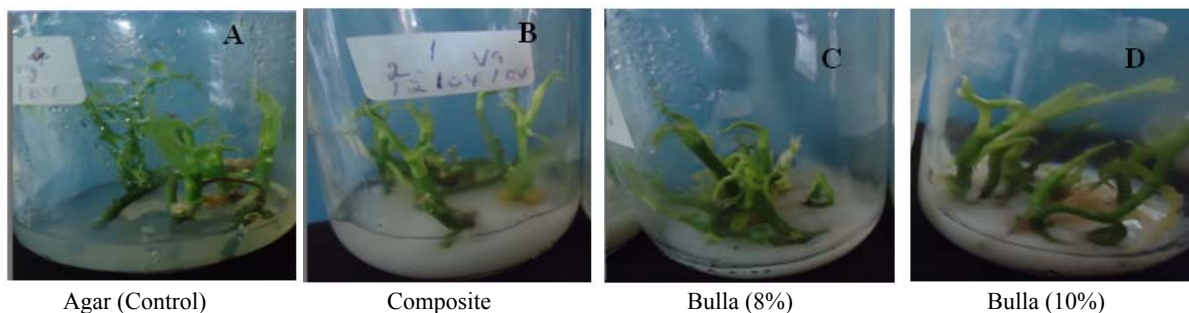


Fig. 1: Different gelling agents influence *in vitro* multiplication potential of vanilla after 60 days of culturing. A) Average shoot numbers, B) average total fresh weight of sample plantlet from multiplication phase, Mean followed by the same letter within the same column are not significantly different

Table 1: Gelling agent affects *in vitro* multiplication

Gelling agent	Shoot height (cm)	Node number	Rooted shoots (%)
Agar (0.7%)	3.06±0.67b	2.53±0.74b	0.0
Composite	3.34±1.02b	2.33±0.82b	26.6
Bulla (8%)	2.92±0.77b	1.93±0.46b	60.0
Bulla (10%)	4.09±0.84a	3.33±0.72a	80.0
CV (%)	24.82*	27.57*	

Mean followed by the same letter within the same column are not significantly different; All growth parameters per gelling agent are mentioned as mean value; Composite: Agar (0.2 %) + Bulla (6%); *: Significant different of $p < 0.05$

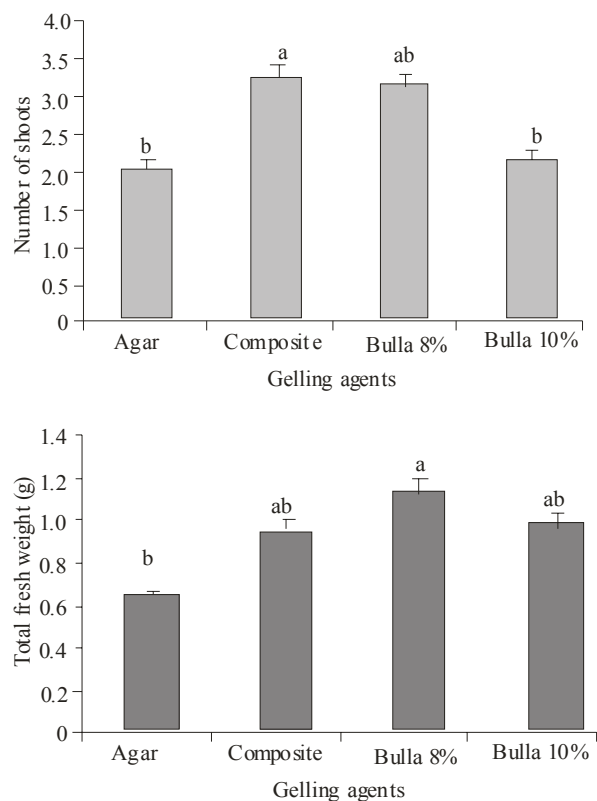


Fig. 2: *In vitro* multiplication of vanilla on media gelled with different gelling agents. A) Agar (0.8%) was used as a control, B) composite was a mixture of agar (0.2%) and bulla (6%). Two different bulla concentrations namely C) 8% and D) 10% were used separately as treatments

is more energy rich (8.5 MJ/kg) (Pijls *et al.*, 2006) may acts as an additional carbon source and adds other ionic supplements. The better response on bulla starch gelled media could also be due to the absence of inhibitors, which have been reported to be present in agar (Debergh, 1983)

Gelling agents were significantly ($p < 0.05$) different for number of shoots and total fresh weight (Fig. 2). Here, the composite and bulla (8%) treatments apparent higher average number of shoots and total fresh weight than both agar and bulla (10%). This increment because

of addition of bulla is might be supplement of additional nutrients, which most probably resulted in improved cell growth and morphogenesis. An increased bulla (10%) level affected the total fresh weight, suggesting the influence of up taking free water coupled with nutrients. This is due to the unbound or available water levels in the culture medium for up take by cultured explants is dependent on the chemical composition of the media and the concentration of gelling agent used to solidify the medium (Mbanaso, 2008).

An increased percentage of rooted shoots was obtained from the media gelled with bulla (10%) followed by bulla (8%) and composite (Table 1). Under normal circumstance, excess number of rooted plantlets was not anticipated since the target was proliferation. Previously, Abebe *et al.* (2009) reported that vanilla explants are easily rooted on hormone free or rooting hormone supplemented media within 30 days after culturing. Rooting can also be observed on multiplication media when the culture is kept for more than 60 days. However, rooted plantlets were obtained on multiplication media gelled with bulla starch within 60 days after culture as compared with agar gelling agent. This most likely could be due to a synergetic effect of bulla that supplements additional energy for rapid root growth.

***In vitro* rooting:** Micro-propagated vanilla plantlets are easily developed roots on hormone free *in vitro* media (George and Ravishankar, 1997; Abebe *et al.*, 2009). In this study, the effects of gelling agents on rooting ability and other subsequent growth of plantlets were evaluated. Non-significant differences for rooting ability, average root number and root length were recorded among the gelling agents (Table 2). The result suggested that utilization of bulla starch as a gelling agent has little effect on vanilla rooting during rooting phase. However, a significant difference ($p < 0.05$) for newly grown shoots was observed among gelling agents. The two tested bulla levels (8 and 10%) enhanced new shoot growth during rooting phase followed by agar and composite treatments (Fig. 3), implying adds additional carbon sources and other

Table 2: *In vitro* rooting of vanilla on culture media gelled with different gelling agents

Gelling agent	Root number	Root length (cm)	Shoot height*	Rooting ability (%)
Agar (control)	1.26±0.74	1.50±0.69	1.06±0.68bc	100
Composite	1.21±0.98	1.54±0.47	1.02±0.29c	100
Bulla (8%)	1.18±0.74	1.45±0.89	1.23±0.82a	100
Bulla (10%)	1.19±0.43	1.51±1.29	1.15±0.81ab	100
CV (%)	7.310	9.190	7.0600	
p-value (5%)	0.358	0.686	0.0001	

Mean followed by the same letter within the same column are not significantly different; Composite: Agar (0.2 %) + Bulla (6%); *: New shoots grown on rooting media

Table 3: Cost comparison of gelling agents supplemented into culture medium for vanilla micro-propagation

Gelling agent	Conc./L (w/v) (%)	Cost (ETB*)/kg	Cost (ETB)/L	Cost saved (%)
Agar	0.8	2500	17.5	-
Composite	6% + 0.2	-	8.6	50.9
Bulla (8%)	8.0	60	4.8	72.6
Bulla (10%)	10.0	60	6.0	65.7

*: 17.20 ETB ~ 1 \$; Composite: 6% bulla + 0.2% agar ; Conc.: Concentration



Fig. 3: *In vitro* rooting ability of vanilla on media supplemented with different gelling agents and acclimatization in greenhouse, A) Agar (0.8%) was used as a control, B) the composite was mixture of agar (0.2%) and bulla (6%). Two different concentrations of bulla, (C) 8% and (D) 10% were used separately. Rooted plantlets were acclimatized for, (E) 15 days and (F) 60 days on soil in greenhouse

mineral nutrients to the culture. Bulla is known by rich in several macro- and micronutrients, which are affected by the cultivars and production location (Atlabachew and Chandravanshi, 2008).

Improved *in vitro* vanilla plantlets performance on onset (bulla) starch gelled medium as compared to agar are an indication of bulla starch potential as agar substitute in plant tissue culture. This result is in line with reports on cassava starch as a suitable gelling agent in plant culture (Gerbe and Sathyanarayana, 2001; Kuria *et al.*, 2008). Bulla starch is a safe product that is biodegradable and environmentally sounds plant

product, which is in agreement with previous report on other plant product called cassava starch (Kuria *et al.*, 2008). The benefit obtained from bulla starch in micro-propagation of vanilla justifies the use of this new gelling agent as a substitute of agar.

Cost analysis of gelling agents: *In vitro* propagation of vanilla is become efficient and promising, but the total cost mostly come from *in vitro* media components that are needed to be reduced by optimizing and/or finding cheap and locally available alternative components. Gelling agent, commonly agar, is known by the top

costly component in the *in vitro* media. As a result, using bulla starch as an alternative gelling agent, 50-72% of the agar cost per a litter was saved in vanilla micro-propagation (Table 3). The result apparent that media gelled with bulla (8%) has saved about 72.6% followed bulla (10%) and composite with the values of 65.7% and 50.9%, respectively. Hence, bulla starch was found as a cheap and locally accessible alternative to agar in developing countries, particularly in Ethiopia, which unable to import required amount or type of agar. This finding is slightly cheaper than cassava starch that is about 50 times cheaper than agar (Kuria *et al.*, 2008).

Plantlet survival in greenhouse: Rooted plantlets grown on different gelling agents were acclimatized in greenhouse with above 90% survival rate (Fig. 3E and F). No survival rate difference was observed among different gelling agents (data not shown). The result is in agreement with Abebe *et al.* (2009), who reported above 85% survival of *in vitro* multiplied vanilla in greenhouse.

CONCLUSION

Micro-propagation of vanilla was efficient using enset starch commonly called 'bulla' that could conveniently serve as a substitute for agar as a gelling agent. Bulla starch is found as a cheap and locally available gelling agent that saved 50-72% of agar cost when using different concentrations bulla and composite of bulla and agar in vanilla tissue culture. Apart from gelling agent, bulla used to improve *in vitro* vanilla plantlets growth performance, which might be the result of additional carbon source. Further study would be needed in the area of bulla powder characterization to ascertain the principal active agents depending on source cultivars and locations as well as on different crops.

ACKNOWLEDGMENT

The authors wish to acknowledge all staffs of plant biotechnology research laboratory, Jimma Agricultural Research Center, Ethiopia for their contribution on the work and manuscript writing up. Our deep gratitude goes to Ms. Roman Getachew for her technical help throughout the experiments in the laboratory and to Mr. Zakir Abbanega for his technical help in greenhouse.

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