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REGULAR ARTICLE

*Ensete ventricosum (*WELW.*)* CHEESMAN: A CHEAP AND ALTERNATIVE GELLING AGENT FOR PINEAPPLE (*Ananas comosus* VAR. SMOOTH CAYENNE) *IN VITRO* PROPAGATION

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ABSTRACT

Different mechanisms are tried so far to reduce the production cost of plant tissue culture through increasing multiplication rate and/or substituting expensive components for wider usage of the technology. Subsequently, replacing the most common gelling agent, agar, took an attention since higher proportion of media cost comes from it. Up to now different alternatives were tried though they have drawbacks due to their inherent chemical characteristics. Therefore the search for new alternatives like *Enset ventricosum* is important. Enset flour, 'Bulla', has been tried in this study for the first time to substitute agar and showed no significant difference for shoots number, root number, shoot height, leaf number and an associated fresh weight of the plantlets besides good gelling ability than Agar. Subsequently, 'Bulla' at 80g L^{-1} gelled well and gave 11.8 shoots with 0.95g and 13.33 roots having 1.37 cm length. This rate can also save up to 76 % cost of gelling though significant difference was found for root length of 3.23 cm with Agar, 8 g L^{-1}. This indicates further study on biochemical and/ or hormonal activity and across crop genotypes to use 'Bulla' as a cheap alternative commercial gelling agent.

Key words: *Ensete ventricosum*; Gelling agents, Bulla; Low cost tissue culture, *Ananas comosus*

INTRODUCTION

Protocols of plant tissue culture media routinely incorporate adding inorganic and organic nutrients, growth regulators, a carbon source, and frequently a gelling agent, Agar, to support and orient the tissue (**Hazel *et al.,* 1994**). The properties of agar that make it the gelling agent of choice are stability, high clarity and resistance to metabolism during cultivation (**Jain and Babbar, 2002**). It is the most frequently used solidifier in plant tissue culture media (**Afrasiab and Jafar, 2011**) and the most expensive component used in plant tissue culture media (**Daud *et al.,* 2011**). Because of the high price of tissue culture grade agar, attempts have been made to identify suitable alternatives like Cassava starch (**Moses and Grace, 2004**), gum katira (**Jain and Babbar, 2002**) guar gum **(Jain *et al.,* 2005)**. Consecutively, it has been used to culture organisms as varied as prokaryotic and eukaryotic microalgae whereby commercially important plants including turmeric, tobacco, blueberry and banana **(Agrawal *et al*., 2010; Atici *et al*., 2008; Ozel *et al.,* 2008; Tyagi *et al.,* 2007)** can be mentioned. However, despite a distinct cost advantage over agar, none is likely to be used as routinely as agar due to some inherent drawbacks such as unstable slant formation and remaining as viscous liquid **(Jain and Babbar, 2011)**. Therefore the search for other alternative gelling agents is mandatory whereby Enset flour, 'Bulla', could be an alternative.

Enset (*Ensete ventricosum* (Welw.) Cheesman) belongs to the family Musaceae **(Cheesman, 1947)** which is one of the seven species of the genus *Ensete* being a monocarpic, perennial giant herbaceous plant reaching 4 - 8 meter or even up to 11 meter in height. It is related to and resembles banana plant produced primarily for the large quantity of carbohydrate-rich food found in a false stem and an underground bulb (corm) **(Pankhurst, 1996)**. 'Bulla' is the small amount of water-insoluble starchy product of Enset that may be separated from mixture of the decorticated (scarped) leaf sheaths and grated corm (underground stem base), kocho, during processing by squeezing and decanting the liquid **(Atlabachew and Chandravanshi, 2007)** to be used as gelling agent.

So far, **Hirose *et al*. (2010)** studied Enset starch for food and industrial uses and found a characteristics of high gelatinization property whereas **Gebre-Mariam and Schmidt (1996)** obtained its use as a binder and disintegrant for tablets. Unfortunately, there is no work done for the potential of this crop as an alternative gelling agent for *in vitro* propagation of crops including pineapple. Pineapple (*Annanas comosus* L.), a member of the botanical family *Bromeliaceae*, is a perennial herb native to the American tropics **(Bartholomew *et al*., 2003)** which is well known for its freeness from harmful phytochemicals **(Mateljan, 2007)**. So far

different mechanisms are tried to reduce the production cost by increasing multiplication rate and/or by substituting expensive components such as agar by liquid culture though initial costs for Temporary Immersion Bioreactors (TIB) are higher **(Firoozabady and Gutterson, 2003)**.

Therefore the objective of this study was to assess Enset flour, 'Bulla', as an alternative gelling agent for pineapple *in vitro* micropropagation and cost reduction incured from using expensive gelling agents for the first time.

MATERIAL AND METHODS

Genetic Materials

Flours of Enset (*Ensete ventricosum* (Welw.) Cheesman, 'Bulla', was collected from supermarket found in Jimma, Ethiopia to be used as alternating gelling agent. Enset flour is prepared by fermenting the starchy extracts with intermittent aeration untill the right stage is reached and dried to obtain 'Bulla' flour. Nutritionally 'Bulla' flour consists of high in carbohydrate mainly and low protein and fat (**Atlabachew and Chandravanshi, 2007; Debebe, 2006**) that resulted in the cohhesive nature of the product.

In this study the most worldwidly accepted pineapple variety, Smooth Cayenne, known for its fresh consumption in addition to their canning quality was brought form horticulture department of Jimma Agricultural Research Centre, Ethiopia.

Preparation of Explants

Slips are one of vegetative propagating materials used in pineapple conventional propagation obtained at the base of the aggregate fruit. Due to better establishment at *in vitro* culture, microbial contamination avoidance and multiplication potential, it is used as an explant sourse in pineapple micropropagation (**Abebe *et al.* 2009)**. Accordingly, the stock plants for the study were raised using selected healthy and uninjured pineapple plantation and the slips (Explants) were prepared for culture according to **Abebe *et al.* (2009).**

Gelling of culture medium

For each concentration of 'Bulla' (60, 80 and 100 gL-1), the flour was first made into a thick slurry with part of the medium to be gelled. The remaining medium was heated to a temperature of 78±1 ^{0}C and the corresponding cold slurry stirred vigorously into it. The medium was dispensed into glass jam jars and autoclaved. For Agar (Sigma Chemicals, Germany), 8g L^{-1} was dissolved in the medium by heating and distributed into glass jam jars prior to autoclaving.

MS basal medium **(Murashige and Skoog. 1962)** supplemented with different mixtures of solidifying agents (Agar 8g L^{-1}; Agar 2g L^{-1} and 'Bulla' 60g L^{-1}; 'Bulla' with 80g L^{-1} and 'Bulla' 100g L^{-1}) at 5.8 pH as treatments. Other media supplements are added according to **Abebe *et al.*, (2009).**

Culture condition and maintenance of cultures

All cultures were maintained in air conditioned growth room at 60-70% relative humidity and 25 ± 2^{0}C with a 16 hours photoperiod from cool white 40 watt florescent bulbs under 2000-3000 lux light intensity. After the plantlets developed roots and sufficiently elongated within four weeks, it were removed from the containers and planted in conical shaped seedling trays filled with a pre-sterilized potting mix of top soil, sand and well decomposed coffee husk at a 2:1:1 ratio **(Abebe *et al.*, 2009)**. Then, they were subjected to red sheath cloth cover to acquire more radiation in the range from 610 to 720 nm for strong photosynthetic activity by chlorophyll and carotenoids absorption **(Taiz and Zeiger, 2004; Ayenew *et al.*, 2012)**. Subsequently the plantlets were planted in a one liter polyethylene sleeves followed by field plantation that give good result and performance at both nursery and field.

Experimental Design, Measurements and data collection

The experiment was laid in Completely Randomized Design (CRD) replicated three times. Shoot number, leaf number, shoot height and fresh weight of plantlets and root data (number and length of roots) were collected after six and four weeks of culture periods respectively. Fresh weight of plantlets were determined by removal of the medium, washed in distilled water and dried with filter paper before measuring at digital sensitive balance

according to **Ayenew *et al.* (2012).** Pictures were taken using Sony® Cyber-shot 14.1 Mega Pixels digital camera and pictures were combined using Adobe Photoshop CS3. Cost saved by different alternative gelling agents used in this study is calculated with the formula:

$$\text{Cost saved} = \frac{\text{Previous Gelling cost} - \text{New Gelling cost}}{\text{Previous Gelling cost}} \times 100$$

Data analysis

The data was analyzed according to **Montgomery (2005)** using SAS, statistical software package (Version 9.1) and mean values were compared using the procedure of REGWQ (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*).

RESULTS AND DISCUSSION

Gelling potential and nutritional supplements

Pineapple slips, explants, were cultured on MS media supplemented with an alternative gelling agent, 'Bulla', at various rates and also in combination with Agar. It was found that, all the treatments performed as a promising gelling agent similarly and provided 100 % semi-solid media that sustained its character during the entire growth period of the culture (Fig 1 and Table 1). This finding is inline with **Hirose *et al.* (2010)** that studied Enset starch for food and industrial uses and found a characteristics of high gelatinization property at a temperature of 64.4 ^{0}C. Moreover, **Gebre-Mariam and Schmidt (1996)** found a better binding ability and disintegrant for tablets than commonly used potato starch.

In addition, 'Bulla' can provide further nutritional supplements as it is chemically analyzed by **Debebe (2006)** and confirmed by **Atlabachew and Chandravanshi (2007)**. The authors indicated the availability of nutrients consistently at higher concentrations for Mg, K, Ca, Na, Fe, Zn, Co, Cu and Mn across different Enset genotypes. Fortunately these nutrients are believed to be involved in plant growth and development **(George *et al.*, 2008; Taiz and Zeiger, 2004)** that enhanced pineapple *in vitro* propagation.

Multiplication of pineapple plantlets

The use of different types of alternating gelling agents showed no significant difference for shoots number and the associated fresh weight of the plantlets (Table 1). It was observed that plantlets derived from the use of 'Bulla' at both concentration levels (80 and 100g L^{-1}) showed well sturdy seedlings which are greener in appearance (Fig 1). Similar observation has been found by **Mbanaso (2008)** for shoots robustness after repeated subcultures derived from starch-gelled medium than agar. This could be due to additional supplements of Mg, constituent of chlorophyll, and Mn which activates several enzymes that are involved in the processes of electron transport system in photosynthesis **(Atlabachew and Chandravanshi 2007)** and also water potential difference. In addition, the explants are established quickly since losses of metallic cations like Na and Ca from cutting edges **(George *et al.*, 2008)** can be replaced from 'Bulla' cultures due to higher concentration in it. This is in contrary to **Nkere *et al.* (2009)** besides Powell and **Uhrig (1987)** that found inhibitory substances from using cassava starches that hinder morphogenesis and reduced growth rate of cultures.

Moreover, the availability of high nutrient concentrations in 'Bulla' are linked with cell wall development, plant signal transduction, parts of enzymes and co-factors affecting the *in vitro* pineapple growth **(George *et al.*, 2008)**. This resulted in 11.8 shoots using 'Bulla' at a rate of 80g L^{-1} as a gelling agent which is cost effective (Table 1 and Table 3) than other treatments. Similarly, better result have been obtained from an alternating gelling agent enriched with starch gelling agent due to an influence in morphogenesis by **Daud *et al.* (2011)**

Table 1 The use of different gelling agent alternatives on shoot development of *in vitro* pineapple

No	Gelling agents	Shoot number	Fresh weight (g)	Gelling potential (%)
1	Agar (8g L^{-1})	12.000 ±2.83a	0.930 ±0.35a	100 a
2	Agar (2 g L^{-1}) + Bulla (60 g L^{-1})	12.133 ±2.47a	1.016 ±0.44a	100 a
3	Bulla 80 g L^{-1}	11.800 ±2.65a	0.95 ±0.30a	100 a
4	Bulla 100 g L^{-1}	11.333 ±6.21a	1.48 ±0.93a	100 a
Coefficient of variation		4.07	1.242	-
P value		0.9755	0.5379	-

Legend: Means with the same letter in a column are not significant and are separated using the procedure of REGWQ (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*).

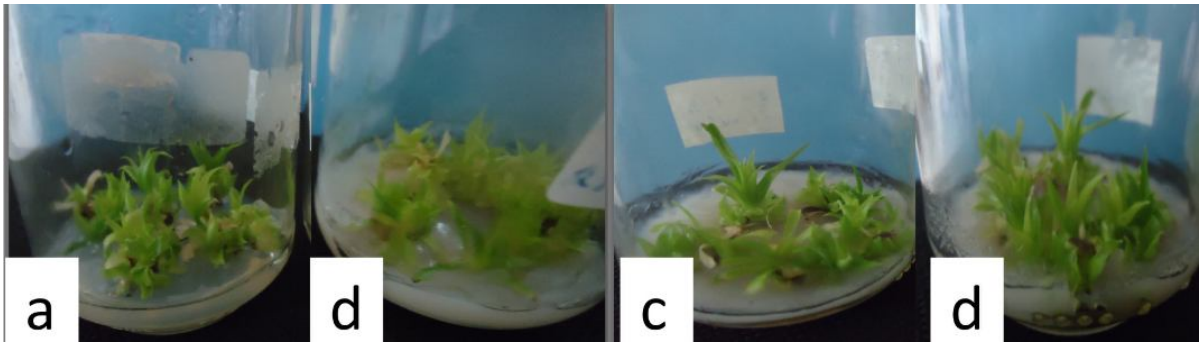

Figure 1 *In vitro* shoot proliferation of pineapple using different gelling alternatives (a) Agar (8g L^{-1}) (b) Agar (2 g L^{-1}) + Bulla (60 g L^{-1}) (c) Bulla 80 g L^{-1} (d) Bulla 100 g L^{-1}

Rooting of pineapple plantlets

The roots developed are not significantly different in their number even if it varies significantly in root length (Table 2). In line with this, using Agar as a gelling agent provided 14 thinner 3.24 cm long roots but alternately 'Bulla' 80 g L^{-1} gave 13 thicker roots which are 1.37 cm long. As it have been found by **Atlabachew and Chandravanshi (2007)** higher Zn concentration, related with biosynthesis of Indoleacetic acid **(George *et al*., 2008)**, have an impact for rooting. Similarly the seedlings obtained at rooting stage showed no significant difference in both seedlings shoot length and number of leaves which have an impact in acclimatization (Fig 2).

Table 2 The use of different gelling agent alternatives on root development of *in vitro* pineapple

Gelling agents	Root number	Root length (cm)	Shoot length (cm)	Leaf number
Agar (8g L^{-1})	14.78±3.64a	3.24± 0.79a	8.12 ±1.07a	13.22 ±2.44a
Agar (2 g L^{-1}) + Bulla (60 g L^{-1})	13.25± 4.19a	1.69±0.58b	5.46 ±1.04a	11.83 ±2.18a
Bulla 80 g L^{-1}	13.33 ±3.34a	1.37±0.51bc	4.93 ±1.31a	12.33 ±1.75a
Bulla 100 g L^{-1}	13.06 ±2.84a	1.21± 0.49c	4.99 ±0.78a	11.72 ±1.45a
Coefficient of variation	3.52	6.34	5.54	1.99
P value	0.4351	<.0001	0.1616	0.1013

Legend: Means with the same letter in a column are not significantly different separated using the procedure of REGWQ (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*).

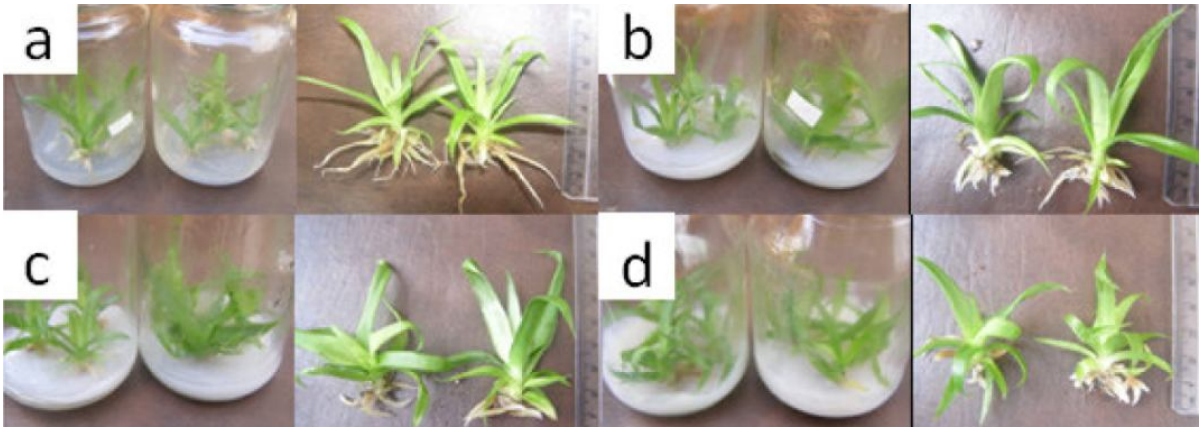

Figure 2 *In vitro* root development of pineapple using different gelling alternatives (a) Agar (8g L^{-1}) (b) Agar (2 g L^{-1}) + Bulla (60 g L^{-1}) (c) Bulla 80 g L^{-1} (d) Bulla 100 g L^{-1}

Cost analysis for different alternative gelling agents

The use of an alternative gelling agent has saved costs of pineapple tissue culture significantly at both pure 'Bulla' (with different concentrations) and in mixture with Agar (Table 3). Therefore, up to 76% cost of gelling agent is saved by the use of 'Bulla' without compromising shoot multiplication and rooting. This study provided an alternative gelling cost reduction from previous study done by **Daud *et al.* (2011)** from potato starch, rice flour, cassava flour and corn flour obtained 66-90% gelling cost reduction. Besides, it supplies nutritional value that should be well studied as shown by **Debebe (2006)** and **Atlabachew and Chandravanshi (2007)** for high concentration of K, Ca, Na, Mg, Fe, Zn, Co, Cu and Mn which is constituent of MS basal media.

Table 3 Cost analysis of different gelling alternatives

Gelling agents	Concentration used	Price per Kg in ETB*	Price for gelling agent per 1L media in ETB*	Price saved per liter
Agar	8g L^{-1}	2500	20	-
Agar + Bulla	2 g L^{-1} & 60 g L^{-1}	-	8.6	57.0
Bulla	80 g L^{-1}	60	4.8	76.0
Bulla	100 g L^{-1}	60	6.0	70.0

Legend: 1 USD = 17.22 ETB* (Ethiopian Birr)

Acclimatization and greenhouse survival

The seedlings showed 90 percent of survival in the green house condition for those seedlings derived from Agar gelling agent and 95 percent from 'Bulla' seedlings (Fig 3). This could be attributed with greener seedlings that have a potential of preparing their own food and easily acclimatizing ability to the outside environment (Fig 4). Findings observed by **Mbanaso (2008)** shows there is higher water content in Agar derived seedlings than from starch which have an impact on ex vitro survival rate. The capacity for water release as a result of explant expansion and expression (**Owens and Wozniak, 1991**) is higher in the agar matrix than in the starch matrix that provides more water to plantlet. The resultant effect would be differential water potential in both systems which would in turn affect water availability. Besides, higher concentration of K in plants occurs in special leaf cells, guard cells, due to media supplement from 'Bulla' around stomata **(George *et al*., 2008)**. The turgor pressure in the guard cells and the degree of opening of the stomata control and thus the level of gas and water vapour exchange through the stomata is regulated which affect acclimatization ability of the seedlings.

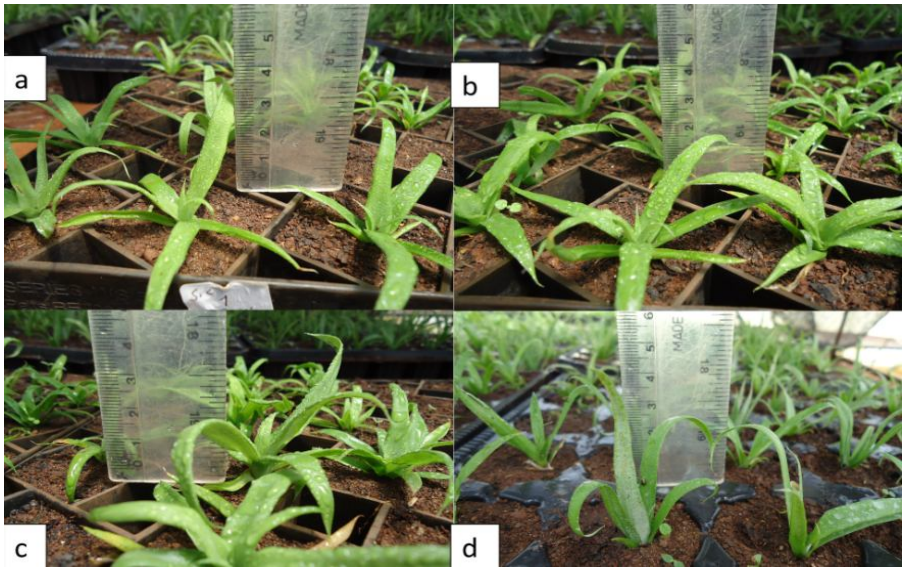

Figure 3 Green house performance of pineapple derived from different gelling alternatives (a) Agar (8g L^{-1}) (b) Agar (2 g L^{-1}) + Bulla (60 g L^{-1}) (c) Bulla 80 g L^{-1} (d) Bulla 100 g L^{-1}

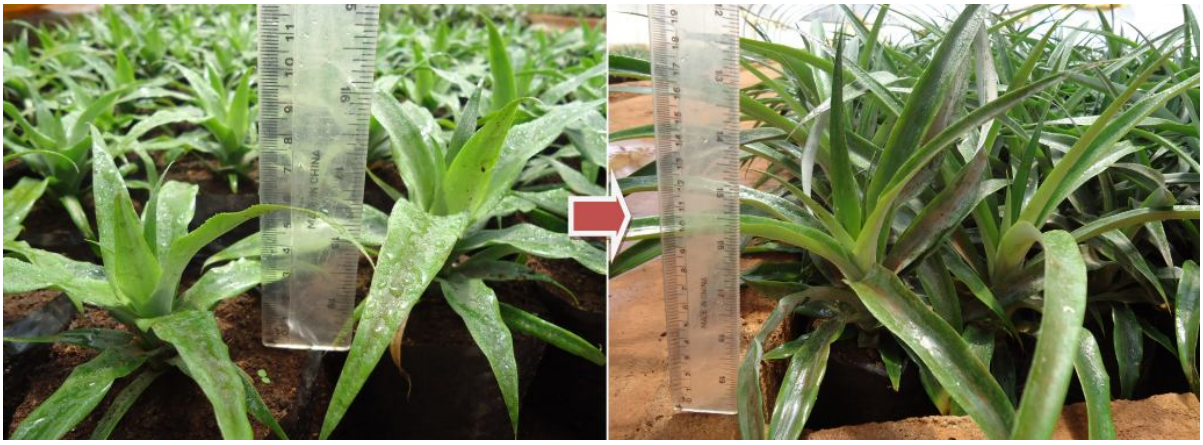

Figure 4 Pineapple seedlings ready for field plantation

CONCLUSION

In this study use of *Enset ventricosum* flour, 'Bulla', have been found a substituting gelling agent for pineapple *in vitro* propagation at different concentration and in mixture with agar. Therefore the use of 'Bulla' at 80g L^{-1} provided 11.8 shoots and 13.33 roots which are equivalent to Agar in other growth parameters except root length. Besides sustaining the cultures, this agar alternative was found cheap and available. The results of this study offer new possibilities of using low cost gelling material as agar alternative which will reduce materials costs considerably and will help in popularizing plant tissue culture techniques.

Even though a promising result is found using 'Bulla' as an alternative gelling agent for pineapple *in vitro* propagation, further studies should be seen on different crop genotypes for wider applicability. In addition, biochemical analysis should be done so that it can be used as an alternative cheap commercial gelling agent in plant tissue culture.

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