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LOW TECHNOLOGY TISSUE CULTURE MATERIALS FOR INITIATION AND MULTIPLICATION OF BANANA PLANTS

N.M. GITONGA, O. OMBORI, K.S.D. MURITHI and M. NGUGI
Tissue Culture Laboratory, Plant and Microbial Sciences Department, Kenyatta University, Kenya
Corresponding author: gitonga35@yahoo.com

ABSTRACT

Tissue culturing has become a routine method for propagating plants in high technology laboratories. The cost of production using conventional tissue culture is, however, high for most of the countries in the sub-Saharan Africa. In this study, we evaluated a micropropagating protocol for local banana (Musa spp.) (Muunju landrace) in Kenya as an alternative to reduce the unit cost of tissue culture micropropagation. Matrices were satisfactory and comparable to the gelling agents. Glass beads were, however, the best matrix in shoot multiplication. Use of support matrices, locally available macronutrients, micronutrients, sugar, equipment and facility reduced the cost of consumable material for banana tissue culturing by about 94%. Putting into account energy, labour and capital investments, the cost dropped from approximately US $ 1.5 to 1.0 per plantlet. Contamination was not observed when the media and equipment were sterilised using a pressure cooker instead of an autoclave. Use of plastic syringes instead of glass cylinders and micropipettes, to measure volumes reduced the cost of the equipment by 96%. The risk of damage and loss due to breakage was eliminated compared to the use of glassware equipment. Shoots were rooted when they were transferred to Murashige and Skoog (MS) medium supplemented with 1 mg l^{-1} napthaleneacetic acid (NAA) or 1 mg l^{-1} Anatone. Acclimatised plants were successfully transplanted and established in the field. There is potential for use of locally available low cost resources as alternatives to the conventional costly laboratory resources.

Key Words: Autoclaves, gelling agents, glass beads

RÉSUMÉ

La culture des tissues est devenue une méthode de routine pour la propagation des plants dans des laboratoires de hautes technologies. Le coût de production utilisant la culture conventionnelle de tissues est par ailleurs élevé pour beaucoup des pays d'Afrique sub-Saharien. Dans cette étude, nous avons évalué un protocol de micropropagation de bananes locales (Muunju landrace) au Kenya comme un coût bas alternatif pour réduire le coût unitaire de la propagation de tissues cultivées. Les matrices étaient trouvées satisfaisants et comparables à l'agent gélant. Les Glass beads étaitent par ailleurs la meilleure matrice dans la multiplication des bourgeons. L'utilisation des matrices de support, macronutriments localement disponibles, micronutriments, sucre, équipement et autre facilités ont réduit le coût du matériel consommable pour la culture d'environ 94% de tissues de banane. Considérant l'énergie, la main d'oeuvre et le capital investi, le coût a approximativement baissé de US $ 1.5 à 1.0 par plantule. La contamination n'était pas observée lorsque le média et l'équipement n'étaient pas stérilisés au pressure cooker à la place d'une autoclave. L'utilisation des syringues plastiques au lieu de cylindres en verre et micropipettes pour mesurer les volumes avaient réduit le coût de l'équipement de 96%. Le risque de damage et perte dû à la casse était éliminé par comparaison à l'utilisation de l'équipement en verre. Les bourgeons étaient enracinés lorsqu' elles étaient transferées au medium de Murashige et Skoog (MS) supplementé avec 1 mg l^{-1} de l' acid napthalénéacétique (NAA) ou 1 mg l^{-1} d' Anatone. Des plants acclimatisés étaient transplantés avec succès et établis dans le champ. Il y a donc une possibilité d'utiliser des resources locales à coût bas comme alternatives aux ressources de laboratoire à coût conventionnel.

Mots Cles: Autoclaves, agent gélant, glass beads

INTRODUCTION

Banana (*Musa* spp.) is an important food crop in sub-Saharan Africa worldwide and ranks the fourth most important staple crop in developing countries (FAO, 2003). Unfortunately, expansion of banana production is frequently limited by costly high quality planting materials. The farmer-produced suckers are good transmitters of insect pests and diseases (Rahman *et al.,* 2004; Haq and Dahot, 2007). This has prompted interest in the use of *in vitro* tissue culture technique. Through meristem micropropagation, pathogen free clones are obtained.

Micropropagation of banana is highly efficient, allowing a large turnover of plants in a very short period of time within very little space (Arias, 1992; Arvanitoyannis *et al*., 2007). Although conventional plant tissue culture has been applied for decades, the high cost of tissue production is a drawback for laboratories with limited resources, especially in the developing countries. In fact, the cost of the micropropagules production precludes the adoption of the technology for large scale micropropagation.

Plant tissue culturing (TC), has three components, namely, nutrients/media chemicals (plant growth hormones, vitamins and minerals nutrients), plant inocula, equipment (culture containers, autoclave, laminar flow, instruments used for micropropagation, pH meter etc) and the structures (media preparation, inoculation, growth and hardening rooms). All these form points of interventions in cost reduction as noted by Ganapathi *et al*. (1995). In addition, the process of tissue culture follows the order of: Stage 1- pre-propagation step or selection and pre-treatment of suitable plants, Stage II - initiation of explants; Stage III - subculture for multiplication/proliferation of explants; Stage IV – shooting and rooting of the explants and Stage V - hardening of plantlets (Ahloowalia *et al.,* 2004). It was estimated that during Stage II, for instance, high costs are encountered in terms of explants, media and disinfectants. The cost of medium preparation (chemicals and energy) can account for 30–35% of the cost of micropropagation of plants (Brink *et al.,* 1998; Savangikar, 2002). On the other hand, the gelling agents such as agar contribute 70% of the total cost of the media (Prakash, 1993; Savangikar *et al.,* 2004). This situation calls for alternative low cost resource to reduce the cost of production and subsequently the cost of plant propagules in sub-saharan Africa. The objective of this work was to evaluate the potential for developing a low cost micropropagating protocol for local bananas in Kenya.

MATERIALS AND METHODS

Plant material. A local variety of banana, Muunju, which is widely grown by smallholder farmers in Meru, Kenya was used as a source material for this study. The plants were maintained in a shade-net in a research farm at Kenyatta University for use as source of explants.

Media preparation. A low cost tissue culture medium was prepared using materials obtained from local agricultural-veterinary (Agrovet) shops and supermarkets; as well as conventional tissue culture medium obtained from chemical suppliers in Kenya. For shoot induction, MS basal salts (Murashige and Skoog, 1962) were supplemented with 6 mg l^{-1} BAP, 3% sugar and 0.3% gerlite (SIM). The shoot multiplication medium comprised of the low cost and conventional media. Tables 1A - C show the macro- and micronutrients and carbon source which were used in the preparation of conventional and the test tissue culture media and their cost. The shoot multiplication media also contained MS vitamins (Murashige and Skoog, 1962), supplemented with 6 mg l^{-1} BAP and 3% sugar alone (liquid medium) or in combination with different gelling agents (agar and gerlite) or support matrices (cotton wool, vermiculite and glass beads) (SMM). Support matrices (cotton wool, glass beads and vermiculite) were considered as low cost materials (Table 1D). Different sources of water, namely tap, rain and distilled, were used to prepare the media for shoot multiplication. For rooting, MS basal salts was supplemented with 3% sugar, and 1 mg l^{-1} NAA (conventional growth regulator) or 1 mg l^{-1} Anatone (low cost

TABLE 1A. Substitution of macronutrients in basal Murashige and Skoog plant culture media

Convectional macronutrient (CM)	Substitute to macronutrient (SM)	Cost (KES kg^{-1})			
		Cost in media (%)	CM	SM	% cost saving (%)
Calcium Chloride ($CaCl_2$)	Calcinit	5.8	1800.0	105.0	94.2
Potassium Hydrogen Phosphate (KH_2PO_4)	Mono potassium phosphate (MKP)	7.1	1700.0	120.0	92.9
Potassium Nitrate (KNO_3)	Potassium fertiliser	5.0	1800.0	90.0	95.0
Magnesium Sulphate ($MgSO_4$)	Epsom Salt	6.9	1300.0	90.0	93.1
Ammonium Nitrate (NH_4NO_3)	Ammonium fertiliser	3.8	3000.0	115.0	96.2
Total cost		5.8	9600.0	520.0	94.2

KES = Kenya shillings (1 US$ = 80 KES); CM = Convectional macronutrients; SM = Substitute Macronutrients

TABLE 1B. Substitution of micronutrients in basal Murashige and Skoog plant culture media

Micronutrient convectional micronutrient	Cost (KES kg^{-1})				
	Substitute to provide micronutrient	Cost in media (%)	CM	SM	% cost saving (%)
Cobalt chloride ($CoCl_2.6H_2O$)	Stanes Micro Food	2.2	10400.0	1200.00	97.8
Copper Sulphate ($CuSO_4.5H_2O$)			9000.0		
Sodium citrate (Na_2EDTA)			2066.0		
Iron Sulphate ($FeSO_47H_2O$)			1400.0		
Hydrogen bor(H_3BO_3)			8260.0		
Potassium Iodate (KI)			4200.0		
Manganese Sulphate ($MnSO_4.H_2O$)			2714.0		
Sodium Molybdate ($NaMo_4.2H_2O$)			15636.0		
Zinc Sulphate ($ZnSo_4.7H_2O$)			944.0		
Total cost		2.2	54620.00	1200.00	97.8

KES = Kenya shillings (1 US$ = 80 KES); CM = Convectional macronutrients; SM = Substitute Macronutrients

TABLE 1C. Substitution of carbon source in basal Murashige and Skoog plant culture media

Carbon source	Cost of carbon source (KES kg^{-1})			
Convectional carbon source	Substitute to provide carbon	Convectional sucrose	Substitute: Table sugar	% cost saving
Sucrose	Table sugar	3500.00	80.0	97.7
Total cost				

(1 US$ = 80 Kenya Shillings (KES))

TABLE 1D. Solidifying agents and support matrices in basal Murashige and Skoog plant culture media

Convectional solidifying agent	Substitute to the solidifying agent	Cost (KES kg^{-1})		
		Convectional substitute	% cost saving per $litre^{-1}$	of media
Gelrite	Glass Beads	26000.00	105.00	94.2
Agar	Vermiculite	29000.00	120.00	92.9
-	Cotton		90.00	93.1
-	Glass Beads		115.00	96.2
Total cost		5500.00	430.00	99.2

(1 US$ = 80 Kenya Shillings (KES))

growth regulator) 3% gerlite (RIM). The media were sterilised using a pressure cooker for 15 minutes after adjusting the pH to 5.8 with 1 N NaOH or 1 N HCL.

Sterilisation and preparation of banana explants. All banana suckers were washed in running tap water for 20 minutes. The ensheathings were removed from the pseudostems to remain with the shoot tip meristem which, was excised. Explants were kept in 1.5% citric acid for 30 minutes. They were surface sterilised in 70% ethanol for 1 minute, followed by 1.54% commercial jik with a 0.1% Ungral for one hour. Commercial bleach (jik) and ungral (an equivalent chemical used in the detergents and soap industry) were used as a substitute for the convectional sodium hypochlorite and Tween 20. The explants were rinsed five times in sterile distilled water.

Shoot initiation. Sterile banana shoot meristems were cultured onto the SIM. One explant was cultured in each culture bottle and this was replicated 20 times. Cultures were incubated at 27±1 °C at 16 hr of fluorescent tube light and 8 hr darkness.

Shoot multiplication. From the same culture, initiated shoots of uniform size, were sub-cultured into SMM. One explant was cultured in each culture bottle, replicated 16 times. The cultures were incubated at 27±1 °C and 16 and 8 hr light and darkness, respectively. After four weeks, the number of shoots produced per explants was recorded.

Rooting. Established shoots were separated and transferred onto RIM. One explant was cultured in each culture bottle, replicated 10 times. The cultures were incubated at 27±1 °C and 16 and 8 hr light and darkness, respectively. The number of days to root initiation was recorded. After three weeks, the number of roots and length of the roots was recorded. The effect of different culture media was tested.

Acclimatisation and transfer of *in vitro* banana plantlets. The opened culture bottles were kept for two days in the shade-net to enable primary acclimatisation of the *in vitro* regenerants. The plantlets were then removed from the culture bottles and washed in running tap water to remove the nutrient media to avoid root fungal attack. Then the plantlets were planted in wooden trays containing rice husks and vermiculite and kept moist for secondary hardening. The use of rice husks and shade-net as alternatives to vermiculite and greenhouse, respectively was again an attempt to reduce the unit cost. After 21 days of hardening, the plantlets were transferred into polythene bags containing a mixture of sterilised garden soil and composite manure in a ration of 1:1. The survival percentage of the plants during hardening was recorded to assess the success of the protocol of using alternative resources. Well established plants were transplanted into the field after 60 days. The locally available low cost equipment used as alternatives to the conventional ones are presented in Table 2.

TABLE 2. Substitution of plant tissue culturing equipment and facilities

Convectional equipment and facility	Cost in KES	Substitute low cost equipment and facility	Cost in KES	% cost saving
Equipment				
Autoclave	70000.00	Pressure cooker	10000.00	87.8
Culture bottles	450.00	Jam jar bottles	15.00	96.7
Micropipette	250.00	Insulin syringes	10.00	96.0
Measuring cylinders	250.00	Vet syringes	10.00	96.0
Petridish	7.00	Office waste papers	1.00	85.7
Aluminium foil	145.00	Office waste papers	1.00	99.3
Subtotal	71102.00		10037.00	85.9
Facility				
Greenhouse	800000.00	Shade net	48000.00	94.0
Total	871102.00		58037.00	93.3

(1 US$ = 80 KES)

Data analysis. The data were analysed using Analysis of Variance (ANOVA) with MINITAB Software, Version 23.22. Means were separated using Tukey's Honest Significant Difference at 5% level.

RESULTS AND DISCUSSION

The substitution of the conventional tissue culture chemicals, equipment and facility with the alternatives available locally in Kenya significantly ($P<0.05$) reduced the cost of initiation and multiplication of Muunju banana variety (Tables 1 and 2). Commercially available macro- and micronutrients occurring in the form of hydro Agri´s fertiliser has also been used previously successfully as an alternative resource for *in vitro mi*cropropagation cassava (Santana *et al.*, 2009).

Our results show that the substitution of sucrose used in conventional tissue culture with table sugar reduced the cost by 97.7% (Table 1C). The use of market sugar instead of sucrose has been reported to reduce the cost of *in vitro* conservation of banana, Karpura chakkarakeli cultivar, with no significant effect on regeneration compared to sucrose (Agrawal *et al*., 2010). According to Goel *et al*. (2007), use sugar in glass beads supported liquid medium caused up to 94% reduction in the cost of the medium used for culturing of *Rauwoflora seperpentina*. The purpose of using sugar in this study was to reduce the overall cost of micropropagating and testing the response of the local banana.

The substitution of macronutrients and micronutrients with the alternatives reduced the cost by 94.2 and 97.8%, respectively (Table 1A-B). Substitution of gelling agents (agar and gerlite) with support matrices (glass beads, cotton wool and vermiculite), conventional equipments (autoclave, culture bottles, micropipette and measuring cylinder) with easily accessible alternatives (pressure cooker, jam jars, insulin and vet syringes) reduced costs by 94.2 and 85.9%, respectively (Table 1A and 2). Support matrix (glass beads) and plastic equipment (jam jars, insulin and vet syringes) were used repeatedly after maintaining and washing them thoroughly before re-autoclaving, thus reducing the cost significantly. Support matrices have been used successfully as a low cost alternative to gelling agents (Bhattacharya *et al.*, 1994; Goel *et al*., 2007). Removing plantlets from the medium with support matrices was easier than with gelling agents. The use of plastic syringes as an alternative for glassware cylinders for measuring solutions during media

preparation eliminated breakages which result from human error.

When the autoclave was substituted with a pressure cooker to sterilise media and apparatus, there was no detectable contamination (Table 1C). This shows that that the pressure cooker can effectively replace the autoclave for this purpose. Office waste papers effectively played the role of aluminium foil in covering instruments during sterilisation process and an alternative for petridishes when culturing banana explants (Table 2). This reduced the expenditure on these materials by 99.3 and 85.7%, respectively (Table 2). Wrapping papers have been reported to substitute aluminium foil during the sterilisation of implements as a way of reducing costs incurred when culturing cassava (Escobar *et al.*, 2006). When a shade-net was used instead of a greenhouse with elaborate controlled conditions for the acclimatisation of *in vitro* regenerants, the associated costs were reduced by 94% (Table 2). Overall, the alternative measures used in producing tissue culture banana plantlets in this study cut down the costs of the conventional process by 93.9% (Tables 1 and 2).

There was no detectable contamination when sodium hypochlorite and Tween 20 were substituted with commercial bleach (jik) and ungral for sterilisation of explants. Again this shows that these alternative materials can be used successfully for sterilisation purposes. The use of commercial bleach also reduced cost of sterilisation by 79.46%.

Shoots were successfully initiated from the shoot tip meristem *via* direct organogenesis. Shoots were initiated directly from the edges (circumference) of the explants. Results obtained showed that Muunju landrace had a high morphogenetic potential during shoot multiplication. On the other hand, the number of shoots produced during shoot multiplication was not affected significantly ($P>0.05$) by different sources of water and type of media used (Table 3). Water is one of the major components used in preparation of the culture media; however, distilled water is expensive in developing countries. Thus, the use of alternative sources of water such as rain or tap water can help to reduce the cost of propagation of local banana.

Multiple shoots were produced when gelling agent (gerlite) and different support matrices (cotton wool, vermiculite and glass beads) were used. The type of gelling agent and support matrices used during the multiplication of shoots in this study had a significant effect ($P<0.05$) on the number of shoots produced (Table 4). This shows that besides the media ingredients, the type of gelling agent or support matrix used influence shoot regeneration. Glass beads as the support matrix produced the highest number of shoots, while the liquid medium produced the lowest (Table 4). With the exception of cotton wool, all support matrices generally produced more shoots than gelling agents (Table 4). This could be due to easy diffusion of nutrients from support matrices to the shoots, hence, promoting rapid shoot multiplication. There is a decrease in water potential in gelling agents compared to support matrices which contributes to the limitation of the nutrient uptake (Bhattacharya *et al.*, 1994). The low number of plantlets produced when cotton wool was used as a support matrix is likely due to phenolic compounds which were exuded

TABLE 3. Effect of different sources of water and type of media on shoot multiplication after 28 days of culture in shoot multiplication medium

Water source	Type of media	
	Conventional tissue culture medium	Low technology tissue culture medium
Tap water	7.63±0.45	6.94±0.48
Rain water	7.31±0.43	7.32±0.51
Distilled water	7.06±0.48	6.75±0.48
	7.33±0.26	7.00±0.28

TABLE 4. Effect of gelling agents and support matrices on shoot multiplication after 28 days of culture in shoot multiplication medium

Gelling agent/support matrix	Mean number of shoots
Gelling agent	
Gerlite	7.71±0.49c
Agar	7.60±0.33c
Support matrices	
Cotton wool	5.20±0.15b
Vermiculite	8.14±0.45c
Glass bead	9.14±0.37c
Liquid medium	1.0±0.01a

Means followed with different letters within the column are significantly different from each other according to Tukey's Honest Significant Difference at 5% level

from the tissues of the cultured banana explants which got stuck onto the cotton matrix and the explants darkened, thus locking the uptake of nutrients. Phenolic compounds are oxidised to quinones which darkens the tissue culture materials and are also known to be highly reactive and toxic to the tissues (Taji and Williams, 1996; Titov *et al.*, 2006; Martin *et al.*, 2007; Ozyigit, 2008). This leads to high mortality of the plantlets in the culture and also inhibits plant regeneration (Ozyigit *et al.*, 2007).

The liquid medium produced the lowest number of shoots without shaking (Table 4). This could be due to rotting of explants caused by super hydration and lack of air for growth to take place. Goel *et al.* (2007) recorded a low shoot multiplication rate of *Rauwolfia serpentine* in a liquid medium, which they attributed to asphyxiation of explants which resulted due to submergence in the medium. Similar results of a low number of shoots when using static liquid medium were reported by Puchooa *et al.* (1999) who worked on the tobacco. This means that liquid media need to be agitated to provide sufficient oxygen to the tissues.

Shoots were rooted successfully in the medium containing both conventional and low technology growth regulators. Root induction of the roots was observed on the 7th day after culture when 1 mg l^{-1} napthaleneacetic acetic acid (NAA) was added into the rooting medium; and on the 5th day with 1 mg l^{-1} Anatone (Table 5). This indicates that Anatone has more strength in the induction of roots compared to conventional NAA. However, there is need to conduct quality analysis to assess the full constituents of Anatone.

The number of roots produced when 1 mg l^{-1} NAA and 1 mg l^{-1} Anatone were added into the medium was 6.00 and 6.46, respectively. The length of the roots produced after 21 days when 1 mg l^{-1} NAA and 1 mg l^{-1} Anatone were added into the medium was 6.17 cm and 6.41 cm, respectively. There was no significant difference ($P>0.05$) between the number and length of the roots formed when NAA and Anatone were used as growth regulators (Table 5). This shows that Anatone is comparable to the NAA in their effect on rooting. This could be due the fact that the anatone has a similar mode of action and strength to NAA which is used in conventional tissue culture. Anatone is a commercial plant regulator which was used as a source of NAA in this study. Therefore, rooting of banana shoots can be optimised using Anatone as an alternative plant growth substance which is less costly.

Plantlets were successfully acclimatised using rice husks and then transplanted into the potted soil in the shade net. Eighty three percent of the plants survived during the acclimatisation procedure when rice husks were used compared to 80% using conventional approach (use of vermiculite). Rice husks which are available and free of charge in the rice growing areas of Kenya can, therefore, be used as an alternative

TABLE 5. Effect of conventional and low cost growth regulator on root formation of Muunju banana variety

Treatment	Days to root emergence	Roots after 10 days of culture	Root length (cm) after 21 days
MS + 1 (mgl^{-1}) NAA	7.00	6.00±0.47	6.17±0.18
MS + 1 (mgl^{-1}) Anatone	5.00	6.46±0.56	6.41±0.51

resource during acclimatisation to reduce costs. Well established plants were successfully transplanted into the field after 60 days. This shows that it is possible to develop a low cost tissue culture protocol for production of banana plantlets within short periods.

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