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Cassava Starch as Alternative Low-cost Gelling Agent for *In Vitro* Micropropagation of Three Musa Genotypes

Victoria Wilson[1]* and Abdou Tenkouano[2]

[1]*Department of Plant Science and Biotechnology, Rivers State University, Port Harcourt, Rivers State, Nigeria*
[2]*CORAF, West and Central African Council for Agricultural Research and Development, Dakar, Senegal*

ABSTRACT

Aims: *This study was conducted to compare the effect of starch from cassava (Manihot esculenta, Crantz) as a gelling agent with that of GelriteTM, a standard gelling agent in the basic MS medium on the rate of shoot initiation, multiplication and regeneration in vitro of three Musa genotypes.*

Study design: *Randomized Complete Block Design with three replications. Analysis of variance (P=0.05) was used to test treatment effects and mean comparison was by Duncan's Multiple Range Test (DMRT) at P=0.05.*

Place and Duration of Study: *The study was carried out at the Plant Tissue Culture Laboratory of the International Institute of Tropical Agriculture (IITA) High Rainfall Station, Onne in Rivers State, Nigeria for a period of ten months.*

Methodology: *The performance of three Musa genotypes [tetraploid plantain hybrid PITA 14 (AAAB) genome, tetraploid cooking banana hybrid BITA 3 (AAAB) genome and a cooking banana landrace Cardaba (ABB) genome] in gelrite, was compared with an alternative gelling agent - starch from cassava (Manihot esculenta Crantz) in a three step micropropagation shoot initiation, multiplication and regeneration cycle*

Results: *The number of shoots initiated in BITA 3, PITA 14 and Cardaba, did not differ significantly with gelrite and starch as gelling agents during shoot initiation. During multiplication and regeneration stages, there were no significant differences in the effect of gelrite and cassava starch on number of shoots produced by BITA 3 and Cardaba. However, plantain hybrid PITA 14 produced significantly higher number of shoots in gelrite than in cassava starch at both stages. Cardaba had significantly lowest number of shoots than the hybrids in all 3 stages.*

Conclusion: *Cassava starch is not an effective low cost substitute for gelrite beyond shoot initiation for PITA 14. Addition of small amounts of gelrite to cassava starch could improve its gelling ability in later stages. Further studies will need to confirm this.*

Key words: Musa Genotypes, Gelling Agent, Low Cost, Multiplication, Proliferation, Regeneration Shoot Initiation

INTRODUCTION

Micropropagation involves the use of small pieces (about 2-5 mm) of plant material in *in vitro* (tissue) culture [1]. In most plants, *in vitro* plant regeneration has been found to depend on many factors. Some of the most important are (a) composition of the basic medium, (b) growth regulators, [2,3]; (c) gelling agent [4], (d) light intensity and quality, (e) photoperiod, (f) temperature, and (g) cultivation vessels and vessel covers [5]. Others include (h) cultivar /genotype (i) explants types and size, (j) pH, (k) surface sterilization and contamination, (l) phenol exudation and its control, (m) carbohydrate/ carbon source and (n) additives, [6-10]. Genotype is important in all biological processes, and the differential response to *in vitro* culture of different genotypes of the same species has been well documented [6,2,11]. A tissue culture study reported [12] that plantain genotypes produced more vigorous-growing healthy shoot tips than the bananas. The most common types of *in vitro* multiplication methods include shoot-tip culture, anther culture, axillary bud culture, leaf disc culture, and protoplast culture. Several studies have shown successful tissue culture

in Musa using apical meristem [13,3], shoot-tip [14-16], floral explants [17-20], protoplast culture [21], embryo culture [22], organ formation [23] and immature fruits as explants [24,25]. Shoot-tip culture, commonly used in Musa micropropagation is simple, easy and used for slow-growth storage of germplasm [1,26]. It was also reported [27] that over 400 Musa accessions of seven genomic constitutions (AA, AAA, AAAA, AAB, AAAB; ABB and BB) have been successfully propagated by *in vitro* shoot tip culture at IITA. The main focus at the time was the disease resistant breeding programme and to supply farmers and research institutes. Interest on low cost *in vitro* alternatives only developed later. Other studies have indicated that the rate of multiplication is high and ranges from two to about ten shoots per subculture at an interval of 30 days [28,29]. More recently up to 10,000 plantlets from a single sucker within eight months have been reported [30]. The advantages of the *in vitro* method over conventional propagules (suckers) include higher rates of multiplication, production of clean and uniform planting materials and the small amount of space required to multiply a large number of plants [31]. Tissue cultured plantlets are lighter than suckers, especially if a light potting substrate is used making transportation easier. They establish faster, grow faster and more vigorously, have a shorter and more uniform production cycle, produce more leaves and suckers and are more genetically uniform than the conventional vegetative suckers [25,31,32]. Tissue cultured plantlets flower earlier than conventional vegetative suckers and give a uniform harvest that makes for better marketing. They have a fruit yield advantage of 20-50% [30,33,34,25] as well as in the following yield components: number of hands in a bunch, number of fingers in a hand, length of the finger, girth of the finger and girth of the pseudostem [32,35]. Moreover, the most reliable technique to reduce risks of transmitting seed-borne pests and diseases is tissue culture [36]. Hence, *in vitro* culture is vital to Musa breeding for multiplication and dissemination of new hybrids and makes tissue culture the best and most prolific technique for Musa multiplication. The main disadvantages of *in vitro* culture are that it requires special skill and sophisticated equipment and high capital outlay [37] that are not readily available to farmers. In addition, explants contamination in commercial and scientific laboratories can be as high as 15% [38-40] the majority of which are caused by fungal, yeast and bacterial contaminants [41]. Also, tissue-cultured plants are always tender and require greater care during the first two months after planting [42]. In order to ameliorate these limitations, attention has been placed on modifying the composition of the growth media so as to lower production costs [43,44] and accommodate a wide range of plants [45,6]. One way of reducing costs of *in vitro* culture in developing countries so that they become more readily affordable is by progressive replacement of most of the very expensive components with low cost alternatives without significant loss in efficacy of the medium and final outcomes [46]. Various gelling agents such as agar, agarose and gellan gum are marketed under trade names such as GelriteTM, PhytagelTM, [47], and 'Gel-GroTM' [48] and used for plant culture media. Gelrite, the conventional standard gelling agent is very expensive, [49] while a commonly used alternative to gelrite, agar, is only slightly less expensive [50-52]. Moreover, although agar was thought to be biologically inert and non-toxic, its adverse effects such as embryo abortion and reduced plantlet production have been reported [53-55]. Gelrite on the other hand induced hyperhydricity on regenerated shoots [56]. Sago has been used as a low cost alternative gelling agent by other researchers [57]. Other studies have tried a combination of corn starch and potato starch [58,59] and cassava starch [60,61,52]. A combination of cassava starch and agar [4,8,52] has also been explored with varying degrees of success. Most researchers agree that cassava starch is odourless; the paste is clear, sticky with gelling properties and contains small amounts of fiber, protein, lipids, minerals, and phosphates [62-69] although one study had reported lipid and protein contents as high as 21% and 2% respectively from cassava obtained from Sokoto, in the Sahel region of Nigeria [70]. Reports of proximal characterization of cassava starch sometimes differ slightly from each other [63,65,66,71]. These differences could be due to several factors that affect the quality of native starch such as genotype, variety, age of the crop at harvest time, type of soil, climatic conditions, vegetative health of the tubers, technology implemented in extraction stage, purification/ contamination, storage conditions, geographic origin, etc [65,71-74]. Pure cassava starch forms a gelatinous matrix that can be autoclaved and stored and thereafter melted by heating [75-77]. In addition, it is cheap, available all year round and being a product of plant origin is biodegradable and poses no threat to the environment when properly disposed of after use. The use of 10% cassava starch as gelling agent compared to 0.8% agar, reduced cost by as much as 42.5% in micropropagation of potato without loss in efficacy [78]. This concentration is lower than the 14% earlier reported [79] for tissue culture of potato and shoot regeneration and higher than the 8% reported [76] for tobacco and chickpea culture. It was pointed [77] out that cassava flour at 8% (w/v) provides optimum gelling; and its quality can be improved by mixing cassava flour with some agar (80 g L^{-1} cassava flour+3.5 g L^{-1} agar). It was also reported [60] that 6 and 7% (w/v) cassava starch gave adequate support and orientation to Musa shoot tip explants but gel stability was only maintained for 7 days. Cassava starch powder at 10% (w/v), has been reported to give satisfactory setting, typical of solid nutrient medium in pour plates and slants for microbial growth [70]. All these studies seem to indicate that the efficacy of cassava starch and the right concentration to be used might depend on the nature of the plant and the plant genotype [11]. Other factors are the quality of the starch and the cassava cultivar from

which the starch is obtained [52,49,80]. However, many of these studies have rarely reported the effects of cassava starch during and over each of the 3 stages of tissue culture, that is, the shoot initiation, multiplication/ proliferation and regeneration stages of growth.

The purpose of this work was to investigate if and how the rate of shoot initiation, multiplication/ proliferation and regeneration *in vitro* of 3 Musa genotypes, would differ significantly when an alternative low cost gelling agent, starch derived from cassava (Manihot esculenta Crantz) was substituted for gelrite, a standard gelling agent in the basic MS medium during each of the stages of tissue culture.

MATERIALS AND METHODS

This study was carried out at the Plant Tissue Culture Laboratory of the International Institute of Tropical Agriculture (IITA) High Rainfall station, Onne (4°51'N, 7° 03'E, 10 m above sea level), in Rivers State, south-eastern Nigeria.

Preparation of explants

Three Musa genotypes formed the source of the explants: a tetraploid plantain hybrid PITA 14 (TMPx 7152-2) (AAAB) genome, a tetraploid cooking banana hybrid BITA 3 (TMBx 5295^{-1}) (AAAB) genome and a cooking banana landrace Cardaba (ABB) genome. Meristematic tissues were obtained from shoot tips of sword suckers of the three Musa genotypes. Aseptic cultures were established by isolating tiny shoot tips of about 5 mm long from buds and suckers of source plants, rinsing with ethanol (95%) and surface disinfecting with 0.05 to 0.75% (w/v) sodium hypochlorite for 15 minutes before washing with autoclaved deionized water.

Table 1. Modified MS Nutrient Culture Medium for Musa shoot multiplication.

Components	Shoot Initiation Medium (per L)	Multiplication/Proliferation Medium (per L)	Regeneration Medium (per L)
MS* basal medium	4.43 g	4.43 g	4.43 g
My-inositol	100 mg	100 mg	Nil
Sugar	30 g	30 g	30 g
NAA (Naphthalene Acetic Acid)	0.18 mg	Nil	Nil
BAP (Benzyl Amino Purine)	2.3 mg	4.5 mg	2.25 mg
IAA (Indole Acetic Acid)	Nil	0.18 mg	0.18 mg
Ascorbic acid	10 mg	10 mg	1 mg
Gelrite	2 g	2 g	2 g
Note: MS* - [81]. Revised medium for Musa [26] shoot multiplication pH = (5.8 ± 0.1); Autoclave at 121°C for 15 mins The culture medium was based on MS [81] medium but modified [26] for Musa shoot multiplication			

Powdered chemicals (Table 1) were each weighed into the beaker following the above checklist. For components requested in very small quantity (growth regulators, vitamins) stock solutions were prepared at an adequate concentration. After all components of the check list had been added to the beaker (except for the solidifying agents, gelrite and cassava starch), this was placed on the magnetic stirrer (model: Cleaver Scientific Ltd) until fully dissolved. The volume of the solution was then adjusted with distilled/ deionized water to 9/10 of the final volume with a measuring cylinder. The pH of the medium was adjusted by stirring and adding droplets of buffer solutions 0.5 M NaOH (below 5.7) or 0.5 M HCl to 5.8 before autoclaving. The pH was allowed to stabilize after each droplet was added in order not to over-acidify or alkalinize the medium. Once the pH was set, the medium was shared into two before the required quantity of each of the solidifying agents was added.

Gelrite

Gelrite (2 g) was weighed into a beaker and distilled water added to reach the final volume. For the gelling agent to dissolve, the mixture was heated on a hot plate (model: Cleaver Scientific Ltd), (20 min at maximum level for 2 L of mixture) and distributed into culture vessels prior to autoclaving using Autoclave model 25X 2389 stainless steel.

Preparation of cassava starch

Freshly harvested tubers (20 kg) of cassava (Manihot esculenta Crantz) cultivar (TME 419) were peeled, washed thoroughly and then crushed. The pulp was suspended in water, sieved and the effluent collected. The effluent was left overnight for the starch to sediment and the supernatant decanted. The surface of the starch was rinsed with clean water and the starch scooped into trays for sun drying at ambient temperature to remove excess moisture. Further drying to constant weight was carried out in a moisture extraction oven (model 655F). The dried starch was milled into powder using a milling machine (Thomas Willey Mill –model Ed-5. Philadelphia U.S.A. 1982) and packaged in

sealed polyethylene bags until required. For each concentration (70 gL^{-1}), the dry cassava starch powder was first made into thick slurry with part of the medium to be gelled. Heat was applied to the remaining medium to a temperature of 78 ± 1°C and the corresponding cold slurry stirred vigorously into it. The medium was dispensed into culture vessels and autoclaved (Table 2).

Table 2. Percentage protein, fibre, lipid and carbohydrate content of cassava (TME 419)

Parameters	%
Protein	1.76
Fibre	2.36
Lipid	0.39
Carbohydrate	86.47

Inoculation and incubation

Inoculation of Musa genotype propagule materials was carried out in a sterile laminar airflow hood chamber (Laboratory Furniture & ASHRAE 110.95 UK tested fume hoods ISO 9001 & ISO 18001). Surface sterilization was achieved through spraying 70% (v/v) ethanol. The block of tissue was surface sterilized (with 7% solution of Sodium hypochlorite plus Tween 80) and transferred to sterile condition. At this stage, shoot-tips in sterile Petri dishes were excised using forceps and scalpel and each shoot-tip (about 5 mm long) was split into two. Each half was inoculated in culture tubes (125 cm × 150 cm), containing 10 ml of MS medium solidified with gelrite or cassava starch, with 3 culture tubes per treatment replicated 3 times giving a total of 54 culture tubes. These were incubated at 22°C ± 2°C under a 16 hour photoperiod with a photosynthetic photon flux density of 40 µMol M-2 S^{-1} provided by overhead white fluorescent tubes. Temperatures were maintained at 27°C ± 2°C by air conditioning units. After 35 days in the shoot initiation culture medium, (stage 1) sub-cultures were carried out by separating shoot clusters developed from explants into individual shoots which were then trimmed down to shoot tips and sub-cultured in the multiplication/ proliferation culture medium (stage 2) and after 35 days sub-cultured again into the regeneration culture medium (stage 3). At the end of 35 days, well-formed shoots were subsequently transferred into rooting medium.

Treatment applications and experimental design

Treatments comprised the three Musa genotypes and the two gelling agents (gelrite and cassava starch) with 3 replications in a randomized complete block design.

Data collection and statistical analyses

Data were collected at the end of each of the 3 stages of the experiment as follows:

Stage 1: Shoot Initiation

Stage 2: Multiplication/ Proliferation

Stage 3: Regeneration

The numbers of fully formed plantlets from shoot-tips were counted at each of the stages. The data were subjected to square-root transformation, prior to analysis of variance (ANOVA) to test treatment effects. All data were analyzed using the general linear model procedure of Statistical Analyses Software (SAS) [82]. Any effects found to be significant have been tested at a significance level of 5% (P =.05) while means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05.

A comparative cost analysis of gelrite and cassava used as gelling agents was carried out to determine if indeed there was a cost reduction from use of cassava starch instead of gelrite.

RESULTS AND DISCUSSION

Shoot initiation stage

The use of gelrite or cassava starch as gelling agent did not produce significant differences in the number of shoots initiated in the first 35 days in each of the Musa genotypes (Table 3). Meaning that although number of shoots initiated in BITA 3, PITA 14 and Cardaba, was less when cassava starch was used as gelling agent, it did not differ significantly from when gelrite was used as gelling agent. It was reported [60] that no significant differences occurred in number of shoots and roots of banana cultured in cassava starch and agar over four subcultures. Potato plantlets cultured in agar were found [78] to have significantly less number of nodes than those cultured in starch and liquid medium. However, potato plantlets cultured on either cassava starch, cassava starch mixed with agar or on liquid medium were

not significantly different in the 3 generations of sub-culturing. Earlier report [4] showed that cassava starch was found to be a suitable substitute for agar in the *in vitro* culture of banana since differences in parameters measured were not significant. With respect to overall performance of genotypes, the number of shoots initiated in the cooking banana landrace Cardaba, was significantly less than those initiated in BITA 3 (cooking banana hybrid) and PITA 14 (plantain hybrid) indicating perhaps greater vigour of the hybrids over the landrace (Table 3).

Table 3. Effects of gelrite and low cost cassava starch as gelling agents on number of *in vitro* shoots in 3 Musa genotypes at shoot initiation stage.

Musa Genotypes	Mean Number of Shoots at Shoot Initiation Stage	
	Gelrite	Cassava Starch
Cooking Banana hybrid BITA 3 (AAAB)	2.90 ± 0.06^{a}	2.29 ± 0.24^{a}
Plantain hybrid PITA 14 (AAAB)	3.06 ± 0.28^{a}	2.66 ± 0.16^{a}
Cooking banana landrace Cardaba(ABB)	1.29 ± 0.12^{b}	1.29 ± 0.19^{b}
NOTE: Means followed by the same letter are not significantly different at p=.05 with DMRT		

Shoot multiplication/proliferation stage

During the multiplication/ proliferation stage, there were no significant differences in the effect of gelrite and cassava starch as gelling agents on the number of shoots produced by the cooking banana hybrid BITA 3 and the cooking banana landrace Cardaba (Table 4). However, for the plantain hybrid PITA 14, gelrite produced significantly higher number of shoots than cassava starch meaning that this Musa genotype performed significantly differently in gelrite and cassava starch at the multiplication/proliferation stage. The implication is that while cassava starch alone could be an ideal low cost substitute for gelrite in stage one for all Musa genotypes and for BITA 3 and Cardaba, in stage 2 at the proliferation/multiplication stage, it is not an effective standalone substitute for gelrite with respect to PITA 14. For this genotype PITA 14, perhaps addition of small amounts of gelrite to cassava starch could improve its gelling ability and increase the production of shoots as recommended [78] in micropropagation of potato (*Solanum tuberosum L.*) and in micropropagation of banana [80]. As in stage one, in comparing overall performance of genotypes, the plantain hybrid PITA 14 produced significantly more shoots than the bananas. Similar findings had been reported [12] that plantain genotypes produced more vigorous-growing healthy shoot tips than the bananas. Cardaba produced significantly fewer shoots than both hybrids in stage 2 (Table 4).

Table 4. Effects of gelrite and low cost cassava starch as gelling agents on number of *in vitro* shoots in 3 Musa genotypes at multiplication/ proliferation stage

Musa Genotypes	Mean Number of Shoots at Multiplication/Proliferation Stage	
	Gelrite	Cassava Starch
Cooking Banana hybrid BITA 3 (AAAB)	8.09 ± 2.00^{bc}	7.81 ± 1.31^{c}
Plantain hybrid PITA 14 (AAAB)	14.44 ± 2.00^{a}	8.42 ± 1.67^{b}
Note: Mean ± standard deviation with 3 replications in each treatment Means followed by the same letter are not significantly different at P=0.05 with DMRT		

Regeneration Stage

The effects of starch and gelrite gelling agents at the regeneration stage are shown in Table 5. The plantain hybrid PITA 14 had significantly more shoots when cultured in gelrite than in cassava starch but the number of shoots produced by BITA 3 did not differ significantly under gelrite and cassava starch, neither did the number of shoots of Cardaba. It is noteworthy that for both bananas, BITA 3 and Cardaba in all three stages, cassava starch could serve as a standalone substitute for gelrite without significant loss in shoot production. However, for PITA 14 plantain hybrid, addition of small amounts of gelrite to cassava starch could be required to improve shoot production in the multiplication and regeneration stages. The superiority of gelrite to cassava for this genotype could be attributed perhaps to an inherent higher level of purity as starch products are not biologically inert [83] making it less inhibitory than the starch to explant proliferation and regeneration under the conditions of these experiments as explained [84]. They reported that gel strength is often an important factor for any gelling agent. In their study, they observed drowning at 70 g/l cassava starch concentration, but when the concentration was increased to 96 g/l, adequate support and orientation of the plantlets was achieved. This is in contrast to 60 g/l cassava starch concentration as reported [85] for *in vitro* propagation of Chrysanthemum mortifolium, but close to 100 g/l reported [59] for *in vitro* potato propagation of Celosia spp. Other reasons may include limited diffusion of nutrients, lateral diffusion of water and presence of impurities [86,87]. Perhaps gelrite retains more water than cassava starch, allowing more water availability for this genotype, implying perhaps that it needs more water than the other genotypes for its more vigorous growth. Comparing genotypes, again as in stages 1 and 2, the plantain hybrid PITA 14 had significantly more shoots than the hybrid cooking banana BITA 3, while the cooking banana landrace Cardaba had significantly fewer shoots than both hybrids in stage 3 (Table 5).

It had been declared [88] that for bananas in general, shoot tips of cultivars having only A genomes produce 2–4 new shoots, whereas cultivars having one or two B genomes produce a cluster of many shoots and buds at each subculture cycle. In this study, all genotypes had at least one B genome, but the presence of the B genome did not show this trend in Cardaba but only in BITA 3 at stages 2 and 3 irrespective of the type of gelling agent. BITA 3 produced only 2-3 shoots in stage 1, while Cardaba consistently produced between 1-4 new shoots at each stage of the culture and not a cluster of many shoots in both gelling agents.

Table 5. Effects of gelrite and low cost cassava starch as gelling agents on number of *in vitro* shoots in 3 Musa genotypes at regeneration stage

Musa Genotypes	Mean Number of Plantlets at Regeneration Stage	
	Gelrite	Cassava Starch
Cooking Banana hybrid BITA 3 (AAAB)	6.90 ± 1.97^{b}	6.40 ± 1.71^{bc}
Plantain hybrid PITA 14 (AAAB)	10.40 ± 2.46^{a}	7.20 ± 0.92^{b}
Cooking banana landrace Cardaba (ABB)	2.10 ± 0.27^{d}	2.10 ± 0.61^{d}

Cost Analysis

The simple comparative cost analysis from use of cassava starch instead of gelrite showed that cassava starch was indeed cheaper than gelrite and resulted in a cost reduction of 85% (Table 6).

Table 6. Comparative cost analysis of use of gelrite and cassava starch as gelling agents in Nigerian Naira (N)

Gelling Agent	Amount Used	Cost (NGN)	Cost Reduction
Gelrite™	2 g/2 litre	130.3	
Cassava starch	70 g/litre	20	85%

Other studies have found reduction in cost of production when agar or gelrite have been wholly or partially substituted by locally produced alternative gelling agents.

CONCLUSION

Cassava starch alone could be an ideal low cost substitute for gelrite in stage one (shoot initiation) for all Musa genotypes, and for BITA 3 and Cardaba in stages 2 and 3 (proliferation/ multiplication and regeneration). However, it is not an effective standalone substitute for gelrite with respect to PITA 14 beyond shoot initiation (stage 1). Perhaps addition of small amounts of gelrite to cassava starch could improve its gelling ability and increase shoot production during the proliferation and root regeneration stages. Further studies will be needed to confirm if this will occur. This study showed that substituting cassava starch for gelrite in the MS medium could result in a cost reduction of up to 85%.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

A. Tenkouano designed the study. V Wilson carried out the field work, collected and analyzed data, and developed the manuscript.

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