USE OF CASSAVA STARCH AS GELLING AGENT DURING *IN VITRO* MICROPROPAGATION OF BANANA

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ABSTRACT

Various alternative gelling agents have been used to substitute the needs of Agar for *in vitro* micropropagation. However, extractions of these alternate gelling agents are costly and required expertise. Thus, the aim of this study was to establish the gelling properties and required amount of locally extracted cassava as an alternative to agar for *in vitro* micropropagation of banana. Furthermore, cassava starch was extracted from fresh cassava root tubers of cultivars AKENA and TM 2961 and their physio-chemical characteristics and gelling potential was evaluated as a gelling supplement with Murashige and Skoog (MS) medium. Various amounts of cassava starch in combination with agar were evaluated for their ability to support *in vitro* growth of Kisansa, Km 5 and FHIA 17 banana cultivars. Results of the study revealed that the physio-chemical characteristics of cassava starch extracted from cultivars AKENA and TM 2961 were different. MS media supplemented with TM 2961 cassava starch significantly ($P<0.001$) influenced the number of shoots and shoot height of all the banana cultivars as compared to AKENA cassava starch. A combination of 50g/l TM 2961 cassava starch and 2g/l agar significantly ($P<0.001$) influenced the number of shoots and shoot height of banana cultivars compared to other combinations, but was not significantly different from media containing 7g/l agar. Addition of small amounts of agar to cassava starch greatly improved the gelling ability. Therefore, cassava starch as a locally available gelling agent can be used to partially substitute agar with good *in vitro* banana growth results.

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1 Introduction

In vitro multiplication of plant tissue is strongly influenced by the physical consistency of the culture medium (George, 1993). Gelling agents are usually added to the culture medium to increase its viscosity and allowing plant tissues or organ parts to remain above the surface of the nutrient medium (Razdan, 1993). Various gelling agents such as agar, agarose and gellan gum are marketed under trade names such as phytigel, gelrite (Babbar & Jain, 1998), and 'Gel-Gro' (Bhattacharya et al., 1994) used for plant culture media. Agar is the most commonly used gelling agent for preparation of solid and semi-solid media (Babbar et al., 2005). However, due to its high cost, it increased the cost of cultural media and the plantlets development. Although banana plantlets tissue culture is quite popular in Uganda but the cost of this production is higher compared to the conventional sucker production, this is making it unaffordable for many poor farmers. Several efforts have been carried out for reducing the cost of culture media, adding of alternative gelling agent is one of them (Gitonga et al., 2010).

Various alternative gelling agents such as semi-solid matrices of galactomanan, guar gum and xanthan gum have been widely used for solidifying the tissue culture medium (Jain et al., 1997; Gonçalves & Romano, 2005; Tyagi et al., 2007). Cassava derivatives including flour (Maliro & Lameck, 2004) and starch (Mbanaso et al., 2001; Dabai & Muhammad, 2005; Kuria et al., 2008) have been also used as a gelling agent. The efficiency of cassava starch in tissue culture media was tested by using industrially derived starch (Mbanaso et al., 2001). Availability of cassava all year around and very low farm gate price (approximately \$ 0.04 per Kilogramme) imperative to test the efficacy of locally extracted cassava starch for in-vitro micropropagation of banana plantlet (Collinson et al., 2000). The gelling characteristics of the locally extracted cassava starch and its influence on *in vitro* growth of banana have not been determined yet. Therefore, this study was conducted to establish the gelling properties and required amount of locally extracted cassava starch that supports *in vitro* growth of banana as an alternative to agar.

2 Materials and Methods

2.1 Extraction and characterization of Cassava starch

100 Kg freshly harvested cassava roots of AKENA and TM 2961 cultivars were collected from the farmers' fields around National Crops Resources Research Institute-Namulonge, Uganda (NaCRRI). Extraction of cassava starch was carried out at NCRRI by using modified Nuwamanya et al. (2009) method. The roots were peeled, washed and crushed by using a mechanical grinder. The pulp was suspended in tap water, sieved twice by using a cheese cloth and the effluent collected in basins. The effluent left overnight for sedimentation and later on the supernatant was discarded. The sedimented starch was scrapped and washed in 1M Potassium chloride to remove

residues. This was left to stand overnight. The resulting supernatant was poured off and settled starch was washed thrice with distilled water at intervals of every four hours. The starch was removed and air dried on a black polythene paper to obtain a powder of starch. The starch was weighed, packaged in air tight polythene bags to prevent re-hydration and stored in a cool dry cardboard.

Cassava starch extracted from each cultivar was characterized for moisture content, solubility temperature, swelling/gelling power, presence or absorbance of amylase, pH, presence or absorbance of amylose, ash and lipid content. Moisture content and temperature at which starch is soluble was determined according to the International Starch Institute (ISI) (1997) method and Autio et al. (1992), respectively. The swelling power was evaluated using Leach et al. (1959) method. The pH, presence and absorbance of amylase were analysed according to Nuwamanya et al. (2009). Starch ash and lipid content were determined according to Helrich (1990).

2.2 Gelling of culture medium using cassava starch

Four levels viz 50, 60, 70 and 80 g/l of cassava starch (from each cultivar) were used in combination with 0, 1, 2, 3 and 4 g/l of agar. Twenty milliliter aliquots of Murashige and Skoog's (1962) media (pH 5.8) prepared with different combinations of cassava starch and agar were melted and dispensed into culture vessels. Media was sterilized by autoclaving at 121°C temperature and 1.05 kg/cm² pressure (103.4 KPa), for 20 min. The media was allowed to cool in a sterile environment provided by laminar air flow hood. Agar at 7 g/l without cassava starch was used as a control.

2.3 Preparation, initiation and subculturing of explants

Sword suckers of banana cultivars Kisansa, Km 5 and FHIA 17 were obtained from a mother garden at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). The pseudo stem at lower part containing the meristematic shoot tip and part of the corm was used as explant. The sheathing leaf bases were removed from the pseudostem leaving the young leaves around the meristem using a knife. The resulting explants were surface sterilized by placing in a beaker containing 5 % commercial bleach supplemented with 3 drops of Tween-20. To ensure proper sterilization, the containers were shaken at the interval of every three minutes for 15 minutes. For poured off used hypochlorite solution, explants were rinsed twice with 70% ethanol for 10 min. The used ethanol was poured off and explants were rinsed thrice with distilled sterile water to rid them of excess ethanol under a laminar air flow hood.

Sterilized explants were reduced to cubes of 2 x 2 x 2 cm by removing all superfluous tissues. Cubes were cut into two through the meristem using sterile surgical blades. The two halves were inoculated into culture vessel containing 20 ml of MS medium (pH 5.8) using sterile forceps. Ten glass culture

vessels per treatment per explant were inoculated and incubated under 14 hr photo period and light intensity of 30-40 $\mu\text{M M}^{-2} \text{S}^{-1}$ supplied by white fluorescent tubes. The temperature was maintained at 25-27 °C in the growth room by air conditioning units. Sub-cultures were carried out each after 4 weeks by separating shoot clusters developed from explants into individual shoots which were then trimmed down to shoot tips and sub-cultured under the same conditions. This process was repeated up to the second sub-culture level (on agar gelled media) after which they were transferred on various treatments of cassava starch gelled medium.

2.4 Experimental design and data analysis

The experiment was laid out in a completely randomized block design in which ten shoot tips of each three banana cultivars namely Kisansa, Km 5 and FHIA 17 were evaluated on each of the different treatments of MS media supplemented with cassava starch at varying concentrations. Shoot clusters from different combinations of gelling concentrations were examined for number of shoots and shoot height. Data of mean shoots number (per explants) and shoot height was subjected to analysis of variance (ANOVA) using Genstat 12th edition (VSN international) for each type of cassava starch under study. Means were separated by Tukey's HSD test ($P < 0.001$). The most appropriate response on the *in vitro* bananas cultured on medium supplemented with a combination of cassava starch and agar was later compared against the response of bananas when cultured on medium supplemented with agar only at 7 g/l (as control), which is commonly used as standard concentration.

3 Results

3.1 Extraction of cassava starch and its physio-chemical gelling properties

The amount of starch recovered from the two cassava cultivars were differed according to the cultivars and it was reported

13.5 Kg and 10.5 Kg for AKENA and TM 2961 respectively. Physico-chemical characteristic of the isolated starch from these two cultivars did not show any significantly different except for pH and amylose absorbance at 70 °C (Table 1).

3.2 Optimization of the cassava starch concentration for *in vitro* banana growth

3.2.1 Number of shoots

Generally the mean number of bananas shoots from explants cultured on MS media supplemented with combination of agar and cassava starch from the two cultivars did not differ significantly ($P > 0.001$).

The highest mean number of shoots was reported from the MS media supplemented with combinations of 50 & 60 g/l AKENA cassava starch and 1 g/l agar (3.89) combination. While in case of TM 2961 cassava starch, combinations of 50 g/l TM 2961 + 2 g/l agar (3.78) and 80 g/l + 1 g/l agar (3.78) (Table 2) showed highest banana shoot production and; this did not significantly differ from 50 & 60 g/l AKENA + 1 g/l agar combination. There was no shoot development observed for each of the concentrations of cassava starch in absence of agar (0 g/l agar) (Table 2). However, the mean number of shoots decreased with increasing concentration of agar beyond 1 g/l for AKENA and 2 g/l for TM 2961 (Table 2).

Furthermore, the three banana cultivars generally exhibited similar number of shoots when cultured on media supplemented with 50, 60, 70 and 80 g/l of cassava starch but with slightly better response at 50 and 60 g/l. Also the banana shoots cultured on media supplemented with AKENA cassava starch combinations were bushy, stunted and had a small girth after the four weeks in culture (hence the high number of shoots as expressed). Yet those cultured on TM 2961 cassava starch combinations gelled media were healthy and were easily cultured on multiplication media.

Table 1 Physico-chemical characteristics of cassava starch extracted from AKENA and TM 2961 cultivars.

Starch property	Cassava cultivars	
	AKENA	TM 2961
Moisture content (g/100g)	16.75	15.76
pH	1.31	0.49
Ash content (g/100g)	0.036	0.037
Lipid content (g/100g)	0.63	0.83
Solubility temperature (°C)	50	50
Gelling temperature (°C)	>60	>60
Presence of amylose at 50 °C	+	+
Amylose absorbance at 620 nm for starch solution at:		
40 °C (nm)	0.00	0.04
70 °C (nm)	0.772	1.260

Table 2 Mean number of *in vitro* bananas shoots production on MS media supplemented with two cassava starch and agar combination at varying concentrations.

Cassava variety	Starch amount (g/l)	Number of shoots*				Average
		Agar amount (g/l)				
		1	2	3	4	
AKENA	50	3.89 ^a	3.67 ^a	3.22 ^{ab}	3.11 ^a	13.89
	60	3.89 ^a	3.44 ^{ab}	3.11 ^{ab}	2.89 ^b	13.33
	70	3.56 ^c	3.44 ^{ab}	2.78b	2.22 ^c	12.00
	80	3.56 ^c	2.89 ^c	2.33c	0.67d	9.45
TM 2961	50	3.56 ^c	3.78 ^a	2.89b	2.8 ^b	13.00
	60	3.56 ^c	3.44 ^{ab}	3.22 ^{ab}	3.00 ^a	13.22
	70	3.56 ^c	3.56 ^b	3.11 ^{ab}	2.67 ^{bc}	12.90
	80	3.78 ^b	3.44 ^{ab}	3.44 ^a	2.44 ^c	13.10

* = Values are means of the three banana cultivars; the response of banana cultivars did not differ. Mean values in column followed by the same letter are not significantly different at $P < 0.001$ according to Tukey's least-significance difference range test.

3.2.2 Shoot height

Generally the mean shoot height of bananas cultured on MS media supplemented with a combination of TM 2961 cassava starch and agar was significantly ($P < 0.001$) higher than those cultured on MS media supplemented with a combination of AKENA cassava starch and agar (Table 3). The mean shoot height of bananas cultured on MS media supplemented with a combination of 50 g/l TM 2961 cassava starch and 2 g/l agar (3.278 cm) was significantly ($P < 0.001$) higher than the cultured on MS media supplemented with other combination levels (Table 3). Also, the mean shoot height of bananas cultured on MS media supplemented with a combination of 50g/l AKENA cassava starch and 1 g/l agar (1.83 cm) was significantly ($P < 0.001$) higher than those cultured on MS media supplemented with various levels in combination with agar (Table 3).

There was no shoot development observed for each of the concentrations of cassava starch in combination with 0 g/l agar (Table 3). Similarly, the mean shoot height of the three banana

cultivars decreased with increasing concentration of the gelling combinations beyond 50 g/l cassava starch.

3.3 Comparison of *in vitro* banana response under cassava starch and agar supplements

When comparing the response of *in vitro* bananas cultured on MS media supplemented with the most appropriate combinations of cassava starch and agar with the control, it was observed that the mean number of shoots did not significantly ($P > 0.001$) differ among the treatments. Mean number of shoots of three banana cultivars cultured on MS media supplemented with 50 g/l AKENA cassava starch and 1 g/l agar and 50 g/l TM 2961 and 2 g/l agar was not significantly ($P > 0.001$) different from that on control media (7 g/l agar) (Table 4). On the other hand, the mean shoot height of *in vitro* bananas kisansa and Km 5 was significantly ($P < 0.001$) higher when cultured on MS media supplemented with 50 g/l TM 2961 and 2 g/l agar compared to when cultured on control media (Table 4). The lowest was observed when cultured on MS media supplemented with 50 g/l AKENA cassava starch and 1 g/l (Table 4).

Table 3 Mean shoot height (cm) of *in vitro* bananas cultured on MS media supplemented cassava starch and various concentration of agar.

Cassava variety	Starch amount (g/l)	Shoot height*				Average
		Agar amount (g/l)				
		1	2	3	4	
AKENA	50	1.83 ^a	1.39 ^a	0.94 ^a	0.56 ^b	4.72
	60	1.39 ^b	1.06 ^b	0.83 ^b	0.67 ^a	3.95
	70	1.17 ^c	0.83 ^c	0.67 ^b	0.50 ^b	3.17
	80	1.11 ^c	0.94 ^c	0.67 ^b	0.28 ^c	3.00
TM 2961	50	1.22 ^d	3.28 ^a	2.83 ^a	1.44 ^a	8.77
	60	1.72 ^a	1.83 ^b	1.28 ^b	1.00 ^b	5.83
	70	1.56 ^b	1.67 ^c	0.83 ^c	0.67 ^c	4.73
	80	1.44 ^c	1.67 ^c	0.83 ^c	0.50 ^d	4.44

* = Values are means of the three banana cultivars; the response of banana cultivars did not differ. Mean values in column followed by the same letter are not significantly different at $P < 0.001$ according to Tukey's least-significance difference range test.

Table 4 Comparison of the mean response of *in vitro* banana cultured on MS media supplemented with cassava starch and agar with control (agar).

Banana cultivars	Mean No. of shoots			Mean shoot height (cm)		
	A	B	C	A	B	C
Kisansa	3.33 ^b	3.33 ^b	3.00 ^b	1.50 ^b	3.17 ^a	3.00 ^a
Km 5	4.67 ^a	4.33 ^a	4.33 ^a	2.00 ^a	3.33 ^a	2.33 ^b
FHIA 17	3.67 ^b	3.67 ^b	3.33 ^b	2.00 ^a	3.33 ^a	3.33 ^a

Here, A = 50 g/l AKENA + 1 g/l Agar; B = 50 g/l TM 2961 + 2 g/l Agar; C = 7 g/l Agar (control). Mean values in column followed by the same letter are not significantly different at $P < 0.001$ according to Tukey's least-significance difference range test.

Discussions

Various alternative gelling agents have been studied for substitute the need of tissue culture grade agar in tissue culture media (Sharifi et al., 2010). This study evaluated the gelling potential of locally extracted starch from two cassava cultivars, their physio-chemical properties and potential to support *in vitro* banana growth. In this regard, AKENA and TM 2961 cassava cultivars yielded 13.5 Kg and 10.5 Kg of dry starch, respectively. According to Morrison & Tester (1993), this yield difference is associated with genetic predisposition. The moisture content AKENA and TM 2961 extracted starch was low and it was in the range described by Nuwamanya et al. (2009). These low moisture ranges are not conducive for microbial growth hence ensuring a long shelf life for the starch types. The pH of both samples of cassava starch was acidic and it was as previously reported by Kasanadze (2000). This implies that the use of cassava starch as a gelling agent in banana *in vitro* propagation may require pH adjustment just after the addition of such gelling agents. The ash content of both cassava starches was low. According to Nuwamanya et al. (2009) these ranges imply that the levels of inherent contaminants like Potassium (K) and Calcium (Ca) were very low and thus do not significantly change the basic salt formulations when supplemented in *in vitro* medium. The lipid content of TM 2961 was higher than that for AKENA. This according to Nuwamanya et al. (2009) leads to TM 2961 trapping more water than AKENA cassava starch.

Since cassava starch does not dissolve in water at room temperature (25-27°C) so solubility temperature was reported 50°C for both the starch samples (Nuwamanya et al., 2009). A gelling temperature of 60°C implied that at low temperatures, gelatinization was not possible. Like other gelling agents for example agar, phytigel and gelrite (Babbar et al., 2005), both samples of cassava starch had to be warmed to a considerable temperature for gelling. Cassava starch were hydrolyzed at 50°C and this process were released amylose and amylopectin (Morrison & Tester, 1993). The differential release of amylose at different temperatures showed that TM 2961 released higher concentration of amylose at 70°C while at same temperature AKENA released lesser amount of amylose.

The response of bananas with respect to shoot height and number differed with the types of gelling agents and its

concentrations. The most appropriate mean number of shoots was 3.78, while for the shoot height this value was 3.28 cm. This highest mean response was observed when MS media was supplemented by the combination of 50g/l TM 2961 cassava starch and 2 g/l Agar and this value is almost at par the result obtained by the recommended concentration of agar (7 g/l). Similar type of observations was made by Maliro & Lameck (2004) and Mbanaso (2008). Result of present study clearly represented that addition of cassava starch reduced the dependency on tissue culture agar.

It is worth noting that the gelling property of both starches was enhanced with the addition of small volumes of agar as compared to when used singly. Findings of the present study are in agreement with Abraham (1993). They reported formation of a weak gel when cassava starch is warmed in solution. Improving of the gelling property of starches using agar was also reported by Kasanadze (2000); Maliro & Lameck (2004), and Getrudis & Wattimena (1994) during *in vitro* propagation of plants. Addition of agar improved the gelling properties of starch in both instances; probably the difference in response as seen for the two varieties could be due to differences in structural properties of the two starches. In this regard TME 2961 structure was not as compact as AKENA cassava starch. This could be due to presence of amylose and amylopectin which are released during warming and are of importance during gelling.

In fact Va et al. (1997) mentioned that the swelling and gelling behavior of un-processed starch is directly dependent upon property of the amylopectin content and amylose acting as an inhibitor of swelling. Furthermore, they reported that when cassava starch warmed with water, starch granules become intact and forming gel. Also Abraham (1993) state that moisture treatment leads to increased amylose susceptibility and decreased crystallinity and water binding capacity. In this respect, probably water is trapped due to the resisting action of amylose. Therefore since TM 2961 starch has more amylose than AKENA starch, it traps more water and hence avail nutrients for banana *in vitro* growth. This property of cassava starch was evidenced at low amounts of starch (50 g/l) compared to high amounts of cassava starch (70 and 80 g/l). In addition, low starch concentration had few amylose molecules which are released. These act to trap some water while leaving more water to dissolve available nutrients for *in vitro* plant

growth. Conversely, at higher concentrations of starch, response reduces. Similar observations were made by Mbanaso (2008) for *Musa* sp shoots.

Since plantlets cultured on AKENA cassava starch-enhanced medium showed the highest number of shoots compared to the medium supplemented with 7 g/l agar and TM 2961, further investigation would be required to evaluate the survival and regenerability of cultures previously maintained on AKENA enhanced medium. This study showed that a combination of locally extracted cassava starch and small amounts of agar can be used for efficient *in vitro* banana growth. This gives more vigorous shoots and with a possibility of reducing the cost of media since cassava starch is cheaper and readily available.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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