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Full Length Research Paper

Cassava starch as an alternative cheap gelling agent for the *in vitro* micro-propagation of potato (*Solanum tuberosum* L.)

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The potential of cassava starch as an alternative and cheap gelling agent for potato *in vitro* culture micro-propagation media was investigated. A two-factor experiment in randomized complete block design was conducted. Four levels of gelling agents; 10% (w/v) cassava starch, 8% cassava starch mixed with 0.25% agar, 0.8% agar and a liquid medium, were evaluated using three selected Kenyan potato cultivars (Tigoni, Asante and Kenya Sifa). Cassava starch at 10% gave adequate support of explants, though it had low viscosity and softened at 42 days after explant inoculation. Cassava starch mixed with 0.25% agar provided the same firmness as 0.8% agar and maintained gel integrity throughout the culturing period of 84 days. Survival *in-* and *ex vitro* was lowest in liquid medium culture. Potato transplants from the liquid medium and cassava starch gelled medium had similar ($p > 0.05$) mean number of nodes and biomass. These mean values were significantly higher compared to the transplants from the agar gelled medium. The use of 10% cassava starch reduced cost by 42.5% in comparison with use of agar.

Key words: Cassava starch, gelling agents, micro-propagation, transplants, potato.

INTRODUCTION

Media for *in vitro* cultures can be classified as liquid or solid. Gelling agents are added to culture medium to increase viscosity wherein explants are not submerged in the medium (Prakash et al., 2000). Like for other plants, culture media used for potato micro-propagation are also gelled with agar at a concentration of 0.8% (w/v) (Prakash et al., 2000; Gebre and Sathyanarayana, 2001). However, establishment of plant micro-propagation laboratories must be based on cost effectiveness. In this respect, agar represents one of the most expensive and commonly used media components, contributing about 70% of the total production cost (Prakash, 1993). Moreover, the exclusive use of agar may result to over-exploitation of its sources (Jain et al., 1997). Although agar was thought biologically inert and non-toxic, its adverse effects have been reported (Debergh, 1983).

Cassava starch, processed from roots of cassava (*Manihot esulenta* Crantz), has a high starch content of over 90% (Onuweme, 1982). Essentially like agar, cassava starch is an acidic polysaccharide consisting in its powdered form of 77% carbohydrate, 21% lipid and 2% protein (Dabai and Muhammad, 2005). The most important characteristics of cassava starch are odourless, paste clarity, stickiness and low impurities like proteins and lipids (Maliro and Lameck, 2004). Pure cassava starch forms a gelatinous matrix that can be autoclaved and stored thereafter melted by heating (Kasanadze, 2000; Nene and Sheila, 1994).

Cassava flour at 8% (w/v) provides optimum gelling and quality can be improved by mixing cassava flour with some agar (80 g l^{-1} cassava flour + 3.5 g l^{-1} agar) (Kasanadze, 2000). Mbanaso et al. (2001) also reported that 6 and 7% (w/v) cassava starch gave adequate support and orientation to *Musa* shoot tip explants but gel stability was only maintained up to seven 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 (Dabai and

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Muhammad, 2005).

Although the cassava starch has been proven to have gelling ability, its use for potato micro-propagation has not been evaluated. Potato is the fourth most important food crop in the world, and is grown in about 140 countries, more than 100 of which are located in the tropics and sub-tropical zones (CIP, 2002). If proven effective to support potato tissue cultures, cassava starch will be a cheap alternative gelling agent especially to developing countries having to import agar while cassava is ubiquitous.

The objective of this study was to evaluate the potential of cassava starch as an alternative low-cost gelling agent in culture medium for potato *in vitro* micro-propagation using nodal cuttings.

MATERIALS AND METHODS

Plant materials

The study was carried out at the plant tissue culture laboratory of the Kenya Agricultural Research Institute (KARI)-National Potato Research Centre Tigoni between June 2005 and April 2006. Three popular Kenyan cultivars viz., Asante (381381.20), Tigoni (381381.13) and Kenya Sifa (720097) were provided by Kenya Agricultural Research Institute Tigoni centre. The plants were certified as disease free and *in vitro* plantlets were initiated through meristem shoot tip culture. They were then routinely multiplied every four weeks using nodal cuttings.

Preparation of MS nutrient medium

Two gelling agents namely Difco Bacto agar (Sigma Chemical Company Germany) and cassava starch extracted from *M. esulenta* Crantz (purchased in Douala, Cameroon), and a liquid medium were used. The liquid media was prepared using full strength Murashige and Skoog basal salts (1962) (MS) supplemented with 3% (w/v) sucrose and 0.001 mg/l gibberellic acid. The pH was adjusted to 5.8 using 1 N NaOH or 0.1 M HCL. For the solid medium, agar was maintained at standard concentrations of 0.8%, cassava starch at 10% and a composite mixture consisting of 8% cassava starch mixed with 0.25% agar. The gelling rates were formulated based on prior preliminary investigations (data not published). Cassava was incorporated as thick slurry into pre-heated medium prior to autoclaving. Ten millilitre of culture medium was dispensed in 20 mm test tubes and covered using aluminium foil preceding autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min.

Inoculation and incubation

Inoculation was carried out in a sterile laminar airflow hood chamber. Surface sterilisation was achieved through spraying 70% (v/v) ethanol. Nodal cuttings were dissected from four weeks-old *in vitro* plantlets using sterile blade and forceps. Single nodal cuttings were inoculated into a test tube containing 10 ml MS medium amended with different gelling agents. These were incubated at 22 ± 2°C under a 16 h photoperiod with a photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} provided by overhead cool fluorescent lamps (Philips, India 30 Watts) for 35 days.

Transplanting

Four weeks old *in vitro* plantlets were transplanted in 15 cm diameter pots half filled with well-watered mixture of steam sterilised soil substrate (forest soil, humus, ballast and sand in the ratio of 4:1:1:1) amended with five grams per pot of Di-ammonium phosphate fertilizer (DAP). The transplants (seedlings) were then kept in glasshouse for observation. The plants were irrigated every alternative day with tap water for two weeks and twice a week thereafter.

Data collection

Collection of data on *in vitro* plantlet performance commenced two weeks after inoculation and on weekly interval for five weeks during incubation. While in the glasshouse the following growth characteristics were determined: transplant survival, number of nodes, plantlet vigour, and percentage plants survived at harvest. Further, a cost analysis benefit of the different media gelling agents was done.

Experimental design and data analysis

A two-factor experiment in a randomized complete block design was conducted with three replicates per treatment and seven test tubes per experimental unit. The *in vitro* plantlets were sub-cultured three times (each lasting 35 days) in the same treatment. Data was subjected to analysis of variance (ANOVA) to test the significance of the differences between treatments with SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, NC, USA) and means separated using the Least Significant Difference at $p < 0.05$.

RESULTS

Medium clarity

Cassava starch gelled medium had poor clarity (Figure 1C) compared to agar (Figure 1A). Clarity of plant tissue culture media is important for prompt detection of microbial contamination. Clarity of the cassava starch gelled medium was satisfactorily improved with addition of 0.25% agar (Figure 1B), and it also improved gel strength. Figure 1D shows fully established *in vitro* plantlets 62 Days after planting.

In vitro plantlet survival

Analysis of variance revealed that, there were no significant interactions during the three generations of sub-culturing hence the means were pooled. The gelled media gave consistently higher percentage plantlet survival than liquid medium in all the three generations (Table 1). The survival of *in vitro* plantlets was not significantly different ($p \leq 0.05$) in media gelled with cassava starch and that gelled with agar.

Number of nodes per *in vitro* plantlet

Medium gelled with agar gave significantly less number of nodes per plantlet than medium gelled with cassava starch and liquid medium respectively (Table 1). However, plantlets cultured on either cassava starch, cassava starch mixed with agar or on liquid medium were not sig-

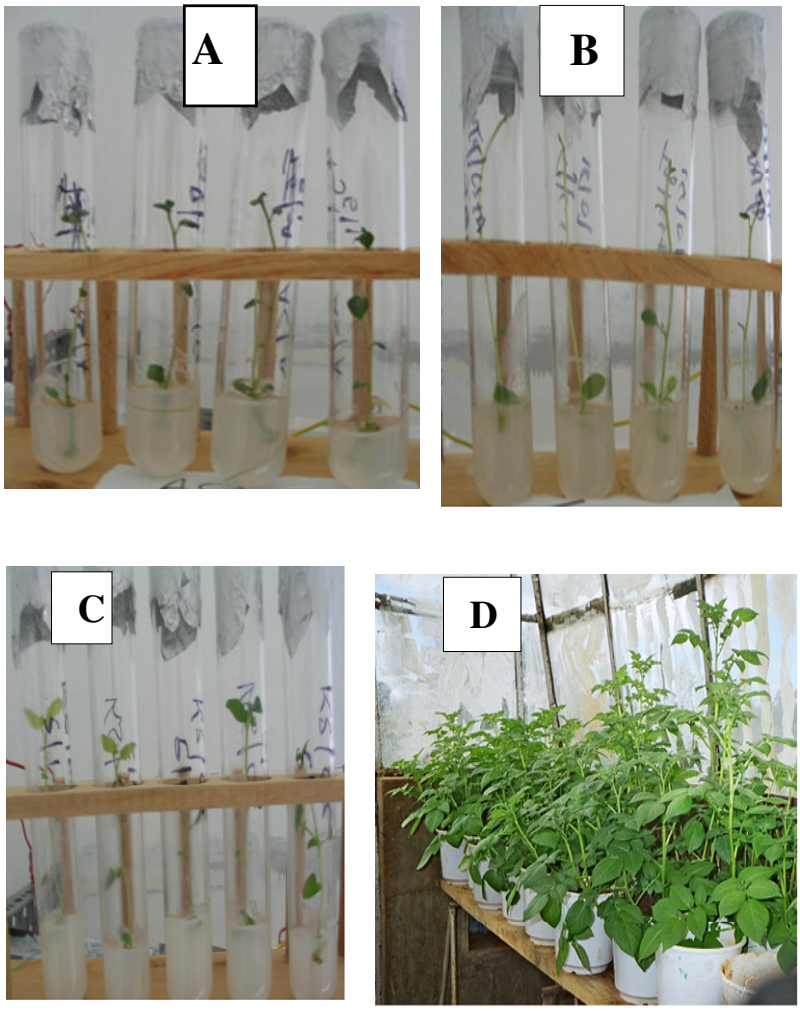

Figure 1. *In vitro* plantlets cultured in (A) 0.8% Difco Bacto agar, (B) 8% cassava starch mixed with 0.25% agar, (C) (10%) cassava starch at 21 days after culture and (D) well established potato plants in glasshouse derived from *in vitro* culture 62 days after transplanting.

nificantly different ($p \leq 0.05$) in terms of number of nodes during the three generations of sub-culturing (Table 1).

In vitro plantlet vigour

Liquid medium gave comparable vigour of *in vitro* plantlets as gelled medium in all the three generations of culture. The plantlets produced were very vigorous attaining a mean score of more than 4 on a “subjective” scale (Table 1).

In vitro plantlet biomass

After 42 DAC, liquid medium promoted significantly higher fresh weight compared to gelled media in cvs. Kenya Sifa and Asante (Table 2). On the other hand for the cv. Tigoni medium gelled with agar produced significantly ($p < 0.05$) lower fresh weight compared to other media formulations (Table 2). The gelling agents did not yield significantly ($p < 0.05$) in terms of fresh weight for cvs. Asante and Kenya Sifa but agar gelled medium was significantly different ($p < 0.05$) from media gelled with cassava starch for cv. Tigoni. The fresh weight observed at 42 DAC ranged 0.514 to 0.718 g.

Transplant survival in glasshouse

Significant difference ($p < 0.05$) was observed between the gelled and liquid media, with plantlets of all the three cultivars cultured in liquid medium having poor survival after transplanting. Gelling agent types were not significantly different ($p < 0.05$) in terms of survival although plantlets derived from medium gelled with a mixture of

Table 1. Pooled means of vigour, number of nodes and survival of *in vitro* plantlets during 1st, 2nd and 3rd generations of culture.

Gelling agent	Vigour	No nodes / plantlet	Survival (%)
Generation I (0 - 35 days)			
Agar	4.4a	7.15b	100a
Cassava starch	4.8a	9.62a	99a
Cassava starch + Agar	4.7a	9.11a	100a
Liquid	4.4a	9.75a	97b
LSD (0.05)	0.40	1.22	1.5
CV%	5.5	2.3	1.6
Generation II (35 - 70 days)			
Agar	4.8a	8.06b	98a
Cassava starch	5.0a	9.2a	99a
Cassava starch + Agar	4.7a	9.5a	99a
Liquid	4.9a	9.89a	94b
LSD (0.05)	0.33	0.96	3.8
CV%	7.0	4.6	4.0
Generation III (70 - 105 days)			
Agar	4.7a	7.38b	100a
Cassava starch	4.9a	9.78a	99a
Cassava starch + Agar	4.9a	8.98a	99a
Liquid	5.0a	9.95a	96b
LSD (0.05)	0.34	0.98	1.89
CV%	7.1	6.4	2.0

Means followed by the same letter within the column are not significantly different at $p \leq 0.05$ using least significant difference.

Table 2. Mean fresh weight (g) of the potato cvs Asante, Kenya Sifa and Tigoni *in vitro* plantlets at 42 days after culturing (DAC) in gelled and liquid medium.

Gelling agent	Fresh weight (g)		
	Asante	Kenya Sifa	Tigoni
Agar	0.546b	0.368b	0.522b
Cassava starch	0.624b	0.500b	0.751a
Cassava starch + agar	0.601b	0.415b	0.721a
Liquid	0.787a	0.774a	0.876a
LSD (0.05)	0.143		
CV%	25.8		

Means followed by the same letter within the column are not significantly different at $P < 0.05$ using Least Significant difference.

cassava starch (8%) and agar (0.25%) enhanced better survival in glasshouse (Table 3).

Cost analysis of gelling agents

The cost of 1 litre MS medium excluding gelling agent worked out to be Kshs. 135, while inclusion of 10% cassava starch in the medium increased the cost to Kshs. 180; amending MS medium with a mixture of 8% cassava starch and 0.25% agar increased the cost to Kshs. 226. Agar on the other hand was the most expensive with one litre MS medium costing Kshs 313 (Table 4). Exclusion of a gelling agent in tissue culture medium resulted to 57% cost reduction. Cassava starch alone at 10% (w/v) resulted in a 43% cost reduction of the media. Cassava starch with 0.25% agar reduced cost of production by 28% (Table 4).

DISCUSSION

Comparative studies of potato micro-propagation in liquid and solid medium revealed that regeneration capacity of

Table 3. Survival of potato *in vitro* plantlets of the cvs Asante, Kenya Sifa and Tigoni after transplanting in glasshouse as influenced by media type.

Gelling agent	Survival (%)			
	Asante	Kenya sifa	Tigoni	Mean
Agar	87.3a	94.0a	98.7a	93.3
Cassava starch	96.0a	93.7a	98.3a	92.7
Cassava starch + agar	96.7a	98.3a	97.3a	97.4
Liquid	65.7b	72.0b	77.3b	71.7
LSD (0.05)	13.5			
CV%	8.6			

Means followed by the same letter within the column are not significantly different at P < 0.05 using least significant difference.

Table 4. Comparative costs of culture medium for *in vitro* propagation of potato using cassava starch as a gelling agent.

Gelling agent	Cost/Kg (KShs)	Conc/L (%, w/v)	Cost/L (KShs)	Cost reductions (%)
Agar	21341	0.8	313	
Cassava starch	442	10	180	43%
Composite		8, 0.25	227	28%
Liquid	135	0	135	57%

KShs 73 = 1US$.
Composite = 8% cassava starch mixed with 0.25% agar

nodal sections was high in both medium as indicated by survival percentage of 96 to 100% (Table 1). The higher mortality of plantlets in liquid media was attributable to physiological anomalies. The affected plants appeared glassy, a bushy habit and thickened and malformed stems and leaves. Rani and Singh (1999) reported hyperhydricity during liquid propagation of potato. Several factors are associated with hyperhydricity such as the level of cytokinin, low light, type of culture vessels, length and number of sub-cultures (Ziv, 1995; Puchooa and Purseramen, 1999). Various remedies to hyperhydricity have been reported including use of solidified media with high concentration of gelling agent or a gelling agent with a higher gel strength (Debergh et al., 1982; Ziv et al., 1983), increasing the calcium and use of fructose or galactose as a carbon source (Debergh et al., 1992).

The mean number of nodes per plantlet was 9.85 in liquid culture compared to 8.47 in solid media (Table 1) over the three generations of culture. The higher number of nodes per plantlet in liquid medium is in agreement with earlier finding by Rani and Singh (1999). They observed significant increase in the number of nodes per plantlet in liquid culture medium (9.8) as compared to solid culture medium (8.1). It was speculated that repeated sub-culturing in liquid medium might diminish the regenerative capacity of shoots due to altered sugar/nitrogen ratio in plantlets. However, we did not observe any loss of regeneration capacity of cultures. Rather, repeated sub-culturing in liquid led to advantage of increase in number of nodes per plantlet as compared to gelled medium but higher proportion of vitrified shoots and low survival after transplanting in the glasshouse.

Vigorous growth was observed in cultures of liquid medium (Table 1) concurring with similar observations on *Pinus caribaea* Skidmore et al. (1988) cultured in liquid. A study by Douglas (1984) reported that *Rhododendron* shoots were ten-fold longer in liquid medium than on agar-solidified medium. The vigorous growth of nodal explants during the three generations of culture may be attributed to the ability of nodal explants to revert to juvenile phase thus regeneration to normal plantlets. Vigorous growth also was an indication of production of high quality plantlets through tissue culture.

Biomass accumulation was better in liquid media (Table 2). Our results suggest that nutrient assimilation was favoured in liquid medium due to increase in the nutrient incorporation rate. Avila et al. (1996) observed two fold dry matter accumulation of potato in liquid than in the solid medium. Growth and multiplication of shoot is enhanced by liquid medium as a consequence of better availability of water and nutrients resulting from a lower resistance to diffusion and closer contact between the explant and the medium (Pierik, 1997). The diffusion of nutrients to roots may decrease with increasing agar concentration in the medium, resulting in lower nutrient availability and, consequently, a reduction in growth. Moreover, an agar-solidified medium has lower water potential (more negative) than its liquid equivalent (Debergh and Zimmerman, 1991). The decrease in water potential resulting from the matrix potential of agar limits

uptake since, nutrient uptake is closely associated with the rate of water influx into tissues (George, 1993). The lower uptake of nutrients could explain the lower development of plantlets in gelled medium as compared to liquid medium. Also lower viscosity of cassava starch gelled medium may explain why plantlets preformed better in medium gelled with cassava starch as compared to plantlets on agar gelled medium. Similar studies have shown that cassava starch at 7% enhanced banana survival *in vitro* and produced consistent results in terms of shoots and roots proliferation as agar over four subcultures (Mbanaso et al., 2001).

The better response on cassava starch gelled media could also be due to the absence of inhibitors which have been reported to be present in agar (Debergh, 1983; Singha, 1984; Pierik, 1997; Puchooa and Purseramen, 1999). Alternatively, the promotory effect of cassava starch may be probably because cassava starch can act as an additional carbon source and the beneficial compounds present which acts as ionic supplements (35% carbohydrates, 1% mineral matter) (Onuweme, 1982) to the medium, which resulted in improved cell growth and morphogenesis. Better performance of plantlets on cassava starch gelled medium as compared to agar is an indication of potential of cassava starch as agar substitute in plant tissue culture.

Gel strength is often regarded as an important criterion for agar quality (Debergh, 1983). Media gelled with 8% cassava starch mixed with 0.25% agar provided the same firmness as media gelled with 0.8% agar and problems of starch softening were solved. Cassava starch 10% (w/v) gave adequate support and orientation of potato nodes. This concentration is lower than 14% earlier reported by Gebre and Sathyanarayana (2001) for shoot regeneration and higher than the 8% reported by Nene and Sheila (1997) for tobacco and chickpea culture.

Cassava starch being 50 times cheaper than agar will find greater acceptability as a cheaper alternative to agar within developing countries needing to import agar. Cassava starch being a product of plant origin is biodegradable and poses no threat to the environment on being properly disposed after use.

Conclusions

Liquid media provide substantially different environment for the *in vitro* plantlets and widespread use is hampered by induction of physiological anomalies, asphyxiation, and low acclimatisation. Agar has remained to be the most expensive constituent of culture media. However, cassava starch at 10% (w/v) can provide alternative low cost medium support. In as much as cassava starch softens in culture medium, gel strength can be maintained by mixing it with 0.25% agar. Although substitutes of agar may differ in their composition, which may affect culture growth, this phenomenon was not observed in potato cultures. Investigating the use of such alternatives on other plant species is crucial.

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