

Improvement of *in vitro* techniques for rapid meristem development and mass propagation of Philippine cassava (*Manihot esculenta* Crantz)

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Received 11 March 2005, accepted 26 November 2005.

Abstract

Cassava (*Manihot esculenta* Crantz cv. Golden Yellow) shoot apical meristems with 1-2 leaf primordia were aseptically isolated and cultured on liquid or solid Murashige and Skoog (MS) medium supplemented with gibberellic acid (GA₃), benzylaminopurine (BAP) and naphthalene acetic acid (NAA) at 0.25, 0.1 and 0.2 mg/L, respectively. The cultures were maintained in an incubation room at a temperature of 25±2°C. Filter paper bridge was used to hold the meristem in liquid medium. Plant regeneration was most effectively promoted by liquid MS medium added with the 3 hormones, producing 2-nodal stage complete plantlets without callus after 3 weeks from inoculation. When used singly, GA₃ did not elicit any growth response while BAP caused shoot formation only. NAA and the solid MS medium added with the 3 hormones induced both shoot and root development but this was preceded by callus production. Using single nodal segments of meristem-derived plantlets, micropropagation experiments showed poor growth response to the CIAT-4E medium containing MS nutrients with GA₃, BAP and NAA additives. Only 13% of the cultures developed into complete plantlets (3-4 nodal stage) after about 2 months of incubation. Increasing the sucrose level of this medium to 3% did not markedly improve plant growth. With the hormone-free MS medium with 2% sucrose (PTCL-1 medium), all cultures developed into complete plants after one month or less. Other PhilRootcrop Tissue Culture Laboratory (PTCL) medium series did not additionally promote shoot, root and complete plantlet development. Agarite and agar-agar as low-cost gelling agents were comparably effective as Sigma agar. Micropropagation rate was estimated at 3¹¹ to 4¹¹ planting materials that can be potentially produced from one nodal explant per year.

Key words: Cassava, *Manihot esculenta* Crantz, meristem, *in vitro* propagation, tissue culture.

Introduction

Cassava (*Manihot esculenta* Crantz) is the leading root crop in the Philippines, which is used as food, animal feed and raw material of industrial products such as starch, flour and pharmaceuticals. Conventional production, which uses stem cuttings for propagation, is beset by problems of lack of planting materials and presence of transmissible diseases, particularly viruses. In systemically infected plants, the pathogen is passed on from one generation to the next through the vegetative propagules. Over time, the entire population of a given clonal variety could be infected with the disease. Even without visible symptom, infected plants exhibit reduced growth and yield performance and could spread the disease to non-target plant species or varieties.

In vitro techniques have been employed to produce disease-free planting materials even from infected mother plants. These materials could then be propagated rapidly. Meristems are usually used to recover clean materials. They are frequently devoid of systemic pathogen due to the absence of differentiated conducting tissues¹. Meristem culture has been shown to eliminate viruses in a number of crops²⁻⁵, including cassava with combined thermotherapy treatment⁶. Meristem culture of Philippine cassava done earlier showed a development process initiated with callus formation followed by rosette shoot formation and complete plantlet production in 6-10 weeks from inoculation⁷. A callus phase in plant regeneration *in vitro* is not desirable due to high frequency

of genetic aberrations⁸. Plants derived from meristems, shoot tip and other tissues without an intermediate callus formation are generally genetically stable and phenotypically homogenous. Production of true-to-type planting materials can rapidly be achieved through *in vitro* propagation, termed also as micropropagation due to the minute size of the plant material used. This propagation method can be done regardless of growing season and could therefore enable the year-round availability of planting materials and the expansion and intensification of production. This study improved the meristem culture technique which eliminated the initial callus phase and optimized the culture conditions for rapid multiplication of meristem-derived plantlets using the 'Golden Yellow' cassava, the number one variety, as test material.

Materials and Methods

Donor plants and sterilization: Freshly harvested stem cuttings were pretreated with insecticide and fungicide solutions at manufacturer's rate, planted in pots and allowed to sprout in a greenhouse. The sprouts were then harvested, expanded leaves and petioles removed, washed with detergent-added water and rinsed with tap water. In a laminar-flow bench, the tissues were soaked in 2% sodium hypochlorite solution for 5 minutes and washed three times with sterile distilled water.

Meristem isolation and culture: Shoot apical meristems consisting of the apical dome with one to two leaf primordia were isolated using a dissecting microscope, sterile hypodermic needle and scalpel. The meristem explant was immediately inoculated onto freshly prepared culture medium to avoid dehydration. The Murashige and Skoog⁹ (MS) nutrients were used as the basic components of the medium added with 2% sucrose. Hormonal additives were tested and included such as gibberellic acid (GA₃) at 0.25 mg/L, benzylaminopurine (BAP) at 0.1 mg/L and naphthalene acetic acid (NAA) at 0.2 mg/L which were applied singly or in combination. Liquid and solid medium formulations were also compared. For the liquid medium, a filter paper bridge was used to hold the meristem (Fig. 1a) while for the solid medium, 0.7% Sigma agar was used as gelling agent. Prior to use, the pH of the culture medium was adjusted to 5.8, dispensed to McCormick vials at 10 ml each, and sterilized in a pressure cooker at 15 psi for 15 minutes. Each vial as culture vessel was inoculated with one meristem. Ten to 15 meristems were used for each treatment. The cultures were incubated in shelves with supplementary illumination from white fluorescent tubes in an air-conditioned room with temperature maintained at 25±2°C. Shoot and root development and callus formation were monitored.

Micropropagation study: Single nodal segments from meristem-derived plantlets were used as explants and inoculated onto freshly prepared culture medium. Different medium formulations were used, namely: CIAT-4E medium containing MS nutrients with 2% sucrose, 0.05 mg/L GA₃, 0.04 mg/L BAP and 0.02 mg/L NAA (personal communication, Dr. Claudia Guevarra, CIAT, Colombia), modified CIAT-4E (MCIAT-4E) medium which is the same as CIAT-4E but with increased sucrose level of 3%, and PhilRootcrops

Tissue Culture Laboratory (PTCL) medium series PTCL-1, PTCL-2 and PTCL-3 containing MS nutrients with 2%, 3% and 5% sucrose, respectively. For all culture media, 0.7% Sigma agar was used as gelling agent. In another experiment, low-cost gelling agents, agarite (Taiwan brand) and agar-agar (Japan brand), were tested as Sigma agar substitute. One nodal explant was inoculated into each culture vessel. For each treatment, 30 nodal cultures were used. Other procedures in medium preparation and incubation of the cultures were the same as described earlier. Shoot and root development, callus formation and complete plantlet production were recorded. Micropropagation rate was also estimated by calculating the potential number of planting materials that can be produced from one nodal explant per year.

Statistical analysis: Standard error (SE) of treatment means was calculated in the meristem culture experiment. In the micropropagation study, variance analysis and mean comparison by the Duncan's Multiple Range Test (DMRT) were performed using MSTAT program.

Results and Discussion

Meristem culture: The meristem explants cultured in liquid MS medium alone or with GA₃ did not show any sign of growth (Table 1). The tissues only swelled and eventually degenerated and turned brown. In BAP-added medium, about 80% of the meristems showed shoot formation after about 10 days from inoculation (Fig. 1b) and 4 days later, 75% of these shoot-forming meristems developed the first open leaf. However, root development was not induced. In NAA-added medium, 60-80% of the meristems were induced to form both shoots and roots after 10 days from inoculation but the shoots did not develop further (Fig. 1c). Shoot

and root development without tissue callusing was affected only when the 3 hormones were combined as medium additives. Shoot initiation was noted in about 80% of the cultures, all of which subsequently developed and formed open leaves by about 2 days earlier than in medium with single-hormone additive. All cultures also developed roots starting after about 13 days from inoculation. Fig. 1d shows the growing meristem-derived plantlet after 19 days from inoculation. These responses were confirmed in a subsequent study (Table 2). The cultures in liquid medium with the 3 hormone additives showed comparable growth response to that in the solid medium but in the latter, callus formation was induced (Fig. 1e) in 61% of the cultures and shoot initiation was relatively delayed. After 21 days of incubation in the liquid culture medium, the plantlets produced 2 nodes which were used in the micropropagation study.

Plant regeneration from meristem *in vitro* usually requires exogenous hormonal supplementation of culture medium. Roca⁶ developed such regeneration medium for cassava meristems at the International Center for Tropical Agriculture (CIAT), Colombia. When tested for the Philippine cassava meristems, it succeeded in regenerating the plant but the development process took 6-10 weeks and was

Table 1. Growth characteristics of cassava meristems in different liquid medium formulations.

Culture medium	Shoot formation		1 st Open leaf		Root formation		Callus formation %
	%	Days	%	Days	%	Days	
MS alone	0	-	0	-	0	-	0
MS+0.25 mg/L GA ₃	0	-	0	-	0	-	0
MS+0.1 mg/L BAP	80	9.8±3.5	75	13.7±3.5	0	-	0
MS+0.2 mg/L NAA	60	10.3±1.5	0	-	80	9.3±1.5	100
MS+ GA ₃ + BAP+NAA	80	8.3±1.5	100	11.5±1.9	100	12.8±3.3	0

Each value represents the mean±SE of 10-15 cultures

Table 2. Growth characteristics of cassava meristems in liquid or solid MS medium added with 0.25 mg/L GA₃, 0.1 mg/L BAP and 0.2 mg/L NAA.

Parameter	Liquid MS	Solid MS
Shoot formation		
% of cultures	85.0	88.9
Days to initiation	7.9±2.4	11.5±5.2
No. of nodes after 21 days	2.1±0.8	2.0±5.9
Root formation		
% of cultures	40.1	61.0
Days to initiation	18.4±3.6	17.8±3.7
Callus formation		
% of cultures	0	61.0
Days to initiation	-	4.8±1.6

Each value represents the mean±SE of 10-15 cultures.

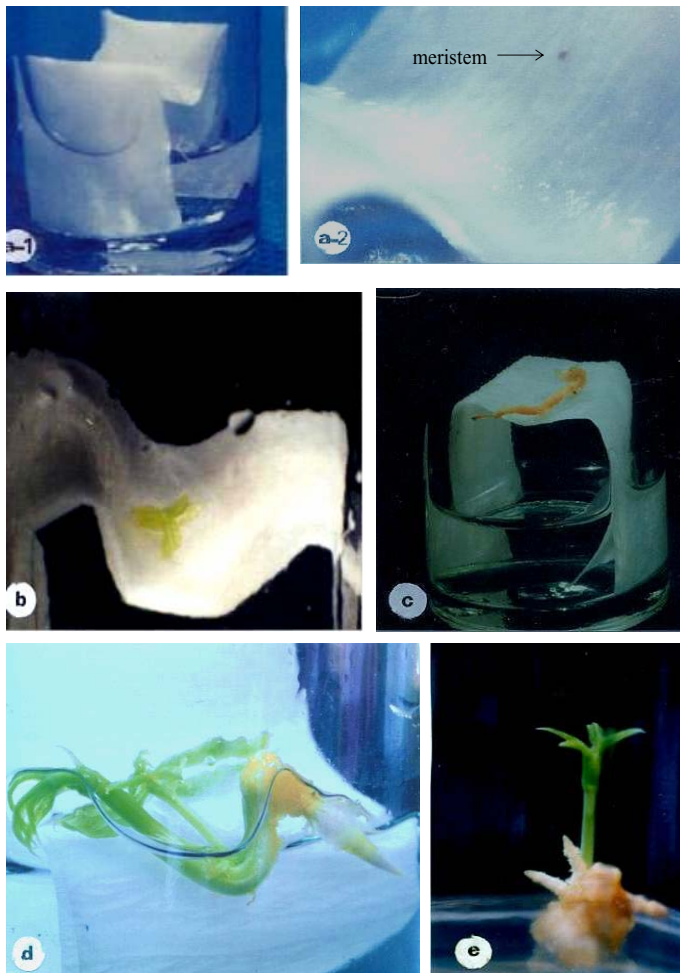


Figure 1. Newly inoculated cassava apical shoot meristem on filter-paper bridge (a-1, a-2) and growth appearance in liquid MS medium added with BAP (b), NAA (c) or combined GA_3 , BAP and NAA (d) and in solid medium with the 3 hormone additives (e).

preceded by callus production⁷. It was also found that apical meristems responded better than axillary meristems and that the optimum meristem size ranged from 0.6-0.8 mm. In the present study, the meristems used were ensured to possess the apical dome and 1-2 leaf primordia, the size of which ranged from 0.1-0.2 mm. Due to the limitation (i.e. callusing) associated with the use of the CIAT medium, other medium formulations were explored. Results show that the regeneration capability of the meristems was best enhanced with the combined use of GA_3 , BAP and NAA as MS medium additives and complete plantlets were regenerated in 3 weeks only. These findings are in agreement with previous results in Indian and Nigerian cassava (*Manihot utilissima*)¹⁰. In an earlier study on sweetpotato meristems, GA_3 at 0.25 mg/L was found to be the optimum level for inducing meristem growth and development¹¹. This was not the case for the cassava meristem as GA_3 supplementation did not induce a growth response possibly because the concentration was already excessive for the tissue. Shabde and Murashige¹² reported that GA_3 could inhibit meristem growth when used at higher concentration. Furthermore, the results demonstrate that the use of the liquid medium favored better meristem development than the solid medium. Tissues in liquid medium have increased uptake of nutrients and hormones due to greater absorptive area¹³. Callusing was not also induced

in contrast to that in solid medium. Callus can physically block the development of root initials, compete with nutrients, and may produce growth inhibitors¹⁴. In addition, a callus phase in plant development could adversely affect the genetic fidelity of *in vitro*-maintained plants⁸. Thus, conditions inducing tissue callusing should be avoided when meristem-derived plantlets are intended for multiplication to produce true-to-type planting materials. These materials could be clean or disease-free as the meristem has still no developed vascular system through which pathogenic microorganisms, particularly viral particles, are conducted¹. For other pathogens such as bacteria that are systemically present in plant tissues, they will develop and proliferate during the culture period and could be promptly discarded.

Micropropagation: Single nodal cultures from meristem-derived plantlets responded differently to the nature of the micropropagation medium. All cultures developed shoots in the five culture media tested but shoot formation occurred earlier in the three PTCL media than in CIAT-4E or MCIAT-4E medium (Table 3). The 3 PTCL media were also very effective in promoting rooting, with all cultures showing root formation after 6-7 days from inoculation. All cultures subsequently developed into complete plantlets with 3-4 nodes each at about one month after inoculation. In CIAT-4E or MCIAT-4E medium, fewer cultures (63-83%) developed roots and this happened at a much later period of about one month from inoculation or longer. They also failed to support further growth and development as only 13-21% of the cultures developed into complete plantlets. The CIAT-4E medium was the least effective among treatments and the only medium that induced callus production.

The results clearly show that the CIAT-4E medium, which is used in the *in vitro* culture of cassava at CIAT, is not suitable for the Philippine cassava or at least for the 'Golden Yellow' variety, even if the sucrose level was increased to 3% (MCIAT-4E). The MS nutrients as basal medium without hormone additive (PTCL media) proved to be sufficient and very effective in multiplying the meristem-derived plantlets. A sucrose level of 2% (PTCL-1) was enough to elicit the desired response. Increasing the sucrose level to 3% (PTCL-2) or 5% (PTCL-3) did not additionally promote shoot, root and complete plantlet development.

In a succeeding experiment verifying the efficacy of the PTCL-1 medium, similar responses were obtained (Table 4). All cultures showed shoot and root formation and later developed into complete plantlets after about 24 days from inoculation. These responses were duplicated by cultures in the same medium but with low-cost gelling agents, agarite and agar-agar (Table 4). Fig. 2 shows the different growth stages of meristem-derived nodal segments in PTCL-1 medium.

After one month of culture, the plantlets had 3-4 nodes (Fig. 2b-c), which can be micropropagated using single nodal segments. Thus, 3-4 new plantlets could be produced from a single nodal explant one month later. Assuming a monthly multiplication cycle and 10% mortality, about 159,432 planting materials can be produced from one nodal explant per year if the plantlets have only 3 nodes per month (Table 4). If the plantlets have 4 nodes per month, the potential number of planting materials is enormous, about 3.8 million. The same number of planting materials are produced with agarite or agar-agar as gelling agent since complete plantlet development was similar to that with Sigma agar.

Table 3. Growth characteristics of cassava meristem-derived nodal tissues in different micropropagation medium.

Culture medium	Shoot formation		Root formation		Callus formation		Complete plant ¹	
	%	Days	%	Days	%	Days	%	Days
CIAT-4E	100	7.1a	63	35.9a	100	7.4	13	56.0a
MCIAT-4E	100	7.1a	83	29.6a	0	-	21	33.8b
PTCL-1	100	5.0b	100	6.0b	0	-	100	29.5b
PTCL-2	100	5.5b	100	6.0b	0	-	100	32.1b
PTCL-3	100	5.0b	100	6.5b	0	-	100	27.7b

¹With roots and shoots having 3-4 nodes

Means in a column with the same letter are not significantly different at 5%, DMRT.

Table 4. Growth characteristics and micropropagation rate of cassava meristem-derived nodal tissues cultured in PTCL-1 medium with different solidifying agents.

Parameter	Sigma Agar	Agarite	Agar-agar
Shoot formation			
% of cultures	100.0	100.0	100.0
Days to initiation	4.8	3.5	3.5
Root formation			
% of cultures	100.0	100.0	100.0
Days to initiation	10.0	10.0	9.5
Complete plant (3-4 nodes)			
% of cultures	100.0	100.0	100.0
Days to development	24.3	25.5	26.7
Micropropagation rate ¹			
With 3 nodes/month	159,432	159,432	159,432
With 4 nodes/month	3,774,873	3,774,873	3,774,873

¹Number of planting materials produced per nodal explant per year, calculated as 3¹¹ for 3-nodal plants/mo or 4¹¹ for 4-nodal plants/mo less 10% mortality.

No significant differences were obtained among means in rows.

Considering that agarite and agar-agar are 90% and 85% lower in price than Sigma agar, respectively, their use could drastically reduce micropropagation cost.

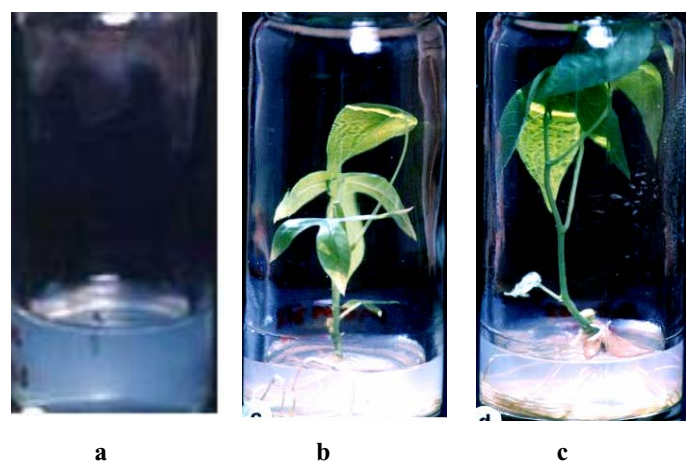


Figure 2. Newly inoculated cassava meristem-derived nodal segment (a) and 3-4 nodal plantlets (b-c) cultured in PTCL-1 medium.

Conclusions

Plant regeneration from shoot apical meristems was best enhanced using liquid MS medium added with GA₃, BAP and NAA. Shoot and root development proceeded without an intermediate callus phase. The meristem-derived plantlets produced after 3 weeks from inoculation were effectively and rapidly micropropagated using single nodal segments and PTCL-1 culture medium (MS nutrients plus 2% sucrose). The CIAT-4E was not suitable as micropropagation medium. Substitution of Sigma agar with low-cost gelling agent, agarite or agar-agar, was highly feasible without adverse effect on plant development and multiplication. Micropropagation cost could possibly be reduced further with the substitution of the other high-cost components of the culture medium. On the other hand, inducing multiple shoot production could increase multiplication rate and cost-effectiveness of micropropagation to mass produce planting materials.

Acknowledgement

Research funding from the Cassava Biotechnology Network (CBN), CIAT, Colombia through Dr. Ann Marie Thro, and Leyte State University (formerly Visayas State College of Agriculture, ViSCA) and the assistance of D. Posas, M. Barosa and N. Davis in the various laboratory activities are gratefully acknowledged.

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