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BRIEF COMMUNICATION

Somatic embryogenesis, plantlet regeneration and micropropagation of cultivars and F_1 hybrids of *Manihot esculenta*

M.N. OGBURIA

Department of Crop/Soil Science & Forestry, Faculty of Agriculture, Rivers State University of Science and Technology, PMB 5080, Port Harcourt, Nigeria

Abstract

Explants of four F_1 hybrids (OMR 36-41/1, OMR 36-41/2, OMR 36-41/4 and OMR 36-41/5) and two cultivars (Rayong 1 and Rayong 60) of cassava (*Manihot esculenta* Crantz) were subjected to different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), kinetin (KIN) and N^6-benzylaminopurine (BAP) to induce somatic embryogenesis, organogenesis and micropropagation. Shoot apices of the F_1 hybrids exhibited higher frequency (62 - 74 %) of proliferation of somatic embryos than the cultivars (21 - 43 %) in Murashige and Skoog basal medium supplemented with 8 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} NAA. Nodal explants of regenerated plantlets were rapidly micropropagated with 90 % efficiency on a medium containing 0.1 mg dm^{-3} NAA and 0.05 mg dm^{-3} BAP irrespective of explant source.

Additional key words: 2,4-dichlorophenoxyacetic acid, *in vitro* growth, 1-naphthaleneacetic acid, organogenesis, phytohormones.

Cassava is native to South America. It is the most important staple food and animal feed crop in Sub-Saharan Africa. Production constraints of this crop are viral diseases, insect pests, low protein content of edible root tubers and the presence of cyanogenic glucosides in both leaves and root tubers (Cock 1985). Conventional breeding of cassava is fraught with many obstacles such as high degree of heterozygosity, polyploidy, low fertility, poor seed set and low rates of seed germination (Byrne 1984). The application of tissue culture techniques to complement conventional breeding methods in regenerating a number of plantlets from the few germinated seeds will be of immense value.

Successful application of this technique is largely dependent on reliable regeneration and micropropagation protocols. There are reports in literature on somatic embryogenesis, micropropagation and plantlet regeneration in cassava (Liu 1975, Stamp 1987, Raemakers 1993, Konan *et al.* 1994). Till date, however, there is no report on *in vitro* embryogenesis and plantlet regeneration of F_1 hybrid seedlings of cassava. This communication reports on the embryogenic, organogenic and micropropagation response of explants derived from seedlings of F_1 hybrids in comparison with explants derived from common cultivars.

Botanical open pollinated mature seeds of two cassava (*Manihot esculenta* Crantz, family *Euphorbiaceae*) clones: OMR 36-41 (derived from CMR 33-30-37) and stem cuttings (30 cm long) of two common cultivars Rayong 1 (local cultivar) and Rayong 60 (M. Col 1984 × Rayong 1; Kawano *et al.* 1990) obtained from Centro International de Agricultura Tropical (CIAT), Thailand were used in these experiments. As a source of explants, stems were planted into soil directly or the seeds were

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - 1-naphthaleneacetic acid; KIN - kinetin; BAP - N^6-benzylaminopurine.

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Fax: (+234) 084 230720, e-mail: mikeogburia@hotmail.com.

germinated in black polythene bags (18 × 18 × 15 cm) filled with non-fumigated topsoil. The seeds were watered regularly and grew in glasshouse with a minimum and maximum temperature of 22 and 35 °C, respectively, until seedlings attained height of 10 cm. Four randomly selected hybrid lines sampled for tissue culture were: OMR 36-41-1, OMR 36-41-2, OMR 36-41-4, and OMR 36-41-5. A minimum of 33 shoot tip explants were cultured for somatic embryogenesis and 15 nodal segments were cultured for plantlet regeneration and micropropagation per each combination of phytohormones and the experiments were arranged in a completely randomized design and replicated 3 times.

The basal medium used was Murashige and Skoog's (1962) with 2 % sucrose and supplemented with different compositions of phytohormones (Table 1). All media were adjusted to pH 5.7 - 5.8, solidified with 0.8 % (m/v) agar (*Naclai Tesque*, Tokyo, Japan) and sterilized by autoclaving at 1.50 kg cm^{-2} and 121 °C for 15 min.

Table 1. Composition of phytohormones [mg dm^{-3}] in the induction (SEM 1 - SEM 6) and regeneration (PRM 1 - PRM 3) media. Phytohormones were added to MS basal medium (Murashige and Skoog 1962) supplemented with 2 % sucrose.

Medium	2,4-D	NAA	KIN	BAP
SEM 1	8.00	0.00	0.00	0.00
SEM 2	8.00	0.50	0.00	0.00
SEM 3	8.00	1.00	0.00	0.00
SEM 4	4.00	2.00	0.50	0.50
SEM 5	4.00	1.00	1.00	1.00
SEM 6	4.00	0.05	0.05	0.05
PRM 1	0.00	0.00	0.00	0.00
PRM 2	0.00	0.10	0.00	0.05
PRM 3	0.10	0.00	0.00	0.05

Callus was initiated from shoot apical meristems of F_1 hybrids and the cultivars. Excised explants were surface sterilized first by dipping in 70 % ethylalcohol, agitated for 1 min and then soaked in 5 % sodium hypochlorite supplemented with 2 - 3 drops of *Tween 20* with occasional agitation for 3 min and rinsed 5 times in double distilled water. All explants were inoculated in 9 cm diameter Petri dishes (*Iwaki Co.*, Tokyo, Japan) containing 20 cm^3 medium and sealed with parafilm M. The cultures were incubated at 24 °C in continuous darkness for 3 - 4 weeks before exposed to continuous light (fluorescent tubes; irradiance of 30 μmol m^{-2} s^{-1}).

Morphogenic reactions were recorded and analyzed as described by Mize and Chun (1988). However, some subjective evaluations such as morphological changes and variations were also conducted.

Callus induction and proliferation commenced approximately after 7 d of culture of apical meristems *in vitro* on all media containing 2,4-D irrespective of the explant source (*i.e.* either from cultivars or F_1 hybrids). After 2 consecutive 7-d subcultures in the same medium, embryogenic structures developed from apical meristems of both explant sources. Genotypic variations were observed in somatic embryogenesis (4 - 9 embryos per explant for the cultivars and 7 - 23 embryos per explant for the hybrid lines) and in percentage plantlet recovery from somatic embryos. 23 somatic embryos per explant were obtained from apical meristem of OMR 36-41/2 on medium with 8 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} NAA compared to 9 somatic embryos per explant of cv. Rayong 1 on the same medium. Shoot apices of the F_1 hybrids exhibited on this medium also higher frequency of proliferation of somatic embryos (62 - 74 %) as compared to that of the cultivars (21 - 43 %) (Table 2).

Normal embryo development was characterised with globular, cotyledonary and torpedo stages before embryo germination (Fig. 1*a*). These embryos regenerated into plantlets upon transfer to regeneration medium (Fig. 1*b*). However, the rate of shoot or plantlet conversion was significantly ($P \leq 0.05$) influenced by media composition. Some of the germinated embryos remained in a trumpet-like state with many cotyledons and distinguishable hypocotyls. These embryos could not differentiate into roots and/or shoots and consequently were regarded as malformed since they differed markedly from normal plantlets with roots, stems and leaves (Fig. 1*c*).

Table 2. Influence of genotype and medium composition on number of embryos per meristem explant in cassava. Mean ± SD of 4 randomly selected responding explants per treatment and data with identical letters in a column are not statistically significant ($P = 0.05$); for media components see Table 1.

	SEM 1	SEM 2	SEM 3	SEM 4	SEM 5	SEM 6
Rayong 1	8.3 ± 2.6b	7.0 ± 2.2b	6.5 ± 1.9b	4.3 ± 2.2c	4.0 ± 2.2b	4.0 ± 2.2b
Rayong 60	9.5 ± 1.0b	5.0 ± 1.8b	5.3 ± 3.0b	5.8 ± 1.7c	3.5 ± 2.4b	5.0 ± 2.2b
OMR 36-41/1	12.0 ± 2.9a	10.3 ± 1.5a	10.5 ± 1.3a	9.8 ± 1.0b	9.5 ± 1.7a	6.5 ± 1.3a
OMR 36-41/2	14.3 ± 3.3a	19.3 ± 3.8a	8.8 ± 3.3a	9.3 ± 2.1b	9.8 ± 1.7a	8.5 ± 1.0a
OMR 36-41/4	14.8 ± 6.0a	14.0 ± 3.6a	13.0 ± 2.9a	12.5 ± 2.4a	6.8 ± 2.1a	8.3 ± 1.5a
OMR 36-41/5	16.0 ± 5.4a	17.5 ± 3.6a	11.8 ± 3.8a	11.5 ± 4.4a	8:5 ± 1.0a	6.8 ± 1.7a

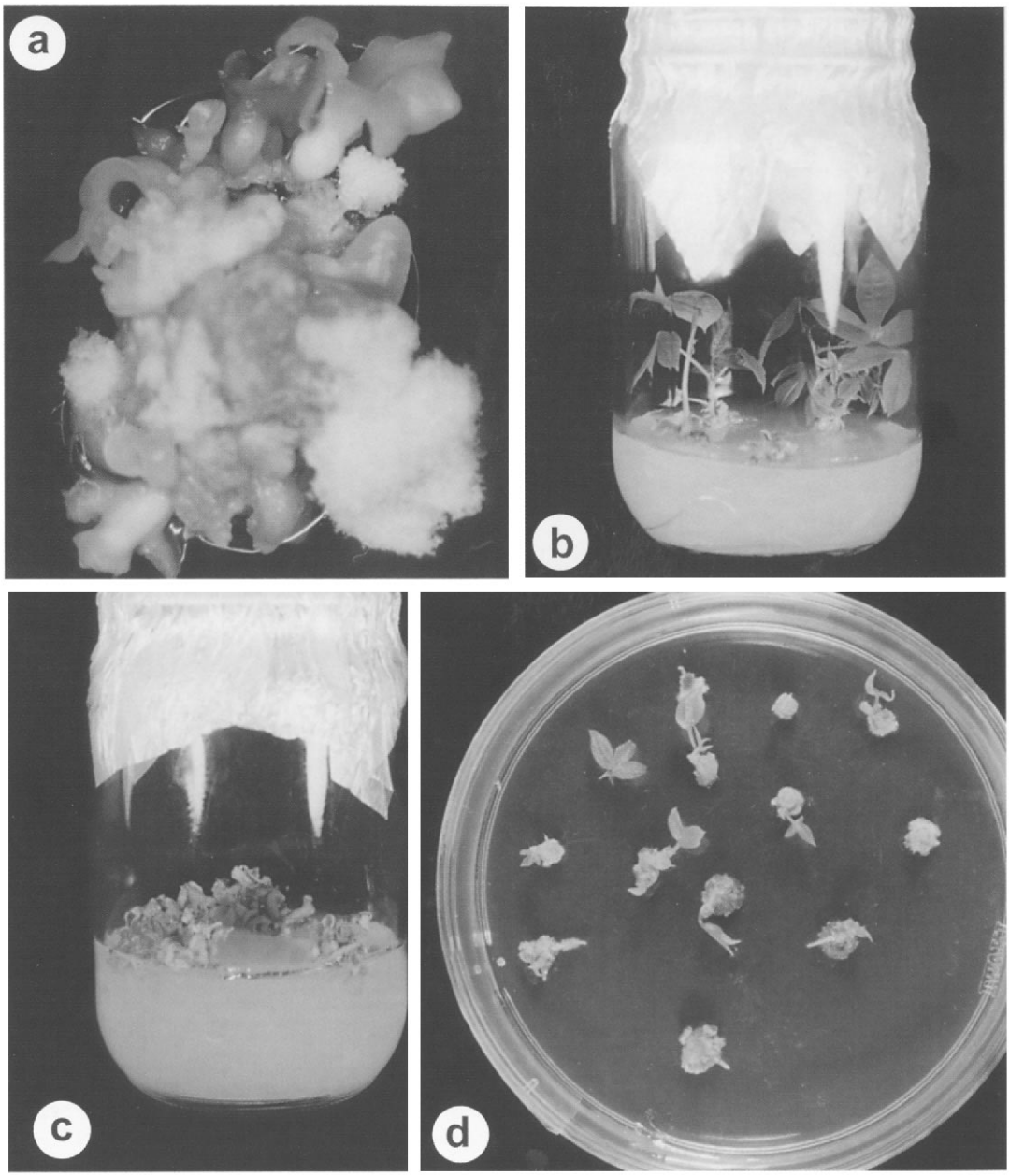

Fig. 1. Somatic embryogenesis and plantlet regeneration in cassava. *a* - embryogenic callus (note globular, heart-shaped, torpedo, and cotyledonary stages), *b* - plantlet regeneration from somatic embryos, *c* - malformed somatic embryos, *d* - rapid nodal micropropagation via nodal segments of regenerated seedlings (note root initials and leaves).

The F_1 hybrid lines produced 7 - 10 % plantlets while the cultivars produced only 1 - 7 % plantlets from primary somatic embryos. Unfortunately, however, about 90 % of the somatic embryos could not be regenerated into full plantlets when transferred into the three regeneration media tested. Rooting frequency was low (12 %) with callus growing at the base of 45 % of the plantlets.

Hormone free medium was least effective in plantlet recovery from germinated somatic embryos obtained from the genotypes as rooting was delayed or never observed. However, the medium (PRM 2) with 0.1 mg dm^{-3} NAA + 0.05 mg dm^{-3} BAP was comparatively effective in shoot induction and promoted rooting.

Since most plantlets developed roots from nodes above the medium level, especially during longer culture (30 - 45 d), rapid micropropagation via nodal segments could be achieved on hormone free (PRM 1) medium (Fig. 1*d*). However, effective rapid micropropagation of plantlets without initial roots *via* nodal segment culture was significantly ($P \leq 0.05$) enhanced on PRM 2 medium

with 90 % efficiency. It was further observed that nodal segments in contact with the medium formed a little callus mass before node sprouting and growth into full plantlets with multiple shoots arising from the base of the initial plantlet. Up to a maximum of 7 shoots could be observed and excised from multiple shoot developing nodal segment. PRM 3 was moderately efficient with 57 % efficiency. Yellow-colouring exudates were found to occur at calli base in touch with the medium and this phenomenon negatively influenced plantlet regeneration from nodal segments. Samples of these exudates were cultivated on freshly prepared medium and were not found to be spreading further on the agar surface, thus the exudates were pathogen-free.

It is evident that the hybrids were superior to the cultivars tested and thus more useful for multiplication of seedlings. This superiority is probably due to heterotic and epistatic effects, characteristic consequences of hybrids *in situ* and the possible loss of these traits in the cultivars. Differences in somatic embryogenic capacity of shoot tips of some cassava genotypes cultured in a medium containing 4 - 16 mg dm^{-3} 2,4-D have been reported by Szabados *et al.* (1987). Similar results of genotype-dependence were also obtained by Raemakers *et al.* (1993). However, the hybrid nature of the plants used by those authors was not specified.

Somatic embryogenesis has been found to be the only routine way for efficient regeneration in cassava and in some other crops (Liu 1975, Stamp and Henshaw 1982, 1986, 1987a, 1987b, Stamp 1987, Mathews *et al.* 1993, Das and Rout 2002, Mohan and Krishnamurthy 2002). According to Konan *et al.* (1994) plantlet recovery from somatic embryos was very low in cassava (0 - 40 %). Therefore, it could be concluded that this is a common phenomenon in tissue culture of cassava since plantlet recovery rate in this study was found to be 1 - 10 %. However, isolated case of an improved conversion rate of somatic embryos have been reported (Mathews *et al.* 1993).

The relatively low conversion rates from somatic embryos makes rapid micropropagation from node segments an attractive alternative. All tested clones showed strong apical dominance *in vitro* and there was no single plant that branched throughout the study. This was particularly true when plantlets were grown on hormone-free medium. In this study, NAA demonstrated the efficacy of nodal segment multiplication. The similar results (*i.e.* nodal segment culture with NAA) have been reported for other Australian genotypes (Smith *et al.* 1986).

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