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Recovery of cassava (*Manihot esculenta* Crantz) plants from culture of immature zygotic embryos

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Abstract

An embryo culture protocol using immature cassava seeds has been developed to enhance successful seed germination and reduce time for population establishment. Embryonic axes were excised from seeds 40 days after pollination and placed on 1/3 MS medium supplemented with growth factors. Fruits were either air-dried at 20 °C to aid dehiscence, or dissected immediately after harvest. Culture of embryonic axes from seeds obtained from mature fruits (90 days after pollination) served as control. Average percent germination and plantlet recovery rate were higher for embryos cultured from non air-dried immature seeds than from air-dried immature seeds. Immature seeds that were air-dried before germination had $\geq$50% reduction in germination rate and $\geq$75% reduction in plantlet recovery rate, indicating that cassava immature zygotic embryos are susceptible to osmotic pressure changes. Genotypic effects were observed in shoot elongation, formation of internodes, and vigor of cultures from both mature and immature seeds. The high percentage of plants recovered from immature seeds through embryo culture opens up opportunities for genetic stock development in cassava that has been previously unexplored.

Abbreviations: MS – Murashige & Skoog (1962); CIAT – International Center of Tropical Agriculture; NAA – 1-naphtaleneacetic acid; GA3 – gibberellic acid-3; NPK – Nitrogen, Phosphorus, Potassium

Introduction

Cassava is a major source of calories and is rapidly becoming a critical factor in economic development in tropical lowlands, and to some extent in tropical highlands. After rice, wheat, maize and potato, cassava is the world's fifth staple and is increasingly used as raw material for industrial production of high quality starches, alcohol, and as base for animal and human food. As demand for cassava in specialized niches intensifies, cassava breeding requires more refined selection parameters, involving a range of plant populations. Low seed set, poor germination rates, and a long growth cycle, i.e. 9–24 months, have been bottlenecks of population development in cassava (Jennings, 1963; Bryne, 1984). Even more debilitating is the effect of inbreeding on seed set, germination, growth and development. Cassava is suspected to be an allotetraploid ($2n$=$4x$=36) (Fregene et al., 1997) and is a highly heterozygous crop that suffers severe inbreeding depression following loss of heterozygosity. Cassava breeding involving backcrossing or full-sib mating leads to unmasking of recessive deleterious alleles which are part of the high genetic load carried by a highly heterozygous polyploid.

Improvement of germination rates holds the greatest promise for resolving some of the compelling problems of cassava population development. Depending on the variety, duration and mode of seed storage, germination rate ranges widely from 0% to 80% in cassava (Biggs et al., 1986). Germination of cassava seeds has been enhanced by various procedures including scarification, treatment by heat and/or acid (Kawano et al., 1978), exposing seeds to red light (Nartey et al., 1974) and more recently by embryo culture from mature (Biggs et al., 1986; Ng, 1989)

and immature seeds (Roca et al., 1988). Embryo culture provides a simple technique for breaking seed dormancy and ensuring a fairly uniform germination rate (Biggs et al., 1986). We have described establishment of a backcross cassava population intended for gene tagging studies using embryo culture of immature seeds, and compared the results with germination from mature seeds of the same population. We also observed the effect of two embryo culture media and the effect of pre-treating the immature fruit by heat, to aid dehiscence.

Materials and methods

Source of seeds

A half-sib backcross mapping population was generated by crossing, in both directions, a set of 5 F_1 plants, chosen for their profuse flowering, to their female parent, cultivar TMS30572, a widely grown improved cassava cultivar from West Africa. The male parent of the F_1 plants is CM2177–2, an improved cassava cultivar adapted to the Colombian Uanos (Savannahs). An initial 400 pollinations, involving 5 F_1 families, from 5–7 month old plants grown at CIAT headquaters, Cali, Colombia, were carried out in June 1995 and mature fruits were harvested 3 months later. Fruits were considered to be mature when the exocarp had dehydrated and had started to detach from the endocarp. Seed pods were air-dried at 20 °C for 48 h to aid dehiscence and were stored in jars at room temperature for 16 months before embryo culture. A second round of 964 pollinations, to further increase the population, involving 2 F_1 families, was made in Jan–Feb., 1997; immature fruits were harvested 40 days after pollination and embryo axes cultured immediately. The initial batch of immature fruits was air-dried as described above to aid dehiscence, but later batches were dissected with a sharp sterile scapel to release the seeds immediately after harvest. Only seeds from two of the BC_1 families common to the 1995 and 1997 pollinations were used in this study.

Excision and culture of embryonic axes

Mature and immature seeds were placed in a 1000 ml glass beaker half-filled with water and those that floated were discarded. Mature seeds were soaked in water to rehydrate for 24 h before culturing, to aid embryo excision. Immature seeds had sufficient moisture and were not rehydrated. The seeds were surface-sterilized by immersion in 70% alcohol for 1 mm, followed by immersion in 0.5% sodium hypochlorite for 6 min, and then rinsed three times with sterile water. Under aseptic conditions, the seeds were split along the longitudinal axis utilizing sterile pliers and the embryonic axes were removed with sterile forceps and scapel. Excised embryonic axes were placed radicle down on 1/3 medium, supplemented with 0.01 mg l^{-1} NAA, 0.01 mg l^{-1} GA_3, 1.0 mg l^{-1} thiamine-HCL, 100 mg l^{-1} inositol, 2% sucrose, 0.7% agar (Sigma Co.) and 25 mg l^{-1} of a commercial fertilizer containing: N 10, P 52. K 10, pH 5.7–5.8 (Roca, 1984). Medium was prepared in 500 ml batches, dispensed, 5 ml per tube, into size number 18 tissue culture test-tubes (Pyrex Inc.), and autoclaved (liquid cycle) for 15 min. A pilot study was earlier conducted to determine that 1/3 MS medium was more appropriate for plantlet development and growth in comparison to the 1/2 MS medium, supplemented with 1 mg l^{-1} GA3, and 2% sucrose. The 1/2 MS medium, with some modifications, was employed in earlier reports of embryo culture in cassava (Biggs et al., l986; Ng, 1989) and, without modification, in the only known report of embryo culture of seeds from immature fruits in cassava (Roca et al., 1988).

Embryo germination and seedling growth

The embryo cultures were incubated under an alternate temperature regime of 35 °C for 16 h and 25 °C for 8 h, in darkness for the first 5 days, to promote growth of the radicle, then under continous illumination from a 40 W fluorescent bulb (5000 μmol m^{-2} s^{-1}) for the next 5 days. The cultures were then transferred to a growth chamber with a 12-h photoperiod (illumination, 5000 μmol m^{-2} s^{-1}) at 27 °C (Marin et al., 1990) and grown for 40 to 45 days. Plants with sufficient growth were micropropagated, by explanting two or three node cuttings for eventual transfer to the greenhouse.

Results

Embryo germination and plantlet development

Embryonic axes of 47 seeds from mature fruits were excised and cultured. Of these 42 embryos or 91% germinated after 2 days. On the other hand, of 438 embryonic axes of seeds from immature fruits that were air-dried prior to culture, only 147 germinated, or

Table 1. Germination rate of cassava seeds using zygotic embryo culture and percent recovery of plants from embryo culture of mature, immature, air-dried seeds at 20 °C, and immature non air-dried seeds of a half-sib backcross population.

Cross	Mature seeds			Immature seeds					
				Air-dried			Non-air dried		
	No. of seeds	% germination	% recovery of plantlet	No. of seeds	% germination	% recovery of plantlet	No. of seeds	% germination	% recovery of plantlet
TMS3O572xCM7857-4	22	90	59	10	100	70	90	100	82
CM7857-4xTMS3O572	–	–	–	51	43	0	29	100	97
TMS3O572xCM7857-77	25	92	76	234	50	25	56	100	86
CM7857-77xTMS3O572	–	–	–	143	33	4	77	100	57
Total	47	91	68	438	45	16	252	100	77

34%. All 252 embryonic axes of seeds from immature fruits that were mechanically opened after harvest, without drying, germinated. A family-by-family account of percent germination of embryonic axes from mature and immature fruits is provided in Table 1. After germination, embryonic axes developed roots after 3–5 days in culture and elongation of hypocotyl was initiated after 10–12 days. Primary leaves could be observed by 20 days after culture, whereas formation of shoots and internodes occurred after 30 days in culture.

Plantlet recovery rate from embryo cultures

Plantlet recovery rate from embryo culture after 2 weeks, described as the number of plantlets obtained as a percentage of total embryonic axes cultured, was tallied, by family, for mature and immature, air-dried or non air-dried, seeds (Table 1). Due to the different pollination events, embryo culture from immature seeds and mature seeds could not be rigorously compared, however percent germination and plantlet recovery were similar between mature and non air-dried immature seeds. Immature seeds that were air-dried prior to embryo culture showed a 75% reduction in plantlet recovery rate compared to when the air-drying pretreatment was eliminated. Of embryonic axes from non air-dried immature seeds that germinated but failed to produce plantlets, 5% lacked root development, although hypocotyls elongated, at times with callus formation, but no further growth, while 14% produced normal roots but with poor shoot development. For immature shoots that were air-dried percentages of the above classes of arrested development of embryonic axes were 19% and 32%, respectively. For mature seeds, there were no embryo cultures lacking roots and 10% had normal root development with delayed growth of shoots.

Micropropagation

Due to heterozygosity of cassava, each seed is potentially a different genotype; therefore maintaining the identity of cultures is essential. Depending upon the development of cultures, at least one single node cutting was obtained from each shoot, providing a multiplication rate of 1:1 to 1:3 after 2 to 4 weeks of culture, respectively.

Genotypic effects on plantlet growth and development

Growth and development of embryonic axes after germination was variable between and within families of the half-sib backcross population for both mature and immature seeds. There was a marked difference in length of shoots and number of nodes of 2-month old plantlets from different families generated from embryo culture of mature and immature seeds. The latter displayed a more drastic disparity between plantlets when the fruits were air-dried prior to culture. The genotypic effect on differential plantlet development was more pronounced when embryonic axes were cultured on 1/2 MS medium supplemented with 1 mgl^{-1} GA_3, and 2% sucrose, 0.7% agar, pH adjusted to 5.6 (data not shown).

Discussion

Germination rate of immature embryo was adversely affected (75% reduction in germination) by air-drying of immature fruits before embryo excision. Air-drying, which was done to aid dehiscence and thus reduce damage to immature seeds during mechanical removal from fruit, appears to reduce moisture and nutrients to the immature, yet growing embryo; 48 h of air-drying was clearly lethal to a majority of the embryos. While germination rates could not be compared between embryo culture of mature and immature seeds, due to different pollination events, the high germination rates and plantlet recovery rate found with immature fruits make it a viable alternative for establishment of cassava populations. Optimizing osmotic pressure potential during embryo culture of seeds from immature fruits might lead to a further improvement of germination and plantlet recovery rate. Cassava somatic embryo cultures are sensitive to differences in osmotic pressure of the culture medium (unpublished results).

Embryos derived from mature fruits that germinated but failed to produce normal plantlets and showed retarded growth at various stages of development were reported in earlier studies of embryo culture in cassava using different culture media (Biggs et al., 1986; Ng, 1989). We have extended these earlier studies to include immature seeds and have described the classes of arrested growth. The first class showed no root formation with little hypocotyl elongation, a second class revealed normal root formation with little or no development of shoots. The first class of arrested development had different percentages, 19% and 5% in embryo culture of immature seeds that were air-dried or non air-dried, but was absent in embryo culture of mature seeds. The widely differing percentages in the two groups of embryo culture from immature

seeds can be attributed to prior air drying, as was also observed in the large differences in germination rate. The low percentage of the first class of arrested development in non air-dried immature seeds and its absence in mature seeds, rehydrated before excision on embryo axes, implies that this class of arrested development may have arisen from damage to the radicle of the immature embryos during embryo excision. The second class with normal root development and little or no shoot growth or normal root development with retarded growth appears to have arisen as a consequence of genotypic effects. The population was a backcross where the effects of inbreeding depression, prominent in cassava, are enhanced. This point is buttressed further by a wide variation in shoot length and number of nodes observed in plantlets recovered from culture of embryonic axes, within families and between families of the half-sib backcross population.

The usefulness of embryo culture, notably the rescue of interspecific hybrids by culture of immature embryos, has been emphasized (Raghavan, 1985; Mejia-Jimenez et al., 1994). We have demonstrated in this study that this can be extended to establishment of cassava populations, where low germination rate, low seed set and the debilitating effects of inbreeding depression prevent establishment of genetic and breeding stocks in numbers adequate for specific breeding goals. The embryo culture protocol employed here provides adequate germination rates but can be optimized further to provide more rapid growth and thus reduce the pronounced genotypic effects encountered in cassava. We are continuing this study to determine more effective growth medium for culture of embryonic axes.

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