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Improvements of cyclic somatic embryogenesis of cassava (*Manihot esculenta* Crantz)

C. J. J. M. Raemakers [1, 2], C. M. Schavemaker [1], E. Jacobsen [1], and R. G. F. Visser [2]

Departments of Tropical Crop Science [1] and of Plant Breeding [2], Agricultural University Wageningen, POB 386, NL-6700 AJ Wageningen, The Netherlands

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Summary In cassava a cyclic system of somatic embryogenesis was developed. Primary (torpedo shaped or germinated) embryos, originating from leaf lobes, could only be obtained after culture on solid medium. Cyclic embryos, originating from embryos, could be obtained in both liquid and on solid medium. The production of embryos in liquid medium was distinctly higher, faster and more synchronized than on solid medium. Lower densities and fragmentation of starting embryos improved the production significantly. The highest production found was 32.1 embryos per initial embryo.
In all treatments the explants initiated multiple embryos. The production of single embryos was achieved by pressing starting embryos through a fine meshed sieve, indicating that embryos can be produced from a piece of tissue with a restricted number of cells. The shoot conversion rate of embryos from liquid medium was comparable with that of embryos from solid medium.

KEYWORDS: direct somatic embryogenesis, primary and higher cycles, liquid culture, shoot development.

ABBREVIATIONS: BM = Basal Medium, MIE = medium volume per initial embryo, E/IE = number of Embryos per Initial Embryo.

INTRODUCTION

Cassava (Manihot esculenta Crantz), also known as yucca, tapioca and manioc, is a perennial shrub of the Euphorbiaceae family, native to Brazil. After rice, maize and sugarcane it is the fourth most important source of energy in the developing countries (Cock, 1985). Especially in Africa cassava is a staple crop of immense importance. Despite the importance for the tropics it did not get much attention of agricultural research centres.
Problems in the cultivation of cassava are virus diseases, insect pests, low protein content of the roots and the presence of toxic cyanogenic glucosides (Cock 1985). The feasibility of overcoming these problems by only classical breeding is hampered because of several problems such as a high degree of heterozygosity, polyploidy, low fertility, poor seed set and low rates of seed germination (Byrne 1984, Martin 1976, Jennings 1963). Therefore, emphasis is being placed on the application of genetic modification to overcome some of these problems. To be able to use this an efficient regeneration and transformation procedure is a prerequisite. The only routine way of regeneration in cassava is by somatic embryogenesis since direct or indirect adventitious shoot formation is seldom observed (Shahin and Shephard 1980, Tilquin 1979). In a number of articles the possibility of somatic embryogenesis of cassava was demonstrated (Raemakers et al. 1992a+b, 1991, Stamp and Henshaw 1987a+b, 1982, Zsabados et al. 1987). The common procedure is induction of primary somatic embryos on leaf lobes or on seed derived cotyledons on 2,4-D containing medium. Somatic embryos can be used as starting explants for a new cycle. In a previous report a method of cyclic embryogenesis on solidified medium was described (Raemakers et al. 1992b). To ensure that such a regeneration system becomes more universal and reproducible, the process should be optimized.
Different factors such as embryo density, fragmentation of embryos and type of medium were varied to study their influence on somatic embryo production for three successive cycles.

MATERIALS AND METHODS

PLANT MATERIAL

The Columbian cassava (Manihot esculenta Crantz) clone M. Col 22 was maintained over 10 years in the greenhouse and brought in vitro two years ago. Plants were maintained on solidified (7 g/l Daichin agar) Basal Medium (BM). BM consisted of Murashige and Skoog (1962) salts and vitamins and 20 g/l saccharose. The temperature in the growth chamber was 30 °C and the light intensity 40 $uEm^{-2}s^{-1}$. All steps were performed in the light (12 hours a day). Explants were placed in 300 ml flasks (liquid culture) or Petri dishes (solid culture). The flasks were put on an orbital shaker (LAB-line instruments inc., model 3519) at 120 rpm.

PRIMARY EMBRYOGENESIS

Forty-eight leaf lobes of 1-6 mm, isolated of in vitro grown plants, were cultured for induction of embryos either in liquid (8 leaf lobes per 50 ml) or on solid step 1 medium (BM with 4 mg/l 2,4-D). The liquid medium was refreshed after 10 days. After 20 days the explants were transferred to step 2 medium (BM with 0.1 mg/l BAP) to allow germination of embryos. Since not enough developed primary embryos could be isolated from leaf lobes for starting the different comparisons, the primary embryos were multiplied by cyclic embryogenesis on solid medium (Raemakers et al. 1992b).

CYCLIC EMBRYOGENESIS

The experiment was set up as a complete randomized design. Every treatment consisted of 4 replications

Correspondence to: R. G. F. Visser

(per replication 10 embryos). The factor, degree of fragmentation, had two levels. Fragmentation was applied by cutting the embryos with a scalpel into small pieces (4-25 mm²) versus intact embryos. The density-factor had three levels; 1, 2.5 and 5 ml of medium volume per initial embryo (MIE). A solid control was added which was exposed to fragmentation. The effect of the different treatments on the production of embryos was studied for three successive cycles.
The liquid step 1 medium (BM with 4 mg/l 2,4-D) was refreshed after 10 days step 1. After 20 days step 1 the content of each flask was divided in two equal parts. One part, cultured in liquid step 2 medium (BM with 0.1 mg/l BAP), was used to start a new cycle after 7 days and the other part, cultured on solid step 2 medium, was used to determine the production of embryos after 21 days. A schematic presentation of the flow of material is given in Figure 1.

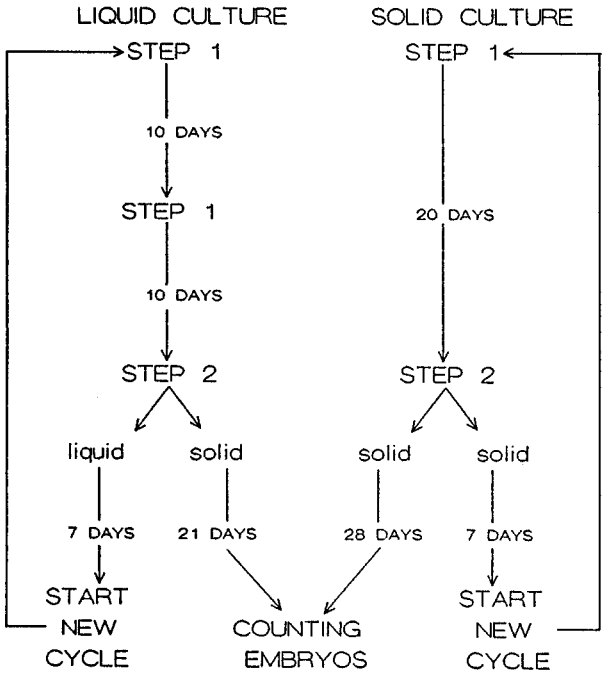

Fig. 1 Schematic representation of the culture flow during the experiments on cyclic embryogenesis.

After 20 days step 1, without refreshing the medium, the content of each Petri-dish was divided in two equal parts and cultured on solid step 2 medium. One part was used to start a new cycle after 7 days and the other part to determine the production of embryos after 28 days. The production was measured as number of newly formed embryos per initial embryo (E/IE). Only stage I and II embryos were counted (Raemakers et al. 1992b). Stage I embryos are torpedo shaped, translucent and possess a distinct hypocotyl. Torpedo shaped embryos (stage I) develop into germinated embryos (stage II) which are characterized by the appearance of green cotyledon tissue.
In the second and third cycle the experiment was extended with a third fragmentation treatment. Embryos were pressed through a fine meshed sieve (0.5 or 0.1 mm²). A statistical analysis was performed using the Manova option of Statistical Package for the Social Sciences (SPSS). Statistically significant mean differences were determined with the LSD test.

SHOOT DEVELOPMENT OF EMBRYOS

Stage II embryos of all treatments of the second and third cycle were cultured on solid BM with 1 mg/l BAP (step 3) for shoot development of embryos. In step 4 shoots were rooted on BM without growth regulators.

RESULTS

INFLUENCE OF MEDIUM TYPE ON PRIMARY EMBRYOGENESIS

Leaf lobes of 1-6 mm, cultured in liquid step 1 medium, formed no embryos or embryo-like structures, only callus proliferation occurred. On solid medium almost all the leaves formed globular shaped embryos but only 30 % of the lobes developed torpedo shaped or germinated embryos. The production was 0.78 embryos per initial leaf lobe.

INFLUENCE OF CYCLE ON CYCLIC EMBRYOGENESIS

The production of somatic embryos was significantly different (p=1%) between the three cycles tested (Table 1). Averaged over all treatments, the production in the first cycle was 7.5 embryos per initial embryo (E/IE). In the second and third cycle it was more than doubled to respectively 18.6 and 20.8 E/IE.
The production of embryos in all liquid treatments of the first cycle (Fig. 2A), except fragmented embryos in 2.5 MIE, was significantly lower (p=10%) than the corresponding treatments of the second (Fig. 2B) and third cycle (Fig. 2C). The production of fragmented embryos in 2.5 MIE differed significantly (p=10%) from only the corresponding treatment of the third and not the second cycle. The two solid treatments of the first cycle had no significantly different production compared to the second cycle, and compared to the third cycle only the production of intact embryos was significantly (p=10%) lower. This differential response of liquid and solid cultured embryos between the cycles was responsible for the significant interaction (Table 1; p=10%) between cycle and medium type.
The production of all second cycle treatments, except intact embryos on solid medium, was not significantly different from the corresponding third cycle treatments.

INFLUENCE OF MEDIUM TYPE ON CYCLIC EMBRYOGENESIS

After 3-5 days step 1 culture of explants turned from green to creamish-white and started to form callus. After 10 days of culture on solid medium most of the explants were covered with callus, whereas in liquid medium only minor amounts of callus were present on the explants, but instead the flasks were filled with a cell suspension. The first torpedo shaped embryos were visible after 10 to 15 days. Almost all torpedo shaped embryos developed cotyledon primordia. The stage I embryos on solid medium had a distinct hypocotyl whereas those in liquid medium had not. The first stage II embryos (germinated embryos) appeared at the end of step 1 especially in the liquid medium, but the majority of the embryos germinated after transfer to step 2. Most of the torpedo shaped embryos developed into germinated embryos. The development of embryos in liquid medium was faster and more synchronized than on solid medium. Another advantage was the ease in which embryos could be isolated of liquid cultured explants compared to solid cultured explants. Averaged over all cycles the production in liquid medium was significantly higher (Table 1; p=10%) than on solid medium, respectively 18.1 versus 10.7 E/IE.

INFLUENCE OF MEDIUM VOLUME ON CYCLIC EMBRYOGENESIS

In the first and second cycle all flasks with 1.0 ml medium per initial embryo (1 MIE) did not produce embryos. In the third cycle the 1 MIE treatment was adjusted by using 20 ml, instead of 10 ml, and the double amount of embryos, so the density level remained unaltered. The production in the adjusted 1 MIE of intact embryos and fragmented embryos was respectively 15.7 and 12.5 E/IE which was significantly lower than the production in the corresponding 2.5 and 5 MIE treatments (Fig. 2C).
Averaged over all three cycles 5 MIE produced significantly more embryos (Table 1; p=1%) than 2.5 MIE. respectively 23.7 and 17.2 E/IE. But analysis of variance of cycles separately showed that only in the second

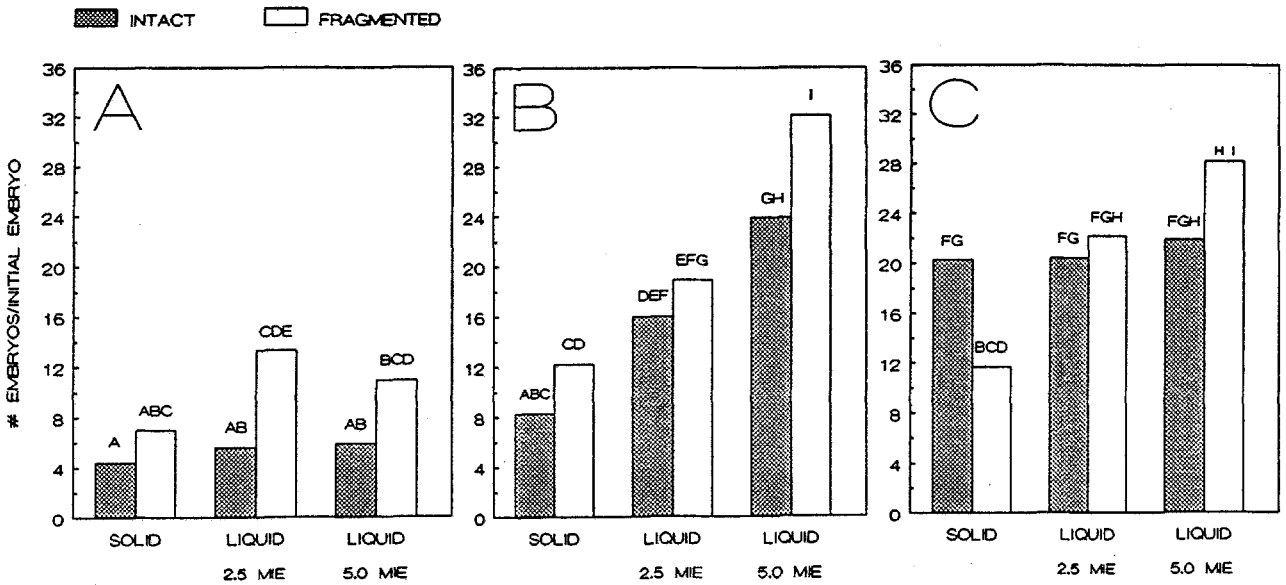

Fig. 2. Influence of fragmentation of initial embryos, medium type and medium volume per initial embryo on the production of embryos in the first (A), second (B) and third (C) cycle (means with the same letter are not significantly different by LSD at 10% level).

Table 1. Multivariate analysis of variance of fragmentation, medium type and medium volume per initial embryo on the production of embryos in the 1st, 2nd and 3rd cycle.

Cycle	1	2	3	1-3
Fragmentation (F)	*	*	ns	*
Medium type (M)	ns	**	*	*
medium volume (MIE)	ns	**	ns	**
Cycle (C)	-	-	-	**
F * M	ns	ns	*	*
F * MIE	ns	ns	ns	ns
F * C	-	-	-	ns
C * MIE	-	-	-	*
M * C	-	-	-	*

*: significant at 10%, **: significant at 1%
ns: not significant

cycle 5 MIE produced significantly more embryos. This explained the significance (Table 1; p=10%) of the interaction between cycle and medium per initial embryo.

INFLUENCE OF FRAGMENTATION ON CYCLIC EMBRYOGENESIS

Averaged over all cycles fragmentation enhanced the production significantly (Table 1; p=10%) from 14.1 to 17.5 E/IE. In only one of the nine combinations presented in Fig. 2A, B and C intact embryos produced more than fragmented embryos (third cycle, solid medium, Fig. 2C). In this treatment the production of embryos was inexplicably high whereas that of fragmented ones was comparable to those obtained in the second cycle. The unusually high production of intact embryos was responsible for the significance (Table 1; p=10%) of the interaction between fragmentation and medium type. in the second and third cycle embryos were pressed through a fine meshed sieve (0.5 mm²) and cultured in liquid medium. A piece of tissue consisted of approximately 200 cells. In the second cycle the production of these sieved embryos (14.2 E/IE) was lower than in all liquid, but higher than in all solid treatments (Fig 2B). In the third cycle the production of sieved embryos, 3.4 E/IE, was lower than in all other treatments. Even embryos fragmented through a sieve with a diameter of 0.1 mm² gave new embryos, but at a very low frequency (0.1 E/IE). The morphology of the embryos produced by the sieved material was different from that of the embryos produced by fragmented and intact embryos. The hypocotyl was very tall and larger than normal, the cotyledons were smaller and the embryo was of a stronger consistency. In Figure 3 the distribution of clustered embryos among the different damaging treatments is given.

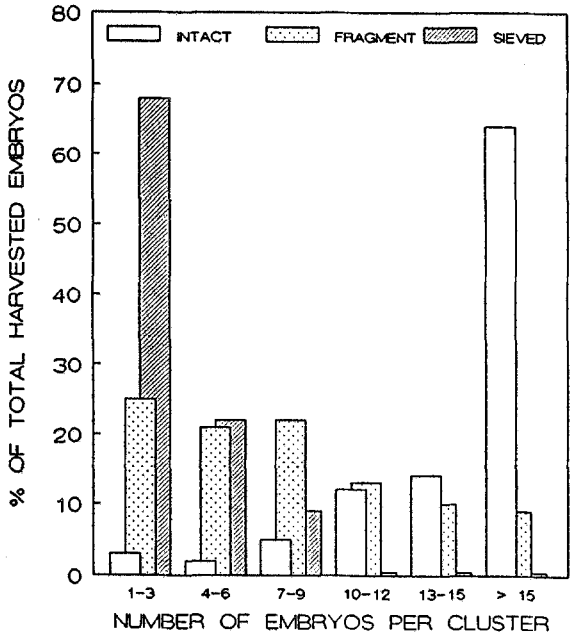

Fig. 3. Influence of different degrees of damaging on the formation of clustered embryos.

Almost 70 % of the total amount of embryos out of the sieved (0.5 mm²) material were produced as single, paired or triple embryos. The clusters of embryos on intact embryos had the widest distribution. It varied from 1 to 44 embryos per cluster. The fragmented embryos had the second widest distribution which varied from 1 to 18.

SHOOT DEVELOPMENT OF EMBRYOS

Embryos of the developmental stage II, from second and third cycle, were cultured for shoot development in step 3 medium. Shoots were characterized by the presence of a distinct stem with, besides the cotyledons,

at least one leaf. Shoots were classified as normal if the leaves had the normal cassava phenotype (a leaf with lanceolated shaped lobes) and as malformed if otherwise. With prolonged culture most of the deformed shoots reverted to normal. In Table 2 the results are summarized according to the type of culture medium used for the production of embryos.

Table 2. Influence of medium type on the development of shoots of second and third cycle embryos.

Step 1	liquid	liquid	solid
Step 2	solid	liquid	solid
number of embryos	47	60	48
percentage shoots	57	60	50
├──>normal	85	80	83
└──>malformed	15	20	17

There seemed to be a positive effect of using liquid medium during step 1 and 2 on the shoot conversion rate (Table 2), however not statistically significant. In all combinations more than 80 % of the obtained shoots had the normal cassava in vitro phenotype. Not only in all medium types, but also with all damaging treatments, including sieving, shoots were obtained.
About 100 normal regenerants, originating from liquid cycles were examined for their growth behaviour in vitro. They showed no phenotypical differences to in vitro multiplied plants. Fifty of the 100 regenerants were multiplied in vitro. During multiplication one regenerant, appeared to have a chimeric nature. Two of the four cuttings of the embryo-derived shoot produced normal shoots and the two other cuttings shoots with variegated leaves. During prolonged multiplications most of these cuttings expressed the variegated phenotype. Some reverted to normal green and others to albino shoots. All shoots even the albino ones formed roots on a medium without hormones.

DISCUSSION

Szabados et al. (1987) and Stamp and Henshaw (1987b) mentioned the possibility of somatic embryogenesis of cassava in liquid medium without showing data. Here we present results showing that in liquid medium, even after three successive cycles, more embryos were produced than on solid medium. Solid medium has some disadvantages compared to liquid medium. On solid medium the explants only make basal contact with the medium, the nutrient and hormone uptake is limited and gradients are formed. This promotes variation and reduced growth which is less obvious in liquid medium. The more homogeneous conditions of liquid medium might also explain the better synchronization of development of embryos in liquid medium. An important advantage of liquid medium, specific for the embryogenic culture of cassava, where the formation of callus suppresses the development of embryos (Raemakers et al 1992a+b), is the mechanical removal of callus developed on the explant due to shaking.
Although not significantly better in all cycles, a density of 1 embryo per 5 ml of medium (5 MIE) gave a higher production than a density of 1 embryo per 2.5 ml medium (2.5 MIE). But irrespective of the density, also the amount of medium used influenced the production. In the 1 MIE treatment with 10 ml medium no embryos were formed whereas in the adjusted 1 MIE treatment (20 embryos in 20 ml) embryo production was established, but lower than at 2.5 or 5.0 MIE. In 10 ml medium the explants were not completely covered with medium during shaking which might be negative for development.
In eight of the ten combinations tested fragmentation of starting embryos enhanced the production compared to intact embryos. Also in soybean fragmentation of starting explants enhanced the production of embryos (Lazzeri et al. 1987). It is likely that on intact embryos, newly induced embryos have to compete for space. By fragmentation of embryos more surface area is created so less newly induced embryos will be suppressed. More surface area also enhances the contact between explants and medium. The embryo production from sieved material was not as abundant compared to fragmented or intact embryos, probably because of excessive damaging of cells. However, sieving provides a relatively large amount of single embryos which might be of importance in specific areas such as cryopreservation, synthetic seed development or automated plant tissue culture.
It was previously shown (Raemakers et al. 1992b) that the production of new embryos depended on the developmental stage of the initial embryos. The production was also influenced by the time period between the transfer of explants to step 2 medium and the start of a new cycle. This is the reason for the lower production of embryos in the first cycle (Fig. 2A), because this cycle was started after 21 days of step 2 with a production of 8.0 E/IE. In the second and third cycle, started after 7 days of step 2, the production was more than doubled.
The shoot conversion rate of embryos originating from liquid medium was comparable with those from solid medium. They both were higherthan those described previously for the first to fifth cycle on solid medium (Raemakers et al. 1992b).
In this report a method is described to obtain routinely embryos of liquid cultures. This type of culture has many advantages compared to a solid culture. The production is higher, the procedure is less time consuming and the development is faster and more synchronized thus providing ideal starting material for transformation experiments.

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