Plant regeneration of cassava (*Manihot esculenta* CRANTZ) plants

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A procedure for plant regeneration of cassava (*Manihot esculenta*) via somatic embryogenesis from immature leaf explants cultured *in vitro* grown plants were developed. The explants showed a differentiated the somatic embryos when cultured on Murashige and skoog medium (MS) with 2% sucrose and the concentration of 2,4-D. The best media for induction of somatic embryogenesis consisted of MS and 2% sucrose supplemented with 7 mg/l 2,4-D. After 10-15 days of culture, the somatic embryo germination varied between 13.6-92.6% and plant conversion percentage between 17.9-42.6%. Whole plants were successfully transferred to soil.

Key words: *Manihot esculenta*, Somatic embryogenesis, plant regeneration, *in vitro*

Abbreviations: MS-Murashige & Skoog (1962), 2,4-D – 2-4-dichlorophenoxyacetic acid, BA– 6-benzyladenine, NAA– napthaleneacetic acid

Introduction

Cassava [*Manihot esculenta* (Crantz)] is grown for its starchy tuberous roots which provide food for over 500 million people, mostly in a small-scale plantation by the farmers in the developing countries (Roca *et al*., 1992). Because of its hardiness and tolerance to the adverse environmental conditions it is a reliable crop, giving adequate yields even it is grown on marginal soils that unable to support other crop plants. Despite its high importance to food security in third world countries, cassava has long been neglected in plant breeding programmes; in addition, the potential for crop improvement by traditional breeding of cassava is constrained by the high heterozygosity, highly outcrossing nature and low natural fertility of the plants. Biotechnology

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could provide an efficient tool to complement the traditional breeding, e.g. by engineering disease and pest resistance, improving the protein content of cassava roots and reducing the cyanogenic glucoside content of the plants. The development of efficient regeneration systems compatible with transformation methods, however, is the first pre-requisite for successful application of genetic engineering techniques, and so far the availability of regeneration systems has been a limiting factor in cassava biotechnology. Regeneration of plants from germinating somatic embryos induced on cotyledons of zygotic embryos, immature leaves or primary somatic embryos has been reported the only reproducible method of de novo regeneration of cassava in vitro (Stamp and Henshaw, 1987; Szabados *et al*., 1987). Recently, regeneration from embryogenic suspensions has also been described (Taylor *et al*., 1996). These methods, however, are both based on germination of somatic embryos to the whole plants, and either require extended tissue culture periods or is poorly compatible with transformation protocols. Axillaries buds have been used to regenerate multiple shoots in cassava (Konan *et al*., 1994), but this system has limited compatibility with the transformation methods. New regeneration modes, suited for both the micro- propagation and transformation would increase the possibility of developing largely genotype independent systems for cassava genetic engineering. Here we report a novel regeneration method for cassava based on direct somatic embryos and shoot organogenesis from immature leaf explants. Plant regeneration of cassava plants was used in the current study because it is suitable for the regeneration of different cassava cultivars and also for their transformation.

Materials and methods

Cassava cultivars K140 were maintained as shoot cultures on basic culture medium (Murashige and Skoog, 1962) with 2% sucrose and then were solidified with 0.6% agarose at pH 5.8 before autoclaving for 20 min at 120°C. The cultures were kept at 25°C at 18/6 h rhythm unless stated otherwise stated stated. Immature leaf lobes, The explants were used for induction of organogenesis and somatic embryos on basic medium supplemented with 1-10mg/l BA– 6-benzyladenine and 1-10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark. Different hormone combinations BA and NAA were tested for their regeneration of plants via germination of somatic embryos. After 15-20 days on embryo induction medium in the dark, the emerging somatic embryos were harvested and transferred to maturation medium [basic medium supplemented with 5 mg/l 6-benzylaminopurine (BA) and 0.1 mg/l NAA] .After 10-15 days, the developing young green cotyledons from the

germinating embryos were harvested. After 20 days of incubation in the darkness, the shoots emerging from the explants were detached and transferred to hormone-free medium for rooting. The rooted shoots were transplanted into soil in the greenhouse.

Results

Effect of cytokinin on organogenesis response

The cytokinin such as BA at different concentrations was tested to compare their effectiveness on organogenesis. After 30 days of culture on the obove induction media, the organogenic callus clumps were transferred to the regeneration medium for shoot development. Within 3–5 days of culture on the induction media containing cytokinin alone, the explants became swollen, and generally formed some callus and small lumps. Within 10–12 days of culture, some buds, shoots and leaf-like structures were directly formed on the surface of the explants or on the callus clumps. In most cases, shoot or multiple-shoots could be formed within 20 days. Different concentrations of BA in the induction media resulted in different induction frequency of shoots and buds. There was a generally increased tendency of organogenesis with the increasing concentration of BA (3-5 mg/l). However, when the concentration of BA increased to 5–10 mg/l, more callus was induced from the explants, and the frequency of organogenesis was not significantly increased (Table 1).

Table 1. Effects of different concentrations of BA on bud organogenesis from the explants.

BA concentrations (mg/l)	Numbers of bud primordial per explants	Frequency of organogenesis (%)
1	3.2	7.4
2	4.8	8.2
3	5.7	21.4
4	6.1	35.5
5	12.4	47.7
6	11.2	46.6
7	10.8	46.5
8	10.4	45.1
9	8.9	45.2
10	8.2	43,6

Effects of auxins on somatic embryogenesis.

When the induction media were supplemented with 2,4-D alone in the dark, callus was formed within one week and somatic embryos were directly induced from the explants within 10–15 days. However, shoot organogenesis was never observed. There was an increased tendency of somatic embryogenesis with the increasing concentrations of 2,4-D in the induction media (Table 2). Different concentration of 2,4-D in the induction media resulted in different induction frequency of embryos. There was a generally increased tendency of somatic embryogenesis with the increasing the concentration of 2,4-D increased to 5-7 mg/l. However, when the concentration of 2,4-D increased to 7-10 mg/l, more callus was induced from TCL explants, and the frequency of somatic embryogenesis was not significantly increased.

Table 2. Effects of different concentrations of 2,4-D on the induction of somatic embryogenesis from the explants.

2,4-D concentrations (mg/l)	Numbers of somatic embryos per explants	Frequency of somatic embryogenesis (%)
1	6.4	13.6
2	7.2	17.3
3	12.8	41.1
4	13.2	43.3
5	15.1	45.5
6	23.6	73.3
7	24.5	92.6
8	22.1	91.1
9	15.9	90.0
10	12.7	86.6

Induction of shoot development from somatic embryos

For improvement of cassava regeneration via germination of somatic embryos, the cytokinin and auxin were combined in the media to induce somatic embryogenesis. The cytokinins were included with 5 mg/l BA and the auxin was included with low concentration of NAA (0.1-0.5 mg/l) (Table 3). When cytokinin and auxin were combined in the induction media, the frequency of shoot development from somatic embryo was significantly improved. Different combinations of BA and NAA resulted in different responses. Among them, 5 mg/l BA in combination with 0.1 mg/l NAA or 0.2 mg/l NAA in the media could induce as high as 33–42% of organogenesis.

However, 5 mg/l BA alone or in combination with 0.5 mg/l NAA could only induce a low frequency and a few numbers of somatic embryos.

Plant regeneration

The regenerated shoots were transferred to the plant growth regulator-free medium for root formation. After 20 days of culture the plantlets were from bottles and the agar was rinsed off with water. After the plant hardened in the water for one week and grew in the nutrient solution for 20-25 days, they were directly transplanted into the fields. It was found out that 93% plantlets had survived and no obvious variation in appearance was observed.

Table 3. Effects of BA in combination with NAA on shoot formation from the explants.

Combination of plant growth regulators (mg/l)	Frequency of shoot development from somatic embryo (%)	Average shoot numbers per explants
5BA, 0.0 NAA	12.3	6.8
5BA, 0.1NAA	42.6	26.7
5BA, 0.2 NAA	33.5	18.1
5BA, 0.5NAA	17.9	11.2

Discussion

The differential response of cassava cultivars to different auxin types and concentrations has been reported previously (Roca and Thro, 1993; Taylor *et al*., 1993). The frequencies of somatic embryo induction can be further increased and the method can be extended to many different cassava cultivars. The efficiency of somatic embryo induction is almost up to two-folder higher than the primary embryo induction in K 140, which confirms the observations that were reported earlier (Raemakers, 1993). The high efficiency of somatic embryo production ensures the production of large numbers of explants for shoot regeneration. This increasing evidence is also seen in the other tested cassava cultivars. The final partially optimised protocol for direct shoot regeneration thus, includes the induction of somatic embryos on 2,4-D depending on the cultivar used, the maturation of somatic embryos and the induction of shoot organogenesis with 0.5 mg/l BA and 0.1 mg/l NAA, followed by transferring of the developing shoots to the elongation and hormone-free medium. One of the reasons in looking for the additional regeneration modes in cassava was due to the observation that the production of transgenic plants was possible by using somatic embryos.

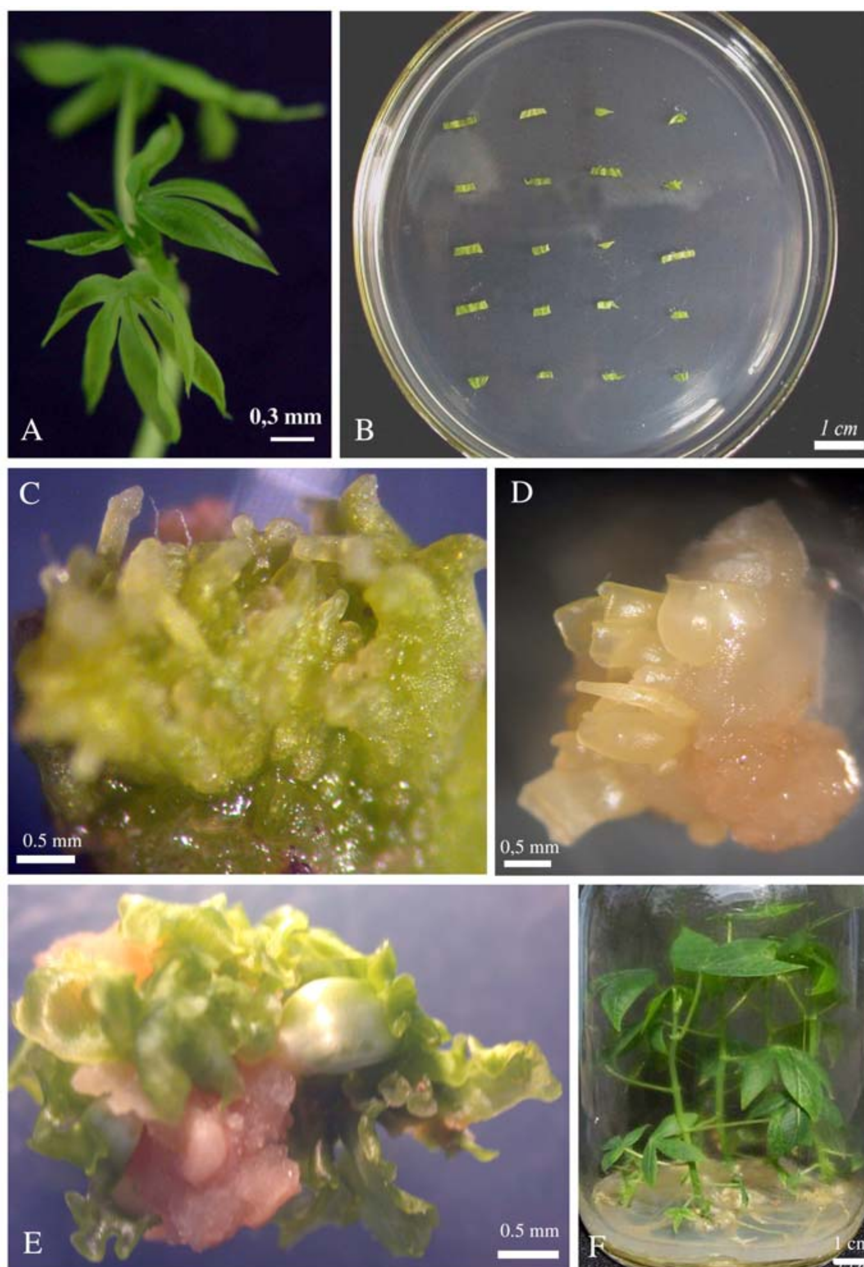

Fig 1. *In vitro* regeneration of cassava. Immature leaf (A) and the explants (B). Shoot organogenesis (C). Cluster of organized embryogenic structure (D) and plantlet regeneration from somatic embryos (E-F).

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