J. Plant Biotechnology(2004) Vol. 6(2). pp. 119~124

Journal of Plant Biotechnology

Somatic Embryogenesis in a Range of Genotypes and Genetic Stability of the Plants Derived from Somatic Embryos Using Morphological and RAPD Markers in Sweet Potato

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Abstract

For long-term conservation of germplasm, somatic embryos of sweet potato are important because shoot tips are not amenable to liquid nitrogen storage. Somatic embryos from different genotypes were used for induction of somatic embryogenesis in a large number of genotypes. Somatic embryogenesis was induced on 2,4-D medium in all the 11 genotypes, collected from geographically distinct locations. Genetic fidelity of the regenerated plants was confirmed by morphological and RAPD markers.

Key words: RAPD markers, somatic embryogenesis, sweet potato, genetic stability

Introduction

Sweet potato is one of the most important tuber crop and seventh most important crop in the world with an annual production of 124 million tones (FAO, 1999). Its rich nutritional content provides enormous potential for preventing malnutrition and enhancing food security in the developing world. Interest in the maintenance, utilization and long-term conservation of its germplasm is therefore receiving increased attention. Somatic embryogenesis can offer a significant approach for germplasm conservation and crop improvement as liquid nitrogen storage of this plant has been extremely difficult. For long-term conservation, induction of somatic embryogenesis in a large number of genotypes and their genetic stability is essential. In earlier studies somatic embryos of different cultivars of sweet potato exhibited a high degree of variation (Chee and Cantlifee 1988; Chee et al. 1990; Mazorrei et al. 1997). The present study was conducted to see the applicability of somatic embryogenesis in a large number of genotypes in sweet potato and to assess the genetic fidelity of the plants generated from somatic embryos. This report deals with somatic embryogensis in 11 genotypes of sweet potato, collected from geographically different locations (Fiji, CTCRI-Trivendrum) and assessment of genetic stability of 50 plants generated from somatic embryos of genotypes (Acc 66) using morphological and molecular (RAPD) markers.

Molecular markers have been effective in evaluating genetic variation within species, because they are not affected by environmental conditions. Of the available molecular markers currently used, RAPD (Random Amplified Polymorphic DNA) is considered to be efficient, simple and cost effective because hybridization steps are not required and only a few nanograms of DNA is needed to detect polymorphism in a short time (Williams et al. 1991). RAPD markers have been routinely used for evaluating genetic fidelity of clonal populations (Tang 2001; Kiss et al. 2001). Morphological characters recorded as germplasm descriptors given by IPGRI were also used to assess morphological stability of the regenerated plants.

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Received Feb. 5, 2003; Accepted Mar. 10, 2004

Materials and Methods

Plant material

Tubers of eleven genotypes of sweet potato, from geographically distinct locations, were obtained form collections in Fiji and CTCRI (Central Tuber Crop Research Institute) Trivendrum and grown in the green house. The genotypes were distinguished on the basis of morphological characteristics as described in the morphological descriptors published from IPGRI. After 10 weeks the nodal segments (1-1.5 cm in size) were excised from the green house grown plants, surface sterilized and cultured onto MS medium supplemented with 0.2 mg L^{-1} Kin and 0.1 mg L^{-1} NAA. Fully-grown *in vitro* plantlets were obtained after 10 weeks of culture and were further used for isolation of axillary meristems. *In vitro* plantlets of these genotypes of sweet potato (Table 1) were multiplied by culturing single node cuttings on MS medium supplemented with 0.2 mg L^{-1} Kin and 0.1 mg L^{-1} NAA.

Somatic embryogenesis

Axillary meristems (0.5-1.00 mm) from shoot cultures of 11 genotypes were isolated under binocular microscope and inoculated onto 20 mL MS medium supplemented with 2,4-D (1.0 mg L^{-1}, 1.5 mg L^{-1}, 2.0 mg L^{-1}). The embryogenic tissues obtained after 4 weeks of culture were selectively sub-cultured every four weeks to fresh MS medium with same concentration of 2,4-D for multiplication. All the cultures were incubated in a culture room (25+2℃) provided with 8/16 h photoperiod with fluorescent lighting of 36 μ moL^{-1} sec^{-2} intensity.

To study the effect of size of the explants on the induction frequency, the axillary meristems from two genotypes, 66 and 67, were grouped into three sizes, > 0.5 mm, 0.5-1.0 mm, 1.1-2.0 mm and cultured onto MS medium supplemented with 1.0 mg L^{-1} 2,4-D. The data were recorded after 4 weeks of culture.

To induce maturation and germination of complete plantlets, embryogenic aggregates of genotype 66 were transferred to MS medium supplemented with 50 mM BAP, 5 mM NAA and 15 mM GA_3 for 10 days prior to transfer to a hormone free MS medium supplemented with 3% sucrose. Fully-grown plantlets obtained on hormone free MS medium after 5 weeks of culture were further multiplied by nodal segment culture on MS medium supplemented with 0.2 mg L^{-1} Kin and 0.1 mg L^{-1} NAA. The identity of individual clones was maintained throughout. The plants were randomly sampled to assess the genetic uniformity.

Table 1. Effect of different concentrations of 2,4-D on induction of somatic embryogenesis in different genotypes of sweet potato.

Genotype No.	Percentage embryogenesis (%)			Time taken for induction (weeks)
	1.0 mg/L	1.5 mg/L	2.0 mg/L	
66	58.3±2.8	45.5±5.0	33.3±5.8	3
67	51.6±2.9	31.6±2.9	20.0±0.0	3
S 256	31.2±2.5	41.6±7.6	60.0±5.0	4
12	28.3±7.6	0.0	0.0	6
267	0.0	0.0	20.0	4
IC 20	0.0	26.6±2.8	20.0±0.0	4
IC 21	10.0	20.0	0.0	7
S 782	30.0±5.0	0.0	0.0	4
76	0.0	18.3±2.8	41.6±2.8	6
IC 44	0.0	10.0	0.0	6
27	11.6±5.8	0.0	0.0	5

Values are means of three replicates ± Standard Deviation

Morphological stability

Sixty *in vitro* grown somatic embryo-derived plantlets (SEP) from genotype 66 were transferred to plastic pots containing 1:1 FYM and soil mixture and kept in shade in the net house for establishment. Initially, to maintain adequate humidity, the pots were covered with polythene bags and made air tight with rubber bands. To reduce excess condensed water, 2 holes (1-2 cm in diameter) were made in the polythene bags. The polythene bags were removed after 10-15 days of transfer and plants transferred to earthen pots (18 inches) after 4 weeks. Frequency of plantlet establishment was deduced based on the total number of plants established / number of plants transferred. These hardened plants were analyzed after 3 months of growth using morphological markers (sweet potato descriptors published from IPGRI). 30 IVP (*in vitro*- control Plants - genotype 66) were also transferred to pots for comparison and analysis of morphological stability.

Molecular stability

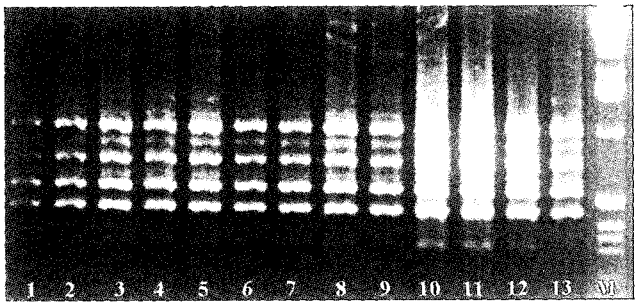
DNA isolation

For assessing the genetic stability, DNA was isolated from leaves of 50 SEP and 20 IVP-control using a Qiagen DNA isolation kit.

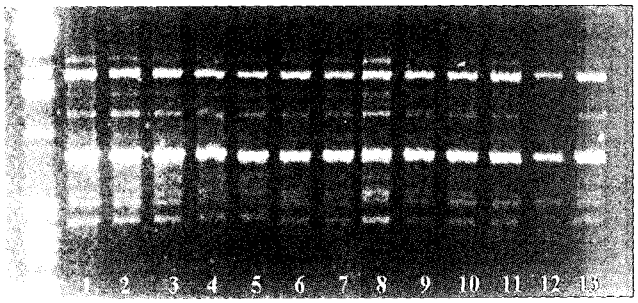
DNA amplification

A set of 20 GC rich ten-mer primers (OPB1-OPB20 Operon tehnologies) were used for PCR amplification. Reaction volume (25 μL) contained 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 2 mM Mg Cl_2, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.8 μM primer (=20 pmol/ reaction), 1.5 U Taq DNA polymerase (Bangalore Genei) and 50 ng template DNA. After initial denaturation step at 94℃, 1min. PCR was run for 40 cycles consisting of a 94℃ denaturing step (30s), a 35℃ annealing step (30s) and a 72℃ elongation step (90s) in a PTC-200 (M J Research) thermocycler. At the end of the run, a final extension period was incubated (72℃, 10 min). Amplification products were separated on 2% agarose gel (Sigma) in TAE buffer (pH 8.0), stained with ethidium bromide and photographed under UV light using red filter.

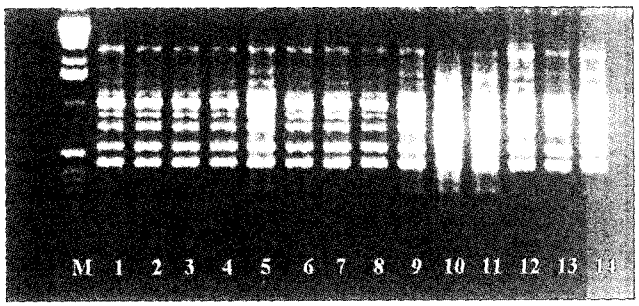
PCR conditions of the RAPD reactions were optimized to yield reproducible results. Only fragments that amplified consistently and were reproducible in a minimum of two replicated reactions were considered. Precautions to avoid the contamination of test specimens with foreign DNA included physical separation of DNA extraction and amplification areas, sterile techniques using laminar flow hood, and the use of sterile tubes and tips.



A. Polymorhic bands generated from primer OPB 12 (5'-3'): Lane M - molecular marker (1Kb), Lane 1-4 IVP control plants, 5-13 SEP.



B. Polymorhic bands as revealed by gel electrophoresis of RAPD fragments generated with primer OPB 15 (5'-3'): Lane M- molecular marker (1 kb), Lane 1-4 IVP control plants, 5-13 SEP.



C. Polymorhic bands as revealed by gel electrophoresis of RAPD fragments generated with primer OPB 7 (5'-3'): Lane M - molecular marker (1kb), Lane 1-3 IVP control plants, 4-14 SEP.

Figure 1. Agarose gel electrophoresis of RAPD fragements of *I. batatas* plants regenerated from somatic embryos/ embryogenic tissues.

Agarose gel electrophoresis

DNA samples were analysed by electrophoresis on 0.7-1.0 % agarose gels. The gel was run in 1X TAE buffer (1 litre 10X buffer contained 48.4 gm Tris-base, 11.4 mL glacial acetic acid and 20 mL 0.5 M EDTA, pH 8.0). Agarose was dissolved by heating in 1X TAE buffer and ethidium bromide was added at a concentration of 0.5 ng/mL after cooling. The gel slabs were prepared by pouring the dissolved agarose into the required casting tray fitted with appropriate comb. The samples were loaded after mixing with 0.1 volume 6X gel loading buffer (30% glycerol, 0.25 % Xylene-cyanol and Bromophenol blue). The gel was run in an horizontal electrophoresis tank in 1X TAE buffer at voltage from 50 to 100 volts. The DNA bands were visualised under UV transilluminator (Photodyne) and photographed. Two sets of PCR were carried out. Only major fragments genetically characterized through segregation analysis were used as markers. Minor fragments, which tend to be unstable in staining intensity and therefore not reliable, were not considered.

Statistical analysis

The values in the tables are the mean values of three replicates along with standard deviation. The experiments were repeated atleast twice.

Results

Somatic embryogenesis

Dicholorophenoxy acetic acid (2,4-D) was effective for inducing embryogenesis in all the 11 genotypes tested, although optimum concentration of the auxin was genotype

Table 2. Effect of size of explant on percentage embryogenesis.

Size of the explant	Percentage embryogenesis (%)	
	66 acc.	67 acc.
> 0.5 mm	10.0±2.9	10.0±5.8
0.5-1 mm	58.3±2.8	51.6±2.9
1.1-2 mm	20.0±3.3	25.0±0.0

Values are mean of three replicates ± Standard Deviation

specific (Table 1). The frequency of embryogenesis also varied depending upon the genotype. Embryogenic tissues were identified by the appearance of organized smooth and compact structures varying from the globular to early torpedo stage of embryos. Almost 70% of axillary bud explants produced embryogenic tissue after 4 weeks. Concentrations above 2.0 mg L^{-1} of 2,4-D produced fast proliferating non-embryogenic light brown callus. High quality embryogenic tissue was induced in genotypes 66, 67 and S 256.

The size of the explant had a critical effect on induction frequencies (Table 2). Axillary buds excised from the genotype 66 were sorted into three sizes, < 0.5, 0.6- 1.0 and 1.1- 2.0 mm and cultured on to MS medium supplemented with 1.0 mg L^{-1} 2,4-D. Only 0.5-1.0 mm explants produced embryogenic tissue at higher frequencies. The smaller and larger explants responded at less than 10% frequency. Buds larger than 1 mm showed greater tendency to form non-embryogenic callus, while those under 0.5 mm failed to grow. The production of mature embryos and their conversion to plantlets were, therefore, investigated in the genotype 66. After 8 weeks, these were scored for formation of green cotyledonary stage. Numerous plantlets (SEP) having well-developed shoot and root system with normal morphology were obtained.

Morphological stability

The regenerated plants were randomly sampled and transferred to pots to assess field performance. Out of 60 SEP and 30 IVP transferred to pots, 50 SEP and 20 IVP established successfully. No variation was observed in all the 50 SEP compared to 20 IVP. They were morphologically similar with respect to all the 17 characters studied (Table 3). The growth pattern and vigor was also similar.

Molecular stability

Total DNA was extracted from leaves of 50 SEP and 20 IVP for RAPD analysis. The comparison showed identical RAPD profiles and band intensity indicating stability of the regenerants.

A total of 20 primers were screened, 10 of which were chosen on the basis of reproducibility of bands. All the 70 plants (50 SEP, 20 IVP) were further analysed using 10

Table 3. Summary of the study of plants for morphological stability.

Gross morphology characteristics	IVP	SEP
Twining	Slightly twining	Slightly twining
Length of the main vine (151-250 cm)	Spreading (178±16.8)	Spreading (200±2.8)
Vine internode length (6-9 cm)	Intermediate (7.2±0.3)	Intermediate (7.5±1.2)
Vine internode diameter (< 4 mm)	Very thin (4±0.8)	Very thin (3.5±0.8)
Vine pigmentation	Green	Green
Secondary vine	Absent	Absent
Vine tip pubescence	Lobed	Lobed
Mature leaf shape	Slight	Slight
Leaf lobe type	3	3
Leaf lobe number	Semi circular	Semi circular
Shape of central leaf lobe	small	small
Mature leaf size (< 8 cm)	Green (7.5±0.08)	Green (8±1.2)
Abaxial leaf vein pigmentation	Green	Green
Foliage colour	Green	Green
Immature leaf colour	Green	Green
Petiole length (< 10 cm)	Very small 9.8±2.8	Very small 10±1.6
Petiole pigmentation	Absent	Absent

primers (OPB1, OPB3, OPB5, OPB7, OPB9, OPB10, OPB11, OPB12, OPB15, OPB17). Each primer generated amplification ranging, 0.28 Kbp in OPB12 to 3.0 Kbp in OPB15. The number of bands for each primer varied from 5 in OPB1, OPB3, OPB5, OPB10 to 9 in OPB17 (Table 4). The 10 primers yielded 62 scorable bands with an average of 6.2 bands per primer. The total number of bands was 4340. All the 10 primers yielded monomorphic bands in amplification. The SEP and IVP appeared homozygous at all the fragments. Out of a total of 4340 bands obtained, no polymorphic band was observed.

Discussion

In earlier studies, attempts to induce embryogenesis in a wide range of genotypes revealed that the majority were either recalcitrant or the response was very low, generally less than 20% (Cavlcante Alves et al. 1994; Zheng et al. 1996; Mazrooei et al. 1997). In the present study, all the 11 genotypes tested responded to embryogenesis with frequencies varying from 12-60%, although the optimum concentration of 2,4-D was genotype specific.

The frequency of embryogenic callus has been reported to decline from 90 to 30% as the number of leaf primordia attached to the apical dome explant increases from 1 to 3 (Cantliffe et al. 1987), which supports the results in the present study also. The 2,4-D concentration was important as explants on media with 0 or 4.0 mg L^{-1} 2,4-D produced either non-embryogenic friable callus or no response at all (Liu and Cantliffe 1984).

The morphological and RAPD analysis revealed no somaclonal variation among the regenerants (SEP, IVP). Genetic markers are considered to be reliable in monitoring stability. Several investigators have applied RAPD to establish genetic stability and found it efficient and reliable (Tang 2001; Kiss et al. 2001; Chowdhury et al. 2001). Using RAPD, other investigators have also reported the absence of genetic variation in regenerants from somatic embryos / cell cultures / anther cultures (Isabel et al. 1993; Valles et al. 1993; Fourre et al. 1997; Tang 2001; Kiss et al. 2001).

The total number of bands scored was fairly high in the present study (4340) as compared to the number of bands (900) obtained by Isabel et al. (1993), who studied genetic stability of SEP *of Picea mariana*. Munthali et al. (1996), Hashmi et al. (1997), Rani et al. (1995) detected 3, 59, and 78 polymorphic bands after scoring a total of 5607, 4180 and 1702 bands, respectively. The total number of bands scored and the number of primers used in our study, thus, seems to be reasonable to detect polymorphism and confirm the stability of plants regenerated from embryogenic tissues.

This report provides an evidence that the plants derived from somatic embryos are genetically uniform and further supports the use of somatic embryos for long-term conservation of germplasm of sweet potato.

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