

MINIMISING SOMACLONAL VARIATION IN TISSUE CULTURES OF SUGARCANE

DL SWEBY¹, BI HUCKETT¹ AND FC BOTHA^{1,2}

¹South African Sugar Association Experiment Station, Mount Edgecombe

²University of Natal

Abstract

A routine *in vitro* culture procedure has been established for sugarcane which uses leaf roll tissue as the source of explant. After four weeks in culture the protocol gives rise to large amounts of contaminant-free, embryogenic callus capable of efficient plantlet regeneration. With variety N18, up to 60 plantlets were obtained from 1 g (fresh mass) of callus. When callus was transferred after four weeks from an initiation medium containing a high level of the hormone 2,4-D (3 mg/l) to a maintenance medium containing a low level of 2,4-D (1 mg/l), callus growth remained just as active. Preliminary analysis of callus DNA using PCR-RAPD technology indicated that, over a three month period in culture, no genetic variation was detectable in calli grown on 2,4-D regimes ranging from 1 mg/l to an extreme of 5 mg/l.

Introduction

Somaclonal variation is a phenomenon of all plant regeneration systems that involve a callus phase, whether regeneration occurs through somatic embryogenesis or by adventitious shoot formation (Larkin and Scowcroft, 1981). It is widespread among plant species, including many important crops. One of the earliest reports of somaclonal variation was in *in vitro*-derived plantlets of sugarcane. Among the regenerated plants there were changes in morphology, such as presence or absence of hairs, differences in isozyme profiles, as well as variation in crop parameters such as cane diameter, stalk length and weight, and cane and sugar yield (Bailey and Bechet, 1989; Lyndsey and Jones, 1989). Somaclonal variation is often heritable (Larkin *et al.*, 1984; Brieman *et al.*, 1987) indicating that it results from genetic change (Karp, 1991). Several types of genetic changes associated with somaclonal variation have been reported, notably variation in chromosome number (Karp *et al.*, 1989; Karp, 1991), gene copy number (Landsmann and Uhrig, 1985; Zheng *et al.*, 1987), DNA mutations (Brown, 1989; Muller *et al.*, 1990) and transpositional changes (Peschke and Phillips, 1991).

Several factors are reported to affect the nature and frequency of somaclonal variation in regenerated plantlets. These include the tissue culture procedure employed (callus, cell suspension or protoplast culture), the time spent in culture, the source of the explant cells, and the composition of the culture medium used, in particular the presence of growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Karp, 1989). Although much effort has been channelled towards understanding how the above factors interact and whether the influence of some are stronger than others, somaclonal variation remains unpredictable because it originates from chance events.

Although much research has focused on the potential of somaclonal variation for the production of agronomically useful mutants (Masirevec *et al.*, 1988), variation can pose a major problem in genetic manipulation systems such as transformation, where specific genetic changes are desired

in otherwise unaltered genomes. The purpose of this study was thus two-fold: first, to establish a rapid and efficient *in vitro* culture procedure for sugarcane and, secondly, to assess the extent of variation produced in the system so that its suitability for use in conjunction with a transformation strategy could be evaluated.

Materials and Methods

In vitro culture

Leaf roll tissue from mature, field-grown sugarcane plants of variety N18 was used as the source of explant. The leaf roll consists of five individual furled leaves and is a young, actively growing part of the sugarcane plant. The leaf roll was cut into sections approximately 1 cm in length and surface sterilised by rinsing in ethanol, followed by a 15 minute incubation in 1,2% (w/v) sodium hypochlorite. The outermost leaf was then removed and the remaining four leaves unravelled and placed on to a solid callus induction medium which consisted of MS nutrients (Murashige and Skoog, 1962) supplemented with 1 g/l casein hydrolysate, 30 g/l sucrose, 3 mg/l 2,4-D and 5 g/l agar, pH 5.8. In this way, many leaf sections suitable for callus proliferation were obtained per individual leaf roll. Cultures were placed in the dark at $\pm 26^\circ\text{C}$. After four weeks, callus was transferred on to a maintenance medium in which the 2,4-D supply was reduced to 1 mg/l. Callus was subcultured at monthly intervals on this medium. For plantlet regeneration, callus was transferred to the same medium lacking 2,4-D and placed in the light at $\pm 26^\circ\text{C}$ under a 14 h light/10 h dark photoperiod regime.

DNA extraction from callus

DNA was extracted from callus according to a modified method of Dellaporta *et al.* (1985). Callus (± 1 g) was harvested, frozen in liquid nitrogen and transferred to a pre-cooled mortar. Tissue was ground to a fine powder, transferred to tubes and mixed with 15 ml DNA extraction buffer (100 mM tris hydroxymethyl-aminomethane (Tris)-HCl pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 500 mM sodium chloride (NaCl), 10 mM 2-mercaptoethanol). After addition of 2 ml 10% (w/v) sodium dodecyl sulphate (SDS) followed by 12 minutes incubation with occasional shaking at 65°C , the solution was thoroughly mixed with 5 ml 5 M potassium acetate (KOAc) and incubated on ice for 30 minutes. After centrifugation at $15\,000 \times g$ for 30 minutes (4°C) to pellet the protein/SDS precipitate, the supernatant was filtered through a glass funnel packed with glass wool. The filtrate was then mixed with 2/3 volume of isopropanol (-20°C) and allowed to stand for 30 minutes at -20°C . Nucleic acids were pelleted by centrifuging at $10\,000 \times g$ for 20 minutes and the pellet redissolved in TE buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). The dissolved nucleic acids were then subjected to extraction of protein with an equal volume of chloroform:isoamylalcohol (24:1) before the addition of sodium acetate to a final concentration of 0.3 M and precipitation

with 2/3 volume of cold isopropanol as previously. Precipitated nucleic acids were pelleted by centrifuging for 10 minutes at 12 500 x g. The pellet was rinsed with 80% EtOH, vacuum-dried and redissolved in 200 μ l sterile water. The DNA yield and purity were analysed both spectrophotometrically and by standard quantitative agarose gel electrophoresis. DNA extracts were stored at -80°C.

PCR-RAPD analysis

The conditions used for PCR amplification of callus DNA for RAPD analysis (Williams *et al.*, 1990; Welsh and McClelland, 1990) were according to the following procedure using arbitrary decamer primers (Operon). The reaction mixture (15 μ l final volume) contained template DNA (3 ng to 30 ng), 10 mM Tris-HCl pH 8,3, 10 mM potassium chloride (KCl), 100 M each of the four common deoxynucleoside 5' triphosphates (dNTPs), 4 mM magnesium chloride (MgCl₂), 0,2 μ M primer, 1U Taq polymerase Stoffel fragment. Thermal cycling conditions were: 1 cycle of 94°C (one minute), 35°C (one minute), 72°C (two minutes); 40 cycles of 94°C (one minute), 35°C (one minute), 72°C (two minutes); 1 cycle of 72°C (seven minutes). PCR products were electrophoresed on 2% (w/v) agarose gels in 0,5 x TBE buffer (45 mM tris-borate, 1 mM EDTA, pH 8,0) at 10 volts/cm, and visualised by UV light at 300 nm following staining with ethidium bromide (1 μ g/ml).

Results

Efficiency of the *in vitro* plantlet regeneration system

The protocol used in this study for the *in vitro* culture of sugarcane was found to yield large amounts of contaminant-free callus with a high regeneration potential. The incidence of microbial contamination was extremely low in the callus cultures initiated from leaf roll tissue, usually about 1%. Callus growth was found to be rapid. Four weeks after culture initiation, leaf sections had given rise to abundant white, compact callus and yellow, fast-growing friable callus. The incidence of grey, non-embryogenic mucilaginous callus was very low. Callus proliferation was not restricted to areas close to the cut surfaces, but was observed anywhere on the leaf section where wounding had occurred. When callus was transferred after four weeks from induction medium on to maintenance medium, callus growth remained as vigorous on the low hormone regime (1 mg/l) as on the high (3 mg/l) regime. Plantlet regeneration potential from the callus was found also to be high. Germination was detected two weeks after callus had been transferred to the regeneration medium in the light, with 50-60 plantlets ultimately being obtained per gram (fresh mass) of callus of variety N18. Preliminary results from studies of long-term regeneration potential have indicated that regeneration from three-month old callus is similar to that of one-month old callus. Likewise, callus grown on 1 mg/l 2,4-D showed the same regeneration potential as callus grown on 3 mg/l 2,4-D.

Optimisation of the experimental conditions for DNA analysis

Some preliminary studies were necessary to optimise the extraction of DNA from callus for subsequent PCR-based experiments. DNA was initially extracted from callus tissue by the method of Dellaporta *et al.* (1985). Although this method resulted in an apparently good DNA yield (200-400 μ g/g fresh mass), it was found that the DNA was heavily contaminated with protein. This was reflected by A_{260/280} ratios of 1,5 to 1,6 and by quantitative agarose electrophoretic gels which indicated an underestimation of DNA concentration. Template concentration is critical for efficient PCR amplification, with 15 ng template DNA being optimal for the chosen reaction conditions. Preliminary PCR experi-

ments, using the DNA extracted according to the protocol, revealed poor amplification in a range calculated to be 3 ng to 21 ng. Thus an additional purification step was incorporated into the DNA extraction method. Treatment of the crude DNA with chloroform:isoamylalcohol to remove protein before final precipitation with isopropanol resulted in A_{260/280} ratios of approximately 1,7 and, consequently, a more accurate assessment of DNA concentration. A comparison between PCR profiles obtained from a DNA extract which had been further protein purified and one which had not is shown in Figure 1.

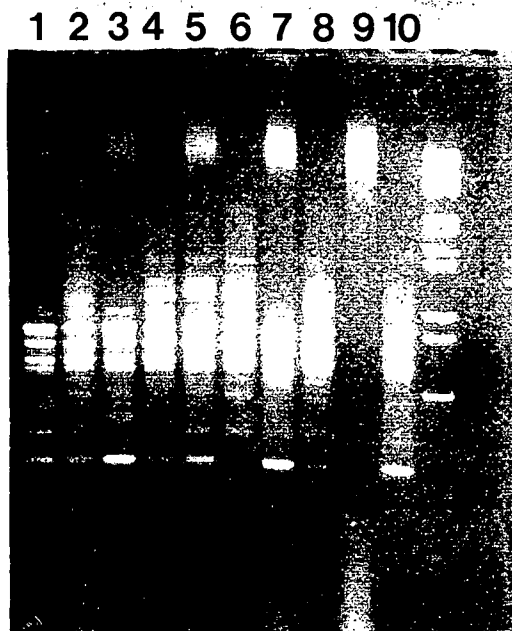


FIGURE 1 Comparative PCR-RAPD profiles of two callus DNA extracts of different purities generated using a single primer. Lanes 1, 3, 5, 7 and 9 represent template DNA, isolated according to Dellaporta *et al.* (1985) at concentrations calculated to be 3, 6, 7,5, 15 and 30 ng respectively. Lanes 2, 4, 6, 8 and 10 represent similar template DNA, further purified by the inclusion of a chloroform step, at concentrations of 3, 6, 7,5, 15 and 30 ng respectively.

The use of template DNA concentrations between 3 ng and 30 ng per 15 μ l reaction was investigated. Figure 1 indicates that for all template DNA concentrations examined, better amplification was achieved for the sample that had been further purified (Lanes 2, 4, 6, 8, 10). The PCR profiles obtained for this sample were cleaner than for the unpurified sample, and more fragments could be detected. Accordingly, all further PCR experiments were performed using 15 ng template DNA which had been obtained using the modified DNA extraction protocol.

Preliminary DNA analyses

So far, data have been obtained from genetic analyses of N18 callus grown on various 2,4-D regimes over a period of three months. Three replicate pieces of callus from each hormone treatment (1 mg/l, 3 mg/l, 5 mg/l 2,4-D) were removed at each monthly subculturing and the DNA extracted from individual calli. Analyses were performed on individual pieces of callus and not pooled samples, because if any genetic variation had occurred it would be random and thus would be reflected as different PCR profiles between individual calli. Figure 2 presents the results of PCR amplification of callus DNA with two random decamer primers.

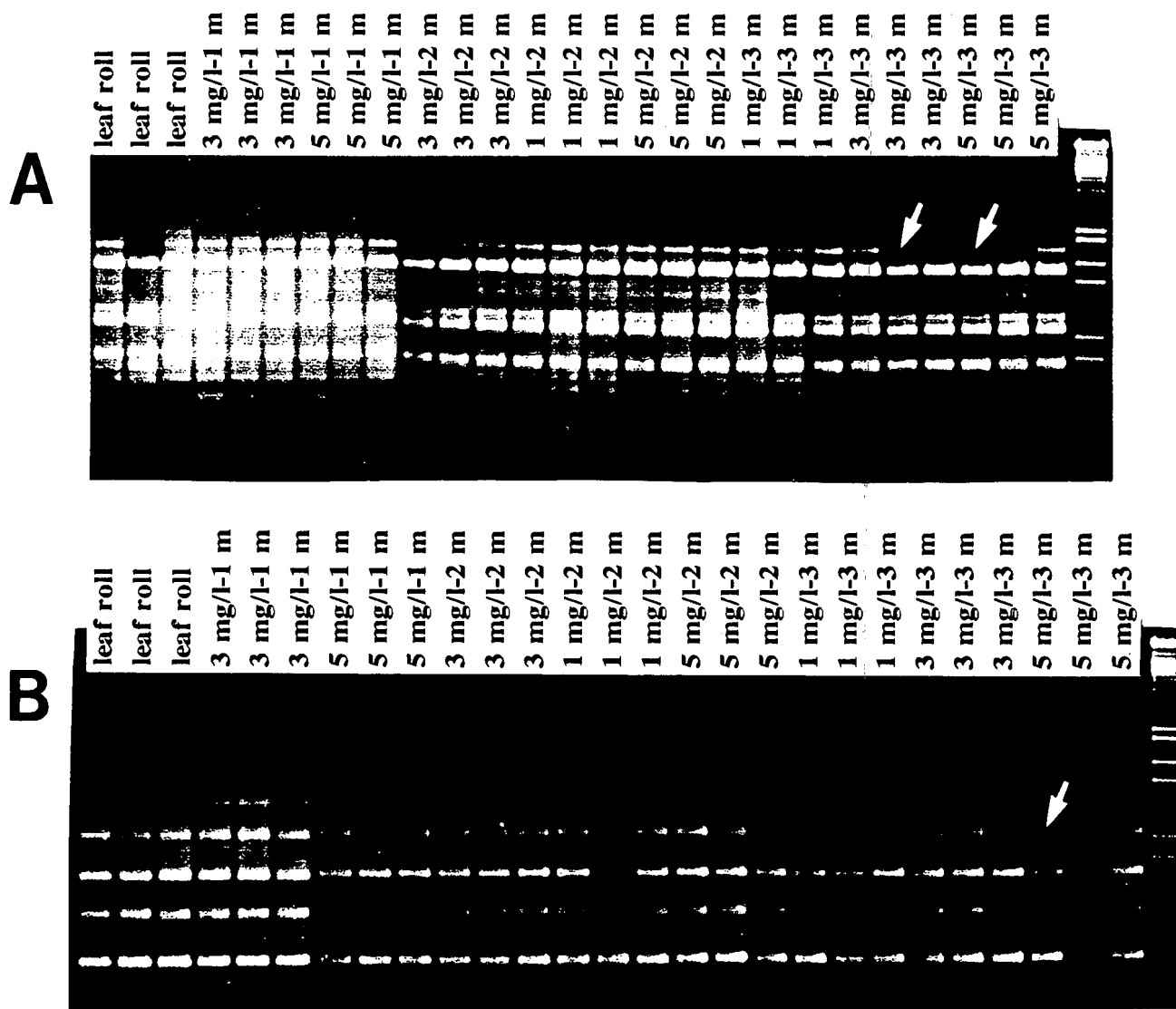


FIGURE 2 Comparative PCR-RAPD profiles of DNA from N18 calli grown on various 2,4-D regimes over a three month period using (a) Operon primer B10 and (b) Operon primer C15. Arrows indicate absent markers which possibly represent induced polymorphisms.

and is representative of the results obtained for all six primers investigated which generated over 40 specific DNA fragments *in toto*.

The most noteworthy feature of these results is the uniformity of the PCR profiles between the various samples. Little genetic variation could be detected between calli grown on the various 2,4-D regimes over a period of three months. Suspected polymorphisms were identified in a sample grown for three months in the presence of 5 mg/l 2,4-D. In this sample, several fragments appear to be missing in PCR profiles generated by primers B10 and C15. If this result represents a true genetic variation, it should become more apparent with further time spent in culture.

Discussion

The rapid production of callus combined with an efficient rate of regeneration is an important feature of an *in vitro* culture system designed to minimise somaclonal variation. In this study, sufficient embryogenic callus for plantlet regeneration was obtained from leaf roll tissue four weeks after culture initiation. This result compares favourably with that of Guiderdoni and Demarly (1988), who reported similar production of friable, embryogenic callus from sugarcane

leaf roll segments after four weeks in culture. Studies have indicated that the choice of explant has a major effect on the nature and rate of callus culture. A comparative study of different explant sources from sugarcane has indicated that the rate of callus development from stem apices is four times slower than from the leaf roll (Chen *et al.*, 1988). A previous *in vitro* culture system for sugarcane, which was established in our laboratories, used two week old shoots germinated from setts as the source of explant (Snyman *et al.*, 1992). That protocol required at least eight weeks before sufficient callus could be obtained for efficient plantlet regeneration as callus was produced from the cut ends only. This was in contrast to the results obtained in this study which have demonstrated callus production not only at the cut edges, but also at any slightly damaged sites on the surface of the leaf roll tissue.

When callus was transferred after four weeks on to medium containing a reduced level of 2,4-D (1 mg/l), callus growth was found to be as vigorous as when grown initially on 3 mg/l 2,4-D. These findings are compatible with the results of Fitch and Moore (1993), who reported that the largest quantities of embryogenic sugarcane callus were obtained when callus was transferred after four weeks from a medium containing 2,76 mg/l 2,4-D to one containing

1 mg/l. In contrast, Chen *et al.* (1988) found that, to obtain sugarcane callus capable of plantlet regeneration, cultures had to be first initiated on a medium containing 1 mg/l 2,4-D and then transferred after four weeks to medium containing 3 mg/l; furthermore, to maintain callus proliferation, callus required alternate subculturing between media containing 3 mg/l and 1 mg/l 2,4-D, respectively. The fact that a completely different range of sugarcane species and varieties was used in each of those studies suggests that conditions for the regulation of callus production and proliferation in sugarcane may be genotype dependent.

The developed *in vitro* culture procedure resulted in the rapid regeneration of green plantlets from embryogenic callus. When four week old callus was transferred to hormone-free medium and placed in the light, plantlets were visible after two weeks. After a further four to six weeks of growth, plantlets had formed roots. These results are comparable to those of Guiderdoni and Demarly (1988), who reported plantlet formation 15 to 20 days after the transfer of calli to regeneration conditions. Such responses are an improvement over those reported by Chen *et al.* (1988) who showed that, for plantlet formation to occur, it was necessary to transfer the callus on to three successive media over a period of six weeks or more. In the present work, as in that of Guiderdoni and Demarly (1988), only one medium formulation was required for plantlet formation.

A number of approaches may be used to assess the genetic integrity of plants that have been generated using *in vitro* cloning techniques. For example, morphology and isozyme analysis were used by Eastman *et al.* (1991) in an evaluation of somaclonal variation during somatic embryogenesis in interior spruce (*Picea glauca engelmannii* complex), and restriction fragment length polymorphism (RFLP) analysis was employed by Chowdhury and Vasil (1993) for an investigation into variation produced in sugarcane by several regeneration systems. In the present study a strategy involving the PCR-RAPD technique was devised to estimate the potential for somaclonal variation in the *in vitro* culture protocol developed for local varieties of sugarcane. PCR-RAPDs have been used recently by Isabel *et al.* (1993) for similar purposes in spruce (*Picea mariana*). Both the RFLP and PCR-RAPD approaches involve the production of DNA fingerprints which are independent of ontogenic expression. However, PCR-RAPD analysis has a number of advantages over all other methodologies: extremely small quantities of material are required, large numbers of samples can be analysed quickly, and extensive portions of the genome can be screened by using a wide range of primers.

In the present work the number of primers used was limited to six, which allowed a total of more than 40 specific DNA marker fragments to be generated. The lack of unambiguous variations in the DNA fragment profiles of callus grown on three different levels of 2,4-D for three months (examples given in Figure 2) suggests that the callus cultures were relatively stable over this period, with a sufficiently low frequency of change for there to be no polymorphisms detectable by this range of primers. In their evaluation of variation using PCR-RAPDs, Isabel *et al.* (1993) used three primers and ten markers, and found that there was no detectable genetic instability among individual somatic embryos within any particular cell line after a maturation period of two years.

Although the generation of variation in cultured cells is well documented (Larkin and Scowcroft, 1981; Karp and Bright, 1985) and considerable morphological, cytogenetic and isozyme variation has been reported in sugarcane clones derived from tissue culture (Heinz and Mee, 1971), there is

increasing evidence that plants regenerated via an embryogenic culture route are likely to be characterised by greater genetic stability than plants generated organogenically (Morrish *et al.*, 1990; Shimron-Abarbanell and Breiman, 1991; Shenoy and Vasil, 1992). Embryogenic cultures are less prone to chromosomal and DNA changes, and are more likely to produce plants that are true to type because of selection pressure in favour of normal cells during somatic embryo formation (Vasil, 1988).

The ultimate goal of this research is to have an *in vitro* culture system for sugarcane which exhibits minimal somaclonal variation, thus making it suitable for use in the production of transgenic sugarcane. The culture protocol developed to date appears to be promising in this regard, but continuation of the current study using combined assessments of genetic and phenotypic changes over a longer period is essential before confident interpretations can be made.

Conclusions

A rapid and efficient *in vitro* culture procedure for the regeneration of sugarcane via somatic embryogenesis has been established. The conditions employed have been shown to induce no DNA variation, as measured by a six primer PCR-RAPD analysis of callus DNA, over a growth period of three months on 2,4-D levels up to 5 mg/l. Although the results do not indicate a total lack of somaclonal variation, they suggest that the culture system supports a low frequency of genetic change. Continuation of the evaluation using a combination of DNA analysis and phenotypic assessment of *in vitro* progeny is required before the culture protocol can be used confidently for the production of transformants.

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