

# TOWARDS A GENE TRANSFER SYSTEM FOR SUGARCANE (*Saccharum* spp. HYBRIDS)

By <sup>1</sup>S. J. SNYMAN, <sup>1</sup>K. G. BLACK, <sup>2</sup>B. I. HUCKETT and <sup>2</sup>M. P. WATT

<sup>1</sup>South African Sugar Association Experiment Station, Mount Edgecombe

<sup>2</sup>UN/FRD PNMRU, Biology Department, University of Natal, Durban

## Abstract

There is a need for *in vitro* culture systems and gene transfer methods to be developed for local sugarcane varieties. In this study, protocols for high yield plantlet regeneration via somatic embryogenesis and cell suspension culture were established for varieties N13 and NCo376. In addition, techniques were developed for the preparation of protoplasts from leaves, callus and cell suspensions. Protoplast culture and transformation were hindered by bacterial contamination, probably derived from an endophytic population. The potential of the seed as a recipient for direct gene uptake was also investigated. Although positive DNA uptake during early imbibition was shown using [<sup>3</sup>H]pBR322, inconclusive results were obtained for transient expression of  $\beta$ -glucuronidase following uptake of pBI221. Evidence is presented for the existence of microbial seed associates. It is proposed that the problem of endogenous microbial contamination be addressed before further work on gene transfer is undertaken using the tested varieties.

## Introduction

Priorities in sugarcane breeding programmes include selection of high sucrose yielding varieties which are insect and disease resistant. However, sugarcane breeders are faced with formidable obstacles such as the narrow genetic base of most commercial cane varieties, the high ploidy of *Saccharum* spp. hybrids, the regular occurrence of aneuploids, inability to control the outcome of crosses and the length of time taken for new genotypes to be characterised. Additionally, in South Africa, environmental conditions must be artificially manipulated to achieve flowering and, therefore, cross hybridisation.

Recent advances in molecular genetics have given rise to a range of techniques that make possible the identification and isolation of genes and their subsequent transfer to recipient genotypes. By means of this recombinant DNA technology, the genetic constitutions of many crop species are being modified in a directed manner and agriculturally useful variants produced. The particular difficulties encountered in the development of new cane varieties in South Africa provide a strong argument for the adoption of such a molecular approach to complement and accelerate conventional sugarcane breeding programmes.

Several methods exist for the introduction of potentially useful genes into plants: (1) *Agrobacterium*-mediated transfer (Hinchee *et al.*, 1988); (2) the uptake of naked DNA by seeds (Topfer *et al.*, 1989); (3) naked DNA uptake by protoplasts via electroporation or chemical induction (Fromm *et al.*, 1985; Hayashimoto *et al.*, 1990); and (4) delivery of DNA to isolated cells or whole tissues via particle bombardment (Klein *et al.*, 1987; Irvine and Almeida, 1991). Usually, the last three options are selected for use with graminaceous species because of the limited natural host range of *Agrobacterium*. One of the aims of this study was to investigate the feasibility of applying two of the simpler trans-

formation systems (DNA uptake by seeds and protoplasts) to local varieties of *Saccharum* (N13, NCo376 and new hybrid Reference No. Y511).

The usefulness of transformed cells and/or tissues is dependent, however, upon the availability of methods for their regeneration into whole plants. Usually, precise methodologies must be established for each variety, explant type and culture system. The range of tissue culture systems for *Saccharum* spp. is fairly extensive and has been reviewed (Liu, 1984) but none of the published protocols has been applied to South African varieties. Because it was envisaged that the transformation work initiated in this investigation would be expanded in the future to include more sophisticated cell or tissue manipulations, the study also includes the development of an array of *in vitro* culture systems for the selected cane varieties.

## Materials and Methods

Field-grown varieties N13 and NCo376 of *Saccharum* species hybrids were used in the establishment of *in vitro* culture systems, and seeds of new hybrid Reference No. Y511 were used in all seed-based DNA uptake experiments.

### Callus cultures

Stalk explants from varieties N13 and NCo376 were surface sterilised in 70% ethanol for 1 min, followed by immersion in 1.5% (w/v) NaOCl and 0.01% (w/v) Tween 80 for 20 min. They were then thoroughly rinsed in sterile water, and cut into approximately 1 cm pieces which were placed on MS medium (Murashige and Skoog, 1962) containing 20-50 g/l sucrose, 2-4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/l inositol, 1 g/l casein hydrolysate and 9 g/l agar (callus induction medium) (Ho and Vasil, 1983a). Calli were maintained in the dark, at 27°C, for two months.

### Plant regeneration via somatic embryogenesis

After two months in culture, calli of N13 exhibiting embryogenic structures were placed on a regeneration medium (callus induction medium without 2,4-D), under a 16 h light/8 h dark photoperiod at 200  $\mu$ E/m<sup>2</sup>/s and 27°C, to induce embryo maturation and germination. Plantlets of 3 cm in height were transferred to fresh solid MS containing 10 g/l sucrose until they attained a height of approximately 10 cm. They were then planted out into sterile potting soil and covered with a sealed plastic bag. Hardening-off was achieved by opening the bags daily, for increasingly longer periods, over a two week period.

### Initiation and maintenance of cell suspension cultures

Cultures were initiated by placing 1 g of two month old friable calli of NCo376 and N13 into 50 ml of MS, 3 mg/l 2,4-D and 30 g/l sucrose (Ho and Vasil, 1983b) and maintained in the dark, at 27°C, on a rotary shaker (120 rev/min). After one month, the single cell suspensions from each culture (approximately 50 ml) were transferred to larger (250 ml) flasks and fresh medium was added to a total volume

of 100 ml. Medium was replaced weekly. Packed cell volume (PCV) was determined after centrifugation (2 000 g for 5 min) of 5 ml culture aliquots.

#### *Isolation of protoplasts and early culture conditions*

Protoplasts were isolated from mesophyll tissue of *in vitro* grown N13 plantlets, cell suspension cultures and calli. The isolation medium contained 0,55-0,65 M sorbitol, 1 mM each  $\text{KH}_2\text{PO}_4$  and  $\text{CaCl}_2$ , 10-50 g/l cellulase Onozuka R-10, 1-4 g/l macerozyme Onozuka R-10 and 2,5-10 g/l hemicellulase (Sigma), at pH 5,6. The material was incubated at a ratio of 1 g f. mass/10 ml medium, for 3-6 h, in the dark, at 26°C. The protoplast suspensions were then sequentially filtered through 1 000, 200 and 88  $\mu\text{m}$  Nybond mesh, after which they were centrifuged for 10 mins at 300 g and re-suspended in a washing solution (0,6 M sorbitol and 1 mM each  $\text{KH}_2\text{PO}_4$  and  $\text{CaCl}_2$ , pH 7,0). The washing procedure was repeated twice to obtain a partially purified protoplast preparation. Protoplast yield and viability were determined using an haemocytometer and Evans blue dye (Larkin, 1976).

When required, protoplasts were cultured, in the dark, for 48 h in MS medium containing 1,5 mg/l 2,4-D and 500 mM mannitol, at a plating density of  $1 \times 10^5$  protoplasts/ml. The protoplast suspensions were incubated in small (3 cm diameter) sealed petri dishes, which were placed on moist filter paper inside larger (11 cm diameter) petri dishes. These, in turn, were maintained in sealed plastic containers over a thin layer of water.

#### *Determination of DNA uptake by seeds*

Plasmid pBR322 (Boehringer-Mannheim) was labelled by the nick translation protocol (Rigby *et al.*, 1977) in the presence of methyl,1',2'- $^3\text{H}$  thymidine 5'-triphosphate and subsequently purified by the spun column method using Sephadex G-50 (Maniatis *et al.*, 1982). The product was stored at -15°C. Specific activity ranged from 0,9-2,7  $\times 10^6$  cpm/ $\mu\text{g}$  trichloroacetic acid (TCA)-precipitated DNA. Dehusked seeds (outer hairs removed) were surface sterilised (0,15% w/v NaOCl, 2 min), washed rapidly with sterile distilled water (x3), then immediately imbibed at 25°C for various time intervals in solutions of [ $^3\text{H}$ ]pBR322 (each sample: 30 seeds/0,345  $\mu\text{g}$  DNA, in a volume of 25  $\mu\text{l}$ ). In some cases the dehusked seeds were scraped to remove the testa prior to imbibition in DNA. Seed samples were removed subsequently to wire baskets (diameter 12 mm, mesh size 100  $\mu\text{m}$ ) and washed under running tap water for 15 min. Washed seeds were crushed in cold TCA (10% w/v, 100  $\mu\text{l}$ ) in Eppendorf tubes using a tapered ground glass rod. Each seed homogenate was washed with cold TCA (5% w/v) on to a glass microfibre filter (Whatman GF/C) held in a single glass filter unit (Millipore) and rinsed under weak suction with cold TCA (5% w/v, 5  $\times$  1 ml aliquots) followed by ethanol (95%, 3  $\times$  1 ml aliquots). Rinsed filters were air dried and counted for radioactivity in scintillation fluid.

#### *Transient expression of DNA incorporated into seeds*

Using the simple transformation method of Hanahan (1985), *Escherichia coli* HB101 was transformed with the pUC19-based expression vector pBI221 (Clontech), which encodes the  $\beta$ -glucuronidase (GUS) gene from *E. coli* flanked in the 5' proximal position by the CaMV 35S promoter sequence and in the 3' proximal position by the nopaline synthase (NOS) polyadenylation terminator region. Large scale culture and caesium chloride density gradient purification of pBI221 was conducted essentially according to Armitage *et al.* (1988). The final product, dialysed against TE buffer (10 mM Tris-HCl, 1 mM EDTA), DNA concentration 380  $\mu\text{g}/\text{ml}$ , was stored at -15°C. Surface sterilised seeds were

imbibed in pBI221 solution (6-10 seeds/3,15  $\mu\text{g}$  DNA, in a volume of 25  $\mu\text{l}$ ) for 24 h in sterile Eppendorf tubes in a moist chamber. Following imbibition, seeds were transferred to a germination medium (MS medium containing 1 g/l casein hydrolysate, 4 g/l sucrose and 9 g/l agar) and maintained in the dark at 25°C for 48 h before being assayed for GUS activity.

#### *PEG-mediated DNA uptake and expression by protoplasts*

Washed, callus-derived protoplasts ( $3 \times 10^6/\text{ml}$ ) were suspended in culture medium (MS containing 1,5 mg/l 2,4-D, 30 g/l sucrose and 500 mM mannitol) containing polyethylene glycol (PEG) (10-30% w/v; MW 6000, Merck) and pBI221 (20  $\mu\text{g}/10^5$  protoplasts), incubated at 25°C for 30 min, washed in culture medium, then treated with DNase (Boehringer-Mannheim) for 30 min following the protocol of Draper *et al.* (1988). Thereafter they were again washed once and maintained in suspension in the culture medium at 25°C for 48 h. Protoplasts were subsequently fixed (0,3% formaldehyde, 500 mM mannitol, 9 mM MES, pH 5,8, for 3 min) and washed with sodium phosphate buffer (200 mM, pH 7,0) before carrying out the GUS assay.

#### *Histochemical assay for $\beta$ -glucuronidase (GUS)*

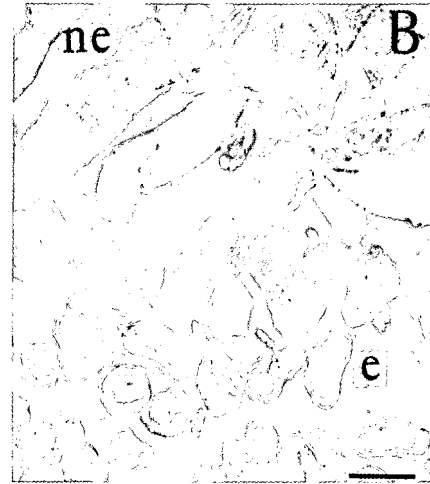
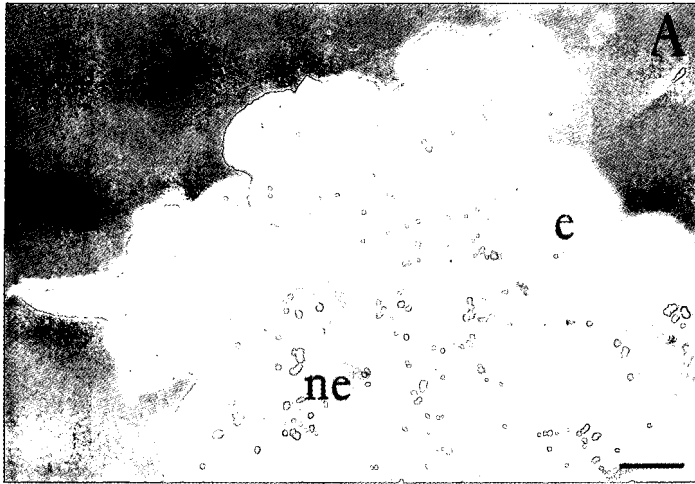
GUS activity was visualised by means of a histochemical assay using 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc) (Sigma) as substrate (Jefferson *et al.*, 1987). Seeds or protoplasts were immersed in the assay medium (2 mM X-Gluc, 0,4 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 0,4 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 0,8 mM EDTA, 400 mM mannitol, 80 mM sodium phosphate, 5% v/v DMSO, pH 7,0) at 32°C overnight. In some cases methanol (20% v/v) was incorporated into the medium before use (Kosugi *et al.*, 1990). Presence of the enzyme was indicated by the appearance of a blue stain in the plant material.

## Results and Discussion

### *Establishment of *in vitro* culture systems*

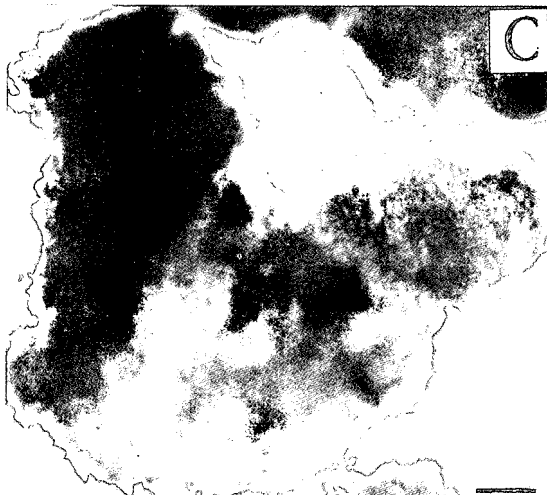
#### *Somatic embryogenesis*

Somatic or asexual embryogenesis is the production of embryo-like structures from somatic cells, a process which can occur directly from an explant or indirectly via a callus stage. The resulting somatic embryos are independent bipolar structures that can develop and germinate to form plants in a manner analogous to their zygotic counterparts (Ammirato, 1987). Some of the developmental stages involved in the process of somatic embryogenesis in variety N13 are presented in Figure 1. Callus production was visible 11 to 14 days after culture initiation and, with continuous proliferation, distinct embryogenic and non-embryogenic regions were clearly recognisable (Figure 1A). As described by many workers (Ho and Vasil, 1983a; Ammirato, 1987), the embryogenic areas were compact, nodular and white and comprised relatively small, thin-walled, richly cytoplasmic, basophilic cells with prominent nuclei, whereas the friable and yellow non-embryogenic calli consisted of large, thick-walled, highly vacuolated and irregularly-shaped cells (Figure 1B). Typical embryogenic heart- and torpedo-shaped structures (Figure 1 C,D) formed after six to eight weeks in culture. At this stage, the calli were transferred to regeneration medium which was devoid of growth regulators, and placed in a 16 h light/8 h dark photoperiod. Embryo germination occurred two to four weeks later (Figure 1E). When plantlets were approximately 3 cm in height they were transferred to tubes containing fresh nutrients but low sucrose levels (10 g/l) (Figure 1F). The protocol used for hardening-off was very effective and resulted in 80% plantlet survival after transfer to greenhouse conditions.

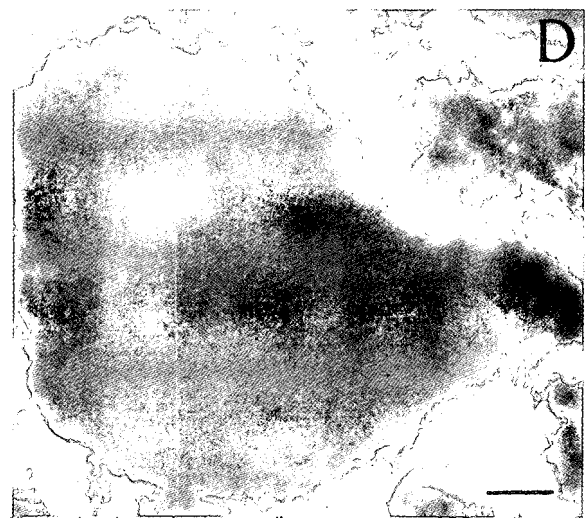


A. Two month old callus showing embryogenic (e) and non-embryogenic (ne) regions (bar represents 1 mm).

B. Two month old callus was squashed, stained with safrin and viewed microscopically. Embryogenic (e) and non-embryogenic (ne) cells are visible (bar represents 50  $\mu$ m).



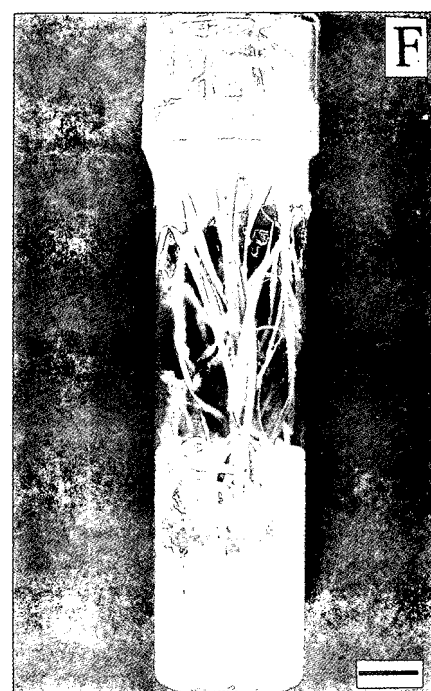
C. Heart-shaped structure (bar represents 100  $\mu$ m).



D. Torpedo-shaped structure (bar represents 100  $\mu$ m).



E. Germinated embryo (bar represents 70 mm).



F. Regenerated plantlets (bar represents 150 mm).

FIGURE 1 Photographic account of stages of somatic embryogenesis in variety N13.

The developmental events which lead to the formation of somatic embryos require ordered expression of morphogenic stimuli. These include endogenous (i.e. cellular and genotype-specific) factors, and an array of applied culture conditions (i.e. medium composition). In this study, the effect of different levels and combinations of 2,4-D (2-4 mg/l) and sucrose (20-50 g/l) on embryogenic callus induction, callus and plantlet yield were investigated by undertaking various diallel experiments (results not shown). The highest yield of plantlet regeneration occurred when explants were supplied with 2 mg/l 2,4-D and 20 g/l sucrose in the induction medium and the same level of sugar in the regeneration medium. Under these conditions callus size was  $0,40 \pm 0,25$  g fresh mass and plantlet yield was greater than 15 plants/callus. With the exception of 50 g/l sucrose, which inhibited callus growth, all of the other tested medium compositions yielded results that were not statistically different from each other. No significantly beneficial effect was observed by the addition of activated charcoal to the regeneration medium (results not shown). Activated charcoal has been shown to adsorb auxin which, at high levels, inhibits embryo maturation and germination (Ammirato, 1983). It seems, therefore, that at the end of the two month period allowed for callus establishment the medium and, hence, the callus cells had become auxin-depleted.

Research on the establishment of *in vitro* culture systems of sugarcane started in the the early 1960s (Liu, 1984) and to date there are numerous reports on successful plant regeneration via both the somatic embryogenic (Ho and Vasil, 1983a; Guiderdoni and Demarly, 1988) and the organogenic (Liu *et al.*, 1972; Nadar and Heinz, 1977; Liu, 1984; Irvine, 1984) developmental routes. However, none of the published protocols has been tested on the varieties most commonly used in South Africa. This shortcoming has been addressed in the present investigation in which relatively high yields of N13 plantlets were obtained, via somatic embryogenesis, using a protocol adapted from Ho and Vasil (1983a). The choice of the somatic embryogenic route of plant micropropagation was intentional due to the well established single cell origin of somatic embryos (Ho and Vasil, 1983a; Ammirato, 1983, 1987) leading to the production of plants that are neither chimaeras nor variants, but true clones which are suitable for genetic analyses and breeding studies.

#### Cell suspension cultures

A cell suspension culture may be defined as a rapidly dividing, homogeneous population of cells suspended in a liquid medium. Most suspension cultures are obtained by transfer of friable callus lumps to an agitated liquid environment. Although morphogenetically competent cell suspension cultures of Gramineae species are rare, there are reported successes using sugarcane (Thom *et al.*, 1981; Ho and Vasil, 1983b; Liu and Shih, 1986; Chen *et al.*, 1988). In this study, all of those four published protocols were tested using calli of varieties N13 and NCo376. Only the medium of Ho and Vasil (1983b) proved effective, such that the calli disaggregated and formed a very fine suspension of rapidly multiplying cells. The doubling time of these cultures was approximately three days (Figure 2). The cultures were maintained as finely dispersed suspensions for a period of four months (subcultured every three to seven days), after which proembryo structures developed. Although these were not plated out, the appearance of these organised structures indicated that the cultures had morphogenic potential.

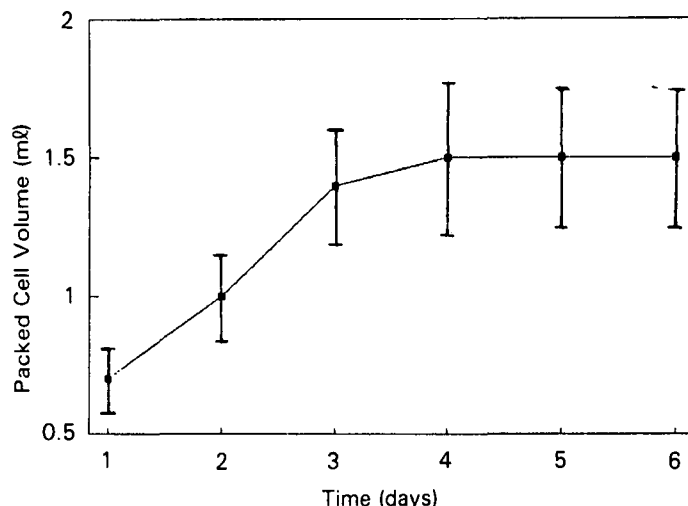


FIGURE 2 The growth of NCo376 cell suspensions measured by changes in packed cell volume over time ( $\bar{x} \pm s.d.$ ).

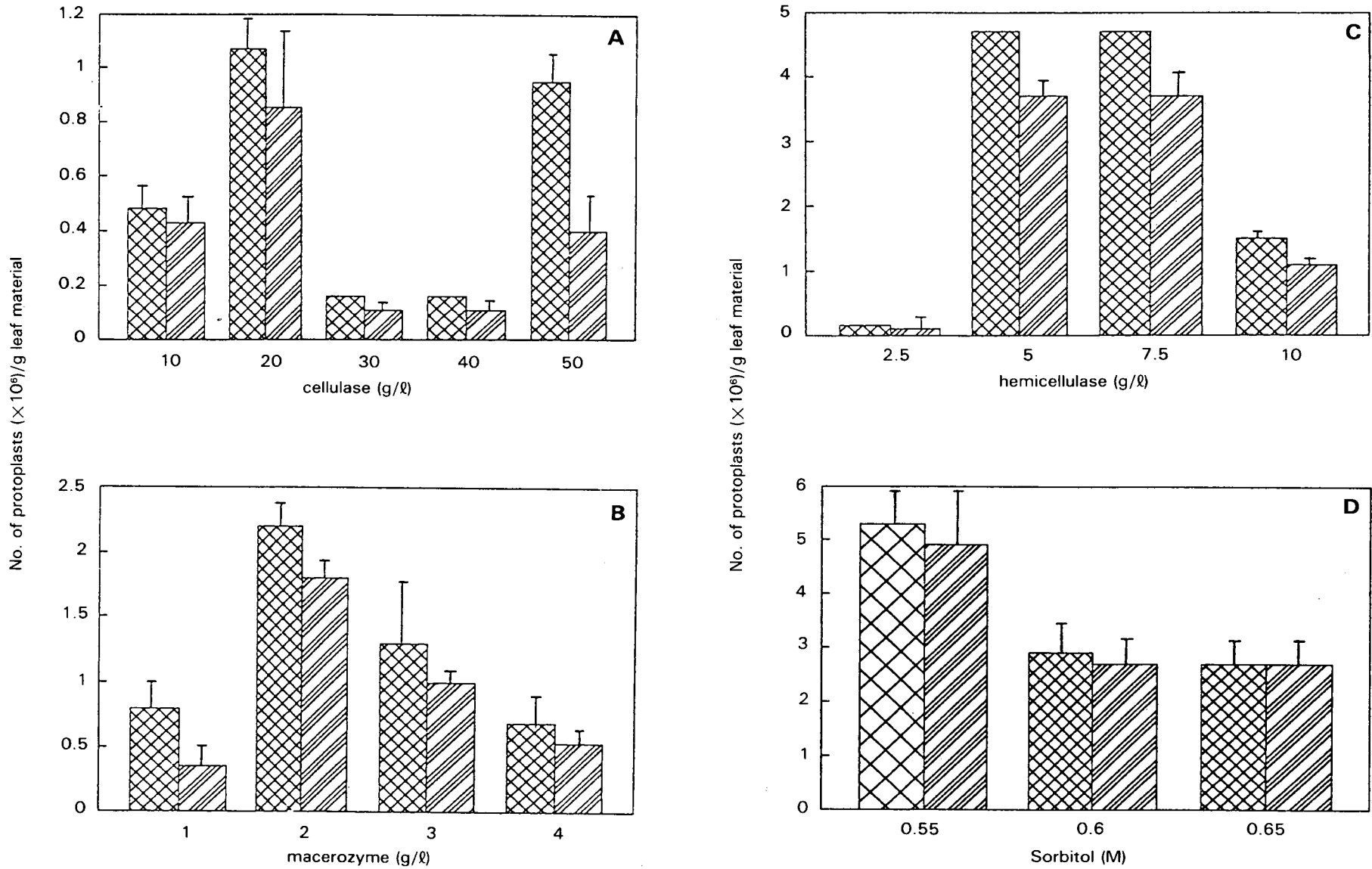
#### Protoplast yield and viability studies

Protoplasts are plant cells from which the cell wall has been removed, usually by enzymatic digestion. The first report on the isolation of sugarcane protoplasts was by Ferezy and Maretzki (1970). As with all *in vitro* culture systems, protocols for the isolation and culture of protoplasts vary with genotype, explant and growth conditions of the donor plant. It was, therefore, necessary to optimise some of the isolation conditions for the production of mesophyll- and callus-derived protoplasts (Figures 3 and 4) from the variety (N13) under investigation. The isolation conditions that yielded the highest number of viable mesophyll protoplasts ( $4,5 \times 10^6$  protoplasts/g f. mass) consisted of a six hour dark incubation in an enzymatic mixture of 20 g/l cellulase, 2 g/l macerozyme, 5 g/l hemicellulase, 0,55 M sorbitol, pH 5,6 (Figure 3). Similar yields ( $4,8 \times 10^6$  viable protoplasts/g f. mass) were obtained from friable calli in a shorter incubation time (3 h) by doubling the cellulase concentration (results not shown). Although Chen and Liu (1974) and Evans *et al.* (1980) have reported plant regeneration from sugarcane mesophyll protoplasts, most workers have chosen embryogenic cell suspension cultures as the source material for protoplast isolation and subsequent regeneration in the Gramineae (Vasil, 1987). In this study, cell suspension cultures proved unsuitable for the production of protoplasts as the resulting suspension was found to be highly heterogeneous and contained odd-shaped, large, multinucleated non-embryogenic cells. Further studies are required to optimise the isolation medium and donor status of the cell suspension cultures.

#### DNA transfer studies

##### Uptake of DNA by seeds

Studies demonstrating passive entry of nonspecific DNA sequences into whole seeds and embryos during imbibition were reported over twenty years ago (Ledoux, 1965; Ledoux and Huart, 1968; Ledoux *et al.*, 1974). The results were criticised on many grounds, including the lack of specific genetic markers by which expression could be monitored (Kleinhofs *et al.*, 1975). Recently, with the availability of more precise molecular tools, renewed interest has been shown in the seed uptake phenomenon as a possible means of germ line transformation in higher plants, particularly in the cereals (Topfer *et al.*, 1989; Heberle-Bors, 1991). In addition, a method of active transfer of naked DNA to graminaceous seeds and embryos by means of electrophoresis has been developed (Ahokas, 1989).



**FIGURE 3** The effect of the components of the protoplast incubation medium on the yield of total (▨) and viable (▧) mesophyll protoplasts ( $\bar{x} \pm s.e.$  at 0.05 level of probability). The following parameters were changed one at a time, while the osmoticum remained the same ( $\text{KH}_2\text{PO}_4$  and  $\text{CaCl}_2$ , 1 mM; sorbitol, 0.55-0.65 M; enzymes at a range of concentrations): cellulase; macerozyme; hemicellulase and sorbitol.

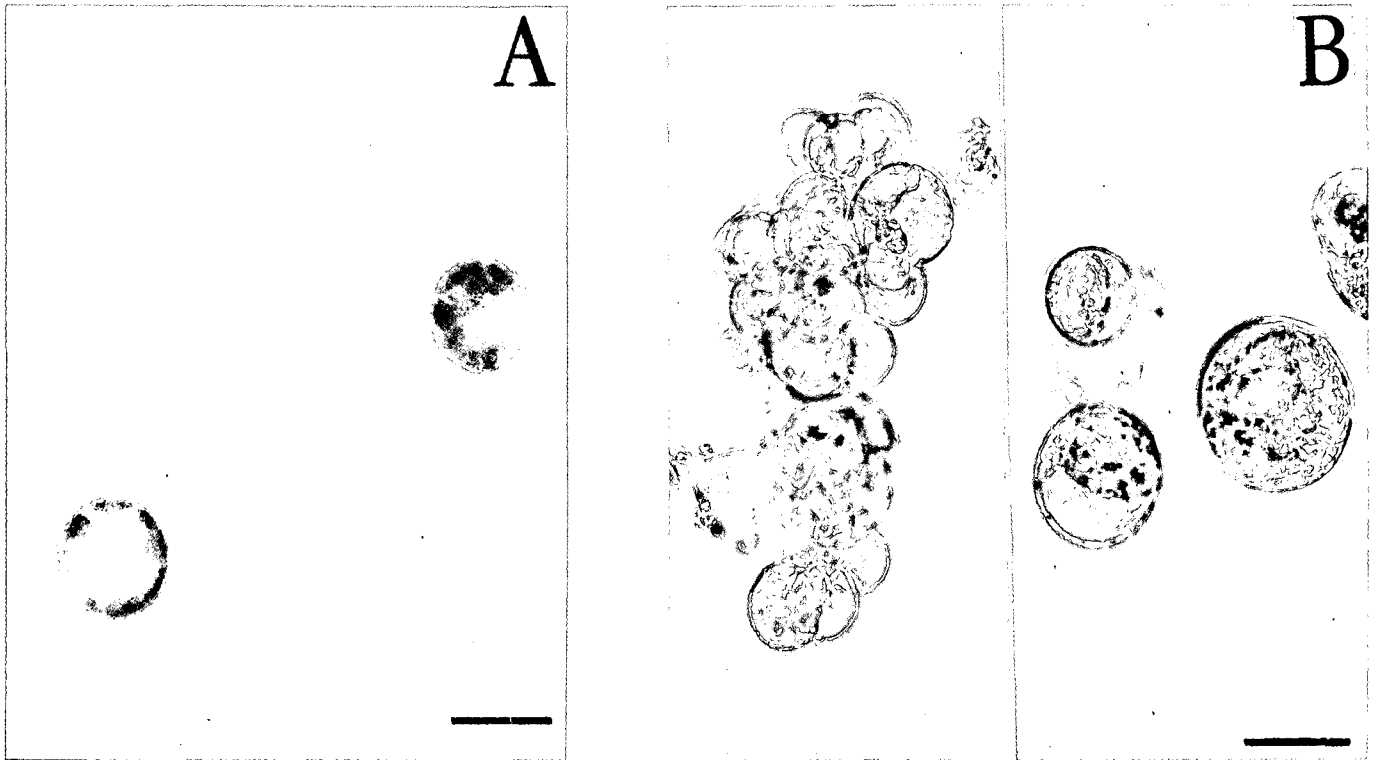
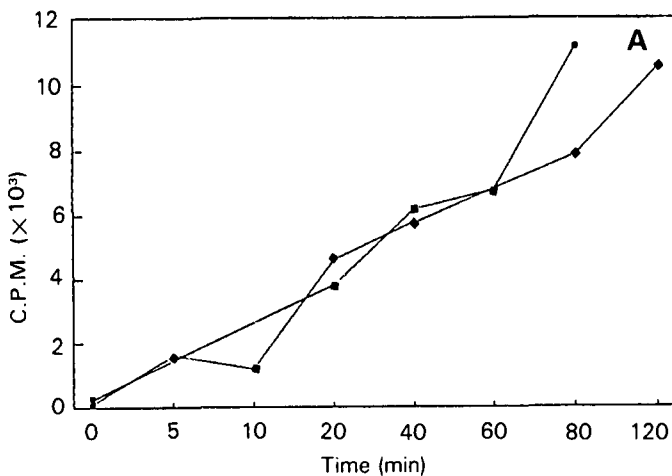


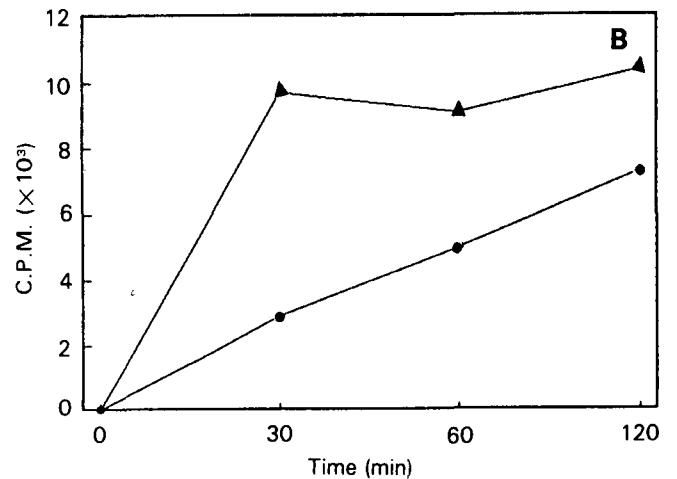
FIGURE 4 Protoplasts isolated from leaf tissue (A) and callus (B) (bars represent 30  $\mu\text{m}$ ).

In order to establish whether DNA can be taken up by sugarcane seeds during imbibition, dry seeds were incubated in a solution of  $[^3\text{H}]\text{pBR322}$  plasmid DNA, and the time course of uptake of TCA-precipitable radioactivity measured. Results showed that intact seeds took up DNA steadily over a 120 min period (Figure 5A), whereas removal of the testa prior to imbibition led to an increase in the rate of uptake and resulted in saturation after 30 min (Figure 5B). The trends shown are similar to those established recently for the uptake of small plasmids by maize caryopses and embryos during imbibition (Coulson, personal communication).

The passage of macromolecular DNA across the barrier of the plasma membrane during early imbibition is probably made possible by the disorganisation of subcellular structure which exists in seeds in the desiccated state (Simon, 1974). Topfer *et al.* (1989) argue that DNA entry into seed tissue under such conditions probably occurs by diffusion along a concentration gradient, in a similar fashion to the loss by leakage of soluble substances in the reverse direction as demonstrated by Simon (1974). Ultrastructural studies by Webster and Leopold (1977) revealed that plasmalemma discontinuities in dry soybean seeds tended to have healed after only 20 min of imbibition. Such observations empha-



A. Typical uptake rates shown by dehusked seeds with testas intact. For each time interval, 10 seeds were incubated in 0,35  $\mu\text{g}$  DNA (specific activities:  $1,4 \times 10^6$  cpm/ $\mu\text{g}$  (■),  $9,2 \times 10^5$  cpm/ $\mu\text{g}$  (●)).



B. Comparison of uptake rates exhibited by seeds with testas intact (●) and testas removed (▲). The testa was removed from dry, dehusked seeds by scraping with a scalpel prior to experimental treatment. For each time interval, 30 seeds were incubated in 0,48  $\mu\text{g}$  DNA (specific activity:  $1,8 \times 10^6$  cpm/ $\mu\text{g}$ ).

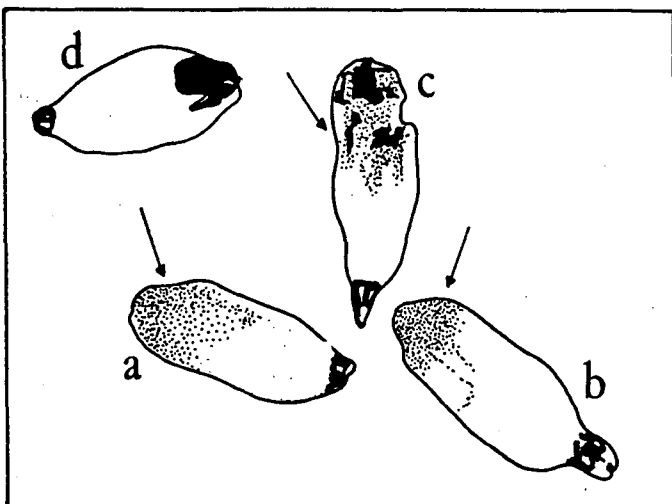
FIGURE 5 Uptake of  $[^3\text{H}]\text{pBR322}$  by sugarcane seeds during early imbibition. Imbibed seeds were thoroughly washed, homogenised in cold TCA and the TCA-precipitable fraction separated by filtration, as described in Materials and Methods.

size the importance of the preliminary stages of the imbibition process for the entry of exogenous macromolecules into the seed interior, and may explain the DNA uptake limit which occurs after 30 min under the conditions of accelerated imbibition achieved by testa removal (Figure 5B).

*Expression of GUS in pBI221 treated seeds*

After establishing evidence for the incorporation of DNA into seed tissue during imbibition, the possibility of subsequent expression was investigated. The expression system chosen for this purpose, the  $\beta$ -glucuronidase vector pBI221, allows localisation of expression to be visualised following uptake, since the GUS gene product may be assayed histochemically. In this study, observations were limited to the detection of transient expression, since this lends itself to rapid analysis in the 24-72 h period following the entry of exogenous DNA into the cytoplasm (Fromm *et al.*, 1985).

Before use, the pBI221 prepared and purified in the laboratory was subjected to restriction analysis and shown to be structurally compatible with the Clontech map (results not shown). Dry sugarcane seeds were incubated for 24 h in a solution of pBI221, then transferred to solid germination medium for 24-48 h, after which time the GUS assay was carried out. A blue colour indicating  $\beta$ -glucuronidase activity was observed among seeds treated with TE buffer only (controls) as well as those treated with pBI221. The blue stain was observed in exactly the same place in both groups of seeds, occurring in a distinct spot under the testa on the uppermost cell layers of the endosperm above the embryo (Figure 6). These results suggested two possibilities: (1) the existence of endogenous sugarcane  $\beta$ -glucuronidase activity in the seed tissue, and (2) the presence of a specific population of microbial seed-associates having a  $\beta$ -glucuronidase-positive genotype.



**FIGURE 6** Diagrammatic representation of sugarcane seeds following GUS assay. Surface sterilised seeds were imbibed for 24 h in either a solution of expression vector pBI221 or TE buffer (controls) and incubated on solid germination medium for 48 h before the GUS assay was carried out. Positive staining was visible as a distinct spot (blue) at the top of the embryo (shown by arrow, a-c) and occasionally around the embryo. A high proportion of control seeds gave similar positive responses. Some seeds exhibited no staining (d).

The enzyme  $\beta$ -glucuronidase is commonly present in micro-organisms but is absent or undetectable in many higher plants (Jefferson *et al.*, 1987); indeed, the success of GUS as a plant reporter gene rests upon this distinction. However,

the presence of substantial endogenous plant GUS activity has been noted by workers in several tissues and organs of plant species from different families (e.g. Kosugi *et al.*, 1990; Alwen *et al.*, 1990). In discussing the issue of background GUS activity, Jefferson and Wilson (1991) comment on the possibility of endophytic microbes being responsible in some cases. They make the point that plants exist only rarely without numerous exo- and endophytic associates, and quote examples of tissues kept in "sterile" culture for years which continue to harbour such symbionts.

Methodological tactics which have been shown to suppress plant GUS analogues, while still allowing activity of the *E. coli* enzyme, include assaying at neutral rather than acid pH (Alwen *et al.*, 1990) and the incorporation of methanol into the assay medium (Kosugi *et al.*, 1990). In the present study, the latter technique was used in conjunction with two other factors, testa removal and an additional sterilisation procedure, to determine more closely the possible cause of the positive response in control seeds. Results are summarised in Table 1.

**Table 1**

Effect of seed pretreatment and assay medium on GUS response of sugarcane seeds in DNA uptake experiments. Seeds were imbibed and assayed essentially as described in Figure 6 and Materials and Methods. Treatments included prior removal of testa by scraping seeds with a scalpel blade, microwaving for 60 s prior to surface sterilisation, inclusion of 20% v/v methanol into the assay buffer, and combinations of these factors. Microwave treatment for 60 s reduced seed germinability to 50% and eliminated seed induced bacterial contamination on nutrient agar. (n = 5-10)

Seed treatment	Seeds demonstrating GUS expression (%)	
	DNA (pBI221)	Control (TE buffer)
testa intact	60	60
testa removed by scraping with scalpel	40	30
testa intact, microwaved for 60 s	90	60
testa removed microwaved for 60 s	60	40
testa removed 20% methanol added to GUS incubation buffer	100	100
testa removed, microwaved for 60 s, 20% methanol added to GUS incubation buffer	100	80

Not surprisingly, testa removal invariably led to a more dramatic blue stain, and this served to confirm the similarity between experimentals and controls. No significant differences were observed in the reactions of DNA-treated and control seeds which had received a methanol assay, suggesting that microbial activity was more likely to be responsible for the observed positive controls than an endogenous plant enzyme. Microwaving seeds for 60 s failed to reduce the number of positive controls. The fact that microwave treatments were applied to seeds in the dry state is of note here, in that the extent of damage would be milder than in hydrated seeds. However, it had already been established that the given microwave dose was sufficient to reduce germination to 50% and eliminate the incidence, on nutrient agar, of bacterial growth from seed squashes (results not shown). Hence, in the context of null plant GUS, the persistent blue colouration in the control seeds could be interpreted as an indication of the resilience of the microbial cells responsible. It is interesting to speculate whether these

might be exocytic spore-formers or, alternatively, endocytic types highly protected from damage by the concentrated proteins in the cytoplasmic milieu of the unimbibed seed.

#### Protoplast viability and DNA uptake in the presence of PEG

The incorporation of naked DNA (direct DNA uptake) by protoplasts was first demonstrated by Davey *et al.* (1980) and Krens *et al.* (1982), using isolated Ti plasmid DNA from *Agrobacterium* and either PEG or poly-L-ornithine as chemical stimulants of endocytosis. The basic method has been used by many workers as a means of transformation, with PEG 6000 having proved to be the most reliable chemical inducer (Draper *et al.*, 1988). Sophistication of the approach has led to improved results, with the frequencies of stable transformation obtained increasing from approximately  $1 \times 10^{-6}$  per developing microcallus to  $1-5 \times 10^{-2}$  (Draper *et al.*, 1988; Saul *et al.*, 1988). Although it is now theoretically possible to transform almost any protoplast system (Draper *et al.*, 1988), the regeneration of transformed plants from protoplasts remains a problem for many species.

The protoplast studies presented here are preliminary, pre-transformation experiments aimed at optimising protoplast viability under the conditions usually required for the uptake of DNA for the purpose of monitoring pBI221 uptake using transient expression of GUS as indicator. Callus-derived protoplast viability was quantified using the dye exclusion method following exposure for 30 min to various concentrations of PEG 6000 in a procedure based on that of Draper *et al.* (1988) for DNA uptake. The PEG concentration range tested was 10-30% w/v (Krens *et al.*, 1982; Draper *et al.*, 1988). PEG at 20% w/v was the highest tested concentration giving greater than 60% viability (results not shown) and was the concentration chosen for use in DNA uptake experiments. Initial trials of the DNA uptake protocol demonstrated that after the required 48 h in culture GUS activity appeared to be negative. However, interpretation of these results was hindered because the protoplast suspensions were invariably heavily contaminated with bacteria. The following steps were taken: (1) The sterility of all reagents was checked, and all manipulations requiring asepsis rigorously monitored; (2) an antibiotic cocktail known to suppress contamination in field grown sugarcane (results not shown) was added to the medium, and (3) individual calli were tested for their potential to induce bacterial growth in nutrient broth, and only "clean" calli used as sources of protoplasts. None of these strategies led to elimination of bacterial contamination. It is notable that calli which yielded highly contaminated protoplast suspensions also served as a source for uncontaminated cell suspension cultures. A possible explanation is that endophytes intimately associated with the cell wall were released into the medium during cell wall digestion, as has been observed in potato (Joughin, 1989).

#### Concluding remarks

The attractive prospect of DNA transfer to seeds, which bypasses *in vitro* regeneration, was found to be problematic due to the presence of seed micro-organisms. Since similar difficulties were experienced in the culture of protoplasts, it is imperative that an effective means of producing plants free of contamination be found if progress in studies of transformation and fusion (somatic hybridisation) is to be made.

A regenerative route for the micropropagation of sugarcane via somatic embryogenesis has been established successfully, and the potential exists for this system to be used in the production of transgenic plants, possibly via particle bombardment. An alternative means of enhancing the genetic base of *Saccharum* spp. could be developed via cell

line selection and germplasm preservation (cryostorage) using the cell suspension culture system established in this investigation.

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