DEVELOPMENT OF *IN VITRO* CULTURE AND GENE TRANSFER TECHNIQUES IN SUGARCANE (*SACCHARUM* SPECIES HYBRIDS)

by

Sandra Jane Snyman

Submitted in partial fulfilment of the
requirements for the degree of
Master of Science,
in the
Department of Biology,
University of Natal
1992

Durban
1992

PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1990 to December 1991, under the supervision of Drs B. Huckett and M.P. Watt.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

ACKNOWLEDGEMENTS

Sincere thanks to Dr Peter Hewitt, Director of the South African Sugar Association Experiment Station for allowing me to present research results carried out while in his employ, in this thesis.

A heartfelt thank-you to my supervisors Drs Barbara Huckett and Paula Watt for their unflagging encouragement and support over the past two years.

I would like to extend a special word of thanks to the following people:

Kevin Black of the SASA Experiment Station for his excellent technical assistance,

Poovie Govender of the SASA Experiment Station, and Felicity Blakeway of the Biology Department, University of Natal, Durban, for their assistance with photography,

Deanne Zeeman of the SASA Experiment Station for her assistance in typing sections of the manuscript,

Staff of the Biometry Department, SASA Experiment Station, for their advice on statistical analyses and use of computer software,

Genetics Department, University of Natal, Pietermaritzburg, for their gift of a sample of pBI221, and

Dr Thomas Pinfold and Jennifer Jacobs for proof-reading the manuscript.

A DEDICATION

Scientific research is like housework;
however resourceful or resolute the
approach, the task is unending.

My thesis is dedicated to the above concept, and to my husband and family who, in the final months, assisted in its completion by doing my housework.

ABSTRACT

In vitro cell and tissue culture systems were developed for sugarcane in order to utilise current transformation techniques to introduce genes to South African sugarcane varieties, which would be difficult, if not impossible to achieve in conventional breeding programmes. Embryogenic calli were initiated in the dark from stem explants of sugarcane varieties NCo376 and N13, on a MS medium containing sucrose (20-50 g/l), 2,4-D (2-4 mg/l), casein (1 g/l), inositol (100 mg/l) and agar (9g/l). After 2 months the somatic embryos were cultured in a light/dark photoperiod for a further 2 months. The best combination of sucrose and 2,4-D for callus initiation, and subsequent plant regeneration, was 20 g/l and 2 mg/l, respectively. Plant yields ranged from 16 to 36 plants per gram fresh weight callus, and the yields were not significantly increased by the addition of activated charcoal to the regeneration medium. When plantlets reached a height of 10 cm, they were transferred to autoclaved soil in pots, hardened-off and placed in the glasshouse.

Suspension cultures were initiated from friable NCo376 calli in liquid MS medium shaken at 100 rev/min in the dark at 27°C, and were subcultured every 3-7 days. Protoplasts from various sources (leaf, calli and suspension cultures) were obtained after enzymatic digestion in cellulase (20-30 g/l), macerozyme (0,2 g/l), hemicellulase (5 g/l), and sorbitol (0,55 M) in a calcium and magnesium salt solution. Protoplasts cultured for 48 h resulted in a loss in viability of 84%.

The potential of the seed as a recipient for direct gene uptake was investigated, as this eliminated the need for *in vitro* culture and plant regeneration. Uptake of [^{3}H] pBR322 DNA by seeds was demonstrated, and seeds with the testa removed exhibited higher initial uptake rates than those with intact seed coats. However, transient expression, using the GUS reporter gene (coding for bacterial ß-glucuronidase) carried on plasmid pBI221, could not be conclusively shown using the histochemical GUS assay, due to GUS activity generated by either microbial contamination or endogenous plant GUS activity. Neither microwaving to eradicate contaminants nor the addition of methanol (20%) to the GUS incubation buffer were successful in overcoming positive results observed in control seeds. An alternative approach to sugarcane transformation, using PEG-mediated DNA uptake and subsequent transient expression of GUS by protoplasts was investigated, but microbial contamination was a persistant problem and no positive results were observed. Further examination and elimination of endogenous contamination is required before transformation studies can be continued.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND SYMBOLS

Cauliflower mosaic virus	CaMV
centimetre	cm
chloramphenicol acetyltransferase	CAT
copy deoxyribose nucleic acid	cDNA
counts per minute	cpm
degrees celcius	°C
deoxyribose nucleic acid	DNA
2,4-dichlorophenoxyacetic acid	2,4-D
figure	Fig.
ß-glucuronidase	GUS
grams per litre	g/l
hour	h
kilobases	kb
messenger ribose nucleic acid	mRNA
micro Einsteins per metre^2 per second	$\mu E/m^2/s$
microgram	μg
micrometre	μm
microseconds	μs
milligram	mg
milligrams per litre	mg/l
millilitre	ml
millimetre	mm
millimolar	mM
minute (time)	min
molecular weight	MW
molar (concentration)	M
MS medium (Murashige & Skoog, 1962)	MS
2-[N-morpholino]ethanesulphonic acid	MES
neomycin phosphotransferase II	NPTII
number	no.
number of observations	n
packed cell volume	p.c.v.
percent/percentage	%
picograms	pg
polyethylene glycol	PEG
revolutions per minute	rev/min
ribose nucleic acid	RNA

second (time)	s
species	sp.
species (plural)	spp.
standard deviation	s.d.
standard error	s.e.
transferred-DNA	T-DNA
trichloroacetic acid	TCA
Tumour-inducing	Ti
ultra violet	u.v.
unit	U
volts	V
volume by volume	v/v
weight by volume	w/v

CHAPTER ONE

GENERAL INTRODUCTION

Sugarcane (*Saccharum* species hybrids), a member of the Gramineae/Poaceae, is a crop of major importance, as it provides about 65% of the sugar produced in the world (Liu, 1984). The zone of sugarcane production characteristically occurs in tropical and subtropical latitudes and the crop is grown in soils and climates displaying great variations (Barnes, 1974). South Africa is the eighth largest producer of cane sugar, after countries such as Brazil, Cuba, India, Australia, United States, Philippines and China, with a production of about 2 million tons per annum (Liu, 1984).

South African sugarcane varieties and their characteristics

Sugarcane varieties bred at the South African Sugar Association Experiment Station at Mount Edgecombe for growth in climatic regions of South Africa, are grown in rainfed conditions on the east coast and also in irrigated plantations in Northern Natal and Eastern Transvaal (Fig. 1.1). The local variety NCo376, which was used in this study, was released for commercial propagation in 1955, and in 1976 it represented more than 60% of the sugarcane harvested in South Africa (Anonymous, 1977). This level decreased to 45% in 1991 due to the release of new varieties that are more resistant to drought and diseases, and also have higher yields (Table 1.1). Variety NCo376 has the distinction of being the most adaptable variety in the South African sugar industry, but other varieties may be more suited to specific conditions (Anonymous, 1991). The most suitable variety for a particular location is normally the one which yields the highest return over a whole crop cycle. Return depends not only on yield of sucrose, but also on the costs of managing and handling the crop (Barnes, 1974). In addition, the problem of disease and soil type vary from area to area and may be the deciding factor in the final choice of variety.

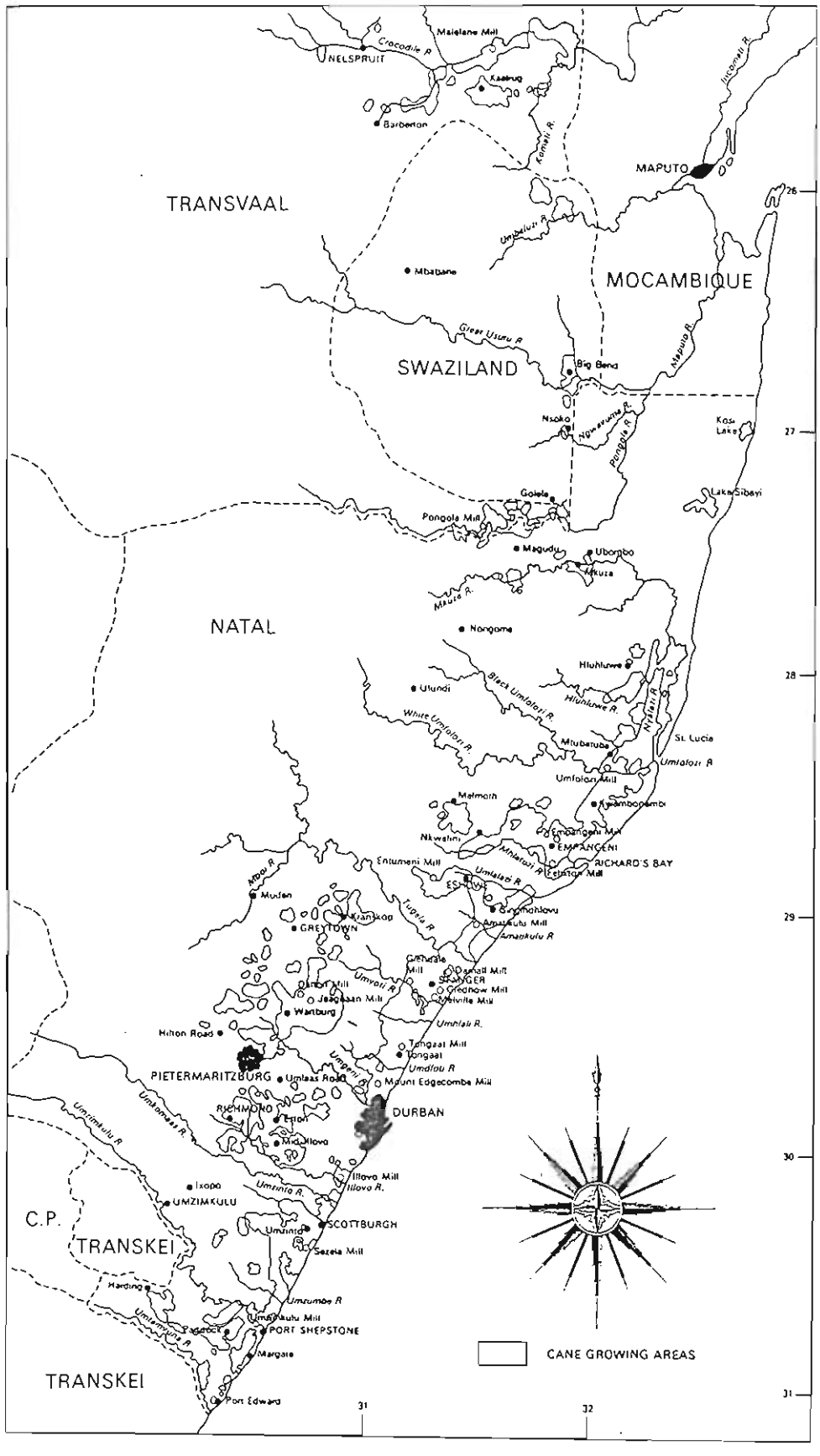


Fig. 1.1 : Map showing South African sugarcane growing areas (reproduced from Anonymous, 1977)

Table 1.1 : Agronomic characteristics of South African sugarcane varieties.

Variety	Year of release	Proportion of 1990/91 crop (%)	Sucrose yield (t suc /ha/yr)	Best features	Worst features
NCo376	1955	45	12	Ratooning ability Adaptability	Drought susceptible Disease susceptible Low sucrose
NCo293	1952	3,3	12	Hardiness at high altitudes	Poor ratooning Mosaic virus susceptible
N12	1979	12,2	12-15	High sucrose yields Drought tolerance	Slow germination
N13	1980	0,6	>12	Rapid growth Annual harvest	Eldana susceptible smut, RSD susceptible
N14	1980	15,1	>13	High yield in irrigated areas	Drought susceptible
N16	1982	1,9	12-22	High yield Rapid growth	Eldana and smut susceptible
N17	1984	1,7	5-22	High sucrose yield Smut resistant	Slow germination
N19	1986	1,3	12-20	High sucrose Resistance to smut	Susceptible to eldana
N21	1989	Data not available	11-13	Eldana resistance	
CP66/ 1043	1987	Data not available	12-18	High yields, smut & mosaic resistance	Requires excellent growing conditions

eldana = Most prevalent insect pest (*Eldana saccharina* Walker)
sugarcane mosaic virus }
smut } = Most prevalent diseases
RSD (Ratoon stunting disease) }

(modified from Anonymous, 1991)

Sugarcane breeding and its problems

Most commercial sugarcane varieties now in use world-wide are descendants of inter-specific hybrids between *S. spontaneum, S. sinense, S. robustum* and *S. officinarum* (Arcenaux, 1965). *Saccharum* species hybrids are highly polyploid and have chromosome numbers ranging from 40 to 120 (Price, 1962). Sugarcane is propagated vegetatively by means of setts, which are segments of stalk which comprise one to five nodes, from which the shoots and roots germinate. Once sugarcane stalks have been harvested, normally twelve to eighteen months after planting, ratooning occurs (the root material which remains in the ground after harvesting, forms new shoot material). The production of fertile seed under normal growth conditions is rare, due to sub-optimal night-time temperatures and day-length during the flower initiation period (Stevenson, 1965).

Priorities in breeding programmes in the major sugar-growing areas world-wide include selection of high sucrose-yielding cane varieties which are also insect and disease resistant. However, sugarcane breeders are faced with formidable characteristics such as the narrow genetic base of most commercial cane varieties, the high ploidy of *Saccharum spp.* hybrids, the regular occurrence of aneuploids and the inability to control the outcome of crosses (Nuss, pers. comm.). Conventional plant breeders have to manipulate environmental conditions to achieve cross-hybridisation between varieties, and in South Africa, specially constructed glasshouses with adjustable photoperiods are necessary to control flowering, collect pollen, and to ensure successful pollination of female plants. Once seed has been produced, a screening and selection programme is initiated, which can take up to 20 years before a new variety is released to growers (Nuss, pers. comm.). Therefore the emergence of new techniques by which plants can be genetically manipulated (reviewed by Vasil, 1987, 1990) may provide an alternative to conventional and somewhat problematic sugarcane breeding.

The introduction of genetic variation by *in vitro* culture

Routes for the introduction of genetic variation include conventional breeding as discussed above, and the more recent techniques of *in vitro* culture of tissue and cells, and gene transfer (reviewed by Ammirato, 1989; Lindsey and Jones, 1989). Sugarcane improvement via cell line selection or gene transfer techniques of necessity includes *in vitro* culture techniques, which have been successfully established in sugarcane varieties grown in other sugar-growing areas in the world. *In vitro* techniques are essential in order to allow researchers to work with single cells or small selected groups of cells in a controlled environment, that can be manipulated.

Research on sugarcane tissue and cell culture began in Hawaii where callus cultures were established (Nickell, 1964). When it was discovered that shoot differentiation occurred from callus (Heinz and Mee, 1969), other researchers began similar studies (Liu, 1971; Nadar *et al.*, 1978; Liu *et al.*, 1982; Ho and Vasil, 1983a). Callus culture is mutagenic in the broadest sense (Liu, 1984), as calli maintained *in vitro* over long time periods are usually cytologically unstable and regenerate into plants that are often characterised by genetic variability (Irvine, 1984). Although this method has given rise to agronomically-useful sugarcane variants in Taiwan (Liu, 1971; Liu and Chen, 1976; Tsay, 1987; Liu, 1990), Brazil (Evans *et al.*, 1980) and Australia (Larkin and Scowcroft, 1981), it has not replaced conventional breeding programmes. The induction of genetic variability in callus cultures can also be achieved by the use of mutagenic agents, such as colchicine and this has been attempted in sugarcane, although no agronomic improvements were evident (Liu, 1990; Irvine *et al.*, 1991).

However *in vitro* callus culture does not only have application for producing somaclonal variants, but also for micropropagation purposes where the resultant plants must be genetically identical to the parent plant, and for the regeneration of transformed cells

or tissues. It has been suggested that the regeneration of sugarcane plants from callus via somatic embryos, rather than via shoot differentiation (Heinz and Mee, 1969; Liu and Chen, 1974; Nadar and Heinz, 1977; Nadar *et al.*, 1978; Ho and Vasil, 1983a; Chen *et al.*, 1988a; Guiderdoni and Demarley, 1988), may limit the genetic variation observed in callus cultures because each embryo arises from a single cell and the route of development follows that of zygotic embryo germination (Ammirato, 1983; Evans *et al.*, 1984a). It is however, essential that callus is not maintained in culture for an extensive time period. The non-chimeric nature of somatic embryogenic clones has been described for other monocotyledonous plants (Botti and Vasil, 1983; Karp and Maddock, 1984; Abe and Futsuhara, 1985; Armstrong and Green, 1985; Bretzinger *et al.*, 1989; Bhaskaran and Smith, 1990).

Another type of *in vitro* culture that has been established for sugarcane is suspension cultures, which may be used as a source of cells for the isolation of protoplasts and the study of plant cell physiology and biochemistry (Ho and Vasil, 1983b; Liu and Shih, 1986; Chen *et al.*, 1988b).

One advantage of using a single cell for transformation purposes is that when it divides, genetic information is passed onto daughter cells and eventually the whole plant. Protoplasts are cells which have had their cell wall enzymatically removed and this facilitates interaction with other organisms or macromolecules. The ability to regenerate plants from single cells has application for genetic engineering techniques, and also for the production of normally incompatible inter- and/or intra-specific hybrids (Ozias-Akins *et al.*, 1986; Tabaeizadeh *et al.*, 1986). Maretzki and Nickell (1973) were the first to isolate sugarcane protoplasts and induce the formation of callus clusters. Protoplasts were isolated from leaves (Chen and Liu, 1974; Evans *et al.*, 1980), but no repeatable regeneration protocols could be established. Later Chen and Shih (1983) were able to obtain callus from protoplasts derived from suspension cells. Regeneration of sugarcane plants from protoplasts was achieved

(Srinivasan and Vasil, 1986; Chen *et al.*, 1988b) but has not yet been repeated.

Plant regeneration from monocotyledonous protoplasts is difficult (Vasil, 1987; Bhaskaran and Smith, 1990). Despite this, protocols for plant regeneration have been established and transgenic rice (Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Datta *et al.*, 1990; Hayashimoto *et al.*, 1990; Raineri *et al.*, 1990) and maize (Rhodes *et al.*, 1988; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990) plants have been produced from transformed protoplasts. A common factor in the regeneration of both the above species is that embryogenic protoplasts (isolated from embryogenic suspension cultures) were the cells targeted for DNA uptake. Although Chen *et al.* (1987) were able to transform sugarcane protoplasts isolated from embryogenic sugarcane suspension cultures, only callus colonies could be recovered. Reports of transformed monocotyledonous protoplasts appear in the literature regularly, but aside from rice and maize, there are not many which can be regenerated to plants (Lorz *et al.*, 1985; Vasil, 1990).

Aims of this study

The aims of this study were to investigate options to supplement the current sugarcane-breeding programme at the Experiment Station at Mount Edgecombe. *In vitro* cell and tissue culture techniques for two South African sugarcane varieties were established, with a view to ultimately being able to use them in conjunction with gene transfer techniques to introduce specific genes into the sugarcane genome. Preliminary work on techiques used for the introduction of DNA into sugarcane was also undertaken.

CHAPTER TWO

DEVELOPMENT OF *IN VITRO* CULTURE SYSTEMS FOR SUGARCANE *(SACCHARUM* species hybrids)

2.1. INTRODUCTORY REMARKS

The overall objectives of the study were to establish protocols for the induction and establishment of callus cultures, suspension culture initiation and maintenance, and the isolation of protoplasts. These *in vitro* systems offer the potential for proliferation of genetically-transformed cells and tissues, and ultimately the regeneration of plants. The establishment of such techniques in sugarcane is one of the steps necessary in any breeding programme which aims to use genetic engineering techniques to insert agronomically useful genes into South African sugarcane varieties.

The varieties used in this study were NCo376, which is widely grown and occupies 45% of the land under sugarcane cultivation, and N13 which only occupies about 1% of land under sugarcane cultivation. The latter variety is a potentially good candidate for improvement by genetic engineering techniques, because it has adequate sucrose yields, but is susceptible to many diseases.

2.2. LITERATURE REVIEW

2.2.1. GENERAL ASPECTS OF *IN VITRO* CULTURE

The cereals and grasses, which constitute the most important group of crop plants, have until recently been recalcitrant to cell culture techniques (Vasil, 1982, 1987; Bhaskaran and Smith, 1990). However, important advances in cell culture have been made, the highlights of which include:

1) the establishment of totipotent cell lines in *Lolium* grass (Jones and Dale, 1982), *Panicum* grass (Lu and Vasil, 1982), sugarcane (Ho and Vasil, 1983a, 1983b) and wheat (Vasil *et al.*,

1990a, 1990b);
2) cell suspension cultures in wheat (Shimada and Yamada, 1979), sugarcane (Ho and Vasil, 1983b) and *Panicum* grass (Karlsson and Vasil, 1986);
3) the development of protoplast culture systems yielding somatic embryos and plants in *Panicum* (Lu *et al.*, 1981), rice (Fujimura *et al.*, 1985) and sugarcane (Srinivasan and Vasil, 1986); and
4) more recently the recovery of somatic hybrid cell lines from *Pennisetum* x *Panicum* (Ozias-Akins *et al.*, 1986) and sugarcane x *Panicum* (Tabaeizadeh *et al.*, 1986), and genetically transformed cell lines in maize and rice (Lorz *et al.*, 1985; Potrykus *et al.*, 1985; Fromm *et al.*, 1986).
As sugarcane is a member of the Gramineae/Paoceae, this literature review will focus largely on the reported developments regarding *in vitro* systems in members of this group.

Plant regeneration from the above-mentioned *in vitro* culture systems can occur via one of two pathways, organogenesis or somatic embryogenesis and either directly from explants with a minimum or absence of callusing, or indirectly via a callus stage first (Fig. 2.1). Callus consists of a mass of tissue, with a low level of organisation, obtained by transferring cut pieces of plant organs to a suitable nutrient medium (Skoog and Miller, 1957). Generally, callus can be maintained indefinitely by regular transfer to fresh culture medium, and plants develop from specific regions of the callus either via somatic embryogenesis or by shoot morphogenesis followed by root development (organogenesis) (Fig. 2.1).

Direct organogenesis (or shoot morphogenesis) involves the development of axillary buds following the inhibition of apical dominance, and indirect organogenesis involves the *de novo* organisation of shoot meristems in callus cultures (Shimada and Yamada, 1979; Evans *et al.*, 1984a; Bhaskaran and Smith, 1990). Organogenic plant regeneration from axillary meristems, which forms the basis of successful large-sale micropropagation in many dicotyledonous herbaceous species (Fig. 2.1), is not common in

cereals and grasses but has been observed in sugarcane (Grisham and Bourg, 1989) and some forage grasses (Ahloowalia, 1984). Organogenic plant regeneration via shoot morphogenesis in callus culture has been observed in graminaceous plants such as maize (Lowe *et al.*, 1985; Woodward, 1989), sorghum (Wernicke *et al.*, 1982), sugarcane (Liu and Chen, 1974; Chen *et al.*, 1988a) and some of the forage grasses (Ahloowalia, 1984). It is important to note that shoot meristems in *in vitro* callus culture have been shown to be multicellular in origin (Steward *et al.*, 1958; Norris *et al.*, 1983; Skene and Barlass, 1983), and may give rise to chimeras (Sacristan and Melchers, 1969; Irvine, 1984), which are unsuitable for clonal propagation, mutation research, genetic analysis and breeding.

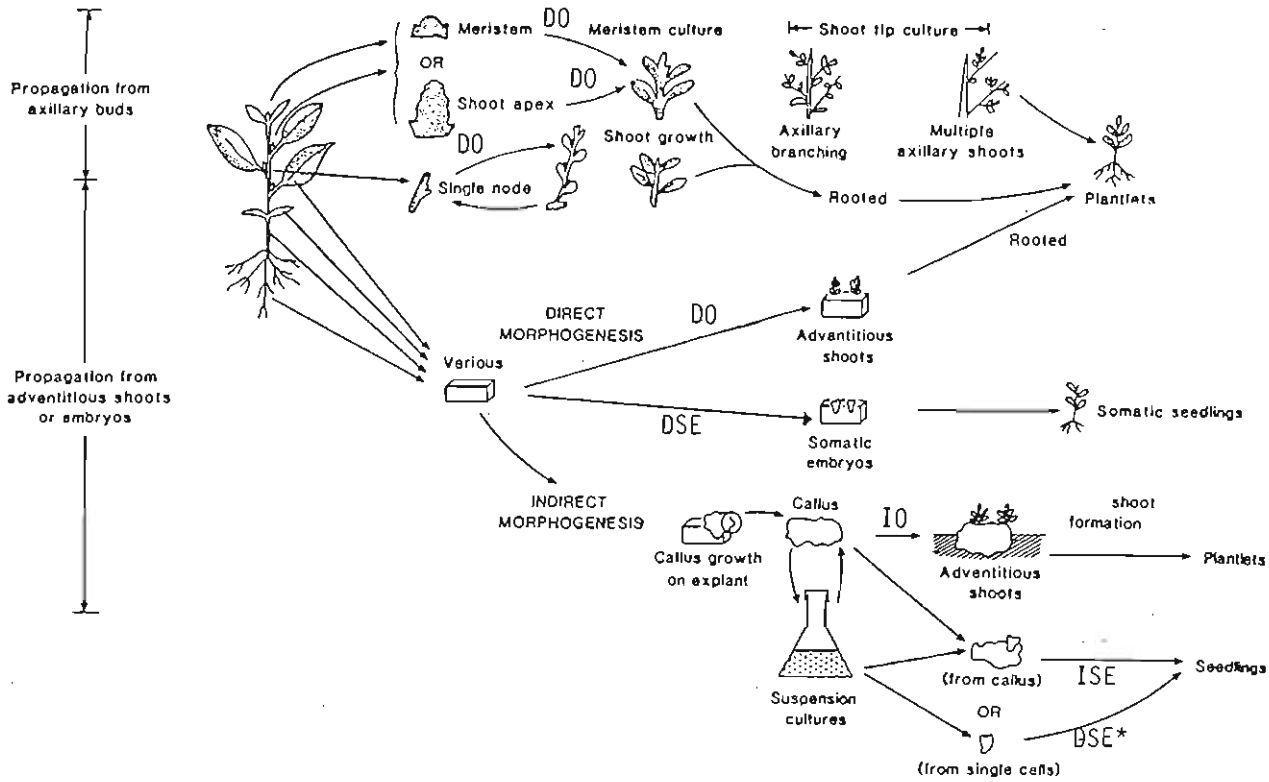


Fig. 2.1 : A diagrammatic representation of the principal pathways of plant regeneration. O, plant regeneration via organogenesis; DO, direct organogenesis; IO, indirect organogenesis; SE, plant regeneration by somatic embryogenesis; DSE, direct somatic embryogenesis; ISE, indirect somatic embryogenesis; *DSE, although these somatic embryos have gone through a callus stage, plant regeneration takes place directly from the embryos without the formation of callus (reproduced from Lindsey and Jones, 1989).

Somatic embryogenesis is the production of embryogenic-like structures from somatic (asexual) cells (Steward *et al.*, 1958). A somatic embryo is an independent bipolar structure, is not physically attached to the tissue of origin, and can develop and germinate to form plants in a manner analogous to germination of zygotic embryos in seeds (Ammirato, 1987). Somatic embryos develop, directly or indirectly (following the formation of a mass of proembryogenic cells), like their zygotic counterparts from single cells (Ammirato, 1983; Vasil *et al.*, 1990a). This analogy in development is shown diagrammatically in Fig. 2.2. Consequently plants derived from embryogenic cultures are neither chimeras nor variants, but true clones.

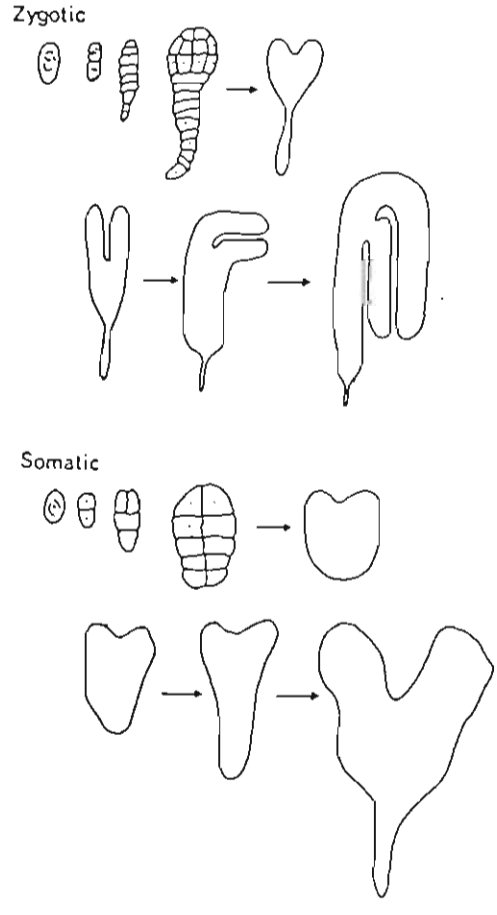


Fig. 2.2: Diagrammatic representation of the stages in zygotic and somatic embryogenesis in carrot. (reproduced from Lindsey and Jones, 1989).

Plant regeneration from callus cultures of sugarcane via somatic embryogenesis was achieved over a decade ago (Liu and Chen, 1974; Nadar and Heinz, 1977; Nadar *et al.*, 1978; Ho and Vasil, 1983a; Chen *et al.*, 1988a; Guiderdoni and Demarly, 1988). Other grasses and cereals have also been regenerated via somatic embryogenesis, for example maize (Lu *et al.*, 1982, 1983; Armstrong and Green, 1985; Woodward, 1989; Ray and Ghosh, 1990), rice (Abe and Futsuhara, 1985; Ling *et al.*, 1983; Abdullah *et al.*, 1986), wheat (Karp and Maddock, 1984; Vasil *et al.*, 1984; Rashid and Quraishi, 1989), oats (Bregitzer *et al.*, 1989), rye (Lu *et al.*, 1984), sorghum (MacKinnon *et al.*, 1986), digitaria grass (Gonzales and Franks, 1987; Watt *et al.*, 1989) and *Pennisetum spp.* (Wang and Vasil, 1982; Botti and Vasil, 1983; Chandler and Vasil, 1984).

Although most graminaceous monocotyledons regenerate via the process of somatic embryogenesis, there are some monocotyledon tissue culture systems where plant regeneration via both somatic embryogenesis and organogenesis has been observed. For example, both embryogenic callus and shoot buds were produced from callus cultures of maize (Lowe *et al.*, 1985; Wooodward, 1989) and sorghum (Wernicke *et al.*, 1982).

2.2.2. FACTORS AFFECTING CELL AND TISSUE CULTURE SYSTEMS

Selection of explants

Callus cultures of graminaceous species have been obtained from a variety of explants including immature embryos, young inflorescences, young leaves, roots and anthers (Vasil and Vasil, 1984; Rout and Sarma, 1991). In embryos, callus originates from peripheral cells in the scutellum, in inflorescences from the floral meristems or from peripheral tissue around vascular bundles, and in leaves from cells of the lower epidermis and mesophyll near vascular bundles (Botti and Vasil, 1983; Vasil and Vasil, 1984; Vasil, 1987). The developmental and physiological stage of graminaceous explants is critical in the establishment of totipotent cultures, and there seems to be a brief period of time

during which embryos, inflorencences and leaves have the competence to form embryogenic cultures (Lu and Vasil, 1982; Botti and Vasil, 1983; Ho and Vasil, 1983a). At this stage selected cells in the explants are meristematic, only partially differentiated and not fully committed to specialised functions. Hence, explants obtained before or after this stage form only non-morphogenic or non-embryogenic cells (Guiderdoni and Demarly, 1988).

In sugarcane there appears to be a close relationship between the state of differentiation of the excised region and the subsequent production of callus, with nodular embryogenic calli being obtained from the bases of fast-growing leaves, while more mature parts of older leaves produced only friable calli (Guiderdoni and Demarly, 1988). The age of the plant does not seem to be as important, as young leaves obtained from mature sugarcane plants have been used successfully for the initiation of callus cultures (Ho and Vasil, 1983a), and this appears to occur in most of the Gramineae (Vasil, 1987; Bhaskaran and Smith, 1990).

The two main explant-sources for the isolation of protoplasts, leaves and suspension cultures, both provide yields of viable protoplasts, but with the former explant source, the search for a subsequent suitable culture method suitable for plant regeneration, has been elusive in the Gramineae (section 2.2.5).

Sterilisation procedures and maintenance of aseptic cultures

Once a tissue, or part of a plant, has been selected for explantation, it has to be excised, disinfected and transferred to a nutrient medium under aseptic conditions. In most cases, a surface-sterilisation procedure is sufficient to remove all surface contaminants. Sterilising agents include sodium hypochlorite, mercuric chloride, silver nitrate, hydrogen peroxide and calcium hypochlorite (reviewed by Constable, 1984). Generally standard surface-sterilisation procedures are followed, for example, plant material is dipped into 70% ethanol, then immersed

in a solution containing a sterilising agent such as sodium hypochlorite, followed by thorough rinsing.

Despite surface-sterilisation procedures, contamination of media on which explants were cultured has been observed, possibly due to 'latent' or endogenous contaminants in the plant material (Leifert and Waites, 1990). The use of antimicrobial agents may overcome *in vitro* contamination problems, especially those contaminants which are difficult to eradicate using sterile techniques only. To be effective, the ideal antimicrobial agent should eradicate contaminants on plant-tissue culture media, have a non-toxic effect on plant cells and have a broad spectrum of activity (Falkiner, 1990).

Pollock *et al.* (1983) investigated the effect of a range of 20 antibiotics against protoplast-derived cells from *Nicotiana plumbaginifolia*. They found that the least toxic antibiotics were the betalactams, such as ampicillin or carbenicillin, which provide a broad spectrum of bacteriocidal activity, and cephalosporins such as cefoxitin and cefotaxime were also recommended. Doses of up to 100µg/ml of each of these antibiotics can be used without apparent ill effect. A combination of the antibiotics cefotaxime, rifampicin, tetracycline and polymixin B in a 'cocktail' mixture has been successful in eradication of bacterial contamination of shoot cultures in apple, walnut, plum, rhododendron and pear (Young *et al.*, 1984).

Yeasts have also been described as contaminants in some plant-tissue cultures, and the most frequently isolated yeast species were *Candida* and *Rhodotorula* (pink yeasts) species (Boxus and Terzi, 1988; Enjalric *et al.*, 1988; Leggatt *et al.*, 1988). These belong to the group of osmophilic yeasts which show high sugar and salt tolerance, and hence are well adapted to grow in plant-growth media and in some plant material such as sugarcane stalks which contain approximately 14% (w/v) sucrose (Alexander, 1985). This, together with their eukaryotic nature, makes yeast contamination very difficult or impossible to eradicate. Successful elimination

of yeast cells from *in vitro* culture was achieved by Shields *et al.* (1984), who tested a number of fungicides and found clotrimazole, miconazole, griseofulvin and fenbendazole to be the most effective against yeast cells, without harming the tissue.

The activity of antimicrobial agents lasts for approximately 48 hours, and so replacement with fresh solutions after this time is recommended (Falkiner, 1990). Antimicrobial substances can be incorporated into liquid or solid culture media. It is necessary to determine conditions which best suit the particular explant and type of media being used.

Composition of the culture medium

Tissues of the Gramineae have been cultured *in vitro* on a range of media, including B5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972), MS (Murashige and Skoog, 1962) and that of Skoog and Miller (1957). These media contain high levels of macro and mico-elements, vitamins, carbon and nitrogen sources, to promote cell proliferation. White's medium (White, 1963) which contains less salts, may be better for suspension cultures where no differentiation of cells is required. A key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate which is thought to be important in embryo formation (Sharp *et al.*, 1980).

The synthetic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is the most commonly used growth regulator for the induction and maintenance of embryogenic callus in graminaceous plants, and concentrations of 0,5-3mg/l have been found to be satisfactory (Abe and Futsuhara, 1985; Bregitzer *et al.*,1989; Yao and Krikorian, 1981). A few other auxins have been used to obtain embryogenesis with greater than or equal success to 2,4-D, for example, Dicamba (2-methoxy-3,6-dichlorobenzoic acid) (Duncan *et al.*, 1985) and CPA (4-chlorophenoxyacetic acid) have been used in maize cultures (Green and Phillips, 1975) and picloram in sugarcane cultures (Fitch and Moore, 1990). 2,4-D is the only

hormone required for somatic embryogenic cultures of sugarcane (Heinz and Mee, 1969; Ho and Vasil, 1983a; Srinivasan and Vasil, 1986). A two-stage production of somatic embryos is common among systems using 2,4-D as the auxin (Sharp *et al.*, 1980). This involves transferring the somatic embryos which were produced on media containing 2,4-D, to media without 2,4-D (Nadar *et al.*, 1978), to promote germination of the embryoids.

The role of cytokinins in conferring competence to regenerate the Gramineae is not clear. Initiation of embryogenic callus from shoot meristem cultures of sorghum required 2,4-D and low levels of kinetin (Bhaskaran and Smith, 1989), but cytokinin alone prevented embryo formation in sorghum callus cultures (Wernicke and Brettell, 1980). In rice, callus cultures initiated on 2,4-D regenerated plants when transferred to a hormone-free medium, while other workers found the addition of a cytokinin in the regeneration medium necessary for shoot formation (Ling *et al.*,1983; Abe and Futsuhara, 1985).

Hormones used in the establishment of *in vitro* systems other than somatic embryogenic cultures, are giberellins for shoot-tip culture and rooting, kinetin and benzylaminopurine for shoot proliferation, indolebutyric acid for root formation, and napthaleneacetic acid for shoot production (Grisham and Bourg, 1989). In liquid culture media employed for suspension cultures, 2,4-D is the hormone most commonly used for graminaceous plant species, and the concentrations used are critical since at low concentrations (0,5-1,5mg/l) root proliferation occurs, and at high concentrations (3-4mg/l) calli do not break up and proliferate (Wernicke and Brettell, 1982; Vasil and Vasil, 1984; Abe and Futsuhara, 1985).

Many different types and concentrations of sugars have been used as carbohydrate sources for tissue culture of the Gramineae. Sucrose appears to be the most effective carbon source for callus growth and for the production of somatic embryos (Sheridan, 1975). Sucrose requirements in sugarcane vary from 2-6% (Ho and Vasil,

1983a). The inclusion of coconut milk (5-10%) and casein hydrolysate (100-500mg/l) have often been found to be helpful, but not essential, during the initial phase of callus induction (Ho and Vasil, 1983a; Chandler and Vasil, 1984; Lu *et al.*, 1984). Similar media are employed for suspension cultures, and manipulation of auxin concentrations can alter the synchrony of the culture, which is important in micropropagation operations (Lindsey and Jones, 1989).

The addition of activated charcoal to the medium has proven useful for somatic embryo development in many cultures, including the date palm, *Phoenix dactylifera* (Tisserat, 1985). Activated charcoal has been shown to adsorb substantial amounts of auxins and cytokinins (Ebert and Taylor, 1990), as well as 5-hydroxymethylfurfural, an inhibitor formed by sucrose degradation during autoclaving (Weatherhead *et al.*, 1978). The beneficial effects of activated charcoal are thought to be due to its adsorption of inhibitors that could prevent growth as well as reducing the level of growth promotors that inhibit embryo germination (Ammirato, 1983).

Media used for the isolation and culture of protoplasts include those of Kao and Michayluk (1975), B5 (Gamborg *et al.*, 1968), and the MS medium. Generally the same osmoticum and high-calcium conditions used in the protoplast isolation medium are retained in the culture medium (Evans and Bravo, 1983). Glucose may be included as a carbon source and as an osmotic stabiliser, until after the cell wall regenerates (Fitter and Krikorian, 1983). Culture of protoplasts occurs in either liquid media or in agar, and cell colonies can be transferred to a variety of culture media for the development and differentiation of plantlets (Lu *et al.*, 1981; Chen and Shih, 1983; Vasil, 1983).

Light regimes in culture

Light regimes in culture vary, with maize callus growing equally well under conditions of alternating light and dark or in

continuous light (Sheridan, 1975), whereas rice callus was better grown in the dark, and subsequent plant regeneration occurred in the light (Abe and Futsuhara, 1985). In sugarcane, regeneration via somatic embryogenesis occurred when callus was incubated in the dark, as opposed to regeneration via organogenesis, when cultures were incubated in continuous light (Liu *et al.*, 1984; Chen *et al.*, 1988a).

Light also appears to influence protoplast culture, and diffuse light seems to be the most popular choice for the culture of protoplasts in the Gramineae (Vasil and Vasil, 1984). Suspension cultures have commonly been incubated in the dark for sugarcane (Ho and Vasil, 1983b), rice (Abdullah *et al.*, 1986) and wheat (Vasil *et al.*, 1990a, 1990b).

2.2.3. REGENERATION OF PLANTS FROM SOMATIC EMBRYOS

Formation and identification of embryogenic cells and structures

Regardless of the explant used (embryo, leaf or inflorescence) to initiate a culture, the first cell divisions often start near developing procambial or vascular tissues (Lu *et al.*, 1982; Vasil, 1982; Guiderdoni and Demarly, 1988). This may be related to the presence of meristematic cells as well as high levels of plant-growth regulators and nutrients in such regions.

During the initial period of culture of the explant in the presence of 2,4-D, embryogenic competence appeared to be conferred on or expressed by only a few cells at specific sites (Vasil, 1987). Thereafter, maintenance of adequate levels of 2,4-D seemed to help perpetuate the embryogenic nature of the cultures, and organisation of somatic embryoid results when 2,4-D levels are lowered (Vasil, 1987; Guiderdoni and Demarly, 1988). Embryogenic calli are characteristically compact with nodular regions and white to pale yellow in colour (Ammirato, 1983; Ho and Vasil, 1983a). Embryogenic calli are often surrounded by a yellowish, friable and translucent non-embryogenic callus and sometimes

pockets of embryogenic cells are randomly distributed within the friable non-embryogenic callus (Bartkowick, 1981; Vasil and Vasil, 1982; Botti and Vasil, 1983). However, soft, friable, but highly embryogenic callus cultures have recently been described in wheat (Vasil *et al.*, 1990a; Vasil *et al.*, 1990b).

Embryogenic cells are easily recognised by their small, thin-walled, tightly-packed, round and densely-cytoplasmic nature, usually with large nuclei which divide frequently (Vasil *et al.*, 1990a; Vasil *et al.*, 1990b). In contrast, the cells of non-embryogenic calli are large, tubular, elongated and vacuolate (Vasil and Vasil, 1982; Nabors *et al.*, 1983). Identification, visual selection and preferential culture of the embryogenic callus at an early stage, are critical in retaining the long term morphogenetic potential of such cultures, and to ensure that plant regeneration is from somatic embryos only (Lu and Vasil, 1982; Vasil and Vasil, 1982; Wang and Vasil, 1982; MacKinnon *et al.*, 1986). The importance of this can be illustrated in maize where regeneration via embryogenesis and organogenesis can occur from the same piece of callus (Woodward, 1989), which may raise questions about the genetic variability of regenerated plants.

Embryo germination

Embryo maturation and germination occurs in the Gramineae only when 2,4-D levels are decreased. Calli can either be placed onto media containing lower 2,4-D levels or left on media containing 2,4-D for a long time-period, so that there is no remaining 2,4-D in the medium (Nadar *et al.*, 1978; Bhaskaran and Smith, 1989).

Within a population of somatic embryos that appear identical to zygotic embryos in all of the traditional globular, heart and torpedo stages (Hammatt and Dacey, 1987), many abnormal forms have been found (Fig. 2.3) (Ammirato, 1987). The abnormal somatic embryos may bear additional and smaller embryos along the axis and this could possibly be a major cause of lack of synchrony in culture. In addition to single embryos, twins, triplets and

highly multiple clusters of embryos can form and these generate multiple shoot plantlets (Ammirato, 1987). Cotyledon and shoot apex development may also be affected and result in the growth of malformed somatic embryos or they may abort prior to plant development. By manipulating the way that somatic embryos are grown, their structure and behaviour can be modified so that the majority develop normally (Ammirato, 1983; Armstrong and Green, 1985; Karlsson and Vasil, 1986).

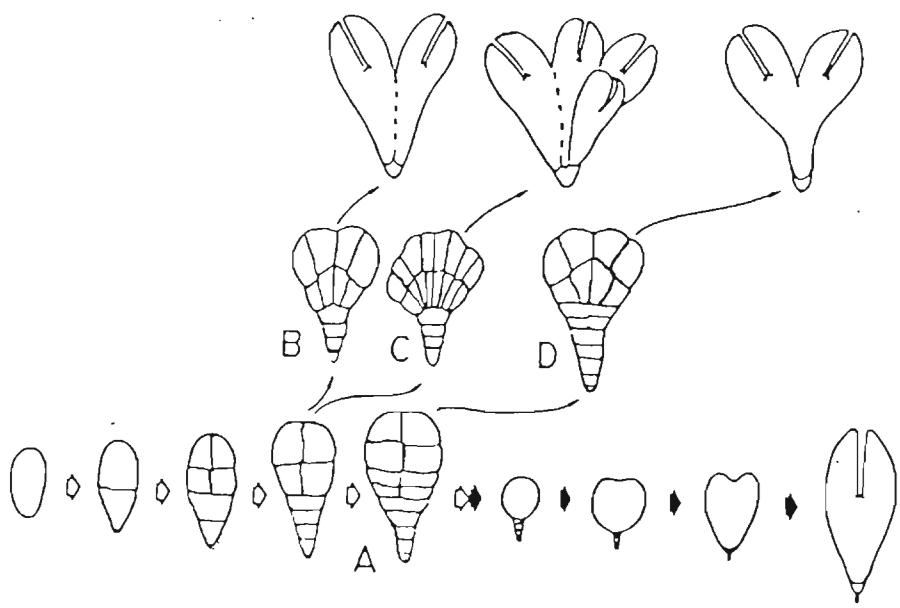


Fig. 2.3: The early stages of somatic proembryo development. A, single proembryo passes through all the typical stages; B, additional divisions in the proembryo, which create two growth-centres that mature into two complete embryos joined at the base; C, more than two centres generate clusters of multiple embryos; D, additional cell divisions and enlargement of apical region results in two shoots, each with cotyledons, on one hypocotyl and radicle. (reproduced from Ammirato, 1987).

Liquid suspension cultures also provide a source of somatic embryos, which germinate to form plants under appropriate conditions (Fig. 2.1) and this is potentially an ideal system for micropropagation purposes (Conger *et al.* 1988). Cell suspension cultures are discussed in more detail in section 2.2.4.

Examples of plants successfully produced via somatic embryogenesis from embryogenic callus in the Gramineae include sugarcane (Nickell, 1964; Heinz and Mee, 1969; Liu and Chen, 1974; Liu *et al.*, 1982; Ho and Vasil, 1983a; Chen *et al.*, 1988a), maize (Lu *et al.*, 1982; Lu *et al.*, 1983; Kamo *et al.*, 1985; Kamo and Hodges, 1986), rice (Abe and Futsuhara, 1985), wheat (Shimada and Yamada, 1979; Karp and Maddock, 1984; Vasil *et al.*, 1990a, 1990b), and napier grass (Vasil and Vasil, 1982; Wang and Vasil, 1982; Botti and Vasil, 1983).

2.2.4. CELL SUSPENSION CULTURES

Suspension culture initiation and maintenance

Cell suspension cultures are most commonly obtained by dispersing friable callus in liquid culture medium, and agitating on a rotary shaker, although they can also be initiated directly from explants (Collin and Dix, 1990). These cultures provide a means of rapid large-scale clonal propagation, they can be used for the isolation of variant or mutant lines, and as a source for the isolation of protoplasts (Lindsey and Jones, 1989).

Although morphogenically-competent cell suspension cultures of Gramineae are difficult to obtain, they have been established in sugarcane (Maretzki and Nickell, 1973; Chen and Shih, 1983; Ho and Vasil, 1983b; Chen *et al.*, 1988b; Liu and Shih, 1986; Srinivasan and Vasil, 1986), wheat (Vasil *et al.*, 1990a, 1990b), maize (Bartowick, 1981; Armstrong and Green, 1985; Kamo and Hodges, 1986), rice (Abe and Futsuhara, 1985), guinea grass (Karlsson and Vasil, 1986), *Digitaria* (Gonzales and Franks, 1987), and napier grass (Lu *et al.*, 1981). In most of these cases embryogenic calli

have been used to establish long-term suspension cultures, from which cells were capable of forming somatic embryos and regenerating into plants.

Suspension cultures consist of small, richly-cytoplasmic, and starch-filled embryogenic cells, present in small groups (Vasil *et al.*, 1990a). As with callus cultures (section 2.2.3), early identification of embryogenic cells, and manipulation of the cultures, ensures the predominance of totipotent embryogenic cells (Vasil and Vasil, 1984; Vasil *et al.*, 1990b). Determination of the growth-rate of suspension cultures has been achieved in a number of ways, for example packed cell volume, fresh and dry cell weights, protein content and cell counts (Ho and Vasil, 1983b; Ryu *et al.*, 1990). Such determinations have been found necessary to determine optimal intervals between subculture and to monitor the state of synchrony and homogeneity of the cells (Ryu *et al.*, 1990). Sieving or filtration of the suspension cultures have been suggested as means to aid selection of the smallest (and presumably single, normal) pro-embryos for transfer to allow for a greater degree of synchrony of maturation of somatic embryo populations (Vasil and Vasil, 1982; Ammirato, 1987).

According to Lindsey and Yeoman (1983), the division rates of suspension culture cells at the exponential phase are typically higher than those of callus cells, but doubling times are slow in comparison with those of microbial cells, and are usually in the range of 24 to 72 hours. There appears to be a critical initial cell density, below which cells transferred to a new medium (liquid or solid) may fail to divide (Ryu *et al.*, 1990). Factors which have been found to influence the minimum size of the inoculum include the culture's physiological characters, the length of time and conditions under which the culture was maintained, and the composition of the fresh medium (Collin and Dix, 1990). Once established, the suspension cell culture proceeds as a series of growth cycles characterised by a sigmoidal nature comprising a lag, cell division, and stationary phase (Ryu *et al.*, 1990).

Somatic embryos in sugarcane up to the globular or early scutellar stage may form in suspension (Liu and Shih, 1986) or the embryogenic cells may be removed from the suspension culture and plated onto solid media for regeneration purposes (Vasil and Vasil, 1982; Ho and Vasil, 1983b). Alternatively, fully mature somatic embryos form in liquid media without plating onto solid media, and although this occurs routinely in a number of herbaceous dicotyledonous plants, *Dactylis glomerata* is the only Graminaceous species in which embryo development has progressed beyond the proembryo stage in liquid culture (Conger *et al.*, 1988).

Plant regeneration from suspension culture cells

Regeneration from suspension cultures of Gramineae has been difficult to achieve and in the first such report only albino plants of *Bromus inermis* were recovered (Gamborg *et al.*, 1970). More recently, embryogenic cell suspension cultures capable of plant regeneration via embryoid formation have been described in some graminaceous plants such as pennesetum (Vasil and Vasil 1982), sugarcane (Ho and Vasil 1983b), maize (Kamo and Hodges 1986; Mitchell and Petolino, 1991), and crabgrass (Gonzales and Franks, 1987).

2.2.5. PROTOPLAST ISOLATION AND CULTURE

Protoplast isolation and purification

Cocking (1960) first used enzymes to release plant protoplasts by applying an extract of hydrolytic enzymes to tomato root tips. Since then the principle of enzymatic cell wall digestion has been applied to plants from a wide taxonomic range, to degrade the three primary components of cell walls, cellulose, hemicellulose and pectin (Evans and Bravo, 1983). The enzymes used are commercially prepared fungal cellulases, pectinases and hemicellulases (Power and Cocking, 1970; Schenk and Hildebrandt,

1971; Pelcher *et al.*, 1974; Vasil and Vasil, 1984).

It is essential that protoplasts are released into an osmotically-balanced medium after the removal of the cell wall, otherwise they will burst (Kao and Michayluk, 1975). Appropriate osmotica consisting of various sugars and sugar alcohols have been added to the isolation solutions to create a hypotonic medium, so that protoplasts do not burst when released into the isolation medium (Evans and Bravo, 1983). Commonly used osmotica in sugarcane include mannitol and sorbitol (Srinivasan and Vasil, 1986; Chen *et al.*, 1988b).

In addition to osmotic strength, other conditions during protoplast isolation can be varied to optimise viability. Calcium and magnesium, in the form of chloride salts may be added to the isolation solution to increase membrane stability of protoplasts (Gamborg *et al.*, 1981). Protoplasts may be isolated in the dark or at low light intensity to avoid starch accumulation, and the pH of the isolation medium is usually between pH 5 and 6, to allow for both enzyme activity and stability of isolated protoplasts (Gill *et al.*, 1981). Gentle shaking can facilitate protoplast isolation by bringing fresh enzyme in contact with cell walls and also by providing a physical force which might cause protoplasts to be discharged from digested tissue, although excessive shaking can result in protoplast destruction (Kao and Michayluk, 1975; Fritter and Krikorian, 1983).

Following enzyme treatment, a mixture of undigested cells, components of broken or burst cells and protoplasts is obtained, which should be purified to remove protoplasts from the enzyme solution (Power and Cocking, 1970; Schenk and Hildebrandt, 1971). Reported purification methods include flotation on dense sucrose solutions (Power and Cocking, 1970), flotation on Ficoll solutions (Schenk and Hildebrandt, 1971), repeated centrifugation and resuspension, and repeated sedimentation without centrifugation (Pelcher *et al.*, 1974).

A number of different methods for determining protoplast viability have been reported and they include the observation of cyclosis as an indication of active metabolism (Pelcher *et al.*, 1974), the exclusion of Evans-blue dye by intact membranes (Kanai and Edwards, 1973), the use of fluorescein diacetate (Larkin, 1976), and photosynthetic activity (Kanai and Edwards, 1973).

Both leaves and suspension cultures have been used as sources for the isolation of protoplasts, though the latter has been more popular in graminaceous species, such as in sugarcane (Maretzki and Nickell, 1973; Larkin, 1982; Chen and Shih, 1983), wheat (Schenk and Hildebrandt, 1971; Okuno and Furusawa, 1977), maize (Rhodes *et al.*, 1988; Shillito *et al.*, 1989), napier grass (Lu *et al.*, 1981; Vasil *et al.*, 1986) and rice (Yamada *et al.*, 1986). The reason for this preference is that protoplasts isolated from embryogenic suspension cultures appear to maintain their totipotency and are capable of regenerating plants (see section below).

Protoplast culture

The osmoticum used for isolation of protoplasts is normally maintained in the culture media, but carbon sources and growth regulators may have to be varied to obtain optimal culturing conditions (Evans and Bravo, 1983). After protoplasts have been purified, the factors that influence culturing are the culture medium (discussed in section 2.2.2) and the plating density (Fitter and Krikorian, 1983; Vasil, 1983) (protoplast density at $1\text{-}4\text{x}10^5$/ml appears to yield maximum cell wall regeneration). Subsequent culturing methods include liquid droplet culture (Kao *et al.*, 1971) and agar culture, with (Cella and Galun, 1980; Shaffler and Koop, 1990) or without (Vasil *et al.*, 1986) a feeder layer. The resulting microcalli colonies are placed on a proliferation medium, and then a regeneration medium to ensure production of callus or plantlets (Vasil *et al.*, 1986; Schaffler and Koop, 1990).

As mentioned previously, the use of mesophyll protoplasts for subsequent plant regeneration has been unsuccessful in the Gramineae. Although Evans *et al.* (1980) and Chen and Liu (1974) reported on colony formation from sugarcane mesophyll protoplasts and Potrykus *et al.* (1977) achieved callus from maize mesophyll protoplasts, plantlet regeneration did not occur. Furthermore, to-date none of these studies have been repeated.

In contrast, the isolation of graminaceous protoplasts from embryogenic suspension cultures and the successful regeneration of plants has been reported for wheat (Shimada and Yamada, 1979; Vasil *et al.*, 1990a, 1990b), rice (Abdullah *et al.*, 1986; Yamada *et al.*, 1986), maize (Shillito *et al.*, 1989; Mitchell and Petolino, 1991), Napier grass (Vasil *et al.*, 1986) and Guinea grass (Lu *et al.*, 1981). However, some graminaceous plant species did not regenerate plants even when embryogenic suspension cultures were used as the source of protoplasts (Lorz *et al.*, 1985; Vasil, 1990). Attempts to recover sugarcane plants from suspension culture protoplasts initially met with little success. Protoplasts isolated from non-morphogenic suspension cultures were found to divide and form either a few small colonies (Larkin, 1982) or calli (Maretzki and Nickell, 1973; Chen and Shih, 1983), but not mature plants. Srinivasan and Vasil (1986) and Chen *et al.* (1988b) isolated sugarcane protoplasts from embryogenic suspension cultures and regenerated plants, but this has not yet been repeated.

2.2.6. HARDENING-OFF AND PLANTING OUT OF REGENERATED PLANTS

Most plantlets derived *in vitro* by organogenesis or somatic embryogenesis, survive and can be grown to maturity after transfer to soil (Vasil and Vasil, 1984; Ziv, 1986). However, never having been exposed to normal environmental conditions, plants produced by tissue culture are accustomed to high humidity levels and aseptic conditions, so they need to be hardened-off with gradual exposure to the environment (Ziv, 1986).

According to reports in the literature, sugarcane and other graminaceous plants are normally transferred to pots containing vermiculite or a mixture of vermiculite and sand (4:1), watered with a nutrient solution of choice, and maintained under a high humidity for about 2 weeks before being transferred to the greenhouse (Liu, 1971; Lu *et al.*, 1982; Lu *et al.*, 1983).

2.2.7. GENETIC STABILITY VERSUS GENETIC VARIATION *IN VITRO*

When plant cells are cultured via some form of unorganised callus phase *in vitro*, the plants that are subsequently regenerated may exhibit various genetic, phenotypic or biochemical charateristics that differ from the parent material. The process whereby such culture-induced variation is generated has been called somaclonal variation (Evans *et al.*, 1984b; Reisch, 1988; Lorz, 1989). The main causes of somaclonal variation are polyploidy, aneuploidy and chromosomal rearrangements (Larkin and Scowcroft, 1981). The major factors affecting the extent of somaclonal variation include the type of explant, medium composition, the time taken to culture the explant and the regeneration pathway (Vasil, 1987; Ammirato, 1989).

Somaclonal variation can serve as a source of breeding for new varieties, but is undesirable if clonal fidelity is required, for example where selected genotypes are being propagated (Ammirato, 1989). Various safe-guards exist to try and minimise unwanted somaclonal variation and these include:

1) selection of an explant which contains meristematic cells and as few differentiated cells as possible, as the latter source of cells could already contain genetic abnormalities and these would be carried over to daughter cells (Vasil, 1987, Ammirato, 1989; Bhaskaran and Smith, 1990);

2) 2,4-D concentrations used for callus induction in the majority of the Gramineae should not be excessively high, so that the rate of cell division is not too rapid for proper repair to be maintained (Evans and Bravo, 1983);

3) exposure to potential mutagenic agents such as colchicine and

ultra-violet or gamma radiation should be avoided (Liu, 1990; Irvine *et al.*, 1991);
4) callus should be kept in culture for as short a time-period as possible to minimise the occurrence of genetic variation (Reisch, 1988);
5) regeneration via indirect somatic embryogenesis, rather than via indirect organogenesis should be encouraged, because somatic embryos arise from single cells which are identical to parent material (Ammirato, 1983; Evans *et al.*, 1984b).

Somaclonal variation has been used to generate agronomically-improved plants in a number of species, and this will be discussed in more detail in section 2.2.8.

2.2.8. POTENTIAL PRACTICAL APPLICATIONS OF TISSUE CULTURE SYSTEMS

In vitro culture has had an impact on conventional plant breeding, particularly in the field of clonal propagation. Further influences on breeding programmes are envisaged using genetic engineering techniques to manipulate plant cells and tissues which subsequently have to be regenerated *in vitro* to obtain transgenic plants. Other potential applications of *in vitro* culture systems in the Gramineae, which will not be discussed below in any detail, include meristem culture for the elimination of plant viruses (reviewed by Evans *et al.*, 1984a; Vasil, 1990), the production of secondary metabolites from plant cells in culture (reviewed by Lindsey, 1986), and the fusion of protoplasts to produce somatic hybrids (Kao, 1977; Ozias-Akins *et al.*, 1986; Power *et al.*, 1986; Tabaeizadeh *et al.*, 1986; Hamill and Cocking, 1988). In the latter instance, very few attempts have been made to obtain somatic hybrids in the Gramineae, because of the difficulties faced in culture and regeneration of grass and cereal protoplasts.

Large scale micropropagation

As discussed previously in section 2.2.7, *in vitro* culture systems that involve an unorganised callus phase may produce variant plants. Thus, in practice, most clonal propagation programmes have made use of: direct organogenesis where axillary buds are multiplied and subsequently rooted; or, direct embryogenesis, where no callus is formed (Fig. 2.1). However, in contrast to herbaceous dicotyledonous plants, many of the Gramineae are propagated vegetatively without any *in vitro* culture being necessary, and this is practically and economically advantageous. Nevertheless, successful micropropagation by direct organogenesis has been reported for a number of forage grasses (Ahloowalia, 1984) and maize (Raman *et al.*, 1980). Sugarcane has been propagated by shoot-tip culture and apical meristem culture (direct organogenesis and direct embryogenesis, respectively) (Grisham and Bourg, 1989). Successful micropropagation via indirect embryogenesis has been reviewed for maize (King and Shimamoto, 1984), oats (Kaur-Sawhney and Galston, 1984) and wheat (Schaeffer *et al.*, 1984).

The synchronous production of somatic embryos in suspension cultures could be used for rapid regeneration, at any time of the year, of a large number of selected genotypes in the Gramineae, especially where somatic embryos are plated out for regeneration purposes; for example bromegrass (Gamborg *et al.*, 1970) and sugarcane (Liu, 1990). Alternatively, entire embryos may form in suspension without the need to plate out, as in *Dactylis glomerata* (orchardgrass) (Conger *et al.*, 1988). Somatic embryos, if protected in some way, could serve as artificial seeds, and be used directly in the field or stored for future use (Ammirato, 1989).

Crop improvement by somaclonal variation

The general principles involved in somaclonal variation have been discussed previously (section 2.2.7). Larkin and Scowcroft (1981) reviewed a number of graminaceous plants (sugarcane, rice, oats, maize and barley) which had been produced by somaclonal variation via callus and protoplast culture. The characteristics displayed by these plants included resistance to diseases, and increases in tiller yield, plant height, fertility and phenotypic variation. Significant improvements among sugarcane variants have been reported, with improved sugar yield (Liu *et al.*, 1984), and increases in stalk number, stalk length, density and weight, percent fibre and improvements in the attitude of the top leaf (Liu and Chen, 1976; Liu, 1990) Anther culture of rice, wheat and maize (Tsay, 1987), resulted in chromosomal changes in plants which are currently being field-tested to determine whether they are superior to plants in commercial production.

Storage of germplasm

The most economical form of storing germplasm for sexually-propagated species is as seeds, but some crops do not produce viable seeds, and some seeds have a limited storage life, as in certain Gramineae. Plant material may be conserved *in vitro* as protoplasts, isolated cells grown in suspension culture, meristem cultures at various stages of development, somatic embryos or organised plantlets (Kartha, 1985; Withers, 1985; Grout, 1990). Plants may be regenerated as required, without risks such as losses due to diseases and pests, associated with maintaining stock plants in the field. The two main approaches for *in vitro* germplasm storage have been slow-growth techniques and cryopreservation.

Various methods have been developed to slow down growth in culture, and these include storage at low temperatures (4-8°C), with a reduced photoperiod, and changing the culture conditions by inducing osmotic stress or adding growth retardants (reviewed by

Kartha, 1985 and Withers, 1985). Somatic embryos of *D. glomerata* (Conger *et al.*, 1988) and wheat have been desiccated, the latter requiring the presence of ABA and sucrose (Carman, 1988), and this has potential for somatic embryogenic storage and perhaps the production of artificial seed.

Cryopreservation is the only approach that completely arrests tissue growth. This occurs at temperatures of -196°C and ensures the maintenance of maximum genetic stability. However most biological material is not resistant to freezing, and it is therefore necessary to develop specific conditions in preparation for freezing, during the freezing and thawing process, and during recovery (Finkle *et al.*, 1985). Cryopreservation has been used to successfully store a wide range of tissue types, including embryos of rice (Bajaj, 1981), wheat and barley (Bajaj, 1985), suspension culture cells in sugarcane (Finkle and Ulrich, 1979), and even protoplasts (reviewed by Bajaj, 1985).

The production of haploid plants

Haploid plants, with gametic chromosome numbers, are particularly useful in plant breeding, both for the rapid production of homozygous lines following chromosome doubling to the original ploidy level (Liu, 1984), for the detection and selection of recessive mutants (Dunwell, 1985) and for genetic transformation purposes (Creissen *et al.*, 1990). *In vitro* techniques can be used for the routine production of haploids and involves the regeneration of plants from cultured anthers, immature pollen or isolated ovules (Vasil, 1990).

Anther-derived haploids in sugarcane were first produced in China (Chen *et al.*, 1979), with only certain genotypes responding to this type of culture. The generation of haploid sugarcane plants has become more streamlined since the discovery that anthers need to be cold pre-treated (Fitch and Moore, 1983; Hinchee *et al.*, 1984). Sugarcane is highly polyploid, so haploids obtained from a single sporophytic development are not likely to carry the basic

haploid number of chromosomes for *Saccharum* spp. Nevertheless, some extent of homozygosity is likely to be achieved by doubling the haploid, and thus plants may become more valuable for conventional plant breeding and for *in vitro* manipulations involving gene cloning.

The majority of the Graminaceous species undergo callus-mediated, haploid plant regeneration. Species capable of haploid plant regeneration, via callus, from cultured anthers include rice (Tsay, 1987), barley (Kasha and Reinbergs, 1980; Creissen *et al.*, 1990), *Datura* sp. (Guha and Maheshwari, 1964) and maize (Sun *et al.*, 1989; Mitchell and Petolino, 1991). Barley anthers in callus culture were transformed by genetic engineering techniques and regenerated plants have produced fertile seed (Creissen *et al.*, 1990). Th e success of this research may encourage the use of haploid culture for future transformation studies in the grasses and cereals.

Regeneration of transformed tissues and/or cells

The potential exists to improve graminaceous monocotyledonous crop plants by the insertion of certain agronomically useful genes which include resistance to herbicides (Comai *et al.*, 1985; Shah *et al.*, 1986; DeBlock *et al.*, 1987; Hinchee *et al.*, 1988; Stalker *et al.*, 1988), insect pests (Fischhoff *et al.*, 1987; Hilder *et al.*, 1989; Vaeck *et al.*, 1987; Herrera *et al.*, 1991; Murray *et al.*, 1991; Vail, 1991), and viruses (Abel *et al.*, 1986; Harrison *et al.*, 1987; Van Dun *et al.*, 1987; Cuozzo *et al.*, 1988; Mayo and Barker, 1990). The availability of an *in vitro* system to allow recovery of plants from transformed cells, tissues and protoplasts is an essential prerequisite to the sucess of any breeding programme that aims to utilise the potential offered by biotechnological advances to crop improvement.

2.3. MATERIALS AND METHODS

2.3.1. *IN VITRO* CULTURE OF SEEDS/ZYGOTIC EMBRYOS

Sugarcane seeds (reference number Y511) were removed from florets, the outer husk and hairs removed (Plate 2.1 A), and the seeds were then surface-sterilised in a sodium hypochlorite solution (0,15%(w/v) for 2mins), followed by three rinses in sterile water. Seeds were aseptically placed into petri-dishes containing a germination medium (Plate 2.1 B), consisting of MS salts and vitamins (Murashige and Skoog, 1962), casein hydrolysate (1g/l) (Sigma, USA), sucrose (4g/l) (Unilab, South Africa), benlate (0,05g/l) (Bayer, SA), and agar (9g/l) (Noble agar-Difco, UK) at pH 5,8, and grown under a 16h light/8h dark photoperiod at 27°C, for 4 days, after which germination and contamination levels were monitored.

2.3.2. PREPARATION OF EXPLANTS FOR *IN VITRO* CALLUS CULTURE

Sugarcane varieties NCo376 and N13 were field grown (Plate 2.2 A) and obtained from the South African Sugar Association Experiment Station, Mount Edgecombe. Stalks (Plate 2.2 B) were cut into single-budded setts, dipped in benlate (0,75g/l) and planted into trays containing autoclaved vermiculite. The setts were grown in the dark for two weeks at 28°C, until the shoots were approximately 15cm long.

The outer leaf sheaths were peeled off the two-week old shoots and the exposed stalk was cut into 1cm long sections (Plate 2.2 C). Each stalk was separately surface-sterilized in ethanol (70% for 1min), followed by a wash in sodium hypochlorite (1,5% (w/v)) plus Tween 80 (0,01% (v/v)) for 20mins, after which it was rinsed three times in sterile distilled water.

Another explant source used was sterile *in vitro* plants which had regenerated from variety N13 via somatic embryogenesis. The stems from these regenerated plants were aseptically removed and were

placed under the same culturing conditions as the surface-sterilised field explants.

2.3.3. GROWTH CONDITIONS FOR CALLUS CULTURE

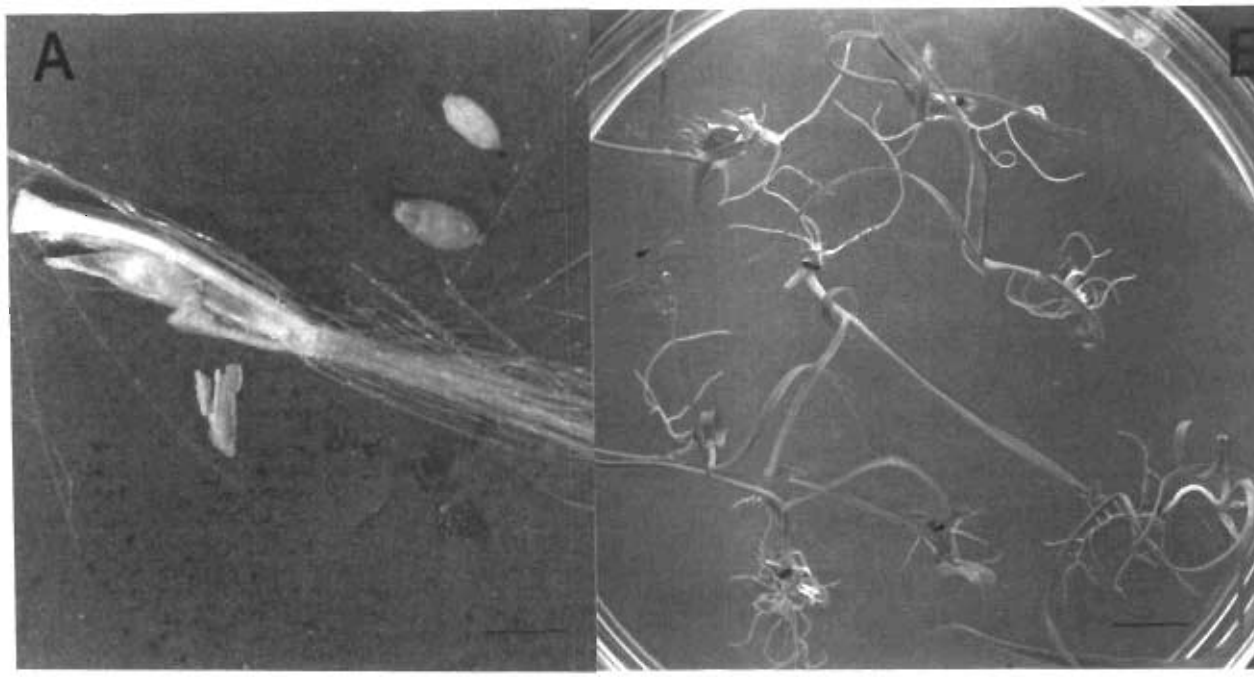
Production of aseptic cultures

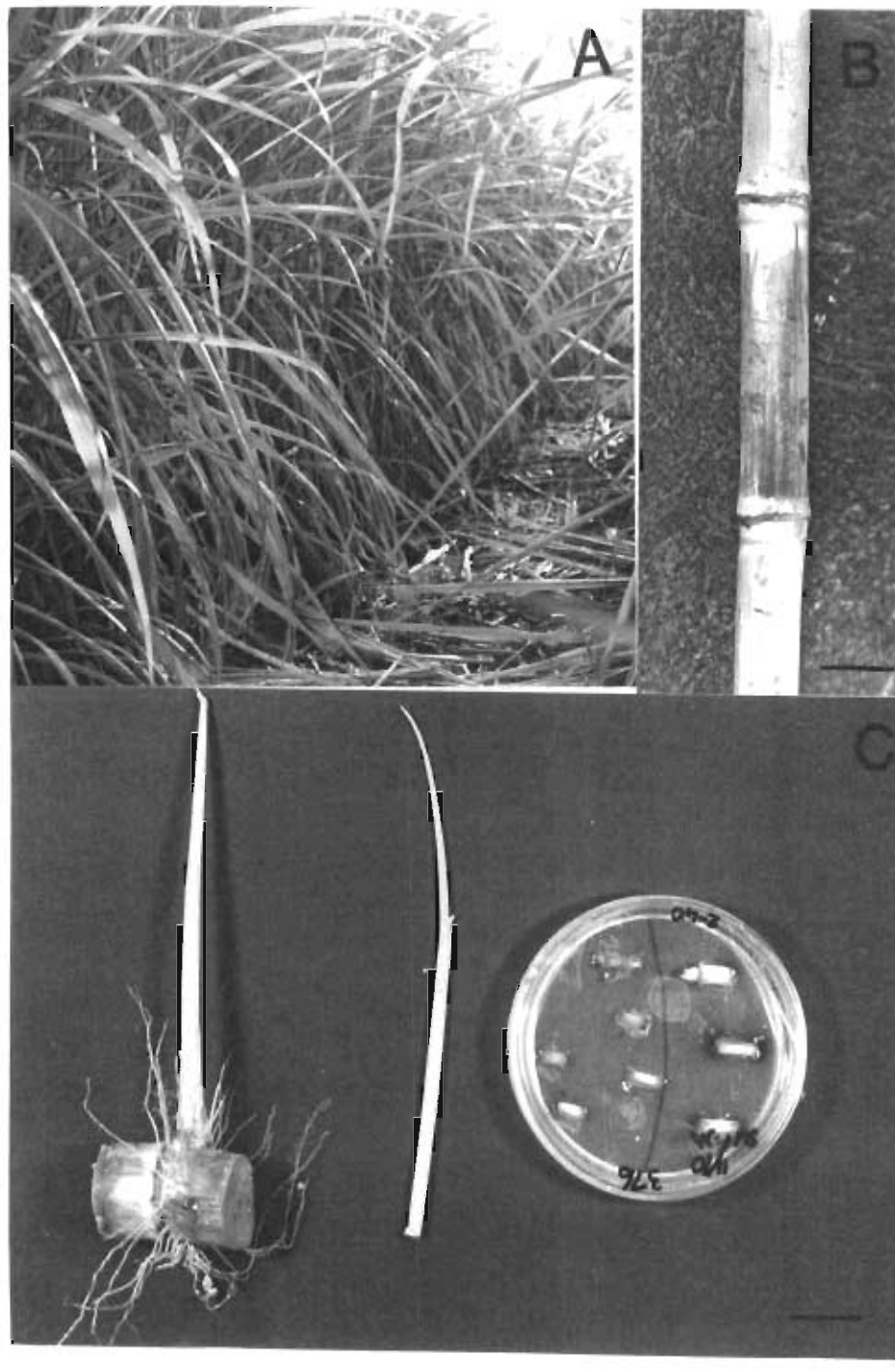
Microbial contamination levels from field-grown material placed in culture were observed to be very high (approximately 65% of the explants were contaminated). Colonies observed to be contaminating the culture media were picked off, Gram stained (Harrigan and McCance, 1966) and observed by light microscopy (Axioskop, Zeiss, Germany). The main contaminant identified was a budding yeast, but there were also Gram positive cocci and rods.

The sensitivity of the contaminants to several antimicrobial agents was tested by means of a zonal inhibition test (Harrigan and McCance,1966). Overnight cultures of the contaminants were spread onto nutrient agar (pH7) (Biolab, South Africa) and MS (pH5,8) plates. Plugs of agar were removed using a number 3 plug-borer, and 0,5ml of each of the antibiotics (at concentrations given below) was filter-sterilised into the wells. Plates were incubated for 24h at 27°C and inhibition zones were measured, from the rim of the well to the outer edge of the inhibition zone, and recorded. From these results, the antimicrobial substances used in an attempt to reduce contamination of field-grown explants were combined in a 'cocktail' mixture. The cocktail, made up in liquid MS medium, contained the antibiotics ampicillin and streptomycin (each at 100mg/l) and rifampicin (30mg/l) and the fungicides clotrimazole and miconazole (each 100mg/l) and nystatin (30mg/l) (Sigma, USA).

Plate 2.1: *In vitro* culture of sugarcane seeds. Sugarcane seeds (zygotic embryos) were 'dehusked', surface-sterilised in sodium hypochlorite (0,15%(w/v)) (A) and germinated *in vitro* (B) on a MS germination medium.
Bars represent A: 2mm
B: 1cm

Plate 2.2: Source of explants for callus induction. Field-grown sugarcane (A) was the explant source for callus induction. The stalks (B) were cut into setts and germinated in the dark, in autoclaved vermiculite for two weeks. The young apical shoots (C) were surface-sterilised and placed onto MS callus initiation medium.
Bars represent B: 5cm
C: 2cm


A
B


A
B
C

Once the field-grown explants had been surface-sterilised (section 2.3.2), they were placed into the MS antibiotic medium (Murashige and Skoog, 1962) for 48h, with shaking. The explants were then aseptically recut, removing 2mm from each end of the 1cm explant, which had been exposed to the surface-sterilants. The explants were aseptically placed on to callus induction medium, and contamination levels and callus production were recorded over a 1 month period. Explants not exposed to antibiotics after surface-sterilisation served as controls.

Callus induction

Stalk explants were aseptically placed on to callus induction medium containing MS salts and vitamins (Murashige and Skoog, 1962), sucrose (20-50g/l), 2,4-D (2-4mg/l) (Sigma, USA), inositol (100mg/l) (Sigma, USA), casein hydrolysate (1g/l) and agar (9g/l). Explants were placed at 27°C in the dark.

2.3.4. PRODUCTION OF SOMATIC EMBRYOGENIC CULTURES

Embryogenic calli were sub-cultured every four weeks on to MS callus induction medium containing 2,4-D (2-4mg/l) and sucrose (20-50g/l). The non-embryogenic areas of the calli were dissected out and discarded.

After 2 months of culture in the dark, most of the calli contained immature embryos, and were sub-cultured onto a regeneration medium of MS salts and vitamins, with sucrose (20-50g/l) and agar (9g/l) at pH5,8, without 2,4-D. In all cases, the regeneration medium contained the same concentration of sucrose as the medium on which the calli were initiated. Cultures were incubated in a 16h light ($200\mu E/m^2/s$)/8h dark photoperiod at 27°C.

Activated charcoal (4g/l) (Unilab, South Africa) was added to the regeneration medium.

2.3.5. HARDENING-OFF AND ACCLIMITISATION OF REGENERATED PLANTS

After 1-2 months of culture on the MS regeneration medium, plantlets (3cm in height) exhibited well-developed root systems, and were transferred to glass tubes containing fresh MS regeneration medium with sucrose at a lower concentration (10g/l). After approximately 2 months (or when they had reached a height of 10cm), the plants were planted out into sterile plastic containers (10x15cm), in autoclaved potting soil. A plastic bag was placed over the plant in the pot to maintain humidity. A gradual decrease in humidity was achieved by opening the bags daily, for increasingly longer periods of time, over a 2 week period.

2.3.6. INITIATION AND MAINTENANCE OF SUSPENSION CULTURES

Four different media were tested to initiate cell suspension cultures (Table 2.1). Cell suspension cultures were initiated by transfer of approximately 1g fresh weight, 2 month-old, friable NCo376 callus (produced as described in section 2.3.3) to 50ml aliquots of medium in 125ml Erlenmyer flasks. The cultures were maintained at 27°C, in the dark, with rotary shaking at 120rev/min. The medium was replaced weekly and aliquots were observed microscopically and monitored for presence of small clumps of embryogenic cells.

After 1 month in culture, the larger pieces of callus were allowed to settle to the base of the container and the lighter, more dispersed cells were transferred to 250ml Erlenmyer flasks, containing a total volume of 100ml of cells and medium. Suspension culture growth-rate was monitored daily over a 2 week period. Samples of 5ml were removed aseptically, and packed cell volume (after centrifugation at 2000g for 5min) was determined.

Table 2.1 : A comparison of the components of four different media recommended for the initiation of suspension cultures of sugarcane.

	Media			
Composition	Ho & Vasil (1983a)	Liu & Shih (1986)	Chen *et al.* (1988b)	Thom *et al.* (1981)
Macroelements	MS	MS	MS	White's basal salts (Nickell and Maretzki, 1969)
Microelements	MS	MS	MS	
Vitamins (µg/l)	MS	MS	MS	thiamine 100 nicotinamide 800 pyridoxine 800
Sucrose(g/l)	30	20	30	18,8
2,4-D(mg/l)	3	3	3	2
Other(mg/l)	casein 500	casein 400 thiamine 900	casein 50 thiamine 900	arginine 57,5 yeast extract 1000

2.3.7. ISOLATION AND CULTURE OF PROTOPLASTS

Protoplasts were isolated from leaves, suspension cultures and calli. Mesophyll protoplasts were isolated from the youngest fully-expanded leaves of N13 hardened-off plants, which were placed in the dark for 24h prior to protoplast isolation. The leaves were cut into squares (0,5cm) and the mid-rib discarded before leaf tissue (1g fresh weight/10ml enzyme solution) was incubated in filter-sterilised enzyme solution. Similarly, 2 month old NCo376 suspension culture cells (2ml settled cell volume/10ml enzyme solution) and 2 month old friable NCo376 or N13 calli (1g/10ml enzyme solution) were used as source material for protoplast isolation.

The protoplast osmoticum contained sorbitol (0,6M), KH_2PO_4 (1mM), $CaCl_2$ (1mM) and $MgCl_2$ (1mM) at pH5,6. Enzymes used for protoplast isolation were cellulase R-10 (10-50g/l) (Onozuka, Japan), macerozyme R10 (1-4g/l) (Onozuka, Japan) and hemicellulase (2,5-10g/l) (Sigma, USA). In order to obtain the optimum enzyme combination for isolation of protoplasts, one enzyme concentration was changed at a time. The enzyme solution containing the protoplast source material was incubated at 26°C in the dark. The protoplast solution was filtered through a series of filters (pore sizes of 1000 and 200μm; Nybond, Switzerland) after 3-6h incubation. The protoplasts were washed in the above-mentioned medium (minus the enzymes; pH7) by centrifugation for 10mins at 100rev/min (MSE Minor, England).

Protoplasts isolated from calli were cultured for 48h at a density of $1x10^5$ protoplasts/ml in MS medium containing 2,4-D (1,5mg/l) and mannitol (0,5M), in the dark, in petri-dishes (3cm in diameter). These were placed on moist filter in larger petri-dishes which in turn, were placed in a plastic container which contained a layer of water at the base.

Protoplast yield was determined by counting protoplasts on a counting chamber (Fuchs-Rosenthal; 0,200mm x 0,0625mm). Viability was assessed by the ability of intact protoplasts to exclude Evan's Blue dye (15g/l) (Larkin, 1976), made up in the osmoticum.

2.3.8. MICROSCOPY

Periodically, small pieces of calli were squashed onto a microscope slide, stained with dilute safranin (Harrigan and McCance, 1966) and viewed by light microscopy to assess the development of embryogenic structures.

2.3.9. STATISTICAL ANALYSES

Results obtained for callus initiation and plant regeneration initiated on media containing a variety of sucrose and 2,4-D concentrations were analysed using an F test, in order to see whether there was an interaction between sucrose and 2,4-D. An F test (Mulder, 1988) was carried out and a regression surface was fitted to the data ($y=a+ b_1x_1+b_2x_1^2 +b_3x_2+b_4x_2^2 +b_5x_1x_2$), and values were weighted to take into account the different precision of points.

The s.e. were calculated by dividing the s.d. by the $\sqrt{n}$. The s.e. obtained were multiplied by values in t-tables, at the 95% confidence level.

2.3.10. PHOTOGRAPHY

Photomicroscopy was undertaken using dissecting and compound light-microscopes.

2.4. RESULTS

2.4.1. PROPAGATION BY MEANS OF ZYGOTIC EMBRYOS/SEEDS

Although sugarcane is propagated vegetatively for commercial purposes, it can also be propagated by true seed. A preliminary investigation involving the uptake of DNA by seeds was undertaken and is described in Chapter 3. To achieve this it was necessary to have seed material that was free of microbial contaminants and which could be cultured *in vitro* in order to monitor DNA expression. Surface-sterilised seeds, placed onto a germination medium, showed 100% germination and no contamination was observed.

2.4.2. CALLUS CULTURE AND PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS IN SOUTH AFRICAN SUGARCANE VARIETIES

Photographic account of plant regeneration via somatic embryogenesis in variety N13

The developmental stages involved in the process of somatic embryogenesis in variety N13, from callus production to regeneration of pantlets from somatic embryos, are shown in Plates 2.3-2.9. It took 11-14 days from the time that the explants were placed on the callus induction medium to the time that callus was first observed. Examination of calli cultured in the dark, showed two distinct regions: the embryogenic regions were compact, nodular and white in colour as opposed to the non-embryogenic ones which were friable and yellowish in colour (Plate 2.3). Microscopic examination of squashed callus revealed that non-embryogenic cells were irregular in shape, larger and less compact than embryogenic cells which occurred in clusters, and appeared to have dense cytoplasmic contents (Plate 2.4). Typical embryogenic heart- (Plate 2.5 A) and torpedo-shaped structures (Plate 2.5 B) were visible toward the end of the second month of incubation in the dark. Embryo germination occurred approximately 14 days to 1 month after dark grown calli were exposed to light (Plate 2.6 A and B). Simultaneous production of roots and shoots occurred on

the regeneration medium (Plate 2.7 A and B). Plantlets 3cm in height with well developed root systems were transferred to tubes containing fresh regeneration medium (Plate 2.8 A and B). Plants were successfully hardened-off and transferred to soil (Plate 2.9 A, B and C).

However not all of the embryos developed normally, and occasionally embryos with clusters of multiple shoots on a single hypocotyl and radicle were observed (Plate 2.10 A and B). Some callus (approximately 2% of the total amount of callus placed in the light) showed precocious root germination when placed in the light (Plate 2.11), and some (approximately 5%) turned black and did not undergo any further development (Plate 2.12).

Antimicrobial treatment of field-derived explants

Although field-grown explants of NCo376 and N13 were surface-sterilised, contamination levels of approximately 65% occurred when explants were placed on the callus induction medium used by Ho and Vasil (1983a). The antimicrobial substances which were found to be successful in eliminating contaminants in many herbaceous plants (Pollock *et al.*, 1983; Shields *et al.*, 1984) were found to be ineffective for reducing contamination levels in sugarcane. Ampicillin, streptomycin, rifampicin, myconazole, clotrimazole and nystatin, when used singly, resulted in no inhibition of microbial growth on agar plates in the zonal inhibition test. A cocktail mixture containing all the above antimicrobial agents resulted in a small zone of inhibition (2mm) when tested on agar plates. However, when the MS-antibiotic cocktail mixture was used on field explants of NCo376 and N13, it did not eliminate contaminants and was found to have an inhibitory effect on callus initiation, with only 6% of explants forming callus, as opposed to 26% of explants forming callus when no antimicrobials were used (Table 2.2). It was therefore decided not to include the antimicrobial treatment of field explants in the protocol of callus induction.

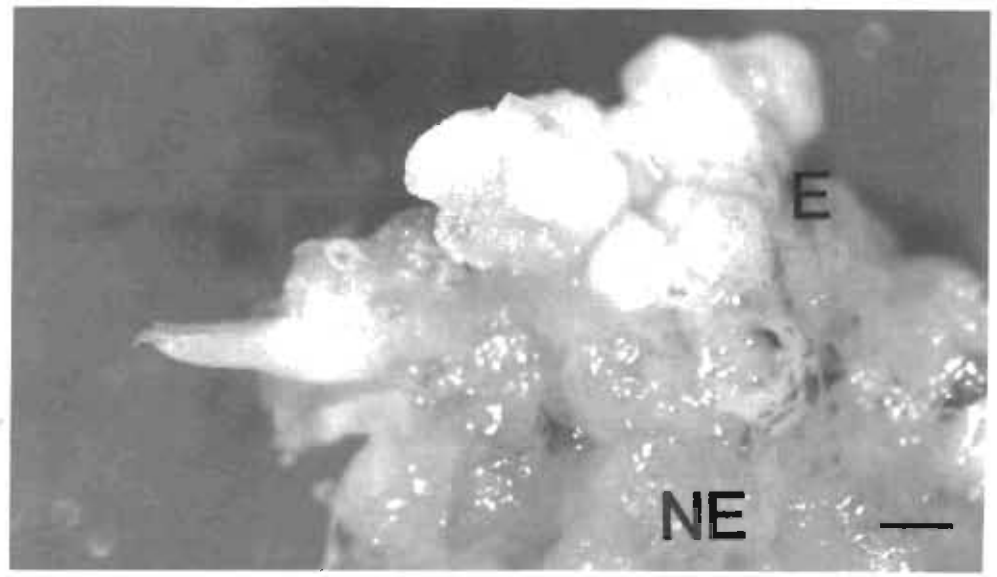


Plate 2.3: Embryogenic and non-embryogenic regions on callus. Embryogenic (E) and non-embryogenic (NE) regions on callus initiated from apical shoots of sugarcane plants.
Bar represents 1mm.

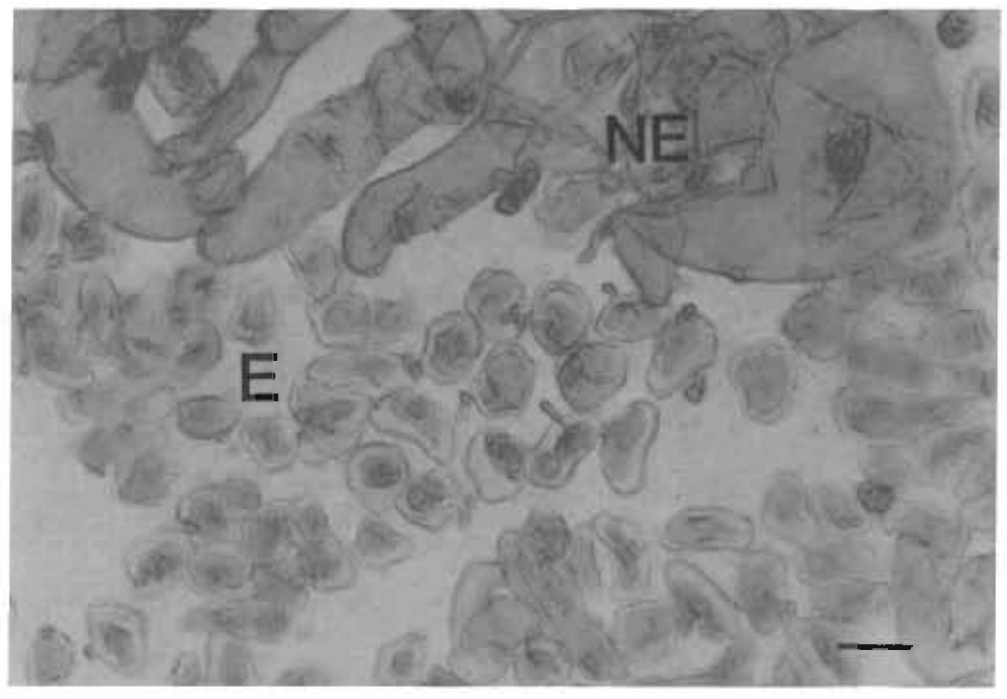


Plate 2.4: Microscopic examination of callus. One month-old callus squashed and stained with safranin exhibited small, dense embryogenic cells (E) and elongated non-embryogenic (NE) cells.
Bar represents 50μm.

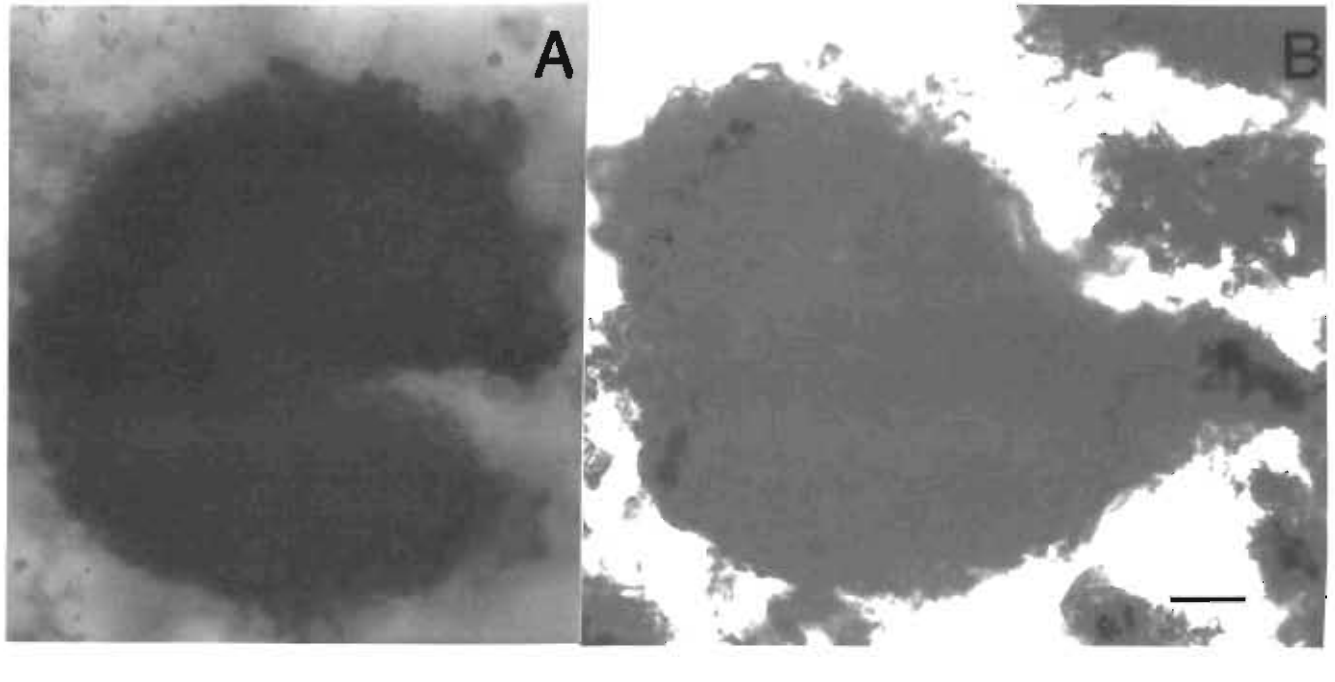


Plate 2.5: Development of pro-embryogenic structures in sugarcane callus. Heart- (A) and torpedo-shaped (B) pro-embryos, characteristic of developing somatic embryos, when 2 month-old callus was squashed, stained with safranin and viewed with a dissecting microscope.
Bars represent 100μm.

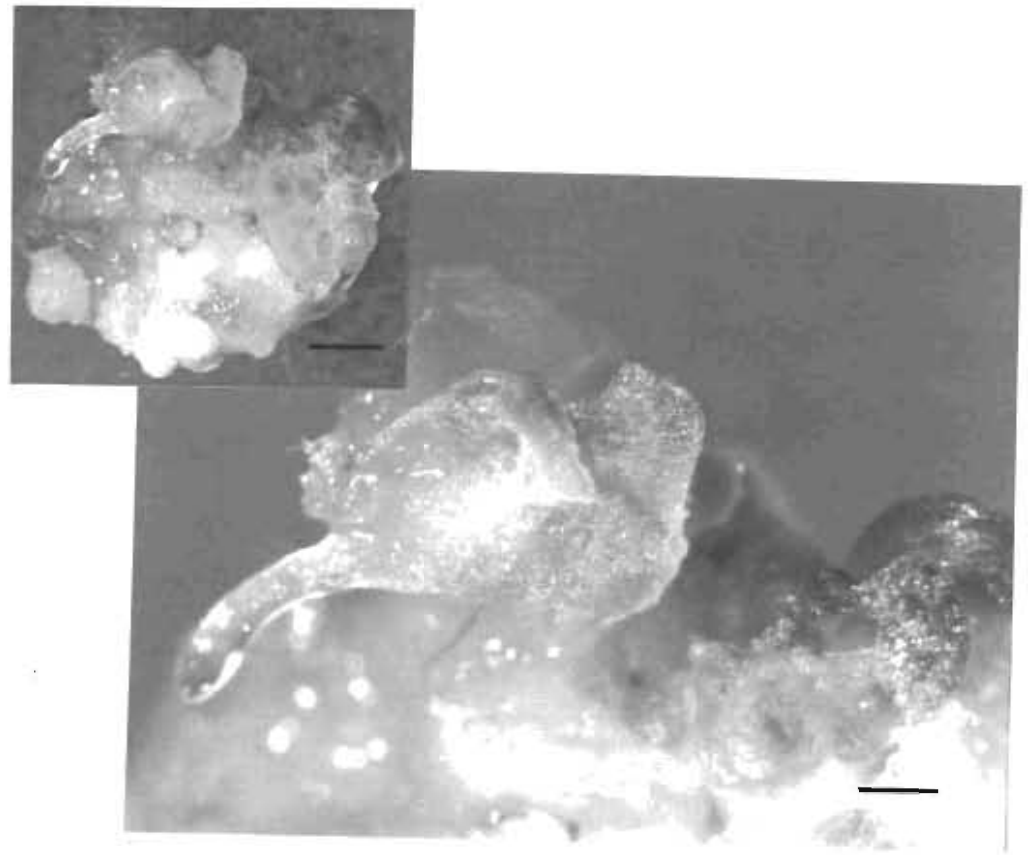


Plate 2.6: Germination of somatic embryos. Callus placed on the MS regeneration medium (without 2,4-D) in a light/dark photoperiod, produced roots and green shoots after 2-4 weeks in culture.
Bars represent (main plate): 2mm
(insert): 30mm

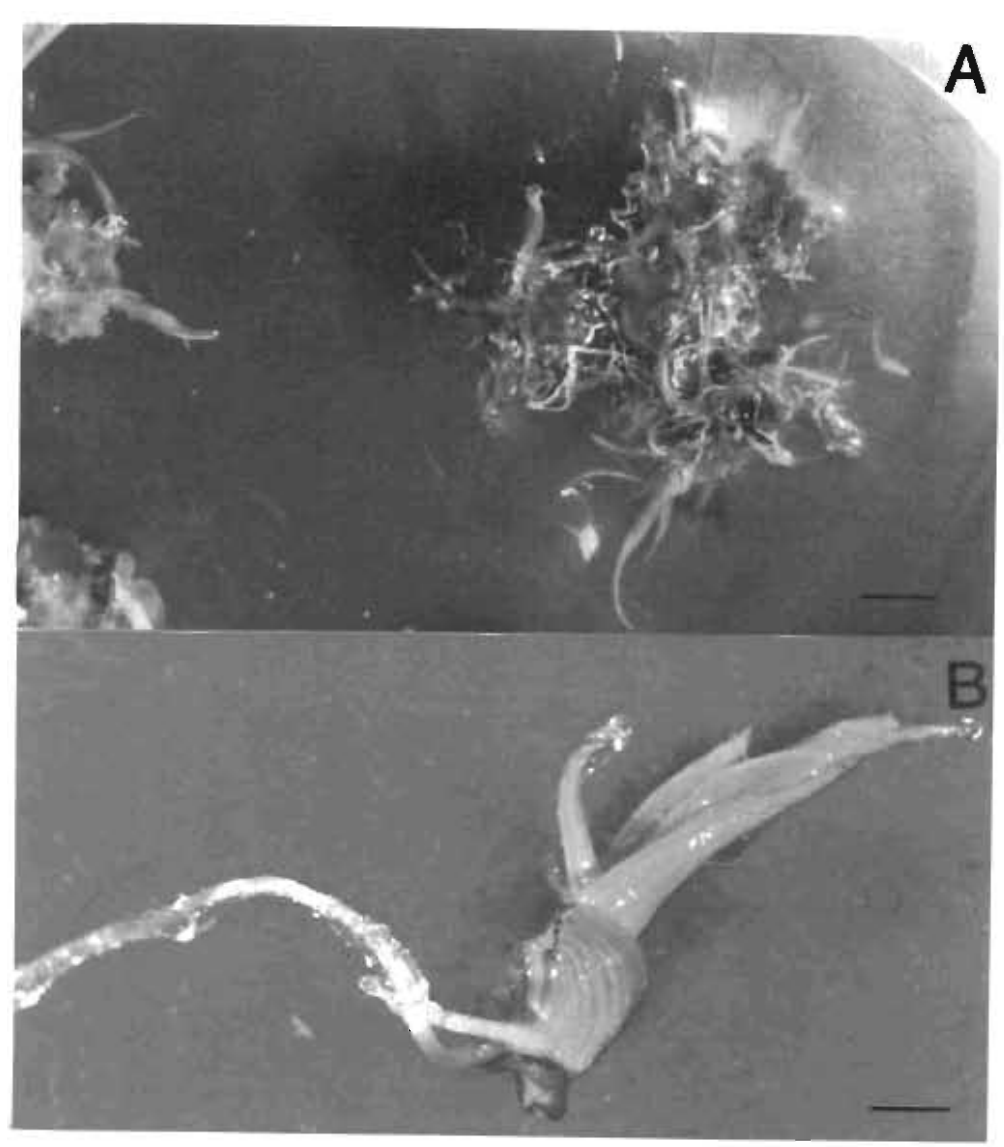


Plate 2.7: Sugarcane plantlets which have been regenerated from callus via somatic embryogenesis. Note the simultaneous production of shoots and roots from embryogenic calli in petri-dishes containing the MS regeneration medium (A), and young plant (B) before being transferred to fresh medium in tubes.
Bars represent A: 5mm
B: 70mm

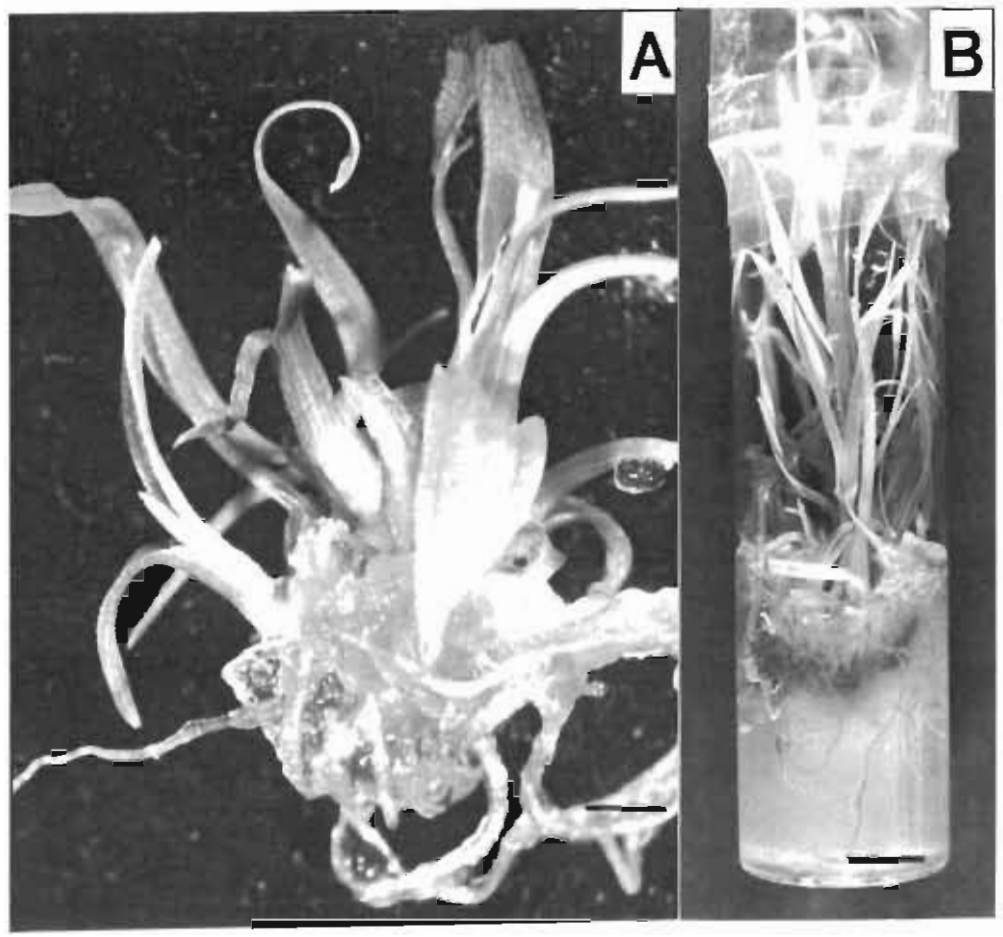


Plate 2.8: Regenerated plants with well-developed shoot and root systems in tubes. Two month-old sugarcane plants with healthy shoots and roots, approximately 3cm in height (A), were transferred to fresh MS medium in tubes (B), containing less sucrose (10g/l).
Bars represent A: 40mm
B: 150mm

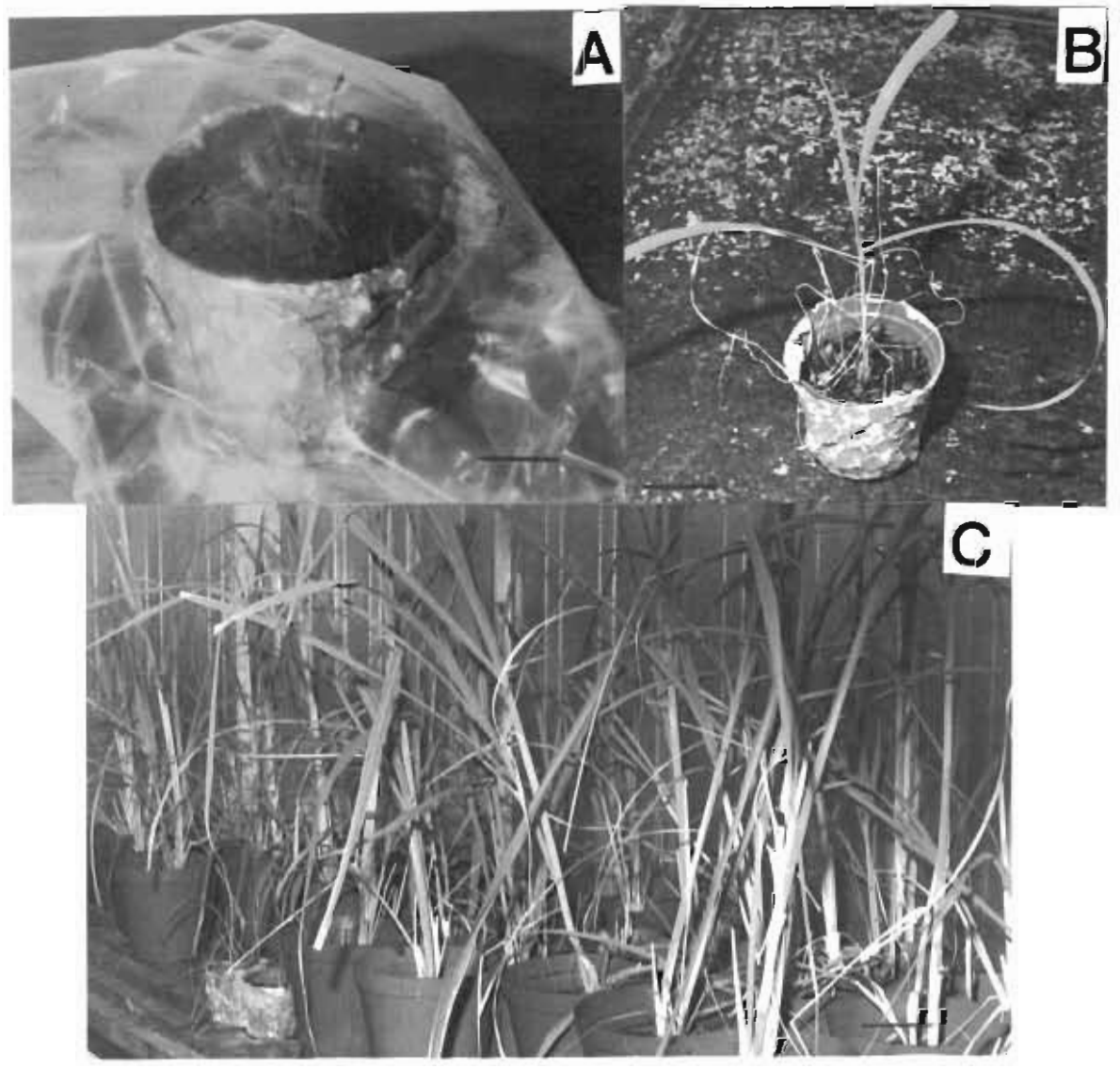


Plate 2.9: Hardened-off sugarcane plants. Regenerated plants were transferred from tubes to pots containing autoclaved potting soil, and were hardened-off by placing pots in plastic bags to maintain high humidity levels (A). The bags were opened for increasing time intervals daily, over a 2 week period. Hardened-off plants were transferred to the glasshouse (B and C).
Bars represent A: 750mm
B: 10cm

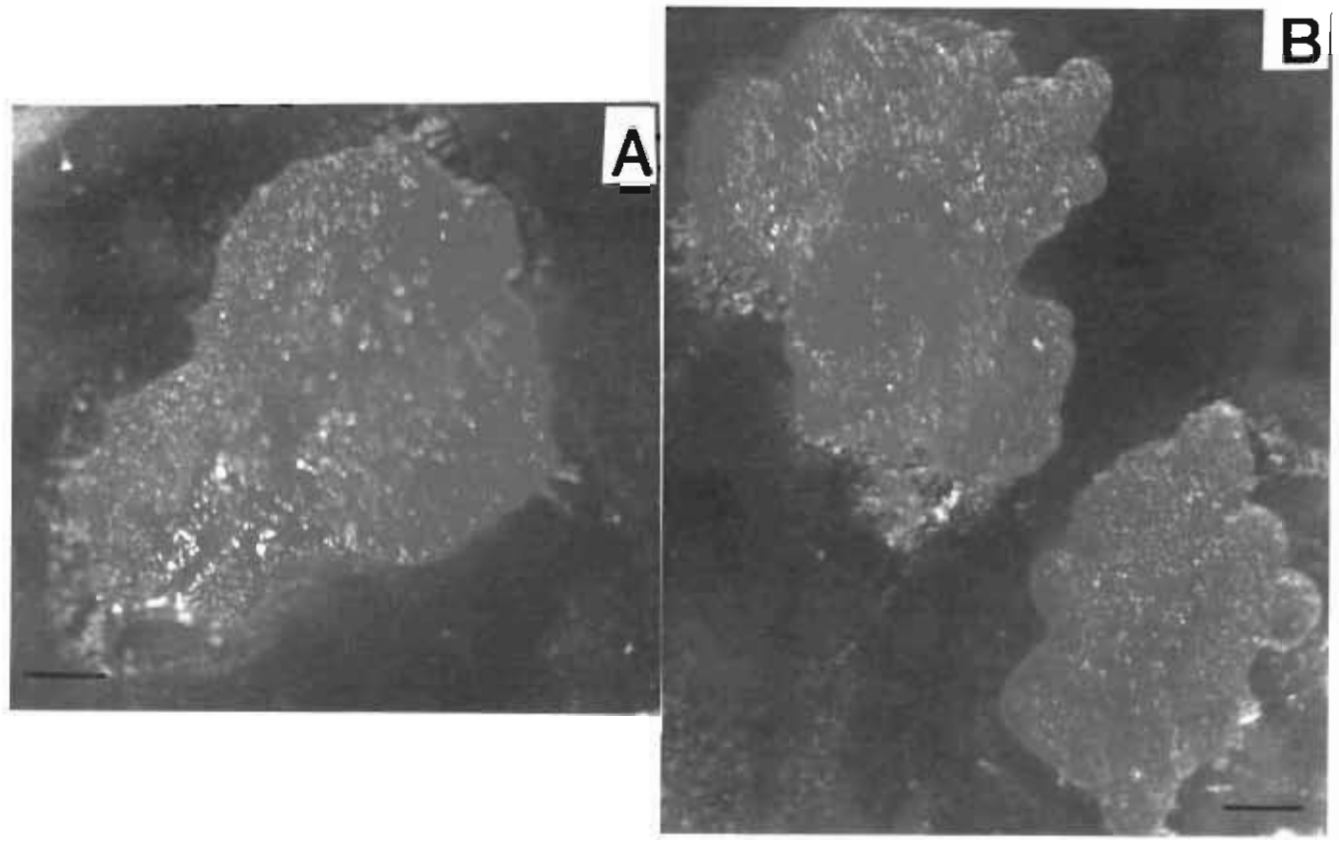


Plate 2.10: The appearance of morphologically abnormal somatic embryos. Normal (A) and abnormal (B) somatic embryos were dissected from 2 month-old callus and stained with safranin. The abnormal embryos had clusters of multiple shoots on a single hypocotyl and radicle.
Bars represent 100μm.

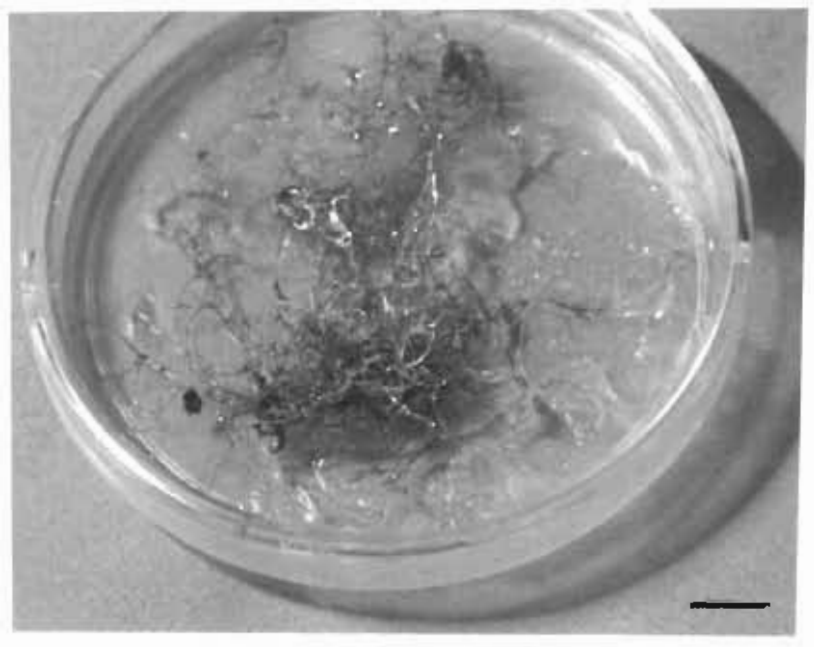


Plate 2.11: Precocious root germination in one month-old calli. Precocious root germination occurred in 2% of calli when placed on the MS regeneration medium in a light/dark photoperiod.
Bar represents 90mm.

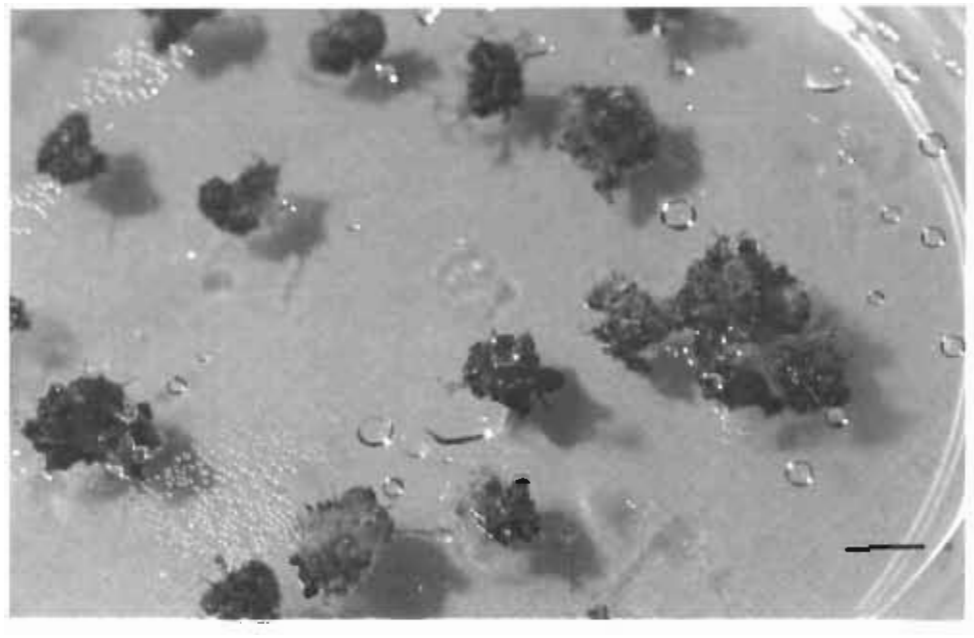


Plate 2.12: Necrosis of calli on regeneration medium. A proportion of the calli (approximately 5%) turned black and did not undergo any further development when placed on the MS regeneration medium in a light/dark photoperiod.
Bar represents 50mm.

Table 2.2: The effect of a cocktail* of antimicrobial substances on contamination levels of NCo376 and N13 explants and callus formation after 1 month in culture (s.e. are at the 0,05 level of probability) (n=6)

Treatment	%contamination	%callus formation
Control	65,5±6	25,7±3,2
Cocktail*	63,6±5,9	5,6±0,7

* cocktail containing ampicillin, streptomycin, myconazole, clotrimazole (each at 100mg/l), rifampicin and nystatin (each at 30mg/l).

Callus was observed on the cut ends of NCo376 and N13 explants, after 11-14 days of culture. Explants were cultured on a MS medium containing 3mg/l 2,4-D and 30g/l sucrose, previously used by Ho and Vasil (1983a) for callus culture of sugarcane. Because of contamination problems, callus was also initiated from stems of N13 regenerated plantlets. Since regenerated plants had been maintained in sterile culture conditions they were free of contaminants, and callus induction on MS medium containing different 2,4-D and sucrose concentrations was carried out using only variety N13.

The effect of sucrose and 2,4-D concentrations on callus induction and plant regeneration

The effect of different sucrose and 2,4-D concentrations on callus induction was investigated for N13 (Table 2.3). The interaction (linear x linear) of the two components was negligible and 2,4-D had little influence on callus formation (no difference between the column means, see Table 2.3). The only major (and statistically significant) effect was sucrose concentration, where callus production was reduced to 33% at a sucrose concentration of 50g/l.

Table 2.3: The effect of a range of 2,4-D and sucrose concentrations on percentage of N13 callus production on the MS medium after 2 months in culture (n=50-90)

sucrose (g/l)	2,4-D (mg/l) 2	3	4	row mean
20	51	43	60	51*
30	54	54	56	55*
50	26	34	38	33*
column mean	44	44	51	

*statistically significant at the 5% level, where the calculated F value > 6,34.

There was no significant difference in the fresh weights of N13 calli grown on different callus induction media for two months (Table 2.4).

Table 2.4: The effect of different 2,4-D and sucrose concentrations on grams fresh weight of N13 calli after 2 months of culture on MS medium (s.e. are at the 0,05 level of probability) (n=7)

sucrose (g/l)	2,4-D(mg/l) 2	3	4
20	0,40±0,25	0,31±0,17	0,38±0,15
30	0,47±0,22	0,38±0,17	0,28±0,1
50	0,31±0,17	0,26±0,17	0,31±0,09

After 2 months of culture in the dark, callus pieces which were observed microscopically to be embryogenic, containing characteristic embryogenic cells and heart-shaped structures, were

placed in the light on a regeneration medium. The regeneration media contained identical sugar concentrations to those used in the induction media. The percentage of N13 calli giving rise to plants is presented in Table 2.5. The greatest number of calli giving rise to plantlets were observed on regeneration media containing 20g/l sucrose (especially from callus initiated on medium containing 2mg/l 2,4-D). There was no statistically significant interaction between sucrose and 2,4-D on the regeneration of plants from calli. However, a significant reduction in the percentage calli producing plantlets occurred at 30 and 50g/l sucrose, when compared to percentage calli producing plants at a sucrose concentration of 20g/l.

Table 2.5: The effect of sucrose levels in the regeneration medium on the percentage of N13 calli which yielded plants (n=9-33)

sucrose (g/l)	2,4-D(mg/l)# 2	3	4	row mean
20	60	47	33	47*
30	33	28	6	22*
50	11	44	19	25*
column mean	35	40	19	

for the purposes of comparison, 2,4-D concentrations on which calli were initiated are included, even though the regeneration medium contained no 2,4-D

* significant effect at the 5% level, where the calculated F value > 5,14

The number of plants produced per gram fresh weight callus was compared, to see whether any particular concentration of sucrose and 2,4-D yielded a greater number of plants (Table 2.6).

Table 2.6: Plantlet yield per gram fresh weight N13 callus grown on regeneration medium for 2 months (s.e. are at the 0,05 level of probability) (n=3-8)

sucrose (g/l)	2,4-D(mg/l)* 2	3	4
20	36±7	18,7±2,3	10,5 ±0,6
30	20,2±4,7	33,8±10	35
50	25,8	16,5±0,8	17,0±3,7

* for the purposes of comparison, 2,4-D levels indicated are those on which calli were initiated as the regeneration medium contained no 2,4-D.

The highest plant yield was produced on regeneration medium containing 20g/l sucrose, but s.e. at the 0,05 level of probability indicated no significant difference between the treatments.

The effect of activated charcoal on plant regeneration

Embryogenic N13 calli were placed on MS regeneration medium with and without activated charcoal and the percentage of calli which yielded plants on each of these media was assessed (Fig. 2.4). Activated charcoal may be beneficial in removing excess growth hormones from calli and/or media (Weatherhead *et al.*, 1978; Tisserat, 1985), therefore the 2,4-D levels on which the calli were initiated were taken into account. High levels (4mg/l) of 2,4-D in the callus initiation medium, inhibited subsequent plant regeneration. Calli induced on MS medium containing 2,4-D at the highest concentration (4mg/l), resulted in a lower average frequency of plant production (20%) than calli induced on media containing lower 2,4-D levels (38% and 43% for 2mg/l and 3 mg/l 2,4-D, respectively), regardless of the presence of activated charcoal. However, the addition of activated charcoal seemed to have the greatest positive effect on plant production from calli

which were initiated on media containing high levels of 2,4-D (4mg/l), with average plant production increasing from 13% to 24%.

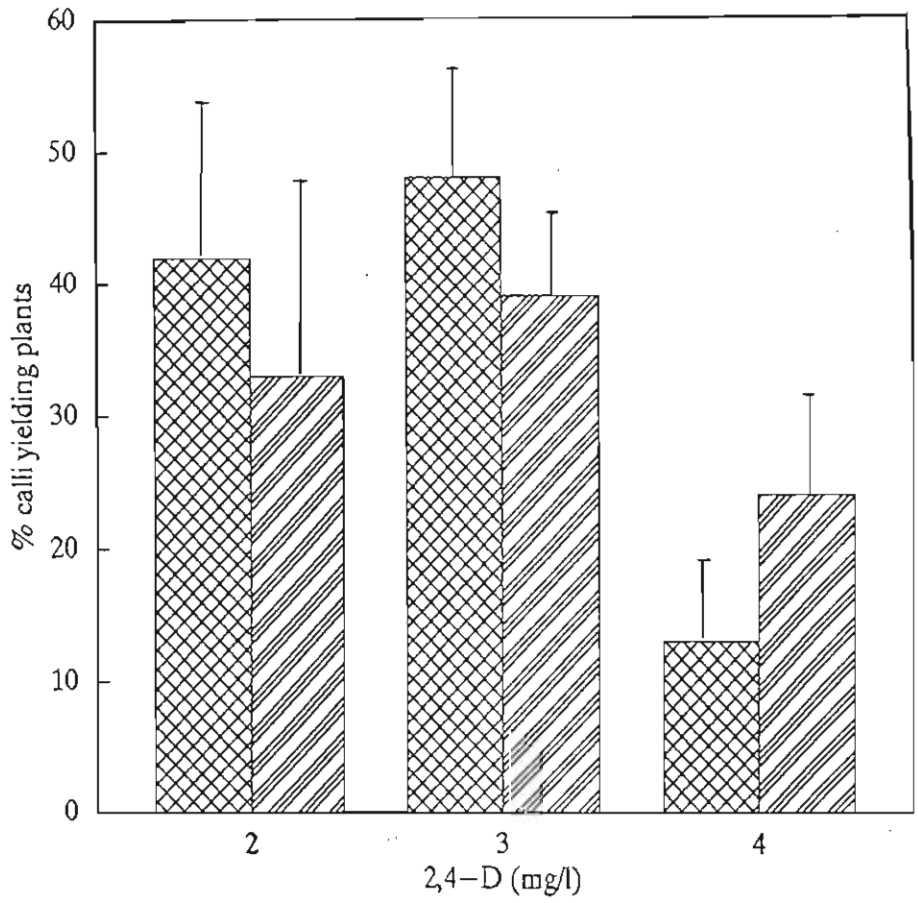


Fig. 2.4 : The effect of the addition of activated charcoal to the regeneration medium, on the percentage of N13 calli producing plants. Calli initiated on MS medium containing a range of sucrose and 2,4-D levels were placed onto MS regeneration medium containing no 2,4-D and the same concentration of sucrose as the medium on which they were initiated. Percentage calli yielding plants on MS regeneration medium in the presence (▨) and absence (▩) of activated charcoal was recorded. (s.e. indicated by lines above bars).

Hardening-off of regenerated plants

When plants are transferred from *in vitro* to greenhouse/field conditions, they are subjected to a decrease in humidity levels as well as to a non-sterile environment (Ziv, 1986), so mortalities can be expected when they are planted out. Sugarcane plants which

reached a height of approximately 10cm in the glass tubes, were hardened-off by placing plants into pots containing autoclaved soil (Plate 2.9). The plants were gradually exposed to greenhouse conditions and a mortality rate of approximately 20% was observed.

2.4.3. INITIATION AND ESTABLISHMENT OF SUSPENSION CULTURES

Calli placed in the Ho and Vasil (1983b) medium disaggregated and formed a very fine suspension (Plate 2.13 A). After 4 months in culture, in addition to a finely dispersed suspension, cell aggregates were also observed (Plate 2.13 B). In contrast, NCo376 calli placed into the media of Liu and Shih, (1986), Chen *et al.* (1988b) and Thom *et al.* (1981), did not disaggregate to form dispersed suspensions in the liquid media and calli turned an orange-brown colour in all of the above media (Plate 2.13 C). An increase in packed cell volume recorded over time (Fig. 2.5) was observed for suspension cultures growing in the medium of Ho and Vasil (1983b). The doubling time of the culture occurred after a period of 3 days, after which a negligible increase in packed cell volume took place over the following 3 days until the cultures were sub-cultured on day 7. However, if the medium was replenished at day 3, then packed cell volume appeared to increase over the next 3 days to levels above those obtained if the cultures were only sub-cultured at day 7.

Microscopic examination of the suspensions showed typical clumps of embryogenic cells, and cytoplasmic streaming and mitotic division were observed, indicating that the cells were actively dividing (Plate 2.14).

NCo376 suspension cultures could be maintained as finely-dispersed suspensions for a period of 4 months, after which time the cells aggregated to form clumps and differentiated into pro-embryos. An increase of the 2,4-D level in the media might prevent the differentiation of cells into pro-embryos. Alternatively suspension cultures at the pro-embryo stage could be used to provide a good source of synchronous embryos for micropropagation

purposes, desiccation of embryos for germ-plasm storage or the production of artificial seed (reviewed by Ammirato, 1989; Lindsey and Jones, 1989).

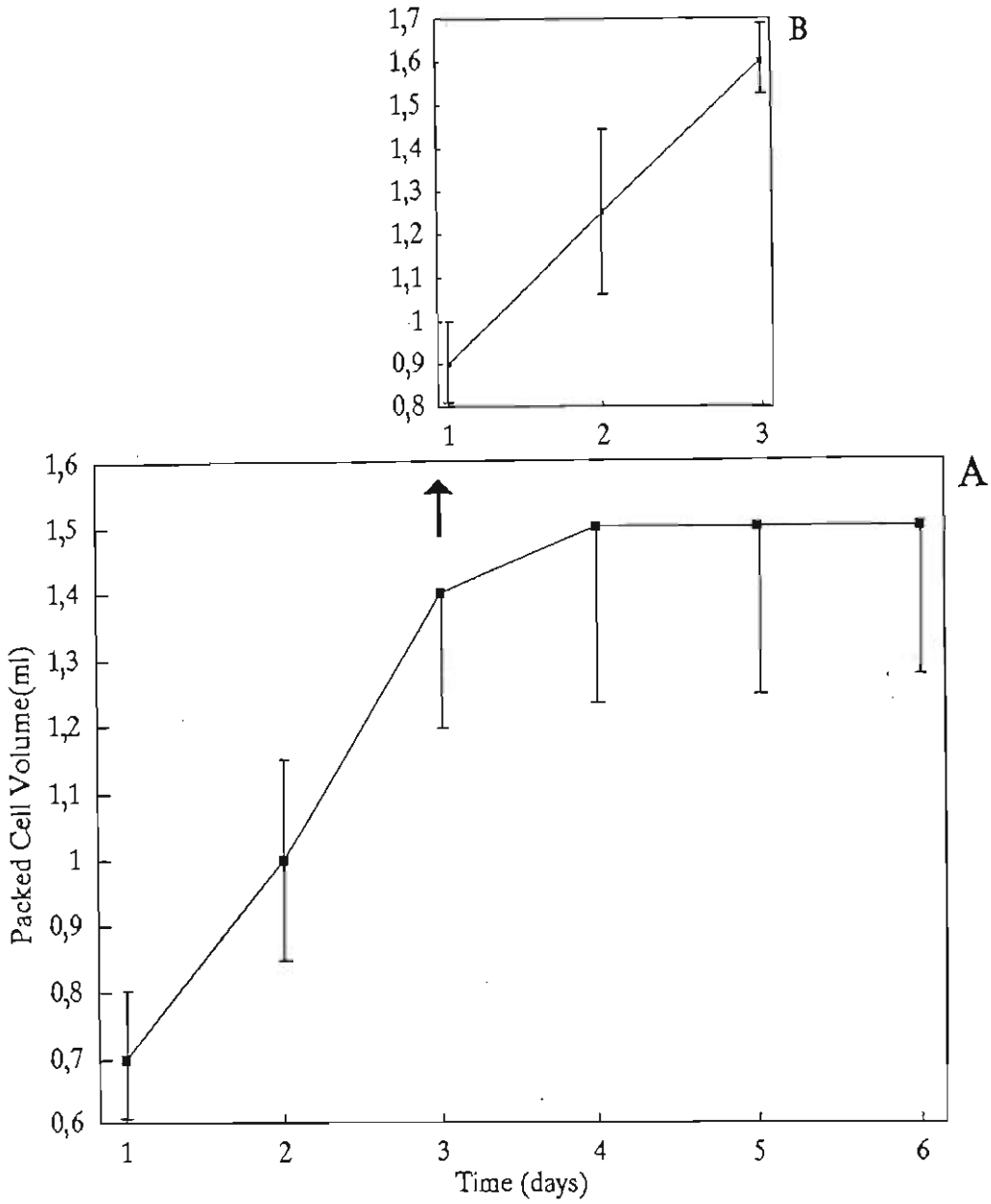


Fig. 2.5: Growth-rate of NCo376 suspension culture cells as determined by an increase in packed cell volume over time. A, suspension culture sub-cultured after 6 days; B, suspension culture sub-cultured after 3 days (↑), which resulted in a higher packed cell volume than that indicated in Fig. 2.5 A, after a total of 6 days in culture. (s.d. indicated by lines on either side of the point).

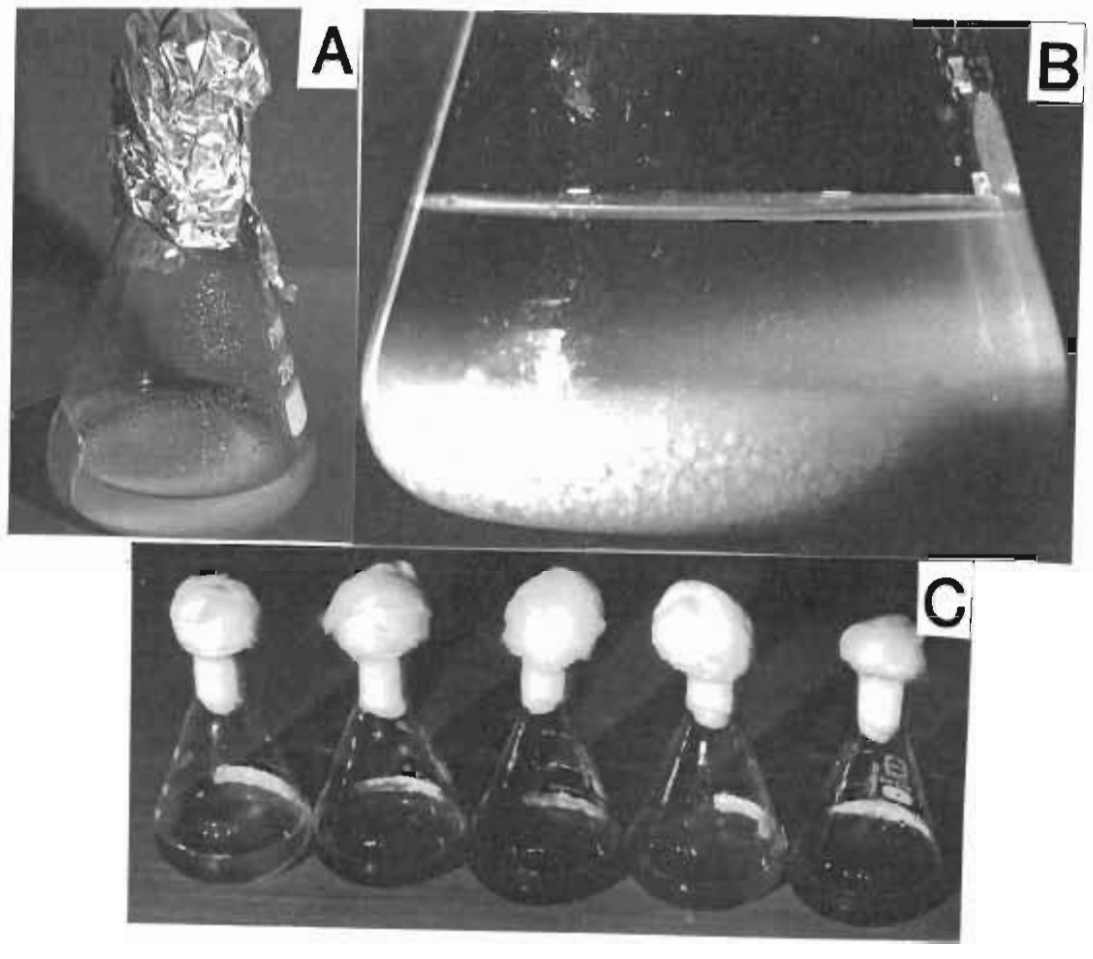


Plate 2.13: Suspension cultures initiated from NCo376 callus. The cells were finely dispersed in the liquid MS culture medium (A). Embryogenic aggregates (B) were observed after 4 months in culture. Other media investigated for the establishment of suspension culture resulted in non-dispersed, discoloured cultures (C).

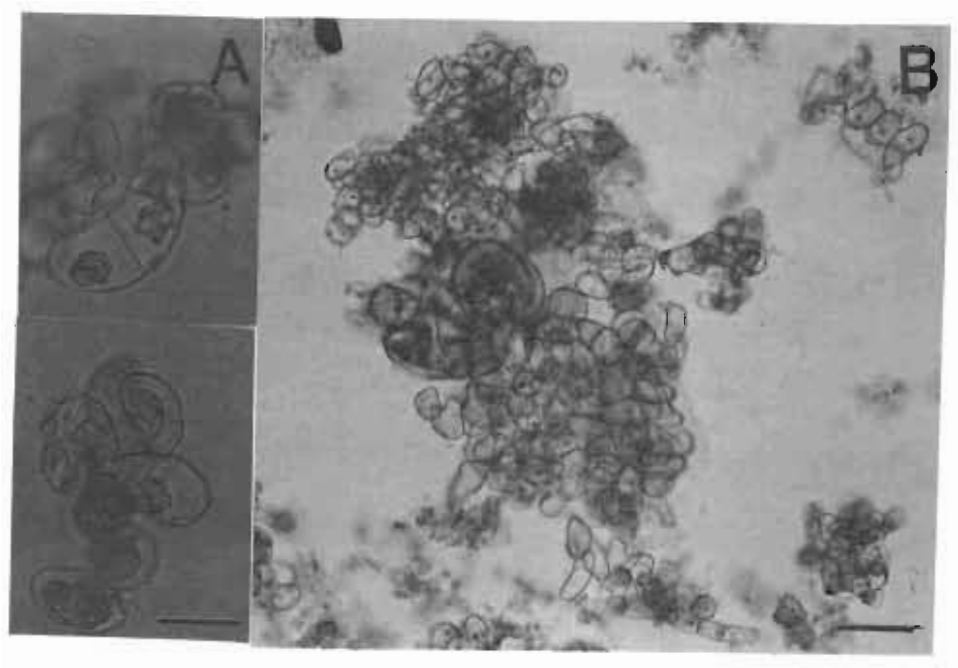


Plate 2.14: Microscopic examination of suspension culture cells. After 2 months in culture, cells containing dense nuclei were actively dividing (A), and were present in small clumps (B).
Bars represent A: 30μm
B: 300μm

2.4.4. PROTOPLAST ISOLATION AND SHORT-TERM CULTURE

Mesophyll protoplasts were isolated in an osmoticum containing cell wall-degrading enzymes at optimal concentrations (Fig. 2.6). The yield of viable protoplasts in each preparation ranged from 50-80% of the total number isolated. Different sorbitol molarities were tested (Fig. 2.7) and 0,55M was found to yield the greatest number of protoplasts. The time of incubation was qualitatively determined by viewing the protoplast solution microscopically every hour for 7h. The best yield ($4,9x10^6$ viable protoplasts/g leaf tissue) of mesophyll protoplasts was found at 20g/l cellulase, 2g/l macerozyme and 5g/l hemicellulase, in 0,55M sorbitol after 6h incubation in the dark.

The protoplast size was estimated to be between 24 and 30μm in diameter. Once the suspension had been filtered and centrifuged, a fairly pure preparation of protoplasts resulted (Plate 2.15 A), with only a few bundle-sheath cells contaminating the preparation.

Protoplast isolation from suspension cultures (under the same conditions as those for mesophyll protoplasts), yielded $1,63x10^6$ ± 1,6 viable protoplasts/2ml settled cell volume, but the resulting suspension was contaminated with odd-shaped, large, multi-nucleate, non-embryogenic cells (Plate 2.15 B), which made this source of protoplasts unsuitable for subsequent DNA uptake studies.

When calli were incubated in the same enzymatic solution used for isolation of mesophyll protoplasts, $1,2x10^6$ protoplasts/g callus were observed, but the solution contained a lot of clumps of cells that had apparently not been digested. A higher concentration of cellulase appeared to digest the cell clumps, and served to reduce the time of incubation from 7h to 3h. Friable N13 calli yielded $4,9x10^6$ viable protoplasts/g callus (Fig. 2.8) in a very homogenous protoplast suspension which could be used in future DNA uptake experiments (Plate 2.15 C).

Protoplasts were cultured for 48h and although 84% mortality occurred after this time period, $1,3x10^6 \pm 0,57$ protoplasts/g of callus were still viable, and could be used for future transient gene expression studies as discusssed in chapter 3.

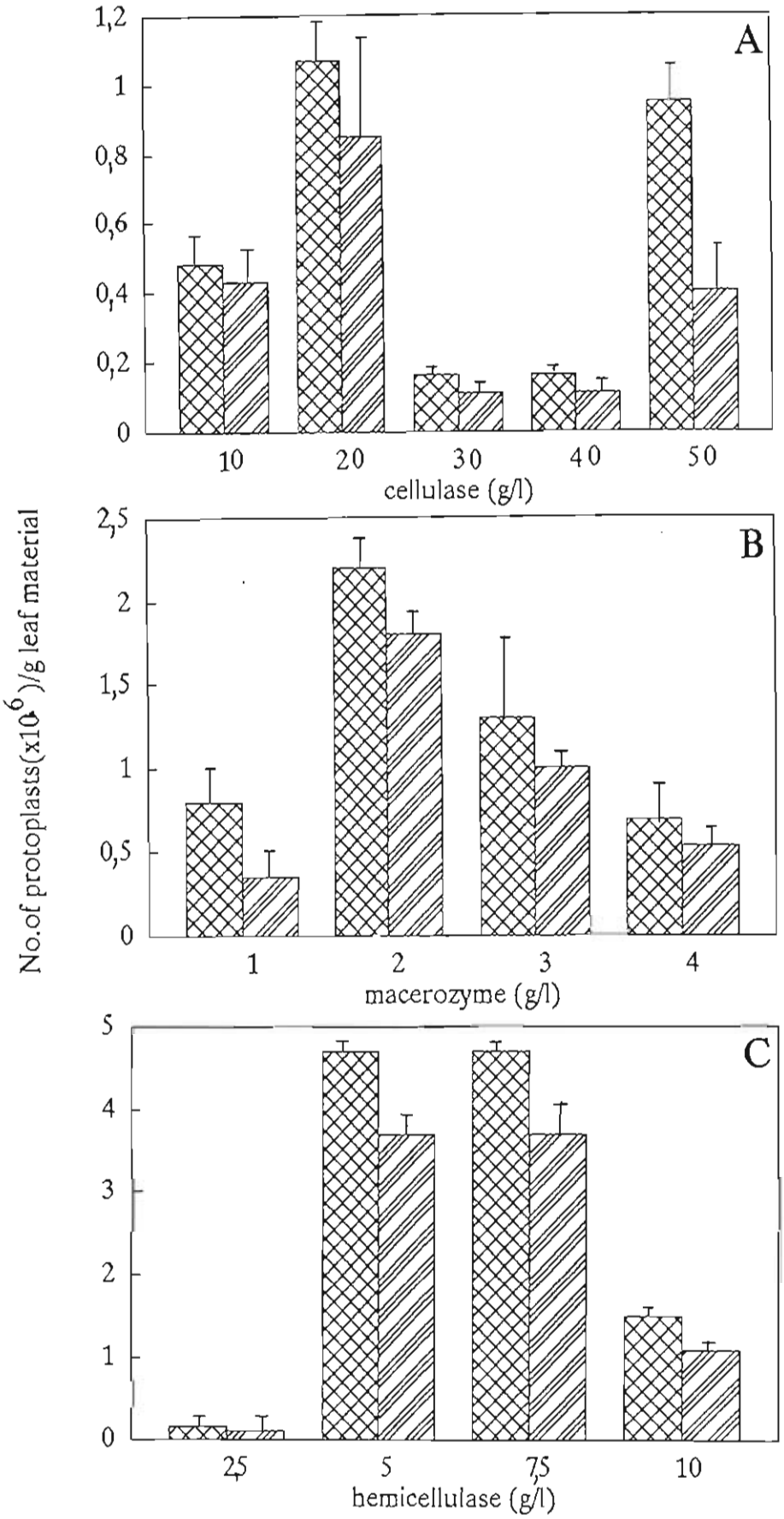


Fig. 2.6 : The effect of the concentration of cell wall degrading enzymes on the yield of total and viable mesophyll protoplasts. Total (⊠) and viable (▨) numbers of mesophyll protoplasts/g fresh weight leaf material isolated at a range of concentrations of cellulase (A), macerozyme (B) and hemicellulase (C) after a 6h incubation period. (bars represent s.e., at the 0.05 level of probability).

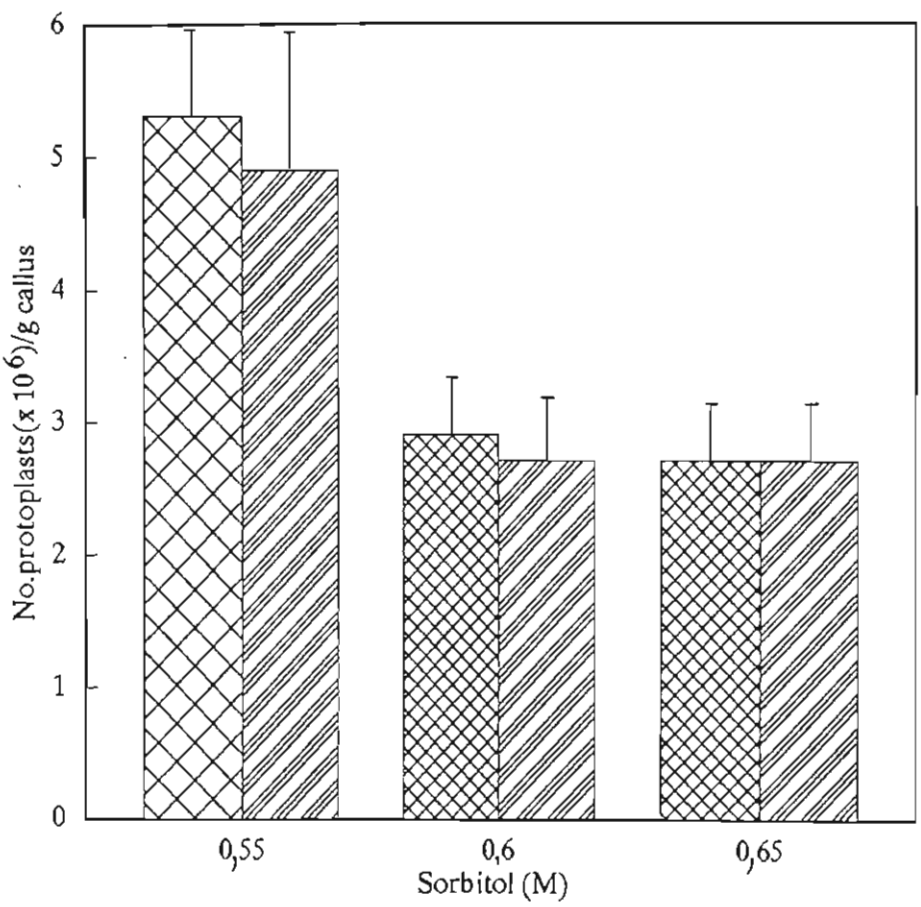


Fig. 2.7 : The effect of sorbitol concentration on yields of total and viable mesophyll protoplasts. Total (▨) and viable (▨) numbers of mesophyll protoplasts isolated per gram fresh weight of leaf material. (lines above bars indicate s.e., at the 0,05 level of probability).

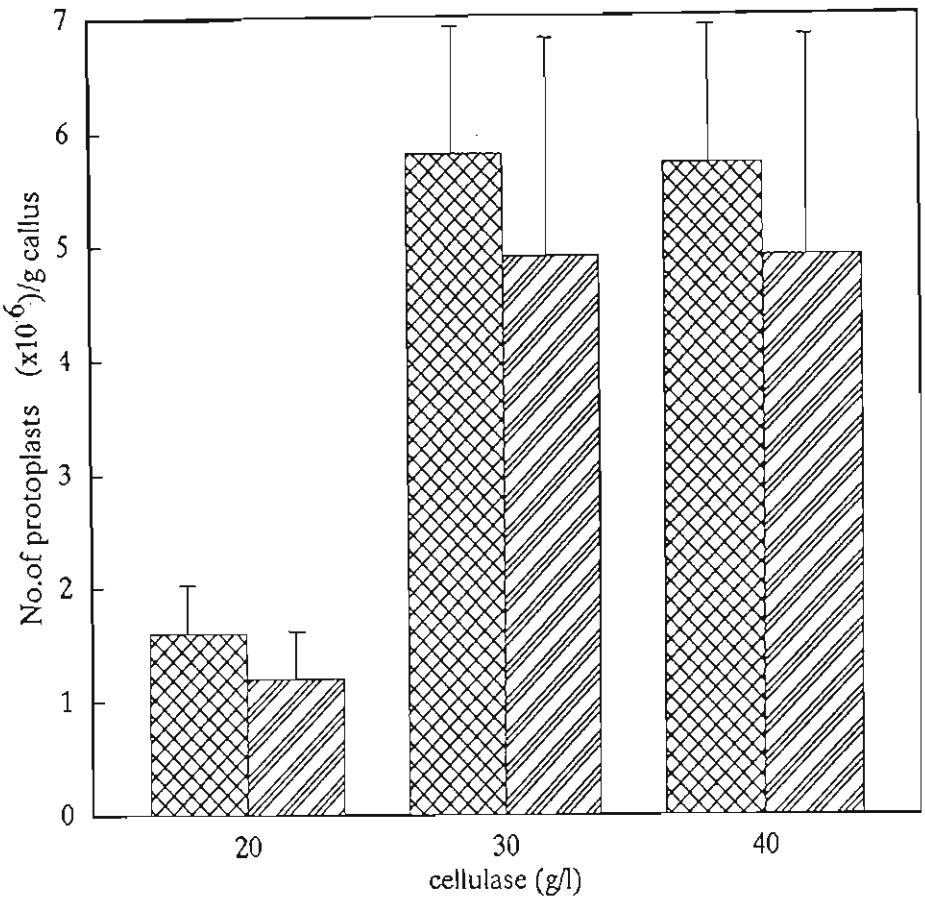


Fig. 2.8 : The effect of cellulase concentrations on the yield of total and viable protoplasts isolated from sugarcane callus. Total (▨) and viable (▨) numbers of protoplasts/g of callus isolated in a medium containing hemicellulase (5g/l), macerozyme (2g/l) and a range (20-50g/l) of cellulase concentrations, after a 3h incubation period. (s.e. are indicated by lines above bars).

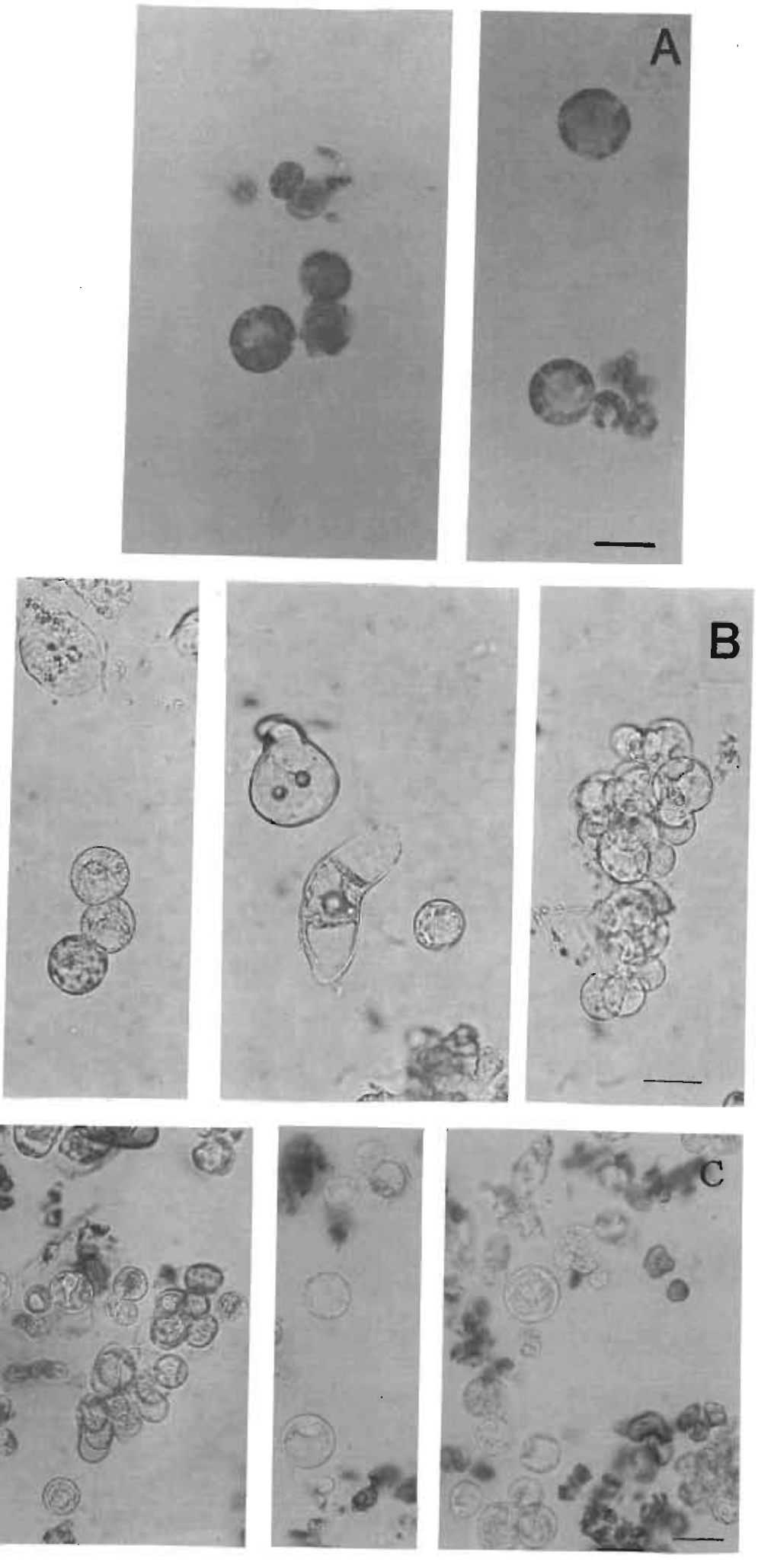


Plate 2.15: Microscopic examination of various sugarcane protoplasts isolated from leaves, suspension cultures and callus pieces. Protoplasts were isolated under the same conditions from sugarcane leaves (A), resulting in a homogenous protoplast suspension, but large non-embryogenic cells were present in the solution of protoplasts isolated from suspension cultures (B), after a 6h incubation. A pure, homogenous suspension of protoplasts was isolated in 3h from callus pieces (C), using a stronger concentration of cellulase.
Bars represent 30μm.

2.5. DISCUSSION

The *in vitro* culture protocols established in this study for South African sugarcane varieties N13 and NCo376, and their potential applications in the sugar industry, are summarised below (Fig. 2.9):

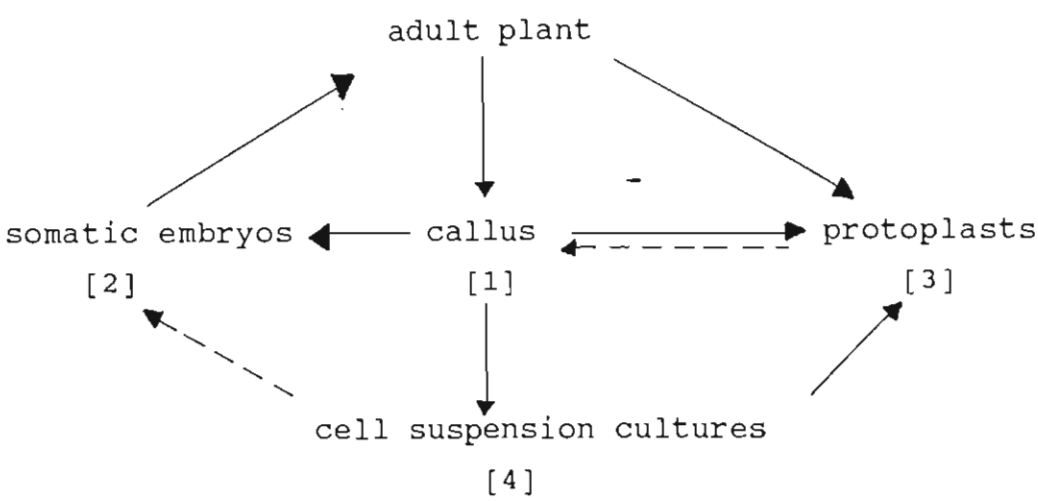


Fig. 2.9 : Summary of relationships between types of *in vitro* cultures established for South African sugarcane varieties N13 and NCo376. (Dotted lines indicate that this type of *in vitro* culture is possible, but was not established in this study).

The potential uses of these systems are:

[1] Callus: propagation of selected genotypes by somatic embryogenesis; initiation of suspension cultures; isolation of protoplasts.

[2] Somatic embryos: propagation of selected genotypes by somatic embryogenesis; investigation into desiccation of somatic embryos for long-term storage of selected genotypes; potential source material for desiccation and uptake of naked DNA during rehydration.

[3] Protoplasts: gene uptake studies; regeneration of plants.

[4] Cell suspension cultures: isolation of protoplasts; source of synchronous somatic embryos.

2.5.1. ADULT-PLANT SOURCE TISSUE

The starting material for some of the *in vitro* culture protocols in this study, were explants from field-derived sugarcane, which were severely contaminated with microorganisms. The use of antimicrobial agents to control contaminants in tissue culture has been well documented (Pollock *et al.*, 1983; Shields *et al.*, 1984; Young *et al.*, 1984; Falkiner, 1990; Leifert and Waites, 1990). However, problems encountered with microbial contamination of sugarcane varieties NCo376 and N13, which resulted in 65% of the explants being discarded due to contamination, were not overcome by the use of any of the tested antimicrobial agents. This was due to an inhibitory effect on callus induction when explants were treated with these compounds (Table 2.2). Consequently, the majority of the investigation into the establishment of *in vitro* cultures was carried out using N13 regenerated plantlets as the major explant source, as these were produced *in vitro* and were therefore free of contaminants. However the need to continually place fresh plant material into culture was recognised, because of the danger of propagating genomic changes. Despite high contamination levels, this was carried out routinely. Because such a high proportion of the explants are discarded, the process of initiating cultures is laborious and time-consuming and could delay the progress of a research programme. One possibility may be to have parent plants in the greenhouse, where they can be treated for contaminants, before being used as explant material. However, such studies were not carried out in this project.

2.5.2. PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS

Microscopic examination of callus which showed the presence of typical embryogenic structures (Plates 2.4 and 2.5), and the observation that simultaneous production of shoots and roots occurred in sugarcane variety N13 on MS regeneration medium (Plate 2.7), suggested that plant regeneration occurred by the process of somatic embryogenesis. The formation of embryogenic callus and

the regeneration of plants via somatic embryogenesis reported here for the South African sugarcane variety N13, corresponds to observations made in other plant regeneration studies, carried out on sugarcane and other grasses. Similar requirements for growth regulators and carbon sources were observed, and the rate of regeneration and yields obtained were also similar. These various aspects are discussed below.

The regeneration of graminaceous monocotyledon plants by the process of somatic embryogenesis does not appear to require extensive manipulation of medium in terms of hormones; 2,4-D is the auxin most commonly used for callus induction and proliferation both in previous work carried out on sugarcane (Heinz and Mee, 1969; Ho and Vasil, 1983a; Chen *et al.*, 1988a; Guiderdoni and Demarley, 1988) and other graminaceous species such as maize (Lu *et al.*, 1982, 1983; Vasil *et al.*, 1984), rye (Lu *et al.*, 1984), *Pennisetum* sp.(Chandler and Vasil, 1984), *Digitaria* sp. (Gonzales and Franks, 1987) and sorghum (MacKinnon *et al.*, 1986). The 2,4-D concentration of 4mg/l which yielded the greatest percentage of calli in sugarcane variety N13 (Table 2.3), gave rise to the lowest number of calli yielding plants (Table 2.5), indicating that although high concentrations of 2,4-D are favourable for callus induction, subsequent regeneration occurs at a higher frequency on media containing low levels of 2,4-D (Ho and Vasil, 1983a; Ahloowalia, 1984; Bhaskaran and Smith, 1989). The best 2,4-D concentration for callus induction, and subsequent plant regeneration in variety N13 was 2mg/l (Table 2.3). The observation in this study that germination of sugarcane embryoids occurred on the removal of 2,4-D from the medium is consistent with the observations in other graminaceous plants (Nadar *et al.*, 1978; Yao and Krikorian, 1981; Abe and Futsuhara, 1985; Bregitzer *et al.*, 1989).

Sucrose concentrations also appear to have an effect on the process of somatic embryogenesis. In this study, the highest concentration of sucrose (50g/l) used resulted in significantly less callus induction (Table 2.3) and subsequent plant

regeneration (Table 2.5), than sucrose concentrations of 20 and 30g/l sucrose. Similarly, Chandler and Vasil (1984) found that high levels of sucrose (60-120g/l) had an inhibitory effect on both callus growth and yield of somatic embryos of *Pennisetum*. However researchers using other material have found that higher concentrations of sucrose were necessary for callus induction, for example in *Digitaria*, 90g/l sucrose was used and this also resulted in the best regeneration yields (Watt *et al.*, 1989). The best sucrose concentration for sugarcane variety N13 callus induction and subsequent plant regeneration was 20g/l (Table 2.5).

The time taken for plant regeneration via somatic embryogenesis varies between species, but generally occurs 1 month after embryogenic calli have been placed on regeneration media in the light (Lu *et al.*, 1984; Bhakasran and Smith, 1988). This is supported by work carried out previously in sugarcane (Chen *et al.*, 1988a; Guiderdoni and Demarley, 1988) and by observations made in this study (Plate 2.7).

When comparing yields of plants produced by somatic embryogenesis, it is difficult to relate the yields of 16-36 sugarcane plants/g fresh weight callus obtained in this study to reports of 25 000 *Pennisetum* sp. plants from a single leaf explant in 7 months (Chandler and Vasil, 1984). In terms of percentage regeneration levels (32%) and the number of *Pennisetum* plants produced per gram fresh weight of callus (1-39), the results compare favourably to those obtained for sugarcane variety N13, in this study (Table 2.6). However, much higher plant yields of 200 plants/g callus for sugarcane (Guiderdonui and Demarley, 1988) and orchardgrass (Hanning and Conger, 1986) have been reported, so the potential does exist for higher plant yields. Since the culture conditions and media used in this study were similar to those used by Guiderdoni and Demarley (1988), variation in plant yield can be attributed to the use of different sugarcane varieties, namely Q75 and CO6415.

In order to determine whether plant regeneration percentages and

yield of plants could be increased, activated charcoal was incorporated into the regeneration medium. Observations by other researchers (Weatherhead *et al.*, 1978; Tisserat, 1985; Ebert and Taylor, 1990) that activated charcoal seemed to remove the inhibitory effect of 2,4-D on embryo germination, were matched in sugarcane variety N13 at the highest 2,4-D concentration, where calli producing plants increased from 13% to 24% in the presence of activated charcoal (Fig. 2.4). However higher regeneration percentages than these were observed from calli initiated on low 2,4-D levels (42% and 48% at 2mg/l and 3mg/l 2,4-D, respectively) without the addition of activated charcoal. Activated charcoal was therefore not routinely added to the regeneration medium.

Somatic embryogenesis has no direct practical application in the field of micropropagation, because sugarcane is propagated vegetatively for commercial purposes. However, the potential for mass propagation of transformed genotypes in a short period of time by the process of somatic embryogenesis is a most important consideration. Other advantages of somatic embryos which may have application in sugarcane-breeding programmes are the desiccation, storage, rehydration and germination of embryos *in vitro*, and their use in germ-plasm storage and artificial seed production (Ammirato, 1989). Of vital significance in all of these applications is the fact that somatic embryos arise from single cells, ensuring the genetic stability and uniformity of the clone (Ammirato, 1983; Evans and Bravo, 1983) provided that callus produced by the indirect somatic embryogenic regeneration pathway is not kept in culture for too long (Larkin and Scowcroft, 1981; Tsay, 1987; Irvine *et al.*, 1991).

2.5.3. ESTABLISHMENT OF SUSPENSION CULTURES

Sugarcane suspension cultures have been established since the 1970's for use in protoplast isolation (Maretzki and Nickell, 1973; Larkin, 1982; Liu, 1988), studies in nutrient transport (Thom *et al.*, 1981) and salt tolerance (Liu and Shih, 1986). Totipotent suspension cultures have been established in genera

within the Gramineae such as wheat (Vasil *et al.*, 1990a, 1990b), maize (Bartowick, 1981; Armstrong and Green, 1985; Kamo and Hodges, 1986) and rice (Abe and Futsuhara, 1985).

Successful establishment of suspensions using friable callus from the South African sugarcane variety NCo376 was achieved in this study (Plate 2.13 A). Packed cell volume measurements indicated that the doubling time for the culture was 3 days (Fig. 2.5). Suspension cultures established in sugarcane previously (Ho and Vasil, 1983b; Srinivasan and Vasil, 1986) had similar doubling times (approximately 4 days), but appeared to reach much higher packed cell volume levels than did the NCo376 culture in this study. Such differences might be attributed to different sugarcane varieties, as the medium used in this study did not differ from the media used in the previous studies (with the exception that coconut water was omitted in this study) or the coconut derivatives might allow greater cell numbers to develop. Coconut milk was omitted from media used in this study because it is difficult to sterilise and could cause increased contamination problems, and results are difficult to reproduce and compare, because the concentration of proteins and growth regulators varies between coconuts.

The NCo376 suspension cultures appeared to be embryogenic in nature after a 4 month period; at this stage the cells aggregated and formed embryogenic structures (Plate 2.13 B). Although the NCo376 suspension cultures were not plated out, they exhibited totipotent tendencies, and would probably be capable of regenerating plants. Organised structures resembling the early stages of embryogeny were reported in previous sugarcane suspension cultures (Ho and Vasil, 1983b; Srinivasan and Vasil, 1986) when 2,4-D levels were lowered to 0,1-1mg/l and sucrose levels increased to 60-100g/l. However the medium used for the establishment and embryogenic development of suspension cultures in this study contained 3mg/l 2,4-D and 30g/l sucrose. Depending on the intended use of the culture, manipulation of 2,4-D and sucrose levels might be used to encourage cells to either disperse

in liquid culture or become organised into embryogenic structures.

In the present study, embryogenic suspension cultures of South African sugarcane varieties were required for the isolation of protoplasts for gene transfer investigation. The embryogenic nature of the cultures gives them the potential to regenerate plants from transformed cells. The cultures could also be used for quick micropropagation of selected genotypes (particularly of transformed cell lines) on a large scale by the production of somatic embryos, which could be plated out subsequently and regenerated into plants.

2.5.4. PROTOPLAST ISOLATION AND SHORT-TERM MAINTENANCE

Protoplasts were isolated from leaves of sugarcane variety N13 in order to establish a general protoplast isolation protocol with readily available material. Mesophyll protoplasts have been isolated from sugarcane leaves by other workers (Chen and Liu, 1974; Evans *et al.*, 1980). Once a general isolation protocol was available protoplasts were isolated from suspension cultures of sugarcane variety NCo376. Yields of $1,6x10^6$ protoplasts/2ml settled cell volume suspension culture were obtained, which compared unfavourably to yields of $4,9x10^6$ mesophyll protoplasts/g leaf material (Figs. 2.6 and 2.7). However, the protoplast preparation obtained from suspension cultures contained protoplasts and undigested cells that varied in size and were difficult to purify. Bartkowiak (1981) encountered similar problems with heterogenous suspension cultures in maize.

Callus provided a good source of protoplasts after slight modification of enzyme concentration and incubation times used for mesophyll protoplast isolation, and yields of $4,9x10^6$ viable protoplasts/g callus were obtained (Fig. 2.8). The protoplasts were homogenous and uniform in size. There is little evidence in the literature to suggest that calli are used routinely as source material for protoplast isolation, but Vasil *et al.* (1990a) found it impractical to obtain a sufficient number of protoplasts from

wheat callus, although no figures for yields were presented.

In most graminaceous monocotyledons, regeneration of plants has not been achieved from mesophyll protoplasts (Vasil, 1987). No reports have substantiated the regeneration of sugarcane plants from mesophyll protoplasts claimed possible by Chen and Liu (1974) and Evans *et al.* (1980). Embryogenic suspension cultures have served as the major source material for protoplast isolation and subsequent regeneration in sugarcane (Srinivasan and Vasil, 1986; Chen *et al.*, 1988b) and the Gramineae in general, for example maize, (Shillito *et al.*, 1989; Mitchell and Petolino, 1991), rice (Abdullah *et al.*, 1986; Yamada *et al.*, 1986; Toriyama *et al.*, 1988; Zhang *et al.*, 1988)), wheat (Shimada and Yamada, 1979) and *Panicum* sp. (Lu *et al.*, 1981).

In order to select the best source of protoplasts for gene uptake work, factors such as the yield, purity and potential regeneration ability of the protoplasts were taken into consideration. Although the NCo376 suspension cultures might provide protoplasts most likely to have totipotent potential, the protoplast suspension consisted of a heterogenous population of cells in terms of cell size, and yields were lower than those obtained from callus and leaves. Therefore no further gene uptake studies were carried out on protoplasts from this source. Mesophyll protoplasts isolated from leaf material provided a homogenous protoplast suspension with good yields of $4{,}9 \times 10^6$ protoplasts per gram fresh weight leaf, but regeneration of plants from this source was unlikely. Callus provided a homogenous protoplast suspension with yields equal to those from leaves, and although the regeneration potential of protoplasts from this source has not been documented, the chances of obtaining cells with regenerative potential is good because the callus is embryogenic in nature. On this basis, callus was chosen as the source of protoplasts for gene uptake studies.

It was the intention that protoplasts isolated from South African sugarcane callus would be used in DNA uptake work and subsequently

cultured in order to monitor transient expression of introduced genes (Chapter 3). Transient expression peaks at approximately 48h, so it is critical to maintain the protoplasts in a viable state for this time period (Draper *et al.*, 1988). There appears to be a critical protoplast density of at least 10^5 protoplasts/ml for gene uptake and subsequent monitoring of expression (Lorz *et al.*, 1985; Potrykus *et al.*, 1985; Larkin *et al.*, 1990). A viable number of $1,3x10^6$ sugarcane protoplasts/g of callus was observed after culture for 48h in this study.

Genetic manipulations of plants at the protoplast stage has been investigated for graminaceous monocotyledons such as maize (Fromm *et al.*, 1986; Rhodes *et al.*, 1988; Huang and Dennis, 1989; Gordon-Kamm *et al.*, 1990), rice (Toriyama *et al.*, 1988; Yang *et al.*, 1988; Zhang *et al.*, 1988; Datta *et al.*, 1990; Hayashimoto *et al.*, 1990; Izawa *et al.*, 1991), wheat (Lorz *et al.*, 1985; Larkin *et al.*, 1990) and sugarcane (Chen *et al.*, 1987). A preliminary investigation into DNA uptake by sugarcane protoplasts isolated from South African varieties is reported in the following chapter.

CHAPTER THREE

DEVELOPMENT OF GENE TRANSFER TECHNIQUES IN SUGARCANE (*SACCHARUM* SPECIES HYBRIDS).

3.1 INTRODUCTORY REMARKS

There is a range of techniques available for gene transfer to plants, and these are reviewed in section 3.2.2. Of all the transformation techniques available, it was decided to use two approaches for the genetic engineering of South African sugarcane varieties, and seeds and protoplasts were used as recipients for transforming DNA. The seed is an attractive option for introducing foreign genes to plants, because no *in vitro* plant regeneration is necesary. Protoplasts provide a simple system for investigating transformation, and despite problems with regeneration to plants, protoplasts have been widely used in transformation studies.

3.2 LITERATURE REVIEW

3.2.1 FACTORS INFLUENCING TRANSFORMATION EVENTS

The successful genetic transformation of a cell or tissue comprises three essential elements: the introduction of foreign DNA into the cell, the integration of the DNA into the host genetic material (nuclear, chloroplastic or mitochondrial) and the controlled expression of the introduced DNA (Lindsey and Jones, 1990). The factors that influence each of these steps in the transformation process will be discussed below.

Factors affecting introduction of DNA into plant cells

Methods used to introduce foreign DNA to plants

Several techniques for the introduction of specific gene sequences into plant cells or tissues exist, and these are reviewed more

extensively in section 3.2.2. The most common method used is mediated by the bacterium *Agrobacterium tumefaciens* , which is the causal agent of crown-gall disease and can insert part of its DNA (transferred or T-DNA) into the plant genome (Chilton *et al.*, 1977; Braun, 1978; Nester *et al.*, 1984). This phenomenon has been exploited and foreign gene sequences have been inserted into *Agrobacterium* T-DNA, which has facilitated their introduction to susceptible plants (Barker *et al.*, 1983; Simpson *et al.*, 1986; Hamill *et al.*, 1987).

Not all plant species are amenable to infection by *Agrobacterium*, but genes can be directly transferred to protoplasts using techniques such as electroporation (Fromm *et al.*, 1985; Shillito *et al.*, 1985; Larkin *et al.*, 1990), microinjection (Lawrence and Davies, 1985; Crossway *et al.*, 1986; Reich *et al.*, 1986) and chemical means (Krens *et al.*, 1982; Lorz *et al.*, 1985; Werr and Lorz, 1986; Meijer *et al.*, 1991). However direct DNA uptake by protoplasts as a means of producing transgenic plants is limited to those plant species that can regenerate from the protoplast stage.

The difference in response to transformation by monocotyledonous and dicotyledonous plants

The majority of monocotyledonous species are not susceptible to infection by *Agrobacterium*, and it has been suggested that monocotyledonous cells may not possess the appropriate cell wall binding sites, which are necessary for *Agrobacterium* attachment (Lippincott and Lippincott, 1978). It is also possible that monocots respond differently at a molecular level to wounding, which is a prerequisite for infection of dicots (Chilton *et al.*, 1977; Nester *et al.*, 1984). However, a recent study carried out by Mooney and Goodwin (1991) indicate that *Agrobacterium* can attach to immature wheat embryos, and that attachment was not wound-dependent (attachment no different between sites enzymatically wounded and those untreated). There is some evidence of *Agrobacterium* T-DNA transfer, if not stable

integration, in some monocots (Hernalsteens *et al.*, 1984; Hooykaas-van Slogteren *et al.*, 1984; Graves and Goldman, 1986; Mooney *et al.*, 1991).

Alternative systems for the introduction of foreign genes to monocots include those direct DNA-uptake methods mentioned earlier and some which make use of regenerable plant material, eg. microprojectile bombardment of tissue and callus (Klein *et al.*, 1987; McCabe *et al.*, 1988; Wang *et al.*, 1988; Mendel *et al.*, 1989; Twell *et al.*, 1989; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990, DNA uptake by pollen of flowering plants (De la Pena *et al.*, 1987; Luo and Wu, 1988; Hess *et al.*, 1990) and uptake of naked DNA into seeds (Ledoux and Huart, 1968, 1969; Ledoux *et al.*, 1971, 1974; Topfer *et al.*, 1989), all of which are discussed further in sections 3.2.2 and 3.2.3.

Factors affecting integration of DNA in the plant genome

There are a number of methods available for transforming plants, and the methods used to insert DNA into cells will be discussed in section 3.2.2. The bacteria of the genus *Agrobacterium*, have a natural ability to insert a particular segment of DNA (and consequently any passenger DNA) into plant genomes, and this type of insertion is under the control of genetic elements within the bacterium (Chilton *et al.*, 1977; Nester *et al.*, 1984). However, *Agrobacterium* does not have the capacity to insert into all plant species, and other transformation techniques rely upon random insertion of DNA into the plant genome (Potrykus *et al.*, 1985). Here, factors such as DNA concentration, the conformation of the DNA (linear or circular), the actual sequence of the transforming DNA and type of vector used (section 3.2.4), are important in determining successful integration.

Factors influencing expression of transforming DNA

Much remains unknown about the factors influencing the expression of foreign genes in plants. Factors such as the position of

insertion of the DNA in the host genome, the copy number, whether DNA methylation occurs, and the transcriptional levels directed by a 'foreign' promoter, can all affect the expression of an introduced gene (Walden, 1988).

Choice of promoter

A promoter can be defined as the DNA region, usually upstream to the coding sequence of a gene or operon, which binds RNA polymerase and directs the enzyme to the correct transcriptional site so that RNA synthesis can be initiated (Oliver and Ward, 1985). Bacterial genes are not transcribed after integration into the plant genome unless they are placed under the control of suitable promoter elements (with the exception of *Agrobacterium* T-DNA, which contains its own promoter elements) (Fraley *et al.*, 1983). Plant genes, however, are usually transcribed and spliced correctly if transferred to a homologous/heterologous host genome (Murai *et al.*, 1983). Different promoters work with different efficiencies, with strong promoters causing mRNA synthesis to be initiated at a high frequency, which enables selection of transformed cells whereas weak promoters direct synthesis of rarer transcripts (Maniatis *et al.*, 1982).

Promoters studied in the most detail are the nopaline synthase (nos) promoter from *Agrobacterium* and the 35S transcript promoter of the cauliflower mosaic virus (CaMV) (Cocking and Davey, 1987). By combining such promoters upstream of the coding sequence of bacterial antibiotic resistance genes, it is possible to produce chimeric genes that function in plants (reviewed by Weising *et al.*, 1988). The bacterial nptII gene from the transposon Tn5 under the control of the CaMV 35S promoter, is a chimeric gene (Paszkowski *et al.*, 1984; Jenson *et al.*, 1986) which has been used to transform many plants (reviewed in more detail in section 3.2.5).

3.2.2. COMMONLY USED METHODS FOR TRANSFERRING GENES TO PLANTS

Agrobacterium-mediated gene transfer

The soil bacterium *Agrobacterium tumefaciens* has the ability to induce tumours (crown galls) on all tested dicotyledonous and some monocotyledonous plants (Braun, 1978; Lippincott and Lippincott, 1978; Hooykas-van Slogteren *et al.*, 1984; Graves and Goldman, 1986). A large (150kb) plasmid called the Ti or tumour-inducing plasmid has been shown to be responsible for tumour formation, due to a remarkable capacity to transfer, insert and express a particular segment of DNA in the plant genome (Chilton *et al.*, 1977; Nester *et al.*, 1984; Shaw *et al.*, 1984; Gardner and Knauf, 1986; Armitage *et al.*, 1988; Herrera-Estrella and Simpson, 1988; Golds *et al.*, 1990). Genetic analysis of the *A. tumefaciens* Ti plasmid reveals that two regions of the plasmid are essential for tumourigenesis, the T-DNA and the virulence (*vir*) region (Chilton *et al.*, 1977; Holsters *et al.*, 1980; DeGreve *et al.*, 1981). The T-DNA encoded genes are integrated and expressed in the plant-cell nucleus, which results in the production of hormones, causing the proliferation of plant cells and prevention of their differentiation (Braun, 1978; Nester *et al.*, 1984; Zambryski, 1988). Other T-DNA encoded genes are responsible for the synthesis of novel amino acid or sugar derivatives, termed opines (Chilton *et al.*, 1977; Barker *et al.*, 1983), and unlike genes in the *vir* region, are not involved in the mechanism of transfer and integration of the T-DNA (Gardner and Knauf, 1986). The mode of transfer of the T-DNA to plant cells is not yet completely understood, but directly-repeated 25 base pair sequences, known as border sequences, have been shown to be essential for T-DNA transfer and integration into the plant genome (Shaw *et al.*, 1984).

In an *Agrobacterium*-mediated gene transfer system utilising Ti plasmid functions, the desired DNA construct is placed between the T-DNA border sequences (Shaw *et al.*, 1984). In addition, since oncogenicity genes on the T-DNA incite a tumorous response in

plant cells, these genes are generally deleted and replaced by an antibiotic resistance gene that can be used as a marker to select transformed cells (Hernalsteens *et al.*, 1984; Zambryski, 1988). The Ti plasmid is too large to manipulate *in vitro* (Zambryski, 1988), so one of two different vector systems is used for plant transformation studies (Fig. 3.1). Cointegrative-type Ti vectors have been constructed in which a gene of interest, cloned on an *E. coli* plasmid, is inserted into the T-DNA by a single recombination event, at a region of common homology (Chilton *et al.*, 1977; Gardner and Knauf, 1986). Binary vectors can also be used, in which the *Agrobacterium* carries two plasmids, one of which has selectable markers and cloning sites between T-DNA borders, which can replicate in *E. coli*, and the other is a Ti plasmid which acts in *trans*, supplying virulence functions to enable the transfer of the desired gene to plant cells (Simpson *et al.*, 1986; Hamill *et al.*, 1987).

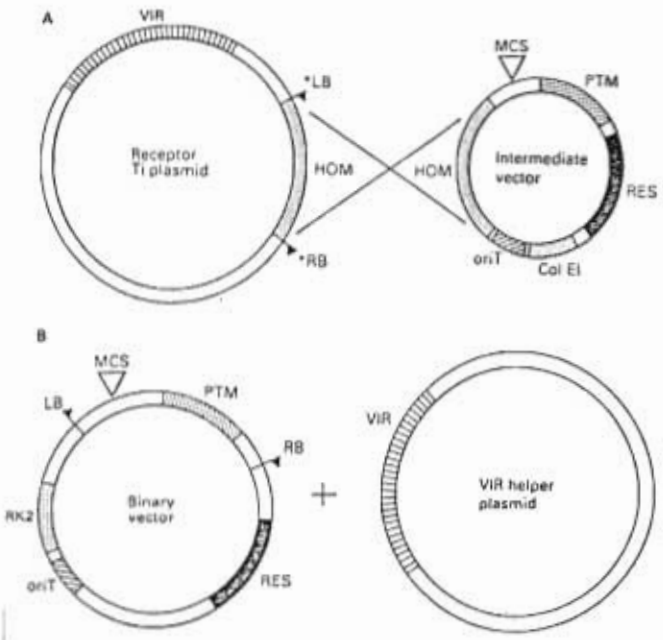


Fig 3.1: Schematic diagram of generalised cointegrative (A) and binary (B) vector systems. VIR, virulence region; HOM, homologous region; LB and RB, left and right borders; MCS, multicloning site; PTM, plant transformation marker; RES, antibiotic resistance marker. (reproduced from Draper *et al.*, 1988)

Plant tissues and cells may be exposed to the *Agrobacterium* vector system in a number of different ways. *In vivo* inoculation of seedlings with *Agrobacterium* cells (Graves and Goldman, 1986; Hinchee *et al.*, 1988; Chee *et al.*, 1989; Raineri *et al.*, 1990) or well-established plants (Murai *et al.*, 1983; Hernalsteens *et al.*, 1984; Hookaas-Van Slogteren *et al.*, 1984; Shaw *et al.*, 1984; Gardner and Knauf, 1986) that have been propagated *in vitro* or *in vivo*, is the traditional way to obtain transformed cells. Selection of the transformed tissues or plants is based on expression of selective marker genes such as antibiotic resistance (Hinchee *et al.*, 1988; Raineri *et al.*, 1990). The leaf-disc technique, where leaf-discs are co-cultivated with a suspension of *Agrobacterium* (De Block *et al.*, 1987), is a simple way to transform cells and these are cultured directly thereafter, going either through a callus stage first or in some cases, by direct regeneration from somatic cells in the leaf discs. This technique is limited to those species that respond to *Agrobacterium*-infection and that also produce plants readily from leaf explants. Protoplasts may also be co-cultivated with *Agrobacterium* (Marton *et al.*, 1979; Van Lijsebettens *et al.*, 1986), and the advantage of this is that large numbers of independent transformation events can occur. However this technique is only useful if the transformed protoplasts can be regenerated to whole plants. A novel approach used by Mooney *et al.* (1991), involved the transfer of *Agrobacterium* to wheat tissues by co-cultivation with the bacterium, following partial enzymatic digestion of the scutellum of immature wheat embryos.

The majority of *Agrobacterium*-mediated gene transfer work has been performed with *A. tumefaciens*, but some work has been done with *A. rhizogenes* (Comai *et al.*, 1985; Simpson *et al.*, 1986; Hamill *et al.*, 1987). Both species transform plant cells in a similar way in terms of DNA transfer and production of novel amino acids in target tissue, but infection by *A. rhizogenes* results in so-called hairy roots as opposed to crown galls, and the T-DNA responsible for transformation is located on the Ri (root-inducing) plasmid (Jensen *et al.*, 1986; McKnight *et al.*, 1987; Stougaard *et al.*,

1987). In view of the possible host-range extension, *A. rhizogenes*-mediated gene transfer should not be overlooked as a means of producing transgenic plants, but will not be discussed in this review.

Most successful *Agrobacterium*-mediated transformations have been achieved in dicotyledonous plants but there have been some reports of monocotyledonous plants being transformed by *Agrobacterium*. Hooykaas-van Slogteren *et al.* (1984) found that stem inoculation of plants from the *Lilliaceae* and *Amarillidaceae* groups resulted in swelling at the site of inoculation, where octopine and nopaline activity was observed, indicating T-DNA transfer to cells. Similarly, asparagus inoculated with *A. tumefaciens* developed tumourous proliferations on the stem, and opine synthesis was detected (Hernalsteens *et al.* 1984). Expression of T-DNA linked markers has been demonstrated in rice cultures (Raineri *et al.*,1990) and in maize seedlings (Graves and Goldman, 1986) transformed by *Agrobacterium*.

The Ti plasmid of *A. tumefaciens* has been used in conjunction with plant-virus DNA to inoculate plants and to monitor the transfer of DNA by the expression of disease symptoms, in a process termed 'agroinfection' which will be discussed later in the review.

Direct gene transfer to protoplasts

The failure to achieve *Agrobacterium*-induced transformation in the majority of monocotyledonous plants, the cereals in particular, has led to increased interest in assessing transformation techniques for direct gene transfer. This field has been extensively reviewed (Cocking and Davey, 1987; Davey *et al.*, 1989; Lorz, 1989; Walden, 1988; Golds *et al.*, 1990). Direct uptake of foreign DNA by protoplasts using techniques such as electroporation, microinjection and chemical methods has resulted in some transformation success (Potrykus *et al.*, 1985; Rhodes *et al.*, 1988; Larkin *et al.*, 1990). However, not many transgenic monocotyledonous plants have been produced using these techniques

because of the difficulties involved in regenerating plants from the protoplast stage (section 2.2.5).

Electroporation

The transformation frequency of cultured mammalian cells exposed to exogenous DNA was found to be significantly increased by electric pulses (Neumann *et al.*, 1982; Potter *et al.*, 1984). Electroporation, as the process has been named, produces transient pores in the plasma membrane when cells are exposed to a suitable electric field, and macromolecules such as DNA can pass into the cell (Shillito *et al.*, 1985). The pores created in the membrane appear to be about 30nm in diameter and persist for several minutes after the pulse, after which the membrane can 're-seal' (Okada *et al.*, 1986a). Field strength (voltage gradient) and pulse duration (decay time) are the two main variables affecting the permeabilisation of the cell membrane by electroporation (reviewed by Draper *et al.*, 1988).

The electroporation pulse is generated by discharging a capacitor across an electroporation chamber, which consists of two flat metal electrodes placed within a sterile plastic cuvette. The protoplasts are suspended between the electrodes in an ionic solution (Larkin *et al.*, 1990; Potter *et al.*, 1984). There are two different approaches to electroporation for the introduction of DNA into plant protoplasts: a) using a high voltage pulse of short duration, for example 1,5kV for 10μs (Shillito *et al.*, 1985), or b) using a lower voltage pulse of a longer duration, for example 350V over 54ms (Fromm *et al.*, 1985).

The advantages of the electroporation system are that it is efficient and convenient, there is a low cell toxicity and it can be used on a wide range of plant protoplasts. These include monocotyledonous plants such as rice (Junker *et al.*, 1987; Toriyama *et al.*, 1988; Yang *et al.*, 1988; Zhang *et al.*, 1988; Izawa *et al.*, 1991), maize (Fromm *et al.*, 1986; Huang and Dennis, 1989) which was successfully regenerated from the protoplast stage

(Rhodes *et al.*, 1988), and dicotyledonous plants such as carrot, tobacco and lucerne (Fromm *et al.*, 1985; Larkin *et al.*, 1990). In a slightly different approach, designed to bypass the requirement for protoplast regeneration, Dekeyser *et al.* (1990) electroporated leaf bases on rice seedlings with a pulse duration ten times higher than that used for protoplasts. It was found that DNA could be introduced into six layers of cells. Using a similar strategy, tobacco-pollen grains were successfully electroporated (Abdul-Baki *et al.*, 1990) and these could be used for *in vivo* fertilisation which could ultimately yield transformed seed.

Polyethylene glycol-induced DNA uptake

Polyethylene glycol (PEG)-mediated protoplast fusion is a method used to generate interspecific plant hybrids (Power *et al.*, 1986), but PEG has also been used to facilitate the entry of DNA molecules into plant protoplasts (Lorz *et al.*, 1985; Werr and Lorz, 1986; Meijer *et al.*, 1991). PEG is a hydrophilic molecule and at high concentrations (30-40%) it appears to minimise charge-repulsion effects between negatively-charged membranes and DNA (negatively charged by virtue of its phosphate groups). It stimulates endocytosis in protoplasts, enabling the uptake of large particulate matter such as whole chloroplasts, liposomes and bacteria (Power *et al.*, 1986; Larkin *et al.*, 1990). In DNA experiments, the total effect of PEG is somewhat unclear, but probably involves the precipitation of plasmid DNA onto the plasma membrane and then the stimulation of its uptake by endocytosis (Freeman *et al.*, 1984; Potrykus *et al.*, 1985).

Krens *et al.* (1982) produced a reproducible method for the stable transformation of tobacco protoplasts using PEG-mediated DNA uptake. Subsequently tobacco protoplasts have been transformed by the PEG-induced DNA technique by several groups of workers (Hein *et al.*, 1983; Kartzke *et al.*, 1990; Larkin *et al.*, 1990). Attempts to transform monocotyledonous protoplasts using PEG-mediated DNA uptake have met with equal success, for example ryegrass (Potrykus *et al.*, 1985), rice (Datta *et al.*, 1990;

Hayashimoto *et al.*, 1990), sugarcane (Chen *et al.*, 1987) and wheat (Lorz *et al.*, 1985; Larkin *et al.*, 1990), but in most of these cases only transformed callus cultures have been recovered. However transgenic rice plants have been produced as a result of PEG-mediated DNA delivery (Datta *et al.*, 1990; Hayashimoto *et al.*, 1990; Meijer *et al.*, 1991).

Microinjection of DNA into protoplasts

Microinjection was originally developed for animal cells and can be used for the introduction of small molecules, macromolecules (DNA, RNA and protein), organelles and virus particles into a wide range of animal cells (Stacey, 1981; Celis, 1984; Hammer *et al.*, 1985). Development of comparable methods for transforming plant protoplasts was attempted by Griesbach (1983) and Steinbiss and Stable (1983), but it is only recently that the feasibility of plant cell transformation by direct microinjection of foreign DNA into protoplasts has been shown (Crossway *et al.*, 1986; Reich *et al.*, 1986). The success of the technique depends on the ability to orientate protoplasts and locate nuclei so that DNA can be injected into the nucleus without damaging it (Lawrence and Davies, 1985). Advantages of microinjection are that removal of the cell wall is not required, and the amount of DNA injected into the cell can be controlled. However it is expensive to set up, requires skilled personnel to carry out the injection, which is a slow process, and the cells are easily damaged (Crossway *et al.*, 1986).

Techniques less frequently used for gene transfer to protoplasts

Direct gene transfer to protoplasts has been achieved by mild sonication of sugar beet and tobacco protoplasts (Joersbo and Brunstedt, 1990), and transient expression was observed in a large proportion of the cells.

The delivery of DNA and other macromolecules to protoplasts by different kinds of liposomes was the subject of early experiments

by Fraley and Papahadjopoulos (1982), but integration of foreign DNA into plant genomes following liposome-mediated transformation, has only recently been demonstrated (Deshayes *et al.*, 1985). Liposomes are artificial vesicles encapsulating DNA or other macromolecules, and the advantages of using this technique is that the membrane encapsidation provides some protection against nucleases and no carrier DNA is required (Hain *et al.*, 1984).

Recently, a study to determine whether silicon carbide fibres could be used to deliver DNA to plant cells was carried out (Kaeppler *et al.*, 1990). This method involves vortexing suspension culture cells with a mixture of plasmid DNA, encoding a selectable or screenable marker gene, and the silicon carbide fibres. Both maize and tobacco cells expressed transient gene activity after the treatment.

Direct gene transfer to intact cells

Microprojectile bombardment of plant cells and tissues

Klein *et al.* (1987) first reported the delivery of nucleic acids to plant cells using high-velocity microprojectiles. The mechanisms involved in the process have been described by Klein and Fromm (1989) and the main points can be summarised as follows: a particle gun is used to accelerate tungsten microprojectiles (4μm in size), under the charge of a .22 calibre blank cartridge. The force of the exploding gun-powder sends a plastic cylinder with DNA-coated particles down the barrel of the particle gun and into a steel plate. The plate stops the cylinder but accelerates the tungsten beads through a small hole in the plate so that they penetrate into cells of leaf, stem or even callus tissue contained in a petri-dish below (Fig. 3.2). The microprojectiles pierce cell walls and membranes and enter intact plant cells without killing them.

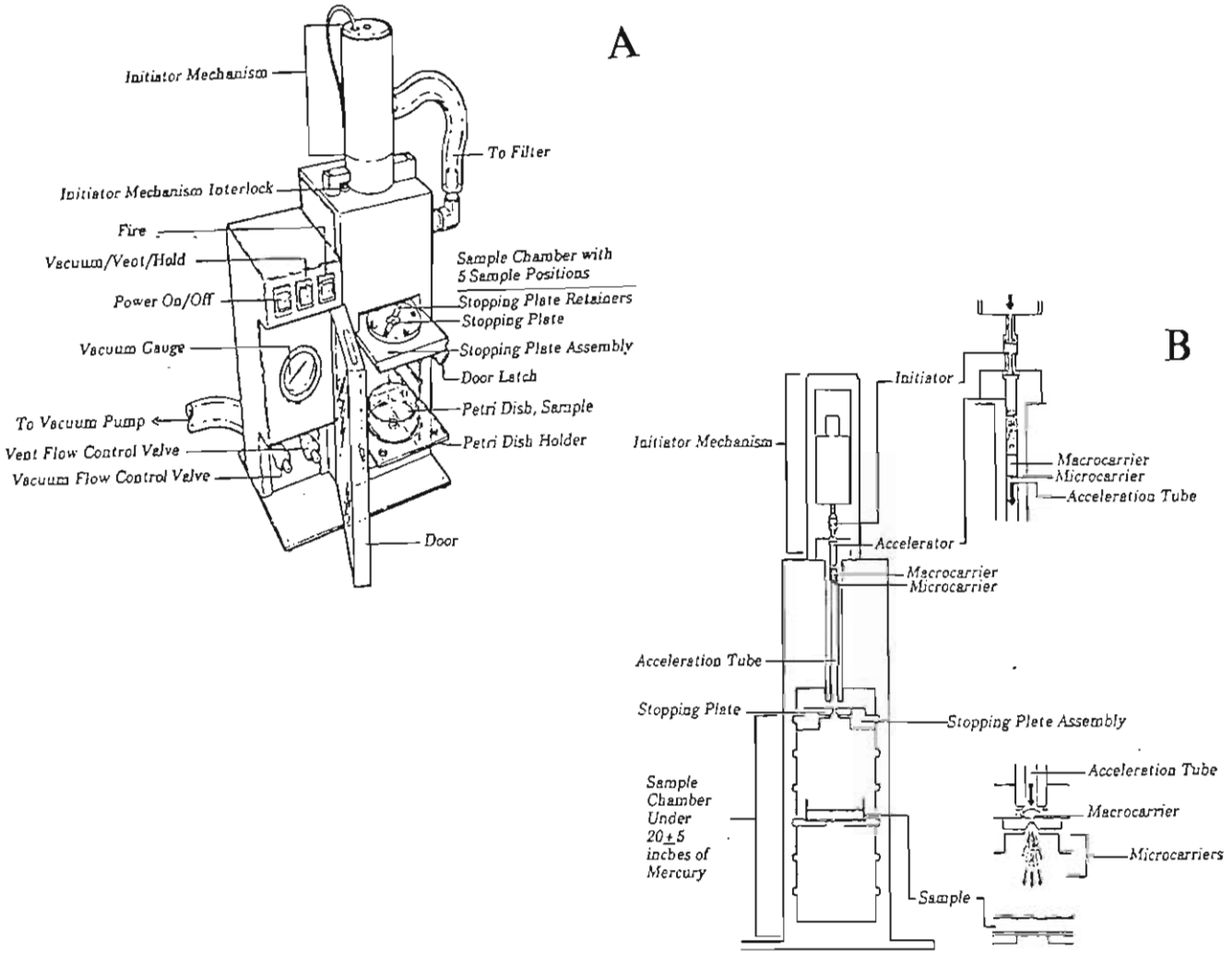


Fig. 3.2: Schematic diagram of the microprojectile delivery system. (reproduced from DuPont Product Bulletin, 1990)

The particle-bombardment process has no host-range limitations, unlike *Agrobacterium*-mediated DNA transfer, and the problems associated with monocotyledon protoplast regeneration might be circumvented by bombarding regenerable tissue such as meristems or even embryogenic callus with DNA-bearing microprojectiles. Creisson *et al.* (1990) have combined both the above-mentioned techniques and have found *Agrobacterium*- plus microprojectile-mediated delivery of viral DNA into microspore-derived callus cultures of barley, to be more successful than co-cultivation of the microspore cultures with *Agrobacterium* alone.

The microprojectile technique has been used to achieve stable transformation of soybean plants, which expressed resistance to the antibiotic kanamycin (McCabe *et al.*, 1988), and wheat which has produced stably-transformed callus lines resistant to kanamycin (Vasil *et al.*, 1991). Fertile transgenic maize plants, expressing luciferase (Fromm *et al.*, 1990) and resistance to the herbicide bialaphos (Gordon-Kamm *et al.*, 1990) were produced by microprojectile bombardment of calli and suspension culture cells, respectively. Transient GUS expression was monitored in suspension culture cells of rice (Wang *et al.*, 1988) and barley (Mendel *et al.*, 1989), as well as tobacco leaves (Tomes *et al.*, 1990), pollen grains (Twell *et al.*, 1989) and maize coleoptiles (Reggiardo *et al.*, 1991) after being subjected to microprojectile bombardment. Sugarcane callus and regenerated plantlets have shown transient GUS expression after being bombarded using a modification of the particle gun, which instead of using the DNA-coated tungsten particles, employs an airless spray-gun to deliver the DNA (Irvine and Almeida, 1991).

Viral-mediated DNA delivery

Plant viruses have received attention as potential vectors for the introduction of foreign genes into plants because their host range includes many of the cereals (Walden, 1988). In view of the difficulties encountered with *in vitro* culture of this group of plants and the fact that they are often not susceptible to transformation using vectors based on the Ti plasmid, viral-mediated DNA delivery is an alternative worth considering. Factors such as the ease of insertion into the plant cell (by mechanical wounding of tissue), ease of manipulation (small genome size: 8kb or smaller), and the fact that viruses replicate in plant cells to achieve a high copy number, make them attractive agents for plant transformation studies (reviewed by Mantell *et al.*, 1985).

The two groups of plant viruses whose genomes comprise DNA are the

caulimovirus family and the gemini viruses. Cauliflower mosaic virus (CaMV), containing double-stranded DNA, has been used to insert foreign genes into whole plants and their correct expression has been obtained (Brisson *et al.*, 1984; Grimsley *et al.*, 1986). Problems with using infectious agents such as viruses are that 1) a systemic infection may result, causing the death of the plant and 2) the genome (particularly of CaMV) is tightly packed, so it is difficult to insert foreign DNA (Walden, 1988). One advantage of using CaMV is that foreign DNA could be transferred to seed of transgenic plants because viral infection is systemic and is passed on to progeny (Grimsley *et al.*, 1986). Another group of plant viruses, the gemini viruses, which contain single-stranded DNA enclosed in a capsid, may be useful for future gene transfer in monocotyledonous plants. Viruses such as maize streak virus (MSV) and wheat dwarf virus (WDV) are normally transmitted to monocotyledonous plants by a species of leafhopper, but transfer has also been achieved by 'agroinfection' (see below).

Viral DNA integrated between border sequences of the *Agrobacterium* Ti plasmid has been transferred to inoculated plants in a process termed 'agroinfection' (*Agrobacterium*-mediated viral infection) (Hohn *et al.*, 1987; Woolston *et al.*, 1988). As naked viral DNA is itself not infectious to plants, successful DNA transfer is confirmed by the development of viral symptoms in the plant (Grimsley *et al.*, 1986, 1987). Inoculation of a plant, susceptible to *Agrobacterium* and sensitive to the introduced virus, leads to the escape of infectious viral nucleic acid from the transforming T-DNA (Grimsley *et al.*, 1986). One or a few copies of successfully transforming viral DNA can spread systemically and cause development of disease symptoms on the whole plant. Agroinfection has been studied and described for CaMV in turnip (Grimsley *et al.*, 1986), maize streak virus in maize (Grimsley *et al.*, 1987; Hohn *et al.*, 1987) and wheat dwarf virus in wheat seedlings (Woolston *et al.*, 1988). It should be emphasised that agroinfection is not an assay for the integration of T-DNA into the plant genome as little is yet known about the

role of the T-DNA transfer process in the initiation of viral infection or the mechanism by which the viral DNA escapes from the T-DNA.

Other mechanisms of viral-mediated DNA delivery include using cDNA from single-stranded RNA plant viruses (French *et al.*, 1986) but this will not be discussed here. Viral cross protection of plants, whereby the introduction of a mild form of the virus protects plants from a more serious infection (Van den Elzen *et al.*, 1985; Van Dun *et al.*, 1987), is discussed later in section 3.2.6.

Pollen-mediated transformation of cells

The 'pollen-tube pathway' involves incubating pollen with transforming DNA and then applying the mixture to the stigma of the plant in order to produce transgenic seeds without involving any tissue culture (Hess *et al.*, 1990). Rice has been transformed using pollen-mediated DNA transfer, and DNA applied to the styles of pollinated rice flowers, after the excision of the stigma, was thought to reach the ovules by flowing down the pollen tubes (Luo and Wu, 1988). De la Pena *et al.* (1987) achieved transformation of rye by injecting DNA into young floral tillers, resulting in seeds and seedlings in which the foreign gene is expressed. Hess *et al.* (1990) pipetted *Agrobacterium* onto wheat spikelets before or during pollination, resulting in the production of transformed seed.

3.2.3. THE POTENTIAL OF THE SEED FOR USE IN GENE TRANSFER TO PLANTS

In a biochemical study of the changes that occur in cell membranes during processes such as desiccation and imbibition, Simon (1974) illustrated the uptake of DNA by dry plant tissues. This phenomenon could offer an alternate and simple way of gene transfer to seeds, particularly of monocotyledonous plants, thereby eliminating tissue culture techniques. It would also

serve to introduce DNA into the germ line of plants (Heberle-Bors, 1991).

Attempts had been made to transfer naked DNA to plant seeds during imbibition as long ago as the 1960's (Ledoux and Huart, 1968, 1969; Ledoux *et al.*, 1971, 1974). However the results obtained in these studies were criticised (Kleinhofs *et al.*, 1975; Kleinhofs and Behki, 1977) because bacterial contamination could not be ruled out as being the source of DNA, and there were no effective molecular markers for monitoring of uptake and expression.

More recently, Feldmann and Marks (1987) used the seed imbibition technique to generate a high frequency of *A. thaliana* T-DNA insertional mutants. The seeds were first imbibed in water and then incubated with *Agrobacterium* containing a disarmed plasmid vector (with kanamycin resistance linked to a Ti plasmid-derived marker). *A. thaliana* plants were selfed, and the resulting seeds grown on kanamycin-containing medium to select transformants. Further evidence for the uptake of naked DNA by seeds was provided by Topfer *et al.* (1989) who demonstrated uptake in seeds of certain cereals (wheat, maize, rice, oat, rye and barley) and legumes (bean and pea). Embryos were removed from seeds either chemically or mechanically, and imbibed in the DNA solution for 2h before being placed onto a MS culture medium. An assay for transient NPTII activity confirmed the presence of the introduced NPTII gene in all the embryos tested. Transient gene expression resulting from contaminating microorganisms was discounted in these experiments, because NPTII signals were only observed when embryos were incubated in a DNA solution, and not in untreated embryos. Embryos which were preimbibed in water yielded no NPTII activity, indicating that it is essential for the membranes to be in a desiccated state for DNA uptake to take place. No NPTII activity was observed in endosperm tissue surrounding the embryo, illustrating that uptake occurs in the actively dividing embryonic cells. The question of DNA penetration through the cell wall and plasma membrane was addressed by Topfer *et al.* (1989), and it was postulated that substances leak out of the embryo

during the initial period of imbibition, after which a gradient may exist for DNA uptake. Still under investigation (Topfer *et al.*, 1989) is the question of whether the transforming DNA is stably integrated and transmitted to progeny.

Another technique for DNA delivery to seeds includes electrophoretic transfection (Ahokas, 1989) which was applied to embryos of barley seeds. Pipette tips which contained DNA, were placed in an 'electroporation-type' chamber, along with the barley embryo (Fig. 3.3), and an electric current facilitated movement of DNA into the embryo, which had been punctured by the pipette tip. Initial experiments showed transient expression of the GUS gene in the germinating embryos.

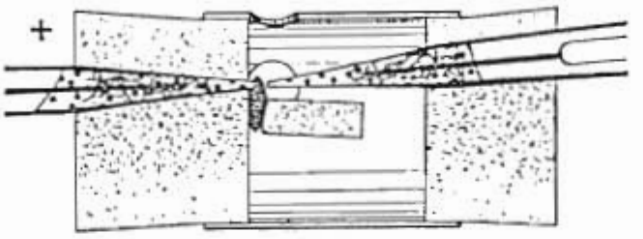


Fig. 3.3: Electrophoretic transfection chamber. The DNA is housed in pipette tips, the barley embryo is depicted in black, and the positive control is on the left. (reproduced from Ahokas, 1989).

As mentioned in the previous chapter, somatic embryos and zygotic embryos (seeds) have similarities in their structure and development (Ammirato, 1983). Although no method has been developed for gene transfer to somatic embryos in particular, it is tempting to speculate upon the possibility of desiccating them and then allowing direct uptake of naked DNA by imbibition during their rehydration, as occurs during imbibition of zygotic embryos. This would overcome the necessity for producing regenerable

protoplasts, and would be particularly advantageous in the graminaceous monocotyledons, many of which regenerate by somatic embryogenesis (Vasil, 1987; Bhaskaran and Smith, 1990).

3.2.4 VECTORS

An important step in any DNA recombination procedure is the selection of a suitable plasmid vector (carrier) into which one or more DNA sequences can be inserted (Maniatis *et al.*, 1982). The vector should contain one or more restriction sites for cloning, and be capable of autonomous replication in a defined host or vehicle organism so that the sequence is reproduced (Oliver and Ward, 1985). There are different types of vectors, for example cloning and expression vectors, and their use is dictated by the nature and size of the gene to be transferred, the host to which it is transferred, and the type of marker genes that are required.

Cloning vectors contain certain DNA sequences which are inserted into complimentary unique (that is there are no duplicate/triplicate sites) restriction sites in the vectors *in vitro* (Maniatis *et al.*, 1982). pBR322 is the most versatile plasmid-cloning vector (derived from *E. coli*), and contains ampicillin and tetracycline resistance and a number of convenient restriction sites (Fig. 3.4). There are several restriction enzymes which have a single target site within one or the other resistance genes, so that insertion of foreign DNA at one site can be detected by insertional activation of that antibiotic resistance, while recombinants can be selected using the other antibiotic (Bolivar *et al.*, 1977; Sutcliffe, 1979). The plasmid is under relaxed control, that is the replication is not coupled to host genome replication, so copy numbers of 10-200 plasmids per cell can occur, and when host protein synthesis is halted by the addition of chloramphenicol, copy numbers of several thousand plasmids can result (Maniatis *et al.*, 1982).

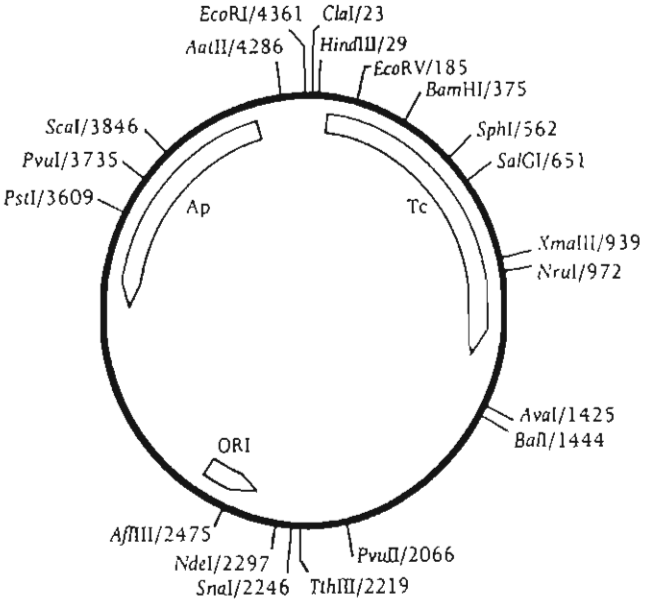


Fig. 3.4: Restriction map of pBP322. Ap, ampicillin resistance; Tc, tetracycline resistance; sites where restriction endonucleases cut are shown. (reproduced from Oliver and Ward, 1985)

Expression vectors contain promoter sequences which facilitate efficient transcription of an inserted gene and this results in cells containing a high concentration of the insert's protein product (Oliver and Ward, 1985). Such vectors should incorporate a selectable plant marker and a site for integration of a 'passenger' (foreign gene) sequence. Different types of vectors can be constructed depending on factors such as the DNA sequence to be transferred, the plant species to which the DNA is being introduced and the size of the DNA (Walden, 1988). Most expression vectors are artificial constructs, and consist of DNA segments obtained from a variety of plasmid sources. For example, the GUS reporter gene casette has been cloned into plasmid pUC19 (derived from pBR322), along with the CaMV promoter and multicloning sites to create pBI221 (Jefferson *et al.*, 1987) (section 3.2.5).

3.2.5. SELECTION OF TRANSFORMANTS BY MEANS OF GENETIC MARKERS

Foreign genes can be introduced into plants in a number of ways, but it is only by investigating the expression of the introduced

DNA that the success of the transformation and the efficiency of particular promoter constructs can be judged. To avoid the lengthy process of selection and bulking up of the tissue for analysis of integrated constructs, it is possible to study transient expression of reporter/screenable genes by immediate analysis (reviewed by Cocking and Davey, 1987; Walden, 1988; Weising *et al.*, 1988).

Detection of transient gene expression

If the methods described previously (section 3.2.2) deliver foreign DNA to plant cells effectively, and a large proportion of the cells take up several copies of the DNA sequence, then transient expression of the DNA may be detectable. Transient expression of the inserted gene occurs cytoplasmically, chromosomal integration is unlikely to occur and is not a prerequisite for expression to take place. Expression normally peaks after 48 hours, after which time the DNA is degraded, or limited integration of concatenated DNA might take place (Walden, 1988).

Transient expression systems require a sensitive method for detection of transformed cells/tissue (Hauptmann *et al.*, 1987). Sensitive assays are available to monitor the expression of reporter genes such as GUS (Jefferson *et al.*, *al.*, 1987), CAT (Gorman *et al.*, 1982) and luciferase (De Wet *et al.*, 1987; Fromm *et al.*, 1990). In the above examples, the gene product is an enzyme, the activity of which can be assayed either in tissue extracts or immunologically.

<u>The main features of the CAT and luciferase systems</u>

The CAT gene, derived from the transposon Tn9, encodes the chloramphenicol acetyltransferase enzyme which catalyses the acetylation of chloramphenicol (Gorman *et al.*, 1982). [^{14}C]-chloramphenicol and acetyl-CoA are supplied as substrates in the assay, and the radioactive acetylated products are separated by

thin-layer chromatography and detected by autoradiography. Transient expression of the CAT gene was first studied in cultured mammalian cells to study promoter function (Gorman *et al.*, 1982) and has subsequently been observed in protoplasts of maize (Fromm *et al.*, 1985), sugarbeet, tobacco (Joersbo and Brunstedt, 1990) and in onion epidermal tissue (Klein *et al.*, 1987).

Luciferases are enzymes which confer upon the host cell the ability to bioluminesce, and two types of genes encoding these enzymes have been cloned in mammalian cells (De Wet *et al.*, 1987), namely bacterial and firefly luciferase. Light emission can be detected by photographic film or by a photometer connected to a recording apparatus. Transient gene expression of firefly luciferase has been monitored in maize (Fromm *et al.*, 1990) and bacterial luciferase in tobacco and carrot (Koncz *et al.*, 1987).

The GUS system

The ß-glucuronidase (GUS) system can be used for the purposes of studying plant promoters (Werr and Lorz, 1986), the activity of chimeric gene constructs (Jefferson, 1987; Jefferson *et al.*, 1987; Dekeyser *et al.*, 1990), evaluating the efficiency of the technique used to introduce foreign DNA (Abdul-Baki *et al.*, 1990; Creisson *et al.*, 1990; Larkin *et al.*, 1990) and, to some extent to establish in which cells or tissues the gene is being expressed (Irvine and Almeida, 1991; Meijer *et al.*, 1991; Reggiardo *et al.*, 1991).

The *E. coli* ß-glucuronidase gene has been cloned and sequenced, and encodes a stable enzyme (Jefferson, 1987; Jefferson *et al.*, 1987). ß-Glucuronidase is a hydrolase, calalysing the cleavage of a wide variety of ß-glucuronides of which many are available commercially as spectrophotometric, fluorimetric and histochemical substrates (Lindsey and Jones, 1990). A general purpose vector for constructing gene fusions has been made (Jefferson *et al.*, 1987), and a derivative of this containing the ß-glucuronidase gene, the CaMV 35S or the nopaline synthase promoter, terminator

sequences and polylinker cloning sites, is pBI221 (Fig. 3.5). This plasmid may be used to transform *E. coli*, which in turn is used to produce plasmids in sufficient numbers to allow for extraction and purification for subsequent use in transformation of target cells or tissue.

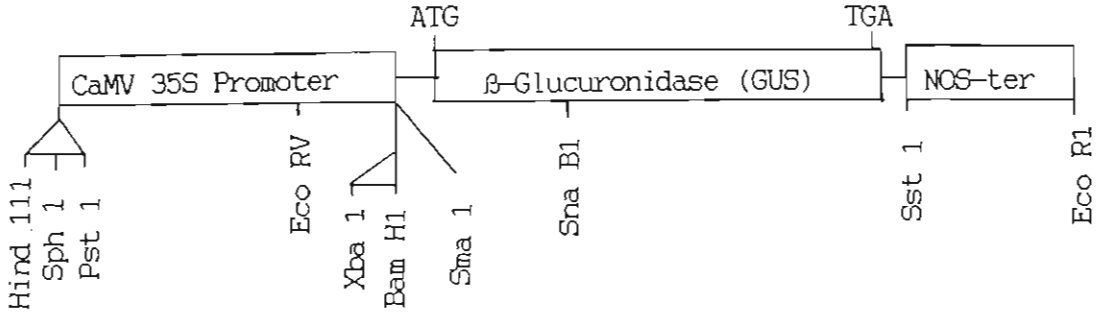


Fig. 3.5 : Structure of pBI221 (3kb), containing the CaMV 35S promoter, the ß-glucuronidase gene, polylinker cloning sites and nopaline synthase terminator (as supplied by Clontech Laboratories).

The principal advantages of the GUS system include the ease of the assay, its sensitivity and the fact that substrates are available (such as X-Gluc; 5-bromo-4-chloro-3-indolyl-b-D-glucuronidide) which give a coloured, insoluble product, used to locate enzyme activity within tissue by histochemistry. Most plants assayed lacked glucuronidase activity, providing a null backround in which to assay chimaeric gene expression (Jefferson *et al.*, 1987). However, problems with the GUS assay have been encountered by some researchers, who have reported endogenous GUS activity in plant tissues (Alwen *et al.*, 1990; Kosugi *et al.*, 1990; Meijer *et al.*, 1991). A prerequisite for the assay is that all endogenous contaminating micro-organisms are eliminated, which may be difficult to achieve in plants that have latent contaminants (Meijer *et al.*, 1991). Modifications to the assay to overcome endogenous plant activity include the addition of methanol to the

incubation buffer (Kosugi *et al.*, 1990) and pH alterations (Alwen *et al.*, 1990).

Despite the abovementioned problems, the GUS assay has been used to test transient expression in a variety of tissues and cells, such as protoplasts (Werr and Lorz, 1986; Larkin *et al.*, 1990; Meijer *et al.*, 1991), pollen grains (Twell *et al.*, 1989), suspension culture cells (Abdul-Baki *et al.*, 1990), callus (Fromm *et al.*, 1990) and intact tissues (Jefferson *et al.*, 1987; Wang *et al.*, 1988; Ahokas, 1989; Creisson *et al.*, 1990; Irvine and Almeida, 1991; Reggiardo *et al.*, 1991).

Detection of stable integration

The development of dominant selectable markers functional in plant tissue, allows the direct selection of transgenic cells by their ability to grow and proliferate under selective conditions, which kill (or prevent the division of) all untransformed cells (Lindsey and Jones, 1990). This ensures the recovery of transgenic material which might only be produced at very low frequencies. The most widely-adopted, stable-integration marker is the neomycin phosphotransferase (NPTII) gene (Beck *et al.*, 1982), which is discussed below.

The NPTII system

Incorporated into vectors with a range of promoter and termination sequences, the NPTII gene has been used with great success for stable transformation of a number of monocotyledons and dicotyledons (reviewed by Weissing *et al.*, 1988; Davey *et al.*, 1989). It is possible to introduce expression vectors which carry both NPTII and GUS encoding genes, for example pBI121 (Jefferson *et al.*, 1987). Using this type of vector, it is possible to rapidly screen all plants which come through the primary selection system, before determining stable transformants by the more time-consuming Southern blots (Tomes *et al.*, 1990).

The NPTII gene from the bacterial transposon Tn5 encodes a small enzyme which confers resistance to a group of aminoglycoside antibiotics which include neomycin, kanamycin and G418, by catalysing their inactivation by phosphorylation (Beck *et al.*, 1982). The reaction involves the transfer of the 8-phosphate group of ATP to the antibiotic molecule, and this addition detoxifies the antibiotic by preventing its interaction with the target site of the ribosome (Beck *et al.*, 1982). Kanamycin is the antibiotic most commonly used, and transformation to resistance can be determined by the phenotypic trait, by Western blotting of the NPTII enzyme or by assaying for NPTII activity in tissue extracts (Reiss *et al.*, 1984).

Although some plants, particularly the graminaceous monocotyledons, exhibit natural resistance to kanamycin (even at concentrations of 800mg/l) (Baribault *et al.*, 1990), gene expression in protoplasts transformed by NPTII and selected on kanamycin-containing medium has been studied for rice (Toriyama *et al.*, 1988; Yang *et al.*, 1988; Zhang *et al.*, 1988), maize (Rhodes *et al.*, 1988; Huang and Dennis, 1989), ryegrass (Potrykus *et al.*, 1985), sugarcane (Chen *et al.*, 1987) and barley (Lorz *et al.*, 1985). In rice, Yang *et al.* (1988) reported regenerated plants which contained the NPTII gene, but only 30% expressed NPTII activity, of which 50% were fertile and produced seed. Other tissues to which NPTII has been successfully delivered, and which expressed resistance to kanamycin, are callus, derived from transformed suspension culture cells of wheat, (Vasil *et al.*, 1991), soybean seed meristems (McCabe *et al.*, 1988), tobacco leaves (Tomes *et al.*, 1990) and wheat pollen grains (Hess *et al.*, 1990).

Other antibiotic agents available for plant selection are hygromycin (resistance is encoded by a hygromycin phosphotransferase gene; van den Elzen *et al.*, 1985; Datta *et al.*, 1990; Hayashimoto *et al.*, 1990) and bialaphos which is produced by the bacterium *Streptomyces hygroscopicus* and has been found to be an effective herbicide (De Block *et al.*, 1987). The BAR gene

which encodes phosphinothricin acetyltransferase and enables the bacteria to resist the effects of their own bialaphos, has been transferred to potato, tomato (De Block *et al.*, 1987), and maize plants (Gordon-Kamm *et al.*, 1990), resulting in the expression of resistance to high levels of the antibiotic.

Herbicides such as the sulphonylureas (Guerineau *et al.*, 1990), imidazoles, and glyphosate are potentially useful as selective agents for transformation studies because they act by inhibiting the activity of a single essential enzyme, and are discussed below.

3.2.6. APPLICATION OF GENETIC ENGINEERING TO PLANT IMPROVEMENT

As the techniques of gene transfer have developed, attention has been focused on their potential application to crop improvement, with the aim of engineering specific traits into a wide variety of plants (reviewed by Walden, 1988, Weissing *et al.*, 1988; Lindsey and Jones, 1989). Unfortunately gene technology has advanced faster than our understanding of plant biochemistry, but it is hoped that in due course these techniques will provide a better comprehension of basic mechanisms involved in plant-gene regulation.

Crop improvement by genetic engineering

Weed control

Resistance to herbicides was among the first traits to which genetic engineering techniques were applied, possibly because it was shown to be a dominant trait exhibiting a simple Mendelian-inheritance pattern in a single nuclear genome (reviewed by Mazur and Falco, 1989). The potential utility of herbicide resistant genes as selectable genetic markers for research in plants, in a manner analagous to that for antibiotic resistance genes, has provided an incentive for research (Weising *et al.*, 1988).

Engineering herbicide resistance/tolerance into plants presents a new alternative for conferring selectivity and enhancing crop safety of herbicides. Research has concentrated on those herbicides with properties such as high unit activity, low mammalian toxicity, low soil mobility, rapid biodegradation and a broad spectrum of activity against various weeds (Gasser and Fraley, 1989). The development of crop plants that are resistant to such herbicides would provide more effective, less costly and more environmentally-attractive weed control (reviewed by Walden, 1988; Lindsey and Jones, 1989). In order to engineer resistance to herbicides into a plant, the molecular basis of its mode of action needs to be well understood. Where the herbicide is known to act on a single biochemical step, several stategies can be adopted in attempting to engineer resistance:

1) selection for resistance in tissue culture, which involves culturing the cells in the presence of the herbicide in order to select resistant cells (reviewed by Lindsey and Jones, 1989; Collin and Dix, 1990),

2) altering the level and sensitivity of the target enzyme for the herbicide (Comai *et al.*, 1985; Shah *et al.*, 1986; Hinchee *et al.*, 1988) and

3) incorporating a gene that will detoxify the herbicide (De Block *et al.*, 1987; Stalker *et al.*, 1988).

The procedure mentioned in the first approach involves either a direct or stepwise selection using callus, suspension culture cells or protoplasts produced *in vitro* (Lindsey and Jones, 1989). Most of the selections for resistance to herbicides involve the direct approach, where callus is exposed to one concentration of the herbicide and the surviving cells are isolated (Collin and Dix, 1990). The alternative is to use the stepwise approach where the surviving callus or suspension culture cells are sequentially transferred to increased levels of the herbicide. Herbicide resistant mutants could be induced by exposure to physical (gamma radiation) or chemical (colchicine) agents (Collin and Dix, 1990).

As an example of the second approach, glyphosate (the active

ingredient of the Roundup herbicide) acts by specifically inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Shah *et al.*, 1986). Glyphosate is active against annual and perennial broadleaf and grassy weeds, has a low animal toxicity, and is rapidly inactivated and degraded in all soils (Mousdale and Coggins, 1984). Resistance to glyphosate has been engineered in various crops by introducing genetic constructs for the overproduction of EPSPS (Shah *et al.*, 1986) or for the glyphosate-tolerant variant EPSPS enzymes (Comai *et al.*,1985; Hinchee *et al.*, 1988).

The third approach is aimed at modifying the herbicide within the cell before it can act on the target. This has been achieved by screening plants that are resistant to the herbicide or micro-organisms that are found in herbicide-contaminated soils, and which degrade the active ingredient of the herbicide (Stalker *et al.*, 1988). If the active ingredient of the herbicide is synthesised by a micro-organism it is likely that the micro-organism will produce a detoxifying enzyme to protect itself (De Block *et al.*, 1987). Resistance to gluphosinate (De Block *et al.*, 1987) and bromoxynil (Stalker *et al.*, 1988) has been achieved by genes coding for enzymes, which inactivate the herbicides by acetylation or nitryl hydrolysis, respectively.

Insect resistance

The production of insect resistant plants is another application of genetic engineering with potential crop improvement envisaged by both the seed and agricultural companies. As well as causing crop damage, insects also act as vectors for viral diseases. Two approaches have been used to engineer resistance to insects in plants. One capitalises on the discovery that the bacterium *Bacillus thuringiensis* (Bt) synthesises a polypeptide lethal to lepidopterous insects (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Murray *et al.*, 1991; Vail, 1991), and the other approach utilises natural defence mechanisms of plants (Hilder *et al.*, 1987).

The Bt endotoxin resides in a parasporal inclusion body which forms during sporulation of the bacterium, and different strains of the bacterium synthesise toxins with different specificities (Dulmage, 1970). Insecticidal proteins ingested by the target insect are solubilised and cleaved by proteases active in the gut, to produce the active toxin. Truncated Bt toxin genes have been introduced into tomato (Vaeck *et al.*, 1987), carrot (Murray *et al.*, 1991), walnut (Vail, 1991), cotton and tobacco (Fischhoff *et al.*, 1987), resulting in transgenic plants exhibiting tolerance to insect larvae in laboratory tests.

Using a slightly different approach, the Bt toxin gene was transferred to epiphytic bacteria which form associations with the roots/leaves of crop plants, thereby ensuring continuous toxin production. The advantage of this is the ease with which bacteria can be transformed, compared to plants. In South Africa, the toxin gene isolated from a Bt causing mortality in a population of stalk-borer pests of sugarcane, was inserted into *Pseudomonas fluorescens*, an epiphyic coloniser (Herrera and Thomson, 1989; Herrera *et al.*, 1991). Laboratory assays indicate successful transfer and expression of the toxin gene, but this has yet to be tested in the field.

A further approach is to enhance the expression of plant genes which are thought to be a naturally-evolved defence response to insect attack, and transfer those genes to plants where they are not normally found; for example proteinase inhibitors, which have a broader spectrum of activity than the *B. thuringiensis* toxin (Hilder *et al.*, 1987). The cowpea trypsin inhibitor has been cloned and inserted into tobacco, where it was found to enhance plant resistance to predatory insects (Hilder *et al.*, 1987).

Disease resistance

Three strategies have been used to engineer plants so that symptoms of a viral infection are reduced. One is an adaptation of viral cross protection (Abel *et al.*, 1986; Van Dun *et al.*,

1987; Cuozzo *et al.*, 1988; Mayo and Barker, 1990); the second utilises symptom modification by viral satellite RNA (Harrison *et al.*, 1987); and the third makes use of anti-sense RNA (reviewed by Lindsey and Jones, 1989).

Cross protection is a phenomenon whereby infection of a plant with one viral strain protects it against superinfection by another, related strain (reviewed by Walden, 1988; Lindsey and Jones, 1989). With the advent of new technologies, it has become possible to transfer specific viral genes to plants in an attempt to confer resistance in a similar way to that of cross protection (Cuozzo *et al.*, 1988). Significant resistance to tobacco mosaic virus (TMV) infection has been achieved by expression of the coat-protein gene of TMV in transgenic plants (Abel *et al.*, 1986), where progeny of transgenic plants either developed delayed viral symptoms upon TMV infection or failed to develop symptoms at all. This approach of coat protein protection produced similar results in transgenic tomato, tobacco and potato plants against a broad spectrum of plant viruses, including alfalfa mosaic virus, cucumber mosaic virus, potato virus X and potato virus Y (Cuozzo *et al.*, 1988; Mayo and Barker, 1990; van Dun *et al.*, 1987).

The second approach involving the suppression of viral infection has been described by Harrison *et al.* (1987), and makes use of the observation that in some plant RNA viruses, certain RNA sequences may ameliorate disease symptoms in infected plants. These sequences, termed satellite RNA, bear no homology to the genomic RNA, and are not required for virus replication and spread. Transformed tobacco plants supplied with a cDNA copy of satellite RNA of cucumber mosaic virus (CMV) developed less severe symptoms than non-transformed plants. It was found that transformed, but non-infected plants, produced only low levels of satellite RNA, but on infection with satellite-free CMV, the synthesis of large amounts of the RNA was induced. The accumulation of satellite RNA was correlated with a reduction in symptom formation.

The third approach to inhibit viral infection involves the

possibility of using anti-sense RNA (minus-strand RNA which binds to plus-strand or mRNA to prevent its translation), which can potentially inhibit the virus replication, packaging and systemic spread in transgenic plants. There is no conclusive evidence yet to suggest that anti-sense RNA can reduce viral symptoms (Lindsey and Jones, 1989).

Application of genetic engineering to basic research

The limitations of genetic engineering systems include the polygenic nature of traits (not knowing the number of genes involved and their precise function), the potential genetic instability of *in vitro* cultured cells and plants, the lack of information on plant metabolism and its regulation (particularly in stress responses), and more specific problems such as random DNA integration and how it influences the expression of other genes (Lindsey and Jones, 1989).

An important contribution of plant-genetic transformation is that it provides a basis for new experimental strategies in plant molecular biology research. These include analysis of viral gene functions by integrating viral DNA (via T-DNA borders) into the plant genome (Grimsley *et al.*, 1986, 1987), inactivating specific genes in the plant genome by the introduction of genes coding for antisense RNA (which interferes by binding to specific mRNA), and the isolation and study of plant genes and promoters (Werr and Lorz, 1986). Applications also lie in the development of strategies such as cDNA cloning, *in vitro* translation and gene library screening (Walden, 1988). Genes responsible for a specific trait may be isolated by the use of transposable elements, which can be used as probes for the isolation of specific genes (transposable elements can modify the regulation of specific genes by inserting into sequences 5' to the coding region) (Haring *et al.*, 1991).

3.3. MATERIALS AND METHODS

Bacterial strains and plasmids used

E. coli HB101 is ampicillin-sensitive (Bolivar and Backman, 1979), and is used routinely for the purpose of plasmid amplification.
pBR322 (Bolivar *et al.*, 1977; Sutcliffe, 1979) is a plasmid used as a multipurpose cloning vector, and in this study was used for radiolabelling purposes.
pBI221 (Clontech, USA) is a plasmid expression vector coding for the ß-glucuronidase enzyme.

Growth and maintenance of bacterial strains

E. coli HB101 was grown on Luria agar (10g/l bacto-tryptone, 5g/l bacto yeast extract, 10g/l NaCl, pH 7,5). Long-term storage of the bacterium was in Luria agar in stab-inoculated bijou bottles, stored at room temperature. Overnight bacterial cultures were grown in Luria broth (5ml), inoculated with a single bacterial colony (Armitage *et al.*, 1988). The overnight cultures were shaken for 12-16h at 37°C on an orbital shaker.

Transformation of *E. coli*

The expression vector plasmid pBI221 (Clontech, USA), was introduced into *E. coli* HB101 (ampicillin-sensitive) by the transformation technique of Hanahan (1985). Transformed cells were selected on Luria agar containing ampicillin (100mg/l).

Generation of expression vector

A technique for large scale plasmid preparation (Armitage *et al.*, 1988) was used to extract pBI221 from transformed *E. coli*. The concentration of purified DNA (determined spectrophotometrically from the absorption reading at 260nm) was 380µg/ml, and the ratio of 260nm to 280nm was 1.8.

Agarose gel electrophoresis of DNA

Plasmids and restriction digests of plasmids were analysed on agarose mini-gels (1% DNA-grade agarose; BioRad, USA), run in TBE buffer (45mM Tris-borate, 1mM EDTA, pH 8) and stained with ethidium bromide (Sigma, USA; 0,5µg/ml). The voltage applied across the horizontal gel apparatus (Hoeffer-HE 33, 7x10cm in size) was 90-100V.

Loading buffer (50% (w/v) sucrose, 4M urea, 0,1% (w/v) bromophenol blue, 50mM EDTA, pH 8) was added to the DNA (in a 1:2 loading buffer:DNA ratio). The DNA plus loading buffer was pretreated at 60°C for 10min after the restriction enzyme digestion to prevent the reannealment of linearised 'sticky' fragments.

Restriction enzyme digestions

Restriction enzyme digestion was performed on Lambda DNA or plasmid DNA with the endonucleases ECoRI, HindIII, BamHI (used singly or in combination) (Boehringer Mannheim, Germany). Each reaction mixture contained DNA (0,2-1µg), 1U of appropriate restriction enzyme, digestion buffer (Boeringer Mannheim, Germany), made up to a final volume of 20µl (Maniatis *et al.*, 1982).

Labelling of plasmid DNA

Labelling of plasmid pBR322 was carried out by nick translation in the presence of [methyl, 1'2'-^{3}H]thymidine 5'-triphosphate ([^{3}H]TTP) according to Rigby *et al.* (1977). A reaction mixture (40µl in total), containing 40µCi [^{3}H]TTP (Amersham, England), unlabelled nucleotides (0,4µg), DNase 1 (121pg) (Sigma, USA), DNA polymerase 1 (16U) (Boehringer Mannheim, Germany) and plasmid DNA (1,25µg) in Tris-HCl (50mM), ß-Mercaptoethanol (1mM) and $MgCl_2$ (10mM) at pH 7,6, was incubated at 15°C for 80 min, after which the DNA sample was applied to a Sephadex G50 spun-column.

Spun-column purification of labelled DNA

Spun-columns were prepared according to Maniatis *et al.* (1982). Syringes (1ml) were plugged at the base with glass wool and packed with Sephadex G50 (Pharmacia, England) which had been hydrated overnight in STE buffer (0,1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8). The columns were suspended in glass test-tubes over open Eppendorf tubes (1,5ml). Eluted DNA was collected by centrifugation (3000rev/min for 5min) after equilibration of the columns with STE under the same conditions. The product was stored at -20°C.

Measurement of specific activity of labelled DNA

Labelled DNA samples were precipitated with trichloroacetic acid (TCA) (Maniatis *et al.*, 1982) in the presence of calf-thymus carrier DNA (30μg carrier DNA/μl labelled DNA) to determine specific activity of high molecular weight fragments. Samples were counted in a Beckman LS 7500 Liquid Scintillation Counter in Beckman 'Ready Value' scintillation cocktail (5ml). Specific activities of 0,9-2,7 x 10^6 cpm/μg DNA were obtained.

Sterilisation of seeds

Seeds were surface-sterilised as described previously (section 1.3.1). The testa of the seed was scraped prior to surface-sterilisation, using a sharp scalpel blade, care being taken to avoid damaging the embryo. Microwave treatments were carried out on seeds placed on filter paper for 5-60s in an Aim Penta microwave, set on high.

Germination of seeds

Seeds were placed aseptically on germination medium (section 2.3.1.).

Protoplast isolation

Protoplasts were isolated from friable achlorophyllous calli, as described in section 2.3.7.

Uptake of labelled DNA by seeds

Seeds were imbibed for various time intervals in solutions of radiolabelled pBR322 plasmid DNA (30 seeds per 25μl; 0,0138μg DNA/μl). After imbibition, seeds were placed in wire mesh (pore size 100μm) baskets suspended above a glass beaker, and were washed extensively under running tap water for 15min. Seeds were crushed in cold TCA (100μl of a 10% solution) in Eppendorf tubes using a tapered glass rod. The seed homogenate was filtered onto a glass fibre filter (Millipore, USA filter unit), and washed with cold TCA (5x1ml aliqouts) and ethanol (3x1ml of a 95% solution) prior to scintillation counting.

Uptake of expression vector by seeds

Seeds were incubated in a solution of pBI221 DNA (6-10 seeds per 25μl; 126μg DNA/ml) for 24h in Eppendorf tubes placed in a sterile, moist container. After imbibition, seeds were transferred to the germination medium and kept in the dark for 48h, before the GUS assay was carried out.

PEG-mediated DNA uptake by protoplasts

Protoplasts ($3x10^6$/ml) were washed and suspended in 2ml of MS culture medium (1,5mg/l 2,4-D, 30g/l sucrose, 0,5M mannitol) containing PEG (10-30% (w/v); MW 6000; Merck, Germany) and pBI221 ($20\mu g/10^5$ protoplasts) for 30min. Thereafter, they were washed and suspended in the above MS culture medium for 48h. In some cases, the broad-spectrum antibiotics penicillin and polymixin B (Sigma, USA; 50μg/ml) were incorporated into the culture medium. Protoplasts were fixed (0,3% formaldehyde, 0,5M mannitol, 9mM MES at pH 5,8) and washed with sodium phosphate buffer (0,2 M) prior

to carrying out the GUS assay.

Assay for transient expression of GUS

Transient expression of ß-glucuronidase (encoded on pBI221) was tested by means of a histochemical assay (Jefferson *et al.*, 1987). Seeds or protoplasts were immersed in the GUS substrate (2mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-Gluc, Sigma), $K_3[Fe(CN)_6]$ (0,1M), $K_4Fe(CN)_6$ (0,1M), Na_2EDTA (0,1M), mannitol (1,23mM) and sodium phosphate buffer (0,2M; pH7) at 37°C overnight. In some cases methanol (20%) was added to the incubation buffer (Kosugi *et al.*, 1990). Presence of the enzyme was visualised by the appearance of a blue stain in the plant material.

Photography

Seeds and protoplasts were photographed as described in section 2.2.9. The agarose gel was photographed under u.v. transillumination using black and white T Max 400 ASA Kodak film and a 1xR red filter.

3.4. RESULTS

3.4.1.UPTAKE OF LABELLED DNA BY SEEDS

In order to establish whether or not DNA could be taken up by sugarcane seeds during imbibition, surface sterilised, dehusked, dry seeds were incubated in [^{3}H] pBR322 plasmid DNA for various time intervals and the radioactivity measured. Seeds took up DNA steadily over a 120min time period (Fig. 3.6A), after which uptake levels decreased (Fig. 3.6B). The sharp decrease in levels of radioactive DNA in the latter seed-batch could indicate that DNA is degraded to some extent and lost by leakage from disaggregated membranes which exist during the process of imbibition.

A comparison was made of DNA uptake by seeds with the testa/seed coat intact and those where the testa had been removed by scraping with a sharp scalpel blade. Fig. 3.7 shows a typical result. It was observed that seeds which had their testa removed, imbibed DNA at a faster rate initially than seeds with the testa intact, but after a 120min time period, the uptake levels were similar.

3.4.2 UPTAKE OF EXPRESSION VECTOR pBI221 BY SEEDS

Having established that DNA could be taken up by seeds, the expression of introduced DNA was investigated. The GUS assay system was chosen to investigate expression by seeds because the assay is simple to perform and has been used to detect transient expression in a number of plants (section 3.2.5; Jefferson *et al.*, 1987). Most plants assayed exhibit no endogenous GUS activity (Jefferson, 1987). The DNA that was introduced into seeds to monitor expression was that of the plasmid expression vector pBI221, which encodes the bacterial enzyme ß-glucuronidase (Fig. 3.5).

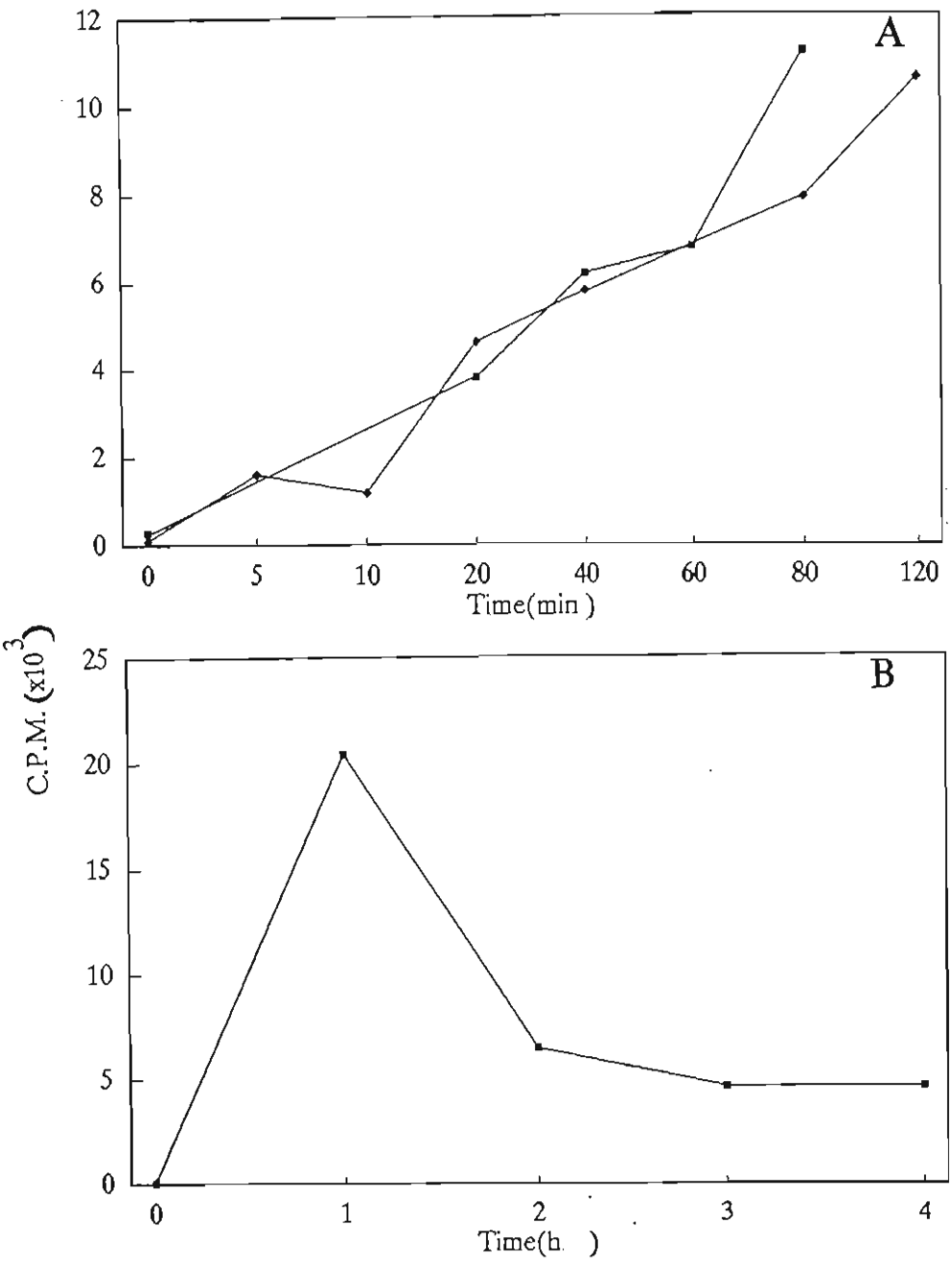


Fig. 3.6 :Uptake of [^{3}H] pBR322 DNA by sugarcane seeds. For each time interval, 10 dehusked seeds were incubated in 0,35μg DNA (specific activity A: (■) 1,4x10^{6}cpm/μg, (◆) 9,2x10^{5}cpm/μg; B: 1,4x10^{6}cpm/μg). Seeds were placed in wire baskets and thoroughly washed under running water for 15min. Subsequently seeds were homogenised in cold TCA, filtered onto a glass fibre filter and washed first with TCA and then ethanol. The filter was air dried and scintillation counted.

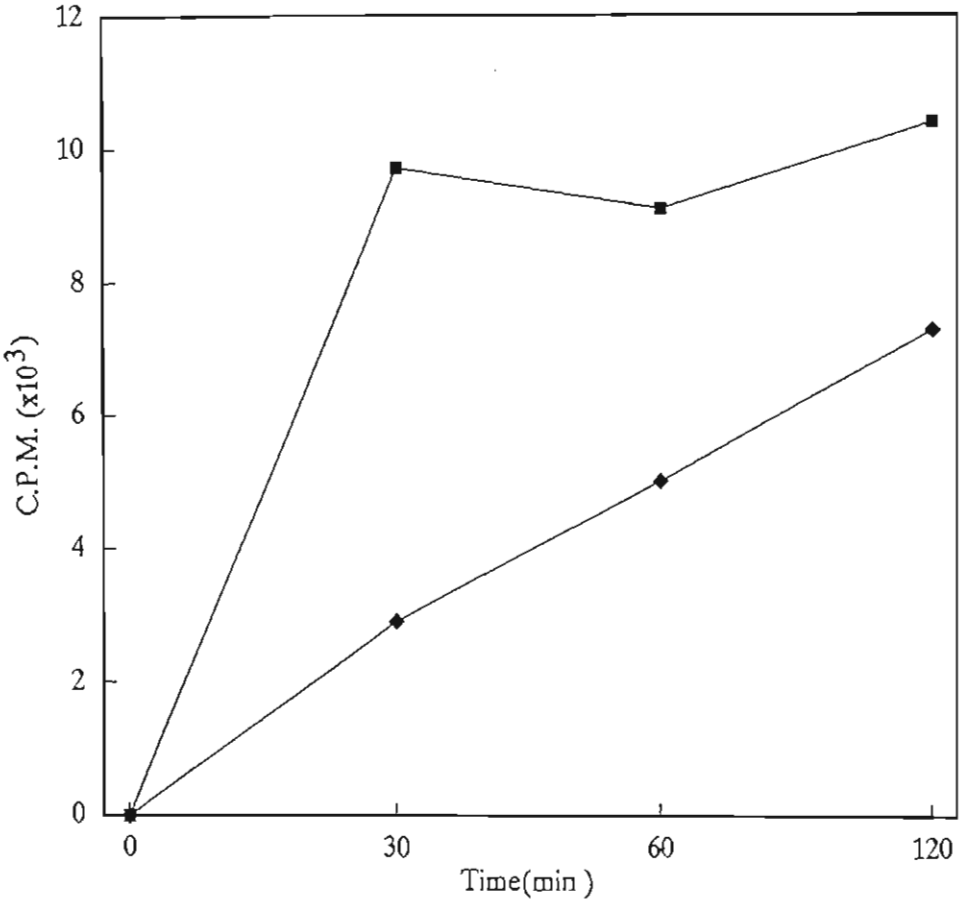


Fig. 3.7 :Rate of uptake of [^{3}H] pBR322 DNA by sugarcane seeds with testa removed (■), and testa intact (◆). The testa was removed from dehusked, dry seeds by scraping with a scalpel blade. For each time interval, 30 seeds were incubated in 0,48μgDNA (specific activity 1,8x10^{6}cpm/μg). Seeds were processed as for Fig. 3.6.

Confirmation of pBI221 structure

Purified pBI221 DNA was restricted, and the digestion fragments analysed by gel electrophoresis in order to confirm that the construct complied with that in the map supplied by Clontech. The pBI221 construct was found to be identical to the original cloned plasmid (Fig. 3.8A and B). Cleavage of pBI221 with EcoR1 linearised the plasmid, resulting in the appearance of a single band (5,7kb). Plasmid digestion with EcoR1 and HindIII separated the CaMV 35S promoter-GUS-Nos-terminator cassette (3kb) from the pUC19 portion (2,7kb), resulting in the appearance of two closely-associated bands. BamH1 cleaved the GUS expression cassette into a further two fragments, the ß-glucuronidase gene (2,2kb) and the CaMV 35S promoter (0,8kb).

Detection of GUS expression in seeds

Sugarcane seeds were incubated in pBI221 DNA in order to see whether transient expression of the DNA could be detected. The expression of DNA was monitored using a histochemical GUS assay. Sugarcane seeds were surface sterilised and incubated in the DNA for 24h before being placed on a germination medium for 48h, after which time the GUS assay was carried out. A blue colour indicating ß-glucuronidase activity was observed in sugarcane seeds incubated in both pBI221 DNA and TE buffer (control), after the GUS histochemical assay was performed (Plate 3.1). The blue colour was observed in exactly the same place in both DNA-treated and control seeds, and occurred under the testa on the uppermost cell layers of the endosperm above the embryo. The presence of the blue stain in control seeds suggested that there was either a microbial seed contaminant present or that there was endogenous GUS activity in the seed tissue.

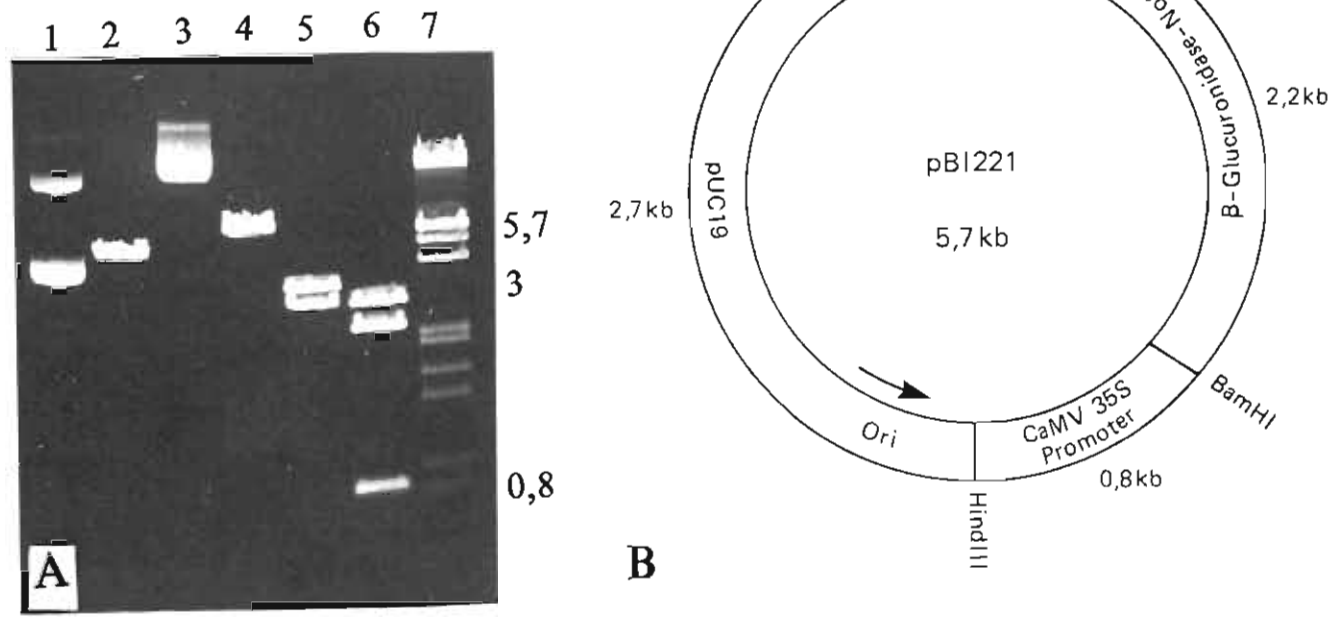


Fig. 3.8: A. Agarose-gel electrophoresis of restriction fragments of pBI221. Agarose (1% (w/v)) gel run at 100V. 1, undigested pBR322 (0,5μg); 2, ECoRI digest of pBR322 (0,425μg); 3, undigested pBI221 (0,7μg); 4, ECoRI digest of pBI221 (0,9μg); 5, ECoRI/HindIII digest of pBI221 (1μg); 6, ECoRI/HindIII/BamHI digest of pBI221 (1μg); 7, ECoRI/HindIII digest of Lambda DNA (0,87μg); size markers are in kilobases.

B. A simplified construct diagram of pBI221 showing restriction sites and sizes of restricted fragments.

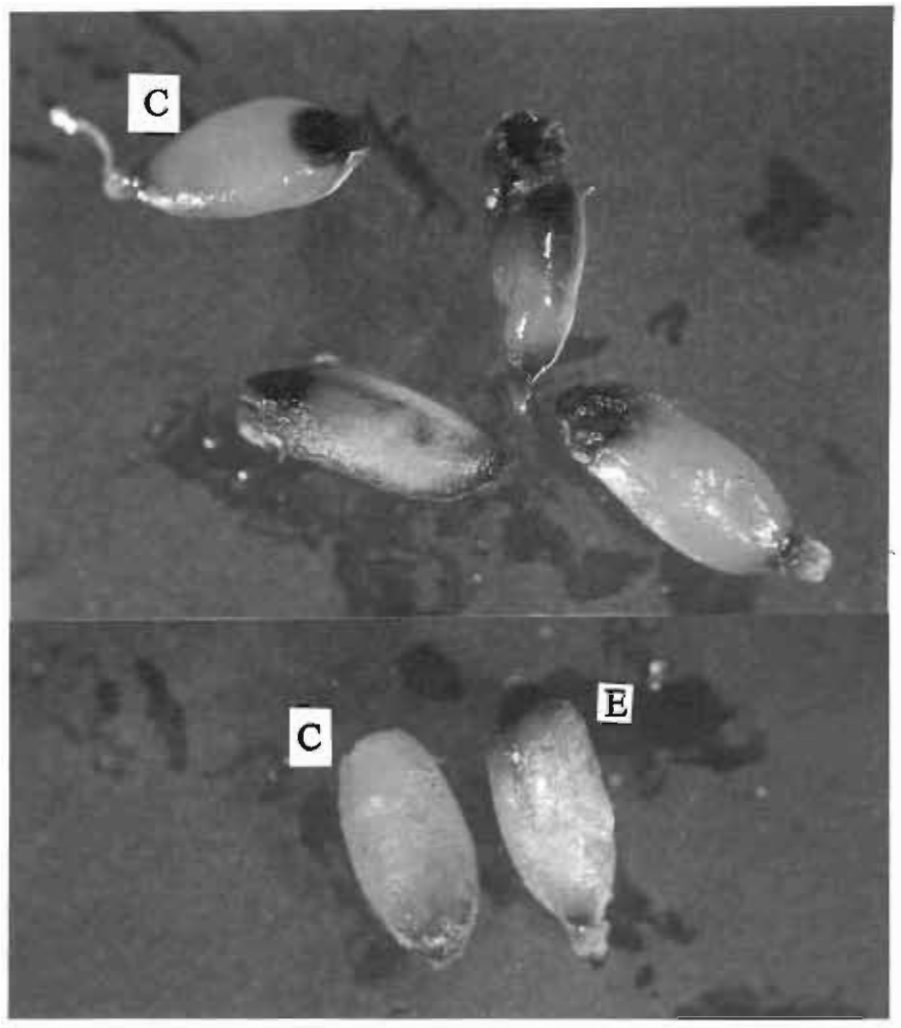


Plate 3.1 : Sugarcane seeds, following uptake of expression vector pBI221 and histochemical GUS assay. Seeds were incubated in DNA for 24h and placed onto germination medium for 48h before the GUS assay was carried out. Note the blue colour in seeds at the top of the embryo, and occasionally around the embryo (E). Although a high proportion of control seeds gave a positive result, some seeds exhibited no blue colour (C). Bar represents 0,7mm

Treatment of seeds in order to overcome positive controls

In an attempt to investigate the two possibilities of microbial seed contamination or endogenous GUS activity in the seed tissue causing positive results in control seeds, a number of variations to seed pretreatment and medium composition of the GUS assay were carried out: the testa was removed in order to see whether this served as a barrier to DNA uptake by the embryo of the seed, more extensive sterilisation treatments were undertaken in the form of microwaving, the addition of methanol to the GUS incubation buffer was investigated, and lastly, a combination of all the above treatments was carried out.

Testa as a possible barrier to DNA entry into embryo

Uptake and expression of pBI221 by seeds which had their testa removed were compared to seeds which had the testa intact, because it was necessary to establish whether or not the testa was a barrier to DNA uptake by the embryo. In addition, previous results with radiolabelled DNA (Figs. 3.6 and 3.7) indicated that seeds with testa removed showed faster initial rates of DNA uptake than seeds with the testa intact. The testa was removed from seeds by scraping it with a scalpel blade, prior to surface sterilisation and incubation in DNA. Seeds with the testa removed resulted in 40% and 30% of seeds expressing ß-glucuronidase in the DNA treated and control seeds, respectively. Seeds where the testa was left intact resulted in 60% of seeds in both the control and DNA-treated seeds expressing ß-glucuronidase activity (summary of treatments in Table 3.2). The blue colour was more visible in seeds which had the testa removed, so this was carried out routinely for subsequent assays.

Elimination of bacterial contamination

In order to try and eliminate microbial contamination from sugarcane seeds, more extensive sterilisation treatments were undertaken. A series of microwave treatments were carried out and

germination and bacterial contamination levels were recorded after microwaving the seeds for various time intervals. There was 100% germinability in seeds not microwaved, but a 50% loss in germinability was recorded when seeds were microwaved for 60s (Table 3.1). All of the microwave treatments reduced contamination levels from 20%, observed in seeds not microwaved, to 0%, except at 15s where 16% contamination was observed (Table 3.1). For further GUS assays, seeds were microwaved for 60s, as this treatment maintained 50% germinability of seeds and seemed to eradicate bacterial contamination.

Table 3.1 : Germination and bacterial contamination levels of sugarcane seeds exposed to microwave treatment for various time intervals. After microwaving, seeds were surface sterilised, squashed in a sterile petri-dish and placed onto nutrient agar for 2 days in order to monitor bacterial contamination. Germination levels were determined by placing whole seeds onto germination medium for 5 days (section 2.3.1). (n=5-6)

Time (s)	% germination	% seeds contaminated
0	100	20
5	100	0
10	80	0
15	100	16
20	66	0
40	40	0
60	50	0

A GUS assay was carried out on seeds that had been microwaved for 60s, and although 90% of seeds incubated in pBI221 were positive for ß-glucuronidase, 60% of seeds incubated in TE buffer also exhibited a positive result (Table 3.2). Seeds which had the testa removed and which were microwaved prior to carrying out the GUS assay, exhibited blue colour in 60% of the seeds incubated in pBI221 and 40% of the seeds incubated in TE buffer (Table 3.2). Although the suspected bacterial contaminant seemed to have been reduced by microwave treatments, positive results were still

observed in control seeds. Therefore, if the positive control results are due to a contamination problem, it is likely that the contaminant is endogenous and is symbiotic in sugarcane seeds.

Table 3.2 : Effect of seed pretreatment and assay medium on sugarcane seeds, as determined by the GUS histochemical assay. Seed treatments included microwaving for up to 60s prior to surface sterilisation, removal of testa by scraping with a scalpel blade, the addition of 20% methanol to the GUS incubation buffer and a combination of the above treatments in order to overcome positive results in the controls. (n=5-10)

Seed treatment	Seeds demonstrating GUS expression (%)		Comments
	DNA (pBI221)	Control (TE buffer)	
microwaved for 60s	90	60	positive controls
testa removed by scraping with scalpel blade vs testa intact	40 60	30 60	blue colour more visible in scraped seeds
testa removed, microwaved for 60s	60	40	positive controls
testa removed, microwaved for 60s, 20% methanol added to GUS incubation buffer	100	80	positive controls
testa removed, 20% methanol added to GUS incubation buffer	100	100	positive controls

Suppression of endogenous plant GUS activity

Kosugi *et al.* (1990) found that the addition of methanol to the GUS incubation buffer was effective at lowering endogenous plant GUS activity in leaves, calli and suspension culture cells of rice, tobacco and bean. Therefore, in an attempt to investigate endogenous GUS activity in seed tissue, 20% methanol was added to the GUS assay incubation buffer. However, in sugarcane seeds, the

problem of a positive response in control seeds was not eliminated by this treatment. All of the microwaved, DNA-treated seeds and 80% of microwaved control seeds, exhibited blue colouration (Table 3.2). Seeds incubated in DNA and TE buffer, and which were not microwaved also exhibited blue colouration. This suggests that sugarcane seeds contain an endogenous microbial contaminant as endogenous plant GUS activity is likely to have been suppresed by the addition of methanol to the incubation buffer.

3.4.3 UPTAKE OF pBI221 DNA BY PROTOPLASTS

There are numerous methods available for protoplast transformation, and the options available for sugarcane include electroporation, microinjection, liposome-mediated DNA delivery and chemical-mediated DNA uptake (reviewed in section 3.2.2). Direct uptake of naked DNA via the last-mentioned method provides the most simple approach, without the need for specialised equipment. Chemical-induced DNA uptake by PEG has been used by numerous workers with considerable success for transformation of protoplasts (reviewed in section 3.2.2). However, PEG at high concentrations has a deleterious effect on protoplasts, and it is necessary to determine the optimum concentration which allows DNA uptake without excessive loss in protoplast viability. The only drawback with this technique is that protoplasts have to be regenerated to plants, and this is a problem in other graminaceous monocotyledons, as well as sugarcane.

Viability studies on protoplasts treated with PEG

The viability of callus protoplasts after exposure to PEG was monitored by the exclusion of Evan's blue dye as described in section 2.3.7. Viability studies using PEG at a concentration range of 10-30% were carried out as this is the range which has had success in previous work on transformation of protoplasts (Krens *et al.*, 1982; Lorz *et al.*, 1985; Werr and Lorz, 1986; Chen *et al.*, 1987). Protoplasts were exposed to PEG for 30min, before the PEG was diluted with culture medium, following the time

intervals and general protocol for PEG-mediated DNA-uptake by Draper *et al.* (1988). PEG at 20% (w/v) resulted in the highest number of viable sugarcane protoplasts ($3,1x10^6$/g callus) with the least loss in viability (38%) (Fig. 3.9) and was the PEG concentration chosen for further DNA-uptake experiments.

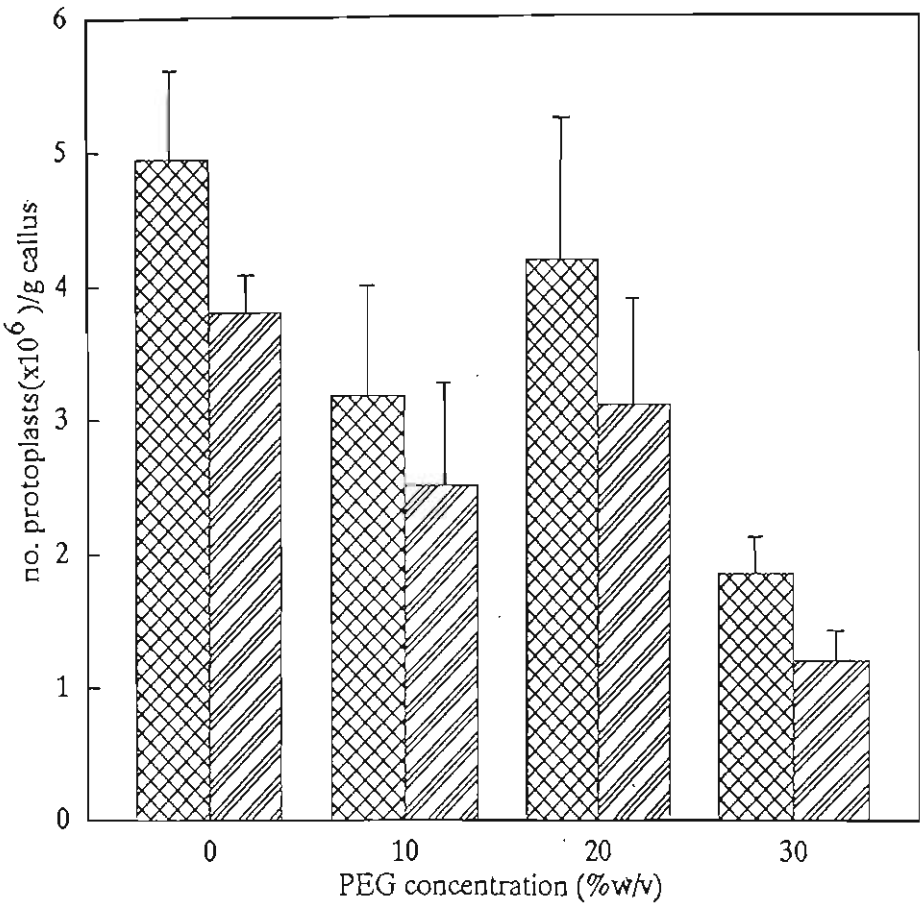


Fig. 3.9 : Total (▨) and viable (▧) numbers of protoplasts, isolated from calli, after exposure to PEG for 30min. The PEG was made up in MS culture medium containing mannitol(0,5M) and 2,4-D (1,5mg/l) (s.e. are at the 0,05 level of probability and are indicated by a line above the bar).

GUS assay for transient expression in protoplasts

Once protoplast viability in different PEG concentrations had been determined, transient expression of transforming pBI221 DNA in protoplasts was investigated. Protoplasts were prepared as discussed in section 2.3.7 and exposed to pBI221 DNA suspended in 20% PEG for 30min, after which time the protoplasts were incubated in MS protoplast culture medium for 48h before the histochemical GUS assay was carried out. Microscopic examination of the protoplasts revealed that the suspensions were contaminated and no blue protoplasts were observed.

Elimination of contamination in protoplasts

In an attempt to overcome the contamination problem, calli were screened for endogenous microbial contamination prior to protoplast isolation. A small piece of the callus to be used was placed into nutrient broth, and turbidity was monitored. Once an uncontaminated source of callus had been obtained, protoplasts were isolated and incubated in a PEG solution containing DNA, after which the GUS assay was carried out. Contamination problems persisted and no blue protoplasts were observed.

In a subsequent step, the antibiotics penicillin and polymixin B were incorporated into the protoplast culture medium after protoplast isolation and DNA incubation. Contamination levels were reduced but not entirely eliminated, and no blue protoplasts were observed after carrying out the GUS assay.

Perhaps the frequency of transformation is too low to pick up transformed cells, and it is possible that even low levels of bacterial contamination could interfere with DNA uptake and subsequent culture of the protoplasts.

3.5. DISCUSSION

The use of the seed in transformation studies

Using radiolabelled pBR322 DNA, it was demonstrated in this study that sugarcane seeds were able to take up naked DNA during imbibition. It was established that DNA was taken up during the first 1-2h of imbibition (Fig. 3.6), and that uptake was more rapid initially in those seeds which had the testa removed (Fig. 3.7). Other researchers have found benefit in doing a preliminary investigation into DNA uptake using radiolabelled DNA. Ledoux and Huart (1968, 1969) imbibed barley embryos in radiolabelled DNA and observed that the DNA was taken up by the seed endosperm and later by cells in the root of the germinating seedling. In another study, radiolabelled DNA was electrophoretically transferred to barley embryos and the results detected by microscopic autoradiography (Ahokas, 1989), and the initial set of results used to improve the uptake procedure. Abdul-Baki *et al.* (1990) investigated uptake of radiolabelled DNA by tobacco pollen grains and subsequently established protocols for the transformation of pollen.

In the present study, the uptake of the GUS expression vector, pBI221, and subsequent transient expression of the ß-glucuronidase gene encoded on the vector, could not be conclusively demonstrated in sugarcane seeds. Blue colouration which is indicative of ß-glucuronidase activity was observed in control seeds incubated in TE buffer which confounded the interpretation of blue colour in the seeds incubated in pBI221 DNA preparations.

Possible reasons for the blue colouration in the control seeds are that there was a microbial contaminant in sugarcane seeds, as has been postulated for causing positive results in early work carried out on barley embryos (Kleinhofs *et al.*, 1975; Kleinhofs and Behki, 1977) or that there was endogenous plant GUS activity, which has been found previously in rice (Kosugi *et al.*, 1990; Meijer *et al.*, 1991) and tobacco (Alwen *et al.*, 1990).

Attempts to eliminate the blue colour in control seeds caused by either of the above two factors included removing testa, microwaving seeds for 60s, the addition of methanol to GUS assay incubation buffer and a combination of the above treatments. Although the microwave treatments appeared to eradicate bacterial contamination detectable by plating onto nutrient agar in most cases (Table 3.1), this did not overcome the problem of positive controls. Bearing in mind that the blue spot was localised (Plate 3.1), and found in both DNA-treated and control seeds (Table 3.2), this brings to light the possibility that the contaminant is an endogenous symbiont, which would be difficult to eradicate. Leifert and Waites (1990) have encountered latent bacteria in plants from a number of species produced *in vitro*, so this seems to support the fact that these types of contaminants are not uncommon, as well as difficult to eradicate. Future verification of this could include microscopic studies, but this is beyond the scope of the present study.

Another possibility for blue colour persisting in the controls is that there was intrinsic plant GUS activity present in sugarcane seed tissue. Similar problems have been encountered in rice (Kosugi *et al.*, 1990; Meijer *et al.*, 1991), and tobacco (Alwen *et al.*, 1990). Alwen *et al.* (1990) found that plant ß-glucuronidase has an optimal activity at pH5, whereas the bacterial ß-glucuronidase is active at pH7, so under appropriate experimental conditions, it might be possible for future GUS assays for bacterial enzyme activity to be carried out without the interference of endogenous plant enzyme activity. Kosugi *et al.* (1990) were able to overcome the endogeous plant GUS activity in rice callus, suspension cells and shoots on partially regenerated plants; tobacco and bean calli and leaf tissue; and maize suspension culture cells, by the addition of methanol to the GUS incubation buffer. This modification also enhanced the blue colour in the histochemical assay. However, in sugarcane seeds, the blue colouration in control seeds was not nullified by the addition of methanol to the incubation buffer (Table 3.2).

Different tissue types could harbour varying levels of endogenous plant GUS activity, and Kosugi *et al.* (1990) found that endogenous GUS activities in calli and suspension culture cells were higher than that of leaves, but a reduction of endogenous activity was observed in all the above tissues when methanol was added to the GUS incubation buffer. This suggests that in sugarcane seeds the endogenous activity may indeed be due to endogenous contaminating microorganisms.

The use of the seed as a recipient for DNA provided a novel and convenient approach for DNA uptake, but it has not been widely used, even though it eliminates the need for *in vitro* regeneration of plants. Reports of successful transformation of seeds which have imbibed naked DNA, include *Arabidopsis* (Feldmann and Marks, 1987), barley (Ledoux and Huart, 1968, 1969; Ledoux *et al.*, 1971, 1974; Topfer *et al.*, 1989) and maize, rice, rye, oats, wheat, bean and pea (Topfer *et al.*, 1989). The recent revival of the seed-imbibition approach by Topfer *et al.* (1989) seemed encouraging, particularly for use with members of the graminaceous monocotyledonous plant group. However taking into account the problems encountered in this study, and the recent observations that so-called transgenic plants produced by Topfer *et al.* (1989) exhibit non-Mendelian inheritance patterns, and microbial contaminants are thought to have affected the interpretation of results (Schell, pers. comm.), the seed is not a likely candidate for DNA uptake at the present time.

Future work in this area may include looking at desiccating sugarcane somatic embryos and investigating naked DNA uptake during the rehydration process. The analogy in development and germination of zygotic and somatic embryos has been discussed (section 2.1.2), and this approach also offers the potential for easy regeneration of plants *in vitro* and provides a novel alternative to using the seed.

Transformation via the protoplast

In this study PEG-mediated DNA uptake and transient expression in sugarcane protoplasts was investigated. No positive results were observed in DNA-treated protoplasts after the histochemical GUS assay was carried out. Problems with contamination of the protoplast suspension were encountered, but the protoplast contaminants did not express any ß-glucuronidase activity, and were therefore different from those observed in the seeds. Contaminants in the protoplast suspension could have bound to the plasma membrane and prevented uptake of DNA. Even if DNA was taken up successfully, contaminants in the culture medium could have altered the medium composition and pH, and caused unfavourable conditions for protoplast growth. Attempts to eliminate contamination by screening calli prior to protoplast isolation and incorporating antibiotics into the culture medium, were only partially successful. Once again the possibility of the presence of an endogenous-contaminating symbiont in sugarcane tissue/cells is raised. The processes necessary for screening and identification of such a symbiont are beyond the scope of this study, but would form an important preliminary study if the work was to be developed further.

Another possible reason for the lack of positive response in protoplasts is that the frequency of transformation was so low that any positive results went undetected. Werr and Lorz (1986) reported successful transformation of wheat protoplasts, but at a low frequency of 1 transformed cell in 10^5. Perhaps a more effective method for screening a low proportion of transformed cells should be investigated. This could include introducing a dominant selectable marker gene like NPTII (section 3.2.5), which would confer the ability to grow in an antibiotic-containing medium on transformed cells and, in addition, may help to overcome contamination problems. Despite problems experienced in this study, protoplasts of a Taiwanese sugarcane variety have been successfully transformed by a research group in England using PEG (Chen *et al.*, 1987). Transformed sugarcane colonies resulted when

these protoplasts were transformed with the selective marker NPTII. Protoplasts were cultured on media containing kanamycin for 8 weeks, but the transformation frequency was still low, approximately 8 in 10^7 cells. Chen *et al.* (1987) reported that glucose was an essential component of the PEG solution, and that it minimised protoplast damage. Perhaps this could be included in future experiments using South African varieties.

Although protoplasts from the grasses and cereals are not regenerated into plants *in vitro* on a regular basis, they are commonly used as recipients for the introduction of foreign genes into plants. Recent success in the regeneration of rice and maize plants from protoplasts (Rhodes *et al.*, 1988; Datta *et al.*, 1990) may encourage continued research on members of the Gramineae. PEG-mediated DNA uptake by protoplasts has been established as a reliable and simple method for the transformation of protoplasts of tobacco (Krens *et al.*, 1982; Hein *et al.*, 1983; Kartzke *et al.*, 1990; Larkin *et al.*, 1990), rice (Yang *et al.*, 1988; Datta *et al.*, 1990; Hayashimoto *et al.*, 1990), ryegrass (Potrykus *et al.*, 1985), maize and barley (Junker *et al.*, 1987), and sugarcane (Chen *et al.*, 1987), so it would seem beneficial to persevere with the same approach in the South African sugar industry.

Transformation alternatives

In the light of results obtained in this preliminary investigation into gene uptake and transient expression in sugarcane plants, it is necessary to consider other transformation methods. Options which are available for protoplast transformation include electroporation but this method does not appear to be superior to PEG-induced uptake for transformation of sugarcane protoplasts (Chen *et al.*, 1987). Microinjection and liposome -mediated DNA delivery are further possibilities (section 3.2.2). However, all of the abovementioned techniques require the regeneration of plants from protoplasts, and so far this has only been achieved on a repeatable basis for rice and maize.

The microprojectile bombardment of tissue/cells has been used with some success in a number of plants, and taking into account the difficulties in regeneration from protoplasts, is an appealing choice (section 3.2.2). A modification of the machine used to carry out microprojectile bombardment has been developed for use in sugarcane (Irvine and Almeida, 1991), and as well as reducing the cost of the equipment, gives promising results (Irvine, pers. comm.).

CONCLUDING REMARKS

Throughout this study contamination problems have persisted. The adult-plant source material for callus induction was severely contaminated and this was not overcome with the addition of antibiotics, which had an adverse effect on callus initiation. Transient expression studies using the seed as a recipient for DNA uptake were unsuccessful because of a suspected endogenous, microbial symbiont. The use of protoplasts isolated from calli for PEG-mediated DNA uptake provided no conclusive evidence to suggest that this system could be used in the transformation of sugarcane, because of contamination problems. Even the use of alternative transformation procedures, such as microprojectile bombardment, may be unsuccessful because of interference from endogenous contaminants. This problem would have to be addressed before any further transformation procedures are investigated.

REFERENCES

Abdul-Baki, A., Saunders, J.A., Matthews, B.F. and Pitarelli, G.W. (1990). DNA uptake during electroporation of germinating pollen grains. *Plant Sci.* 70: 181-190.

Abdullah, R., Cocking, E.C. and Thompson, J.A. (1986). Efficient plant regeneration from rice protoplasts through somatic embryogenesis. *Bio/Technology* 4: 1087-1090.

Abe, T. and Futsuhara, Y. (1985). Efficient plant regeneration by somatic embryogenesis from root callus cultures of rice (*Oryza sativa* L.). *J. Plant Physiol.* 121: 111-118.

Abel, P.P., Nelson, R.S., De, B., Hoffman, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat-protein gene. *Science* 232: 738.

Ahloowalia, B.S. (1984). Forage grasses. In: *Handbook of Plant cell culture.* Vol. 3. Eds: P.V. Ammirato, D.A. Evans, W.R. Sharp and Y. Yamada. MacMillan Publishing Co., New York. pp 91-125.

Ahokas, H. (1989). Transfection of germinating barley seed electrophoretically with exogenous DNA. *Theor. Appl. Genet.* 77: 469-472.

Alexander, A.G. (1985). *The energy cane alternative.* Elsevier Science Publishers, The Netherlands. pp 31-33, 233.

Alwen, A., Vicente, O. and Heberle-Bors, E. (1990). Use of *E. coli* GUS as a reporter gene in plants: possible interference of endogenous ß-glucuronidase. Book of Abatracts: VII Int. Congress of plant tissue and cell culture, Amsterdam, June.

Ammirato, P.V. (1983). Embrogenesis. In: *Handbook of plant cell culture : Techniques for propagation and breeding.* Ed: D.A. Evans, W.R. Sharp, P.V. Ammirato, Y. Yamada. Macmillan Publishing Co., New York. pp 82-122.

Ammirato, P.V. (1987). Organizational events during somatic embrogenesis. In: *Plant Tissue and Cell Culture.* Ed: C.E. Green, D.A. Somers, W.P. Hackett, D.D. Biesboer. Alcu R. Liss, New York. pp 57-81.

Ammirato, P.V. (1989). Recent progress in somatic embrogenesis. *IAPTC Newsletter* 57: 2-16.

Anonymous. (1977). *Sugarcane production in South Africa.* Bulletin No. 1. Published by Experiment Station of the Sugar Association of South Africa. 44pp.

Anonymous. (1991). *History of varieties.* Notes from Certificate Course in sugarcane agriculture. Published by South African Sugar Association Experiment Station. 31pp.

Arcenaux, G. (1965). Cultivated sugarcanes of the world and their botanical derivation. *Proc.* 12th *Congr. Int. Soc. Sugar Cane Technol.* Puerto Rico. 844-854.

Armitage, P., Walden,R. and Draper, J. (1988). Vectors for the transformation of plant cells using *Agrobacterium*. In : *Plant genetic transformation and and gene expression: A laboratory manual*. Eds.: J. Draper, R. Scott and R. Walden. Blackwell Scientific Publications, UK. pp 1-68.

Armstrong, C.L. and Green, L.E. (1985). Establishment and maintenance of friable, embrogenic maize callus and the involvement of proline. *Planta* 164: 207-214.

Bajaj, Y.P.S. (1981). Growth and morphogenesis in frozen (-196°C) endosperm and embryos of rice. *Curr. Sci.* 50: 947-950.

Bajaj, Y.P.S. (1985). Cryopreservation of embryos. In: *Cryopreservation of plant cells and organs*. Ed: K.K. Kartha. CRC Press, Boca Raton, U.S.A. pp 227-240.

Baribault, T.J., Skene, K.G.M., Cain, P.A. and Steele Scott, N. (1990). Transgenic grapevines: regeneration of shoots expressing ß-glucuronidases. *J. Exp. Bot.* 41: 1045-1049.

Barker, R.F., Idler, K.B., Thompson, D.V. and Kemp, J.D. (1983). Nucleotide sequence of the T-DNA region from the *A. tumefaciens* octopine Ti plasmid pTi 15955. *Plant Mol. Biol.* 2: 335-350.

Barnes, A.C. (1974). *The Sugar Cane*. 2nd Ed. Leonora Hill Books, U.K. pp 20-33.

Bartkowiak, E. (1981). Tissue culture of maize: Selection of friable callus lines. *Plant Cell Rep.* 1: 52-55.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. and Schaller, H. (1982). Nucleotide sequence and exact localisation of the neomycin phosphotransferase gene from transposon Tn5. *Gene*. 19: 327-336.

Bhaskaran, S. and Smith, R.H. (1988). Enhanced somatic embryogenesis in *Sorghum bicolor* from shoot tip culture. *In vitro Cell and Dev. Biol.* 24: 65-70.

Bhaskaran, S. and Smith, R.H. (1989). Control of morphogenesis in sorghum by 2,4-D & cytokinins. *Ann. Bot.* 64: 217-224.

Bhaskaran, S. and Smith, R.H. (1990). Regeneration in cereal tissue culture: A review. *Crop Sci.* 30: 1328-1336.

Bolivar, F. and Backman, K. (1979). Plasmids of *Escherichia coli* as cloning vectors. *Methods in Enzymol.* 68: 245-248.

Bolivar, F., Rodriquez, R.L., Green, P.J., Betlack, M.C., Heynecker, H.L. and Boyer, H.W. (1977). Construction and characterisation of new cloning vehicles. II. A multi-purpose cloning system. *Gene* 2: 95-113.

Botti, C. and Vasil, I.K. (1983). Plant regeneration by somatic embrogenesis from parts of cultured embryos of *Pennisetum americanum* (L). K. Schum. *Z. Pflanzenphysiol.* 111: 319-325.

Boxus, P. and Terzi, J.M. (1988). Control of accidental contamination during mass propagation. *Acta Hort.* 225: 189-193.

Braun, A.C. (1978). Plant tumors. *Biochim. Biophys. Acta.* 516: 167-191.

Bregitzer, P., Somers, D.A. and Rines, H.W. (1989) Development and characterization of friable, embryogenic oat callus. *Crop Sci.* 29: 798-803.

Brisson, N., Paszkowski, J., Penswick, J.R., Gronenborn, B., Potrykus, I. and Hohn, T. (1984). Expression of a bacterial gene in plants by using a viral vector. *Nature* 310: 511-514.

Carman, J.G. (1988). Improved somatic embryogenesis in wheat by partial simulation of the in-ovulo oxygen, growth-regulator and desiccation environments. *Planta.* 175: 417-424.

Cella, R. and Galun, E. (1980). Utilization of irradiated carrot cell suspensions as feeder layers for cultured *Nicotiana* cells and protoplasts. *Plant Sci. Lett.* 19: 243-252.

Celis, J.E. (1984). Microinjection of somatic cells with micropipettes : Comparison with other transfer techniques. *Biochem. J.* 223: 281-291.

Chandler, S.F. and Vasil, I.K. (1984). Optimization of plant regeneration from long term embryogenic callus cultures of *Pennisetum purpureum* Schum. (Napier Grass). *J. Plant Physiol.* 117: 147-156.

Chee, P.P., Fober, K.A. and Slightom, J.L. (1989). Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. *Plant Physiol.* 91: 1212-1218.

Chen, W.H., Davey, M.R., Power, J.B., and Cocking, E.C. (1988a). Control and maintenance of plant regeneration in sugarcane callus cultures. *J. Exp Bot.* 39: 251-261.

Chen, W.H., Davey, M.R., Power, J.B. and Cocking, E.C. (1988b). Sugarcane protoplasts: factors affecting division and plant regeneration. *Plant Cell Rep.* 7: 344-347.

Chen, W.H., Gartland, K.M.A., Davey, M.R., Sòtak, R., Gartland, J.S., Mulligan, B.J., Power, J.B. and Cocking, E.C. (1987). Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. *Plant Cell Rep.* 6: 297-301.

Chen, W.H. and Liu, M.C. (1974). Isolation of protoplasts from young sugarcane leaves. *Rep. Taiwan Sugar Res. Inst.* 64: 1-10.

Chen, W.H. and Shih, S.C. (1983). Isolation of protoplasts from cell culture and subsequent callus regeneration in sugarcane. *Int. Sug. Technol. Ass.* (18th Congress): 93-109.

Chen, Z.X.Q., Wang, C., Su, I.C.O., Chen, F and Deng, Z. (1979). The induction of pollen plants in sugarcane. *Acad. Sin. Inst. Genet. Annu. Rep.* pp 91-93.

Chilton, D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and Nester, G.W. (1977). Stable incorporation of plasmid DNA into higher plant cells : the molecular basis of crown gall tumorigenesis. Cell 11: 263.

Cocking, E.C. (1960). A method for the isolation of plant protoplasts and vacuoles. *Nature* 187: 962-963.

Cocking, E.C. and Davey, M.R. (1987). Gene transfer in cereals. *Science* 236: 1259-1262.

Collin, H.A. and Dix, P.J. (1990). Culture systems and selection procedures. In: *Plant cell line selection: procedures and applications*. Ed: P.J. Dix. VCH Publishers, Germany. pp 1-17.

Comai, L., Facclotti, D., Hiatt, W.R., Thompson, G., Rose, R.E. and Stalker, D.M. (1985). Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature* 317: 714-744.

Conger, B.V., Triglano, R.N. and Gray, D.J. (1988). Cell culture of the *Poaceae* (Gramineae). In: *Plant cell biotechnology*. Eds: M.S.S. Pais, F. Mavituna and J.M. Novais. Springer-Verlag, Berlin, Germany. pp 49-61.

Constable, F. (1984). Callus culture: induction and maintenance. In: *Cell Culture and somatic cell genetics of plants. Vol 1: Laboratory procedures and their applications*. Ed: I.K. Vasil. Academic Press, London. pp27-35.

Creisson, G., Smith, C., Francis, R., Reynolds, H. and Mullineaux, P. (1990). *Agrobacterium*- and microprojectile - mediated viral DNA delivery into barley microspore-derived cultures. *Plant Cell Rep.* 8: 680-683.

Crossway, A., Hauptli, H., Houck, C.M., Irvine, J.M., Oakes, J.V. and Perani, L.A. (1986). Micromanipulation techniques in plant biotechnology. *Biotechniques* 4: 320-324.

Cuozzo, M., O'Connell, K.M., Kaniewski, W., Fang, R.-X., Chua, N.H. and Tumer, N.E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* 6: 549-557.

Datta, S.K., Peterhans, A., Datta, K. and Potrykus, I. (1990). Genetically engineered fertile India-rice recovered from protoplasts. *Bio/Technology* 8: 736-740.

Davey, M.R., Rech, E.L. and Mulligan, B.J. (1989). Direct DNA transfer to plant cells. *Plant Mol. Biol.* 13: 273-285.

De Block, M., Botterman, J., Vandeweile, M., Dockx, J., Thoen, C., Gossele, V., Rao Movva, N., Thompson, C., Van Montagu, M. and Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* 6: 2513-2518.

De Greve, H., Decraemer, H., Seurinck, J., Van Montagu, M. and Schell, J. (1981). The functional organisation of the octopine *A. tumefaciens* plasmid pTi B653. *Plasmid* 6: 235-248.

Dekeyser, R.A., Claes, B., De Rycke, R.M.U., Habets, M.E., Van Montagu, M.C. and Caplan, A.B. (1990). Transient gene expression in intact and organised rice tissues. *The Plant Cell* 2: 591-602.

De la Pena, A., Lorz, H. and Schell, J. (1987). Transgenic rye plants obtained by injecting DNA into young floral tillers. *Nature* 325: 274-276.

Deshayes, A., Herrera-Estrella, L. and Caboche, M. (1985). Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. *EMBO J.* 4: 2731-2737.

De Wet, J.R., Wood, K.V., DeLuce, M., Helsinki, D.R. and Subramani, S. (1987). Firefly luciferase: structure and expression in mammalian cells. *Mol. Cell Biol.* 7: 725-737.

Draper, J., Scott, R., Kumar, A. and Dury, G. (1988). Transformation of plant cells by DNA-mediated gene transfer. In: *Plant genetic transformation and gene expression : A laboratory manual.* Eds: J. Draper, R. Scott, P. Armitage and R. Walden. Blackwell Scientific Publications, Oxford. pp 69-160.

Dulmage, H.T. (1970). Production of spore-δ-endotoxin variants of *Bacillus thuringiensis* in two fermentation media. *J. Invertebr. Pathol.* 16: 385-389.

Duncan, P.R., Williams, M.E., Zehr, B.E. and Wiaholm, J.M. (1985). The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. *Planta* 165: 322-332.

Dunwell, J.M. (1985). Haploid cell cultures. In: *Plant cell culture: a practical approach.* Ed: R.A. Dixon. IRL Press, Oxford, England. pp 20-36.

Ebert, A. and Taylor, H.F. (1990). Assessment of the changes of 2,4-D concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell Tissue and Organ Cult.* 20: 165-172.

Enjalric, F., Carron, M.P. and Lardet, L. (1988). Contamination of primary cultures in tropical areas: The case of *Hevea brasilfiensis*. *Acta Horticulturae* 225: 57-65.

Evans, D.A. and Bravo, J.E. (1983). Protoplast isolation and culture. In: *Handbook of plant cell culture : Techniques for propagation and breeding.* Ed: D.A. Evans, W.R. Sharp, P.V. Ammirato, Y. Yamada. Macmillan Publishing Co, New York. pp 124-176.

Evans, D.A., Crocomo, O.J. and de Carvalho, M.T.V. (1980). Protoplast isolation and subsequent callus regeneration in sugarcane. *Z. Pflanzenphysiol.* 98: 355-358.

Evans, D.A., Sharp, W.R. and Bravo, J.E. (1984a). Cell culture methods for crop improvement. In: *Handbook of Plant Cell Culture. Vol 2: Crop Species.* Ed: W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada. Macmillan Publishing Co, New York. pp 47-68.

Evans, D.A., Sharp, W.R. and Medina-Filho, H.P. (1984b). Somaclonal and gametaclonal variation. *Am J. Bot.* 7: 759-774.

Falkiner, F.R. (1990). The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. *IAPTC Newsletter* 60: 13-23.

Feldmann, K.A. and Marks, M.D. (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. *Mol. Gen. Genet.* 208: 1-9.

Finkle, B.J. and Ulrich, J.M. (1979). Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. *Plant Physiol.* 63: 598-601.

Finkle, B.J., Zavala, M.E. and Ulrich, J.M. (1985). Cryoprotective compounds in the viable freezing of plant tissues. In: *Cryopreservation of plant cells and organs*. Ed: K.K. Kartha. CRC Press, Boca Raton, U.S.A. pp 75-107.

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Meyer, E.M., Rochester, D.E., Rogers, S.C. and Fraley, R.T. (1987). Insect tolerant tomato plants. *Bio/Technology* 5: 807.

Fitch, M.M. and Moore, P.H. (1983). Haploid production from anther culture of *Saccharum spontaneum* L. *Z. Pflanzenphysiol.* 109: 197-206.

Fitch, M.M. and Moore, P.H. (1990). Comparison of 2,4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. *Plant Cell Tissue and Organ Cult.* 20: 157-163.

Fitter, M.S. and Krikorian, A.D. (1983). *Plant protoplasts: Some guidelines for their preparation and manipulation in culture.* Calbiochem-Behring, Hoescht Corp, New York. 28pp.

Fraley, R.T. and Papahadjopoulos, D. (1982). Liposomes: the development of a new carrier system for introducing nucleic acids into plant and animal cells. *Curr. Tech. Microbiol.* 96: 171-191.

Fraley, R.T., Rogers, S.G., Horsch, R.D., Sanders, P.R., Flick, J.S. *et al.* (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci.* USA 80: 4803-4807.

Freeman, J.P., Draper, J., Davey, M.R., Cocking, E.C., Gartland, K.M.A., Harding, K. and Pental, D. (1984). A comparison of methods for plasmid delivery into plant protoplasts. *Plant Cell Physiol.* 25: 1353-1365.

French, R., Janda, M. and Ahlquist, P. (1986). Bacterial gene inserted in an engineered RNA virus : efficient expression in monocotyledonous plant cells. *Science* 231: 1294-1296.

Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. and Klein, T.M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* 8: 833-839.

Fromm, M.E., Taylor, L.P. and Walbot, V. (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci.* USA 82: 5824-5828.

Fromm, M., Taylor, L.P. and Walbot, V. (1986). Stable transformation of maize after gene transfer by electroporation. *Nature* 319: 791-793.

Fujimura, T., Sakurai, M., Akagi, H., Negishi, T. and Hirose, A. (1985). Regeneration of rice plants from protoplasts. *Plant Tissue Culture Lett.* 2: 74-75.

Gamborg, O.L., Constabel, F. and Miller, R.A. (1970). Embryogenesis and production of albino plants from cell cultures of *Bromous inermis*. *Planta* 95: 355-358.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.

Gamborg, O.L., Shyluk, J.P. and Shahin, E.A. (1981). In: *Plant tissue culture-methods and applications in agriculture*. Ed: T.A. Thorpe, Academic Press, New York. pp 115-154.

Gardner, R.C. and Knauf, V. (1986). Transfer of *Agrobacterium* DNA to plants requires a T-DNA border but not the vir E locus. *Science* 231: 725-727.

Gasser, C.S. and Fraley, R.T. (1989). Genetically engineering plants for crop improvement. *Science* 244: 1293-1299.

Gill, R., Rashid, A., and Maheshwari, S.C. (1981). Dark requirement for cell regeneration and colony formation by mesophyll protoplasts of *Nicotiana plumbaginifolia* Viv. *Protoplasma* 106: 351-354.

Golds, T.J., Davey, M.R., Rech, E.L. and Power, J.B. (1990). Methods of gene transfer and analysis in higher plants. In: *methods in molecular biology plant cell and tissue culture*. Vol 16. Ed: J.W. Pollard and J.M. Walker. Havana Press, England. pp 341-371.

Gonzales, R.A. and Franks, E. (1987). Induction of morphogenically competent callus and suspension cell cultures of *Digitaria sanguinalis*. *In vitro Cell. Dev. Biol.* 23: 581-584.

Gordon-Kamm, W., *et al.* (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell* 2: 603-618.

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2: 1044-1051.

Graves, A.C.F. and Goldman, S.L. (1986). The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*. *Plant Mol. Biol.* 7: 43-50.

Green, C.E. and Philips, R.L. (1975). Plant regeneration from tissue cultures of maize. *Crop Sci.* 15: 417-421.

Griesbach, R.J. (1983). Protoplast microinjection. *Plant Mol. Biol. Rep.*1: 32-37.

Grimsley, M., Hohn, B., Hohn, T. and Walden, R. (1986). 'Agroinfection', an alternative route for viral infection of plants by using the Ti-plasmid. *Proc. Natl. Acad. Sci.* USA 83: 3282-3286.

Grimsley, N., Hohn, T., Davies, J.W. and Hohn, B. (1987). *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. *Nature* 325: 177-179.

Grisham, M.P. and Bourg, D. (1989). Efficiency of *in vitro* propagation of sugarcane plants by direct regeneration from leaf tissue and by shoot-tip culture. *J Am. Soc. of Sug. Technol.* 9: 97-102.

Grout, B.W.W. (1990). Genetic preservation *in vitro*. In: *Progress in plant cellular and molecular biology*. Eds: H.J.J Nijkamp, L.H.W. van der Plas and J. van Aartrijk. Kluwer Academic, The Netherlands. pp 13-22.

Guha, S. and Maheshwari, S.C. (1964). *In vitro* production of embryos from anthers of *Datura*. *Nature* 204: 497.

Guiderdoni, E. and Demarly, Y. (1988). Histology of somatic embryogenesis in cultured leaf segments of sugarcane plantlets. *Plant Cell Tissue Organ Cult* . 14: 71-88.

Guerineau, F., Brooks, L., Meadows, J., Lucy, A., Robinson, C. and Mullineaux, P. (1990). Sulfonamide resistance for plant transformation. *Plant Mol. Biol.* 15: 127-136.

Hain, R., Steinbiss, H.-H. and Schell, J. (1984). Fusion of *Agrobacterium* and *E. coli* spheroplasts with *Nicotiana tabacum* protoplasts - direct gene transfer from micro-organisms to higher plants. *Plant Cell Rep.* 3: 60-64.

Hamill, J.D. and Cocking, E.C. (1988). Somatic hybridisation of plants and its use in agriculture. In: *Plant cell biotechnology*. Eds: M.S.S. Pais, F. Mavituna and J.M. Novais. Springer-Verlag, Berlin, Germany. pp 21-41.

Hamill, J.D., Prescott, A. and Martin, C. (1987). Assessment of the efficiency of cotransformation of the T-DNA of disarmed binary vectors derived from *Agrobacterium tumefaciens* and the T-DNA of *A. rhizogenes*. *Plant Mol. Biol.* 9: 573-584.

Hammatt, N. and Dacey, M.R. (1987). Somatic embryogenesis and plant regeneration from cultured zygotic embryos of soybean (*Glycine max* L. Merr.). *J. Plant Physiol.* 128: 219-226.

Hammer, R.E., Pursel, V.G., Rexnroad, C.E., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. and Brnster, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* 315: 680-683.

Hanahan, D. (1985). Techniques for simple transformation of *E. coli*. In: *DNA Cloning I*. Ed: D.M. Glover. IRL Press, Oxford. pp 109-135.

Hanning, G.E. and Conger, B.V. (1986). Factors influencing somatic embryogenesis from cultured leaf segments of *Dactylis glomerata*. *J. Plant Physiol.* 123: 23-29.

Haring, M.A., Rommens, C.M.T., John, H., Nijkamp, J. and Hille, J. (1991). The use of transgenic plants to understand transposition mechanisms and to develop transposon-tagging strategies. *Plant Mol. Biol.* 16: 449-461.

Harrigan, W.F. and McCance, M.E. (1966). *Laboratory Methods in Microbiology.* Academic Press, London. pp 8-10, 154.

Harrison, B.D., Mayo, M.A. and Baulcombe, D.C. (1987). Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. *Nature* 328: 799-802.

Hauptmann, R.M., Ozias-Akins, P., Vasil, V., Tabaeizadeh, Z., Rogers, S.C., Horsch, R.B., Vasil, I.K. and Fraley, R.T. (1987). Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. *Plant Cell Rep.* 6: 265-270.

Hayashimoto, A., Li, Z. and Murai, N. (1990). A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. *Plant Physiol.* 93: 857-863.

Heberle-Bors, E. (1991). Germ line transformation in higher plants. *IAPTC Newsletter* 64: 2-10.

Hein, T., Prezewozny, T. and Schieder, O. (1983). Culture and selection of somatic hybrids using an auxotrophic cell line. *Theor. Appl. Genet.* 64: 119-122.

Heinz, D.J. and Mee, G.W.P. (1969). Plant differentiation from callus tissue of *Saccharum* sp. *Crop Sci.* 9: 346-348.

Hernalsteens, J.P., Thiu-Toong, L., Schell, J. and Van Montagu, M. (1984). An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis*. *EMBO J.* 3: 3039-3041.

Herrera, G., Snyman, S.J. and Thomson, J.A. (1991). Development of a potential biological agent for the sugarcane borer *Eldana saccharina*. *Frontiers of biotechnology in Agriculture*. Satellite Meeting of the 15th International Congress of Biochemistry. Israel, August 1991, p19.

Herrera, G. and Thomson, J.A. (1989). Biological control of *Eldana saccharina* using cloned *Bacillus thuringiensis* genes. Cloning of delta-endotoxin DNA sequences from *B.thuringiensis* 234. *Proc. S. Afr. Sug. Technol. Ass.* 63: 189-191.

Herrera-Estrella, L. and Simpson, J. (1988). Foreign gene expression in plants. In: *Plant molecular biology : A practical approach*. Ed. C.H. Shaw. IRL Press, Oxford. pp 131-160.

Hess, D., Dressler, K. and Nimmrichter, R. (1990). Transformation experiments by pipetting *Agrobacterium* into the spikelets of wheat (*Triticum aestivum* L.). *Plant Sci.* 72: 233-244.

Hilder, V.A., Gatehouse, A.M.R. and Boulter, D. (1989). Potential for exploiting plant genes to genetically engineer insect resistance, exemplified by the cowpea trypsin inhibitor gene. *Pestic. Sci.* 27: 165-171.

Hinchee, M.A., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S.J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T. and Horsch, R.B. (1988). Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Bio/Technology* 6: 915-922.

Hinchee, M.A., Dela Cruz, A. and Maretzki, A. (1984). Developmental and biochemical characteristics of cold-treated anthers of *Saccharum sponteneum*. *J. Plant Physiol.* 115: 271-284.

Ho, W. and Vasil, I.K. (1983a). Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma* 118: 169-180.

Ho, W. and Vasil, I.K. (1983b) Somatic embryogenesises in sugarcane (*Saccharum officinarum* L): Growth and plant regeneration from embryogenic cell suspension cultures. *Ann. Bot.* 51: 719-726.

Hohn, B., Hohn, T., Boulton, M.I., Davies, J.W. and Grimsley, N. (1987). Agroinfection of *Zea mays* with maize streak virus DNA. *Plant Mol. Biol.* 10: 459-468.

Holsters, M., Silva, B., Van vliet, F., Genetello, C. and De Block, M. (1980). The functional organization of the nopaline A. *tumefaciens* plasmid pTiC58. *Plasmid* 3: 212-230.

Hookyaas-Van Slogteren, G.M.S., Hooykaas, P.J.J. and Schilperoot, R.A. (1984). Expression of Ti-plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefacines*. *Nature* 311: 763-764.

Huang, Y. and Dennis, E. (1989). Factors influencing stable transformation of maize protoplasts by electroporation. *Plant Cell Tissue and Organ Cult.* 18: 281-296.

Irvine, J.E. (1991). Texas Agricultural Experiment Station, Texas, U.S.A.

Irvine, J.E. (1984). The frequency of marker changes in sugarcane plants regenerated from callus tissue. *Plant Cell Tissue Organ Cult.* 3: 201-209.

Irvine, J.E. and Almeida, C.G. (1991). Delivery of plasmid DNA through particle bombardment with an airless sprayer. Texas Agricultural Experiment Station. Leaflet No. MP-1726. 5pp.

Irvine, J.E., Benda, C.T.A., Legendre, B.L. and Machado, G.R. (1991). The frequency of marker changes in sugarcane plant regenerated from callus culture. II. Evidence for vegetative and genetic transmission, epigenetic effects and chimeral disruption. *Plant Cell Tissue and Organ Cult.* 26: 115-125.

Izawa, T., Miyazaki, C., Yamamoto, M., Terada, R., Iida, S. and Shimamoto, K. (1991). Introduction and transposition of the maize transposable element Ac in rice (*Oryza sativa* L.) *Mol. Gen. Genet.* 227: 391-396.

Jefferson, R.A. (1987). Assaying chimeric genes in plants : The GUS gene fusion system. *Plant Mol. Biol. Rep.* 5: 387-405.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusions : ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907.

Jensen, J.S., Marcker, K.A., Otten, L. and Schell, J. (1986). Nodule-specific expression of a chimaeric soybean leghemaglobin gene in transgenic *Lotus corniculatus*. *Nature* 321: 669-674.

Joersbo, M. and Brunstedt, J. (1990). Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Rep.* 9: 207-210.

Jones, M.G.K. and Dale, P.J. (1982). Reproducible regeneration of callus from suspension culture protoplasts of the grass *Lolium multiflorum*. *Z. Pflanzenphysiol.* 105: 267-274.

Junker, B., Zimmy, J., Luhrs, R. and Lorz, H. (1987). Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake. *Plant Cell Rep.* 6: 329-332.

Kaeppler, H.F., Gu, W., Somers, D.A., Rines, H.W. and Cockburn, A.F. (1990). Silicon carbide filter-mediated DNA delivery into plant cells. *Plant Cell Rep.* 9: 415-418.

Kamo, K.K., Becwar, M.R. and Hodges, T.K. (1985). Regeneration of *Zea mays* L. from embryogenic callus. *Bot. Gaz.* 146: 327-334.

Kamo, K.K. and Hodges, T.K. (1986). Establishment and characterisation of long-term embryogenic maize callus and cell suspension cultures. *Plant Sci.* 45: 111-117.

Kanai, R. and Edwards, G.E. (1973). Purification of enzymatically- isolated mesophyll protoplasts from C3, C4 and crassulacean acid metabolism plants using an aqueous dextran-polyethylene glycol 2-phase system. *Plant Physiol.* 52: 484-490.

Kao, K.N. (1977). Chromosomal behaviour in somatic hybrids of soybean - *Nicotiana glauca*. *Molec. Gen. Genet.* 150: 225-230.

Kao, K.N., Gamborg, O.L., Miller, R.A. and Keller, W.A. (1971). Cell divisions in cells regenerated from protoplasts of soybean and *Haplopappus gracilis*. *Nature* 232: 124.

Kao, K.N. and Michayluk, M.R. (1975). Nutritional requirements for growth of *Vicia hajastona* cells and protoplasts at a very low population density in liquid media. *Planta* 126: 105-110.

Karlsson, S.B. and Vasil, I.K. (1986). Morphology and ultrastructure of embryogenic cell-suspension cultures of *Panicum maximum* Jacq. (Guinea grass) and *Pennisetum purpureum* Schum. (Napier grass). *Am. J. Bot.* 73: 894-901.

Karp, A. and Maddock, S.C. (1984). Chromosomal variation in wheat plants regenerated from cultured immature embryos. *Theor. Appl. Genet.* 67: 249-255.

Kartha, K.K. (1985). *Cryopreservation of plant cells and organs.* CRC Press, Boca Raton, USA. 276pp.

Kartzke, S., Saedler, H. and Meyer, P. (1990). Molecular analysis of transgenic plants derived from transformants of protoplasts at various stages of the cell cycle. *Plant Science* 67: 63-72.

Kasha, K.J. and Reinbergs, E. (1980). Achievements with haploids in barley research and breeding. In: *The Plant genome.* Eds: D.R. Davies and D.A. Hapwood. The John Innes Charity, Norwich. pp 215-230.

Kaur-Sawhney, R. and Galston, A.W. (1984). Oats. In: *Handbook of plant cell culture. Vol 2. Crop species.* Eds: W.R. Sharp, D.A. Evans, R.V. Ammirato and Y. Yamada. MacMillan Publishing Company, New York. pp92-107.

King, P.J. and Shimamoto, K. (1984). Maize. In: *Handbook of Plant cell culture. Vol 2. Crop species.* Eds: W.R. Sharp, D.A. Evans, R.V. Ammirato and Y. Yamada. MacMillan Publishing Company, New York. pp 69-91.

Klein, T.M. and Fromm, M.E. (1989). Blast those genes! *Agricultural Res.* 37: 14-15.

Klein, T.M., Wolf, E.D., Wu, R. and Sanford, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327: 70-73.

Kleinhofs, A. and Behki, R. (1977). Prospects for plant genome modification by nonconventional methods. *Ann. Rev. Genet.* 11: 79-101.

Kleinhofs, A., Eden, F.C., Chilton, M.D. and Bendich, A.J. (1975). On the question of the integration of exogenous bacterial DNA into plant DNA. *Proc. Natl. Acad. Sci. USA* 72: 2748-2752.

Koncz, C., Olsson, O., Langridge, W.H.R., Schell, J. and Szalay, A.A. (1987). Expression and assembly of functional bacterial luciferase in plants. *Proc. Natl. Acad. Sci. USA* 84: 313-315.

Kosugi, S., Ohashi, Y., Nakajima, K. and Arai, Y. (1990). An improved assay for ß-glucuronidase in transformed cells : methanol almost completely suppresses a putative endogenous ß-glucuronidase activity. *Plant Science* 70: 133-140.

Krens, F.A., Molendijk, L., Wullems, G.J. and Schilperoort, R.A. (1982). *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. *Nature* 296: 72-74.

Larkin, P.J. (1976). Purification and viability determinations of plant protoplasts. *Planta* 128: 213-216.

Larkin, P.J. (1982). Sugarcane tissue and protoplast culture. *Plant Cell Tissue Organ Cult.* 1: 149-164.

Larkin, P.J. and Scowcroft, W.R. (1981). Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60: 197-214.

Larkin, P.J., Taylor, B.H., Gersmann, M. and Brettell, R.I.S. (1990). Direct gene transfer to protoplasts. *Aust. J. Plant Physiol.* 17: 291-302.

Lawrence, W.A. and Davies, R.D. (1985). A simple method for the microinjection and culture of protoplasts at very low densities. *Plant Cell Rep.* 4: 33-35.

Ledoux, L. and Huart, R. (1968). Integration and replication of DNA of *M. lysodeikticus* in DNA of germinating barley. *Nature* 218: 1256-1259.

Ledoux, L. and Huart, R. (1969). Fate of exogenous bacterial deoxyribonucleic acids in barley seedlings. *J. Mol. Biol.* 43: 243-262.

Ledoux, L., Huart, R. and Jacobs, M. (1971). Fate of exogenous DNA in *Arabidopsis thaliana*. *Eur. J. Biochem.* 23: 96-108.

Ledoux, L., Huart, J. and Jacobs, M. (1974). DNA-mediated genetic correction of thiaminless *Arabidopsis thaliana*. *Nature* 249: 17-21.

Leggatt, I., Waites, W.M., Leifert, C. and Nicholas, J.R. (1988). Characterisation of microorganisms isolated from plants during micropropagation. *Acta Hort.* 225: 93-102.

Leifert, C and Waites, W.M. (1990). Contaminants of plant tissue cultures. *IAPTC Newsletter* 60: 2-13.

Lindsey, K. (1986). The production of secondary metabolites by immobilised plant cells. In: *Secondary metabolism in plant cell cultures.* Eds: P. Morris, A.H. Scragg, A. Stafford and M.W. Fowler. Cambridge University Press, England. pp143-155.

Lindsey, K. and Jones, M.G.K. (1989). *Plant Biotechnology in Agriculture.* Open University Press, Milton Keynes. pp 57-85.

Lindsey, K. and Jones, M.G.K. (1990). Selection of transformed cells. In: *Plant cell line selection Procedures and applications.* Ed: P.J.Dix. VCH Publishers, Germany. 241pp.

Lindsey, K. and Yeoman, M.M. (1983). The relationship between growth rate, differentiation and alkaloid accumulation in cell cultures. *J. Exp. Bot.* 34: 1055-1065.

Ling, O.H., Chen, W.Y., Chen., M.F. and Ma, Z.R. (1983). Somatic embrogenesis and plant regeneration in an interspecific hybrid of *Oryza* L. *Plant Cell Rep.* 2: 169-171.

Lippincott, B.B. and Lippincott, J.A. (1978). Cell walls of crown-gall tumors and embryogenic plant tissues lack *Agrobacterium* adherence sites. *Science* 199: 1075-1078.

Liu, M. (1971). A new method for sugarcane breeding - tissue culture technique. *Taiwan Sugar* 18: 8-10.

Liu, M.C. (1984). Sugarcane. In: *Handbook of plant cell culture.* Vol 2. Ed. W.H. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada. MacMillan Press, New York. pp 572-605.

Liu, M.C. (1988). High frequency callus formation from protoplasts of sugarcane suspension cultures. *Taiwan Sugar* (Sept.-Oct.): 9-14.

Liu, M.C. (1990). Selection of agronomically useful mutants through plant cell culture systems. *Taiwan Sugar* 37: 8-13.

Liu, M.C. and Chen, W.H. (1974). Histological studies on the origin and process of plantlet differentiation in sugarcane callus mass. *Proc. 15th Int. Soc. Sug. Technol.* Durban. 118-121.

Liu, M.C. and Chen, W.H. (1976). Tissue and cell culture as aids to sugarcane breeding 1. Creation of genetic variation through callus culture. *Euphytica* 25: 393-403.

Liu, M.C., Chen, W.C. and Shih, S.C. (1982). Histogenesis of sugarcane callus originating from young leaf and stem tip explants. *Can. J. Bot.* 60: 2889-2895.

Liu, M.C. and Shih, S.C. (1986). Salt stress-induced increases in nitrate reductase activity and protein content in sugarcane cell culture. *Proc.* 19th *Int. Soc. Sug. Technol.* Jakarta, Indonesia. 263-273.

Liu, M.C., Yeh, H.S. and Chen, W.H. (1984). A high-sucrose and vigorously growing calliclone 71-4829. *Rep. Taiwan Sugar Res. Inst.* 102: 1-11.

Lorz, H. (1989). *In vitro* manipulation of cereal plants. In: *2nd International symposium on genetic manipulation in crops.* Lisbon. Eds: A. Mujeeb-Kazi and L.A. Sitch. pp 93-101.

Lorz, H., Baker, B. and Schell, J. (1985). Gene transfer to cereal cells mediated by protoplast transformation. *Mol. Gen. Genet.* 199: 178-182.

Lowe, K., Taylor, D.B., Ryan, P. and Paterson, K.E. (1985). Plant regeneration via organogenesis and embryogenesis in the maize inbred line B73. *Plant Sci.* 41: 125-132.

Lu, C., Chandler, S.F and Vasil, I.K. (1984). Somatic embryogenesis and plant regeneration from cultured immature embryos of rye (*Secale cereale* L.) *J. Plant Physiol.* 115: 237-244.

Lu, C. and Vasil, I.K. (1982). Somatic embryogenesis and plant regeneration from tissue cultures of *Panicum maximum*. *Am. J. Bot.* 69: 77-81.

Lu, C., Vasil, I.K. and Ozias-Akins, P. (1982). Somatic embryogenesis in *Zea Mays* L. *Theoret. Appl. Genet.* 62: 109-112.

Lu, C., Vasil, V and Vasil, I.K. (1981). Isolation and culture of protoplasts of *Panicum maximum* Jacq. (Guinea grass): somatic embryogenesis and plantlet formation. *Z. Pflanzenphysiol.* 104: 311-318.

Lu, C., Vasil, V., and Vasil I.K. (1983). Improved efficiency of somatic embryogenesis and plant regeneration in tissue culture of maize (*Zea Mays* L.) *Theoret. Appl. Genet.* 66: 285-289.

Luo, Z. and Wu, R. (1988). A simple method for the transformation of rice via the pollen-tube pathway. *Plant Mol. Biol. Rep.* 6: 165-174.

MacKinnon, C., Gunderson, G. and Nabors, M.W. (1986). Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. *Plant Cell Rep.* 5: 349-351.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning : A laboratory manual.* Cold Spring Harbor Laboratory Press, New York. 545pp.

Mantell, S.H., Mathews, J.A. and McKee, R.A. (1985). *Principles of plant biotechnology: An introduction to genetic engineering in plants.* Blackwell Scientific Publications, Oxford. pp 89-158.

Maretzki, A. and Nickell, L.G. (1973). Formation of protoplasts from sugarcane cell suspensions and the regeneration of cell cultures from protoplasts. *Colloq. Int. C.N.R.S.* 212: 51-63.

Marton, L., Wullems, G.J., Molendijk, L. and Schilperoot, R.A. (1979). *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. *Nature* 277: 129-130.

Mayo, M.A. and Barker, H. (1990). Induction of resistance to plant viruses by transformation with virus genes: the poacher turned gamekeeper. In: *Aspects of Applied Biology* 24. Association of Applied Biologists, England. pp 65-78.

Mazur, B.J. and Falco, S.C. (1989). The development of herbicide resistant crops. *Annu. Rev. Plant Physiol. and Plant Mol. Biol.* 40: 441-470.

McCabe, D.E., Swain, W.F. and Christou, P. (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* 6: 923-926.

McKnight, T.D., Lillis, M.T. and Simpson, R.B. (1987). Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains. *Plant Mol. Biol.* 4: 439-445.

Meijer, E.G.M., Schilperoot, R.A., Rueb, S., Van Os-Ruygrok, P.E. and Hensgens, L.A.M. (1991). Transgenic rice cell lines : expression of transferred chimeric genes. *Plant Mol. Biol.* 16: 807-820.

Mendel, R.R., Müller, B., Schulze, J., Kolesnikov, V. and Zelenin, A. (1988). Delivery of foreign genes to intact barley cells by high velocity microprojectiles. *Theor. Appl. Genet.* 78: 31-34.

Mitchell, J.C. and Petolino, J.F. (1991). Plant regeneration from haploid suspension and protoplast cultures from isolated microspores of maize. *J. Plant Physiol.* 137: 530-536.

Mooney, P.A. and Goodwin, P.B. (1991). Adherence of *Agrobacterium tumefaciens* to the cells of immature wheat embryos. *Plant Cell Tissue and Organ Cult.* 25: 199-208.

Mooney, P.A., Goodwin, P.B., Dennis, E.S. and Llewellyn, D.J. (1991). *Agrobacterium tumefaciens*-gene transfer to wheat tissues. *Plant Cell Tissue and Organ Cult.* 25: 209-218.

Mousdale, D.M. and Coggins, J.R. (1984). Purification and properties of 5-endolpyruvylshikimate 3-phosphate synthase from seedlings of *Pisum sativum* L. *Planta* 160: 78-83.

Mulder, J.C. (1988). *Statistical techniques in education.* HAUM Educational Publishers, Pretoria, South Africa. 251pp.

Murai, N., Sutton, D.W., Murray, M.G., Slighton, J.L., Merlo, D.J. *et al.* (1983). Phaseolin gene from bean is expressed after transfer to sunflower via tumour-inducing plasmid vectors. *Science* 222: 476-482.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473-497.

Murray, E.T., Rocheleau, T., Eberle, M., Stock, C., Sekar, V. and Adang, M. (1991). Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. *Plant Mol. Biol.* 16: 1035-1050.

Nabors, M.W., Heyser, J.W., Dykes, T.A. and DeMott, K.J. (1983). Long duration, high-frequency plant regeneration from cereal tissue cultures. *Planta* 157: 385-391.

Nadar, H.M. and Heinz, D.J. (1977). Root and shoot development from sugarcane callus tissue. *Crop Sci.* 17: 814-816.

Nadar, H.M., Soepraptopo, S., Heinz, D.J. and Ladd, S.L. (1978). Fine structure of sugarcane (*Saccharum* sp) callus and the role of auxin in embroygenesis. *Crop Sci.* 18: 210-216.

Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F. (1984). Grown gall : A molecular and physiological analysis. *Ann. Rev. Plant Physiol.* 35: 387-413.

Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982). Gene transfer into mouse myloma cells by electroporation in high electric fields. *EMBO J.* 1: 841-845.

Nickell, L.G. (1964). Tissue and cell cultures of sugarcane: Another research tool. *Hawaii Plant Rec.* 57: 223-229.

Norris, R., Smith, R.H. and Vaughn, K.C. (1983). Plant chimeras used to establish *de novo* origin of shoots. *Science* 220: 75-76.

Nuss, K.J. (1991). South African Sugar Association Experiment Station, Mount Edgecombe.

Okada, K., Nagata, T. and Takebe, I. (1986). Introduction of functional RNA into plant protoplasts by electroporation. *Plant Cell Physiol.* 27: 619-626

Okuno, T. and Furusawa, I. (1977). A simple method for the isolation of intact mesophyll protoplasts from cereal plants. *Plant Cell Physiol.* 18: 1357-1362.

Oliver, S.G. and Ward, J.M. (1985). *A dictionary of genetic engineering.* Cambridge University Press, England. 153pp.

Ozias-Akins, P., Ferl, R.J. and Vasil, I.K. (1986). Somatic hybridization in the Gramineae: *Pennisetum americanum* (L). K. Schum. and *Panicum maximum* Jcq. (Guinea grass). *Molec. Gen. Genet.* 203: 365-370.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B. and Potrykus, I. (1984). Direct gene transfer to plants. *EMBO J.* 3: 2717-2722.

Pelcher, L.E., Gamborg, O.L. and Kao, K.N. (1974). Bean mesophyll protoplasts: production, culture and callus formation. *Plant Sci. Lett.* 3: 107-111.

Pollock, K., Barfield, D.G. and Shields, R. (1983). The toxicity of antibiotics to plant cell cultures. *Plant Cell Rep.* 2: 34-39.

Potrykus, I., Harms, C.T., Lorz, H. and Thomas, E. (1977). Callus formation from stem protoplasts of corn (*Zea mays* L.). *Molec. Gen. Genet.* 156: 347-350.

Potrykus, I., Saul, M., Petruska, J., Paszowski, J. and Shillito, R.D. (1985). Direct gene transfer to cells of a graminaceous monocot. *Molec. Gen. Genet.* 199: 183-188.

Potter, H., Weir, L. and Leder, P. (1984). Enhancer-dependent expression of human immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc. Natl. Acad. Sci.* USA 81: 7161-7165.

Power, J.B. and Cocking, E.C. (1970). Isolation of leaf protoplasts: macromolecule uptake and growth substance response. *J. Exp. Bot.* 21: 64-70.

Power, J.B., Davey, M.R., Freeman, J.P., Mulligan, B.J. and Cocking, E.C. (1986). Fusion and transformation of plant protoplasts. *Methods Enzymol.* 118: 578-594.

Price, S. (1962). A modified leaf-squash technique for counting chromosomes in somatic cells of *Saccharum* and related grasses. *Proc.* 11th *Congr. Int. Soc. Sugar Cane Technol.* Reduit, Mauritius. 583-585.

Raineri, D.M., Bottino, P., Gordon, M.P. and Nester, E.W. (1990). *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). *Bio/Technology* 8: 33-38.

Raman, K., Walden, D.B. and Greyson, R.I. (1980). Propagation of *Zea mays* L. by shoot tip culture: a feasibility study. *Ann. Bot.* 45: 183-189.

Rashid, H. and Quraishi, A. (1989). High frequency embryogenic callus induction and its regeneration in three wheat cultivars. In: *Second International Symposium on Genetic Manipulation in crops*. Lisbon. Eds: A. Mujeeb-Kazi and L.A. Sitch. pp 205-215.

Ray, D.S. and Ghosh, P.D. (1990). Somatic embryogenesis and plant regeneration from cultured leaf explants of *Zea mays*. *Ann. Bot.* 66: 497-500.

Reggiardo, M.I., Arana, J.L., Orsaria, L.M., Permingeat, H.R., Spitteler, M.A. and Vallejos, R.H. (1991). Transient transformation of maize tissues by microprojectile bombardment. *Plant Sci.* 75: 237-243.

Reich, T.J., Iver, V.N. and Miki, B.L. (1986). Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti-plasmids. *Biotechnology* 4: 1001-1004.

Reisch, B.I. (1988). Genetic instability in plant cell cultures: utilisation in plant breeding and genetic studies. In: *Plant Cell Biotechnology*. Eds: M.S.S. Pais, F. Mavituna and J.M. Novais. Springer-Verlag, Berlin, Germany. pp 87-96.

Reiss, B., Sprengel, R., Will, H. and Schaller, H. (1984). A sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. *Gene* 30: 211-218.

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D. and Detmer, J.J. (1988). Genetically transformed maize plants from protoplasts. *Science* 240: 204-207.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labelling deoxyribonucleic acid to a high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113: 237-251.

Rout, J.R. and Sarma, N.P. (1991). Anther callus induction and green plant regeneration at high frequencies from an interspecific rice hybrid *Oryza sativa*. L x *O. rufipogon*. Grieff. *Euphytica* 54: 155-159.

Ryu, D.D., Lee, S.D. and Romani, R.J. (1990). Determination of growth rates of plant cell cultures: comparative studies. *Biotechnol. and Bioeng.* 35: 305-311.

Sacristan, M.D. and Melchers, G. (1969). The karyological analysis of plants regenerated from tumorous and other callus cultures of tobacco. *Molec. Gen. Genet.* 105: 317-333.

Schaeffer, G.W., Lazar, M.D. and Baenzinger, P.S. (1984). Wheat. In: *Handbook of Plant cell culture. Vol 2. Crop species.* Eds: W.R. Sharp, D.A. Evans, R.V. Ammirato and Y. Yamada. MacMillan Publishing Company, New York. pp 108-136.

Schaffler, G. and Koop, H, (1990). Single cell nurse culture of tobacco protoplasts : physiological analysis of conditioning factors. *J. Plant Physiol.* 137: 95-101.

Schell, J. (1991). Max-Planck Institute, Germany.

Schenk, R.U. and Hildebrandt, A.C. (1971). Production, manipulation and fusion of plant cell protoplasts as steps towards somatic hybridisation. *Coll. Int. CNRS* 193: 319-333.

Schenk, R.U. and Hildebrandt, A.C. (1972). Medium and techniques for induction of growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* 50: 166-204.

Shah, D.M., Horsch, R.B., Klee, H.J., Kishore, G.M., Winter, J.A., Tumer, N.E., Hironaka, C.M., Sanders, P.R., Gasser, C.S., Aykent, S., Stiegel, N.R., Rogers, S.G. and Fraley, R.T. (1986). Engineering herbicide tolerance in transgenic plants. *Science* 233: 478.

Sharp, W.R., Sondahl, M.R., Caldas, L.S. and Maraffa, S.B. (1980). The physiology of *in vitro* asexual embryognesis. *Hort. Rev.* 2: 268-310.

Shaw, C.H., Watson, M.D., Carter, G.H. and Shaw, C.H. (1984). The right hand copy of the nopaline Ti-plasmid 25 bp repeat is required for tumour formation. *Nucleic Acids Res.* 12:6031-6041.

Sheridan, W.F. (1975). Tissue culture of maize I. Callus induction and growth. *Physiol. Plant* 33: 151-156.

Shields, R., Robinson, S.J. and Anslow, P.A. (1984). Use of fungicides in plant tissue culture. *Plant Cell Rep.* 3: 33-36.

Shillito, R.D., Carswill, G.K., Johnson, C.M., DiMaio, J.J. and Harms, C.T. (1989). Regeneration of fertile plants from protoplasts of elite inbred maize. *Bio/Technology* 7: 581-587.

Shillito, R.D., Saul, M.W., Paszkowski, J., Muller, M. and Potrykus, I. (1985). High efficiency direct gene transfer to plants. *Biotechnology* 3: 1099-1103.

Shimada, T. and Yamada, Y. (1979). Wheat plants regenerated from embryo cell cultures. *Jpn. J. Genet.* 54: 379-385.

Simon, E.W. (1974). Phospholipids and plant membrane permeability. *New Phytol.* 73: 377-420.

Simpson, R.B., Spielmann, A., Margossian, L. and McKnight, T.D. (1986). A disarmed binary vector from *Agrobacterium tumefaciens* functions in *A. rhizogenes*. Frequent cotransformation of two distinct T-DNA's. *Plant Mol. Biol.* 6: 403-415.

Skene, K.G.M. and Barlass, M. (1983). Studies on the fragmented shoot apex of grapevine. *J. Exp. Bot.* 34: 1271-1280.

Skoog, F, and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* 11: 118-130.

Srinivasan, C. and Vasil, I.K. (1986). Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). *J. Plant Physiol.* 126: 41-48.

Stacey, D.W. (1981). Microinjection of mRNA and other macromolecules into living cells. *Methods in Enzymol.* 79: 76-88.

Stalker, D.M., McBride, K.E. and Malyj, L.D. (1988). Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. *Science* 242: 419-423.

Steinbiss, H.H. and Stable, P. (1983). Protoplast derived tobacco cells can survive capillary microinjection of the fluorescent dye Lucifer Yellow. *Protoplasma* 116: 223-227.

Stevenson, G.C. (1965). *Genetics and breeding of sugarcane*. Longmans, London. 284pp.

Steward, F.C., Mapes, M.O. and Mears, K. (1958). Growth and organised development of cultured cells. II. Organisation in cultures grown from freely suspended cells. *Am. J. Bot.* 445: 705-708.

Stougaard, J., Abildsten, D. and Marcker, K.A. (1987). The *Agrobacterium rhizogenes* pRi TL-DNA segment as a gene vector system for transformation of plants. *Mol. Gen. Genet.* 207: 251-255.

Sun, C.S., Prioli, L.M. and Sondahl, M.R. (1989). Regeneration of haploid and dihaploid plants from protoplasts of supersweet (sh2 sh2) corn. *Plant Cell Rep.* 8: 313-316.

Sutcliffe, J.G. (1979). Complete nucleotide sequence on the *E. coli* plasmid pBR322. *Cold Spring Harbor Symp. Quant. Biol.* 43: 77-90.

Tabaeizadeh, Z., Ferl, R.J. and Vasil, I.K. (1986). Somatic hybridization in the Gramineae: *Saccharum officinarum* L. (sugarcane) + *Pennisetum americanum* (L). K. Schum. (pearl millet). *Proc. Natl. Acad. Sci.* USA 83: 5616-5619.

Thom, M., Maretzki, A., Konor, E. and Sakai, W.S. (1981). Nutrient uptake and accumulation of sugarcane cell cultures in relation to the growth cycle. *Plant Cell Tissue Organ Cult.* 1: 3-14.

Tisserat, B. (1985). Embryogenesis, organogenesis and plant regeneration. In: *Plant Cell Culture*. Ed: R.A. Dixon. IRL Press, Oxford. pp 79-105.

Tomes, D.T., Weissinger, A.K., Ross, M., Higgins, R., Drummond, B.J., Schaaf, S., Malone-Schoneberg, J., Staebell, M., Flynn, M., Anderson, J. and Howard, J. (1990). Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves. *Plant Mol. Biol.* 14: 261-268.

Töpfer, R., Gronenborn, B., Schell, J. and Steinbiss, H. (1989). Uptake and transient expression of chimeric genes in seed-derived embryos. *The Plant Cell* 1: 133-139.

Toriyama, K., Arimoto, Y., Uchimiya, H. and Hinata, K. (1988). Transgenic rice plants after direct gene transfer into protoplasts. *Bio/Technology* 6: 1072-1074.

Tsay, H.S. (1987). *Crop improvement through somaclonal variation*. ROC-USA Agricultural Biotechnology Workshop. Tapei, Taiwan. pp 1-15.

Twell, D., Klein, T.M. Fromm, M.E. and McCormick, S. (1989). Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. *Plant Physiol.* 91: 1270-1274.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montegu, M. and Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* 328: 33-37.

Vail, P.V. (1991). Mothproofing walnut trees of the future. *Ag. Res.* June 1991: 10-11.

Van den Elzen, P.J.M., Townsend, J., Lee, K.Y. and Bedbrook, J.R. (1985). A chimaeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Mol. Biol.* 5: 299-302.

Van Dun, C.M.P., Bol, J.F. and Van Vloten-Doting, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* 159: 299-305.

Van Lijsebettens, M., Inze, D., Van Montagu, M. and Schell, J. (1986). Transformed cell clones as a tool to study T-DNA integration mediated by *Agrobacterium tumefaciens*. *J. Mol. Biol.* 188:129-145.

Vasil, I.K. (1982). Plant cell culture and somatic cell genetics of cereals and grasses. In: *Plant improvement and somatic cell genetics*. Ed: I.K. Vasil, W.R. Scowcroft and K.J. Frey. Academic Press, Orlando USA. pp 179-204.

Vasil, I.K. (1983). Isolation and culture of protoplasts of grasses. In: *Plant Protoplasts*. Ed: K.G. Giles. Academic Press, London pp 79-90.

Vasil, I.K. (1987). Developing cell and tissue culture systems for the improvement of cereal and grass crops. *J. Plant Physiol.* 128: 193-218.

Vasil, I.K. (1990). The contributions of biotechnology and its challenges. *IAPTC Newsletter* 62: 2-11.

Vasil, V., Brown, S.M., Re, D., Fromm, M.E. and Vasil, I.K. (1991). Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. *Bio/Technology* 9: 743-747.

Vasil, V., Redway, F. and Vasil, I.K. (1990a). Regeneration of plants from embroygenic suspension culture protoplasts of wheat (*Triticum aestivum L.*). *Bio/Technology* 8: 429-432.

Vasil, V. and Vasil, I.K. (1982). The ontogeny of somatic embryos of *Pennisetum americanum* (L). K. Schum. *Bot. Gaz.* 143: 454-465.

Vasil, V. and Vasil, I.K. (1984). Induction and maintenance of embryogenic callus cultures of Gramineae. In: *Cell culture and somatic cell genetics of plants Vol 1: Procedures and their applications*. Ed: I.K. Vasil. Academic Press, London. pp 37-40.

Vasil, V., Vasil, I.K. and Lu, C.H. (1984). Somatic embryogenesis in long-term callus cultures of *Zea mays* L. (Gramineae). *Am. J. Bot.* 71: 158-161.

Vasil, I.K., Vasil, V. and Redway, F. (1990b). Plant regeneration from embryogenic calli, cell suspension cultures and protoplasts of *Triticum aestivum* L. (Wheat). In: *Progress in plant cellular and molecular biology*. Eds: H.J.J. Nijkamp, L.H.W. van der Plas and J. von Aartrijk. Kluwer Academic, The Netherlands. pp 33-37.

Vasil, V., Wang, D. and Vasil, I.K., (1986). Plant regeneration from protoplasts of *Pennisetum purpureum*. Schumm (Napier grass). *Z. Pflanzenphysiol.* 111: 233-239.

Walden, R. (1988). *Genetic transformation in plants*. Institute Biology : Biotechnology series. Open University Press, Milton-Keynes, U.K. pp 70-81.

Wang, Y.C., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C. and Wu, R. (1988). Transformation of rice, wheat and soybean by the particle bombardment method. *Plant Mol. Biol.* 11: 433-439.

Wang, D. and Vasil, I.K. (1982). Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpurea* Schum. (Napier or Elephant grass). *Plant Sci. Lett.* 25: 147-154.

Watt, M.P., Mycock, D.J. and Cresswell, C.F. (1989). Plant regeneration by somatic embryogenesis from leaf explants of *Digitaria eriantha* (Stead) subsp. *eriantha*. *XVI Int. Grasslands Conference Nice, France*.pp 421-422.

Weatherhead, M.A., Bardon, J. and Henshaw, G.G. (1978). Some effects of activated charcoal as an additive to plant tissue culture media. *Z. Pflanzenphysiol.* 89: 141-147.

Weising, K., Schell, J. and Kahl, G. (1988). Foreign genes in plants : Transfer, structure, expression and applications. *Annu. Rev. Genet.* 22: 421-477.

Wernicke, W. and Brettell, R.I.S. (1980). Somatic embroygenesis from *Sorghum bicolor* leaves. *Nature* 138: 138-139.

Wernicke, W., Potrykus, I. and Thomas, E. (1982). Morphogenesis from cultured leaf tissue of *Sorghum biocolor* (L.). The morphogenetic pathways. *Protoplasma* 111: 53-62.

Werr, W. and Lorz, H. (1986). Transient gene expression in a Gramineae cell line : A rapid procedure for studying plant promoters. *Mol. Gen. Genet.* 202:471-475.

White, P.R. (1963). *A handbook of plant and animal tissue culture*. Jaques Cattel Press, USA. 214pp.

Withers, L.A. (1985). Cryopreservation and storage of germplasm. In: *Plant cell culture: A practical approach*. Ed: R.A. Dixon. IRL Press, Oxford, England. pp 169-191.

Woodward, B.R. (1989). Somatic embryogenesis in maize (*Zea mays* L). *MSc thesis.* 195pp.

Woolston, C.J., Barker, R., Gunn, H., Boulton, M.I. and Mullineaux, P.M. (1988). Agroinfection and nucleotide sequence of cloned wheat dwarf virus DNA. *Plant Mol. Biol.* 11: 35-43.

Yamada, Y., Yang, Z. and Tang, D. (1986). Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.) *Plant Cell Rep.* 5: 85-88.

Yang, H., Zhang, H.M., Davey, M.R., Mulligan, B.J. and Cocking, E.C. (1988). Production of kanamycin resistant rice tissues following DNA uptake into protoplasts. *Plant Cell Rep.* 7: 421-425.

Yao, D.Y. and Krikorian, A.D. (1981). Manipulation of rice (*Oryza sativa* L.) from aseptically cultured nodes. *Ann. Bot.* 48: 255-259.

Young, P.M., Hutchins, A.S. and Canfield, M.L. (1984). Use of antibiotics to control bacteria in shoot cultures of woody plants. *Plant Sci. Lett.* 34: 203-209.

Zambryski, P. (1988). Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu. Rev. Genet.* 22: 1-30.

Zhang, H.M., Yang, H., Rech, E.L., Golds, T.J., Davis, A.S., Mulligan, B,J., Cocking, E.C. and Davey, M.R. (1988). Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. *Plant Cell Rep.* 7: 379-384.

Ziv, M. (1986). *In vitro* hardening and acclimitization of tissue culture plants. In: *Plant tissue culture and its agricultural applications.* Ed: L.A. Withers and P.G. Aldeson. University Press, Cambridge. pp 187-196.