DIRECT TRANSFORMATION OF MAIZE (*Zea mays* L.) TISSUE USING ELECTROPORATION AND PARTICLE BOMBARDMENT, AND REGENERATION OF PLANTLETS

by

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THESIS
Submitted in partial fulfilment of the
requirements for the Degree of
MASTER OF SCIENCE AGRICULTURE
in the Department of Genetics
University of Natal
PIETERMARITZBURG

January, 1996

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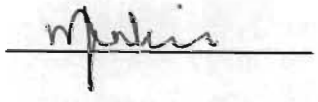
DECLARATION

I hereby declare that, unless specifically indicated, this thesis is the result of my own investigation.



Megan Joy Jenkins
January 1996

ACKNOWLEDGMENTS

Sincere thanks are due to many individuals and organisations who have contributed towards this project:

Dr P.E. Shanahan for his supervision and enthusiasm;

The staff of the Genetics Department, especially Mrs P. Wallis for her support and technical assistance;

Professor Gewers for his kind donation of caryopses and advice concerning the background of the inbreds used;

Mr K. Hosking for his work in construction of the controlled environment chamber;

Mr M. Savary for his assistance with microscopy;

The staff of Audio Visual for their assistance with photography;

Professor F. Botha and his staff, especially Mrs G. Meyer and Mrs S. Snyman, of the Biotechnology Department of the South African Sugar Association Experiment Station for their assistance with particle bombardment;

The FRD and University Bursary Scheme for financial support;

Miss N. Warburton for her friendship;

My parents and family for support throughout my study years;

Mr W. Brunkhorst for his constant support and belief that I could do it.

LIST OF ABBREVIATIONS AND DEFINITIONS OF SCIENTIFIC TERMS

2,4-D - 2,4-Dichlorophenoxyacetic acid
ABA - abscisic acid
ADH1 - alcohol dehydrogenase
a.i. - active ingredient
ALS - acetolactate synthase
ASA - acetylsalicylic acid
A. tumefaciens - *Agrobacterium tumefaciens*
B. thuringiensis - *Bacillus thuringiensis*
BAP - N^6-benzylamino-purine
CaMV 35S - cauliflower mosaic virus fragment, containing 400-1000 bp of 35S upstream sequence
CAT - chloramphenicol acetyltransferase
Competence - the ability of a cell to respond in desired ways to specific stimuli during development
dicamba - 3,6-dichloro-*o*-anisic acid
DMSO - dimethyl sulphoxide
E. coli - *Escherichia coli*
EDTA - Ethylene diamine tetra acetic acid
Embryogenic - capable of regeneration into plants; regenerable maize cultures are generally classified as Type I (non-friable; regenerates through both somatic embryogenesis and organogenesis) or Type II (very friable; regenerates almost exclusively via somatic embryogenesis)
epi-genetic - non-genetic factors
Explant - any portion of a plant which is placed *in vitro* on a nutrient medium
FDA - fluorescein diacetate
FITC - Fluorescein isothiocyanate
Friability - degree of physical association between cells
GUS - β-glucuronidase
Immature embryo - zygotic embryo taken 10 to 14 d after pollination when the endosperm is in the "milk" stage
KM - Kao and Michayluk
Leaf base - the lower third of the leaf blade and the leaf sheath

LS - Linsmaier and Skoog

MDMV - maize dwarf mosaic virus

MES - 4-Morpholine-ethanesulfonic acid

Morphogenesis - differentiation of plant organs from cell culture which includes the independent initiation of shoot or root primordia as well as somatic embryogenesis

MS - Murashige-Skoog

MS-CM medium - MS Callus maintenance medium

MUG - 4-methyl umbelliferyl-glucuronide

N6(0.6) - N6 Protoplast culture medium solidified with 6 g L^{-1} agar and supplemented with 11% (m/v) mannitol, 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D; pH 5.8

N6-PC - N6 Protoplast culture medium

NAA - napthalene-1-acetic acid

Non-embryogenic - incapable of regeneration. Non-embryogenic cells are usually elongated, vacuolated and irregularly shaped NOS - nopaline synthase

NPT II - neomycin phosphotransferase II

PAT - phosphinothricin acetyltransferase

PEG - polyethylene glycol

PIG - particle inflow gun

Plantlet - an apparently functioning connected shoot and root which develop either by the germination of a somatic embryo or by two separate events, shoot formation and subsequent root initiation

PPT - phosphinothricin

Precocious germination - early germination

Protoplast - in the context of cell culture, a protoplast is a plant cell from which the cell wall has been removed by mechanical or enzymatic processes

rpm - revolutions per minute

SASEX - South African Sugar Association Experiment Station (Mt Edgecombe, South Africa)

Somaclonal variation - genetic variation observed amongst plants regenerated from tissue cultures

Somatic embryogenesis - the development of an embryo from cells other than gametes or their fusion products (zygotes) by a

process which more or less recapitulates zygotic embryogenesis

T-DNA - transfer DNA

Transformation frequency - number of transformed calli per unit surviving protoplast (relative transformation frequency) or per unit initial protoplast (absolute transformation frequency)

Ubi1 - ubiquitin gene

X-Glu - 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid

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ABSTRACT

In vitro callus cultures of 10 South African elite maize inbreds were established from immature embryos and immature leaf bases excised from *in vitro* germinated seedlings. The callus was initiated and maintained on Murashige and Skoog (MS) medium supplemented with modified concentrations of the following supplements: glycine (7.7 mg L^{-1}), nicotinic acid (1.3 mg L^{-1}), thiamine-HCL (0.25 mg L^{-1}), pyridoxine-HCL (0.25 mg L^{-1}), calcium pantothenate (0.25 mg L^{-1}), casein hydrolysate (200 mg L^{-1}), L-asparagine (198 mg L^{-1}), sucrose (20 mg L^{-1}), 2,4-D (3 mg L^{-1}) and agar (10 g L^{-1}); pH 5.8. Callus cultures were incubated at 27±1°C under a 12 h light/12 h dark photoperiod. Friable callus was used to initiate cell suspension cultures, which were maintained in liquid MS callus maintenance medium supplemented with sucrose (20 mg L^{-1}) and 2,4-D (3 mg L^{-1}); pH 5.8, and incubated in the dark at 27°C in an orbital shaker water bath operating at 150 rpm. Greenhouse grown leaf tissue, callus cultures and cell suspensions were used as sources for the isolation of maize protoplasts. An efficient protocol for the isolation of maize protoplasts from callus was established, with an enzyme combination of 2.5% (m/v) Cellulase Onozuka R-10, 0.5% (m/v) Macerase® Pectinase and 0.5% (m/v) Hemicellulase (Sigma®) being most effective. Subsequent to static digestion for 16 h, the digested callus was gently teased apart to release the protoplasts. An optimum concentration of mannitol (11% (m/v)) was determined for use as osmotic agent. Callus cultures, cell suspension cultures, and protoplasts isolated from maize callus cultures, were used in preliminary investigations into the direct transfer of genes into maize tissue. Successful transformation of maize callus was obtained with electroporation, using a Pro-Genetor™ electroporator set to a voltage of 350 V, of 50 ms duration. The pBI221 (β-glucuronidase) plasmid construct was utilized. Maize callus was also successfully transformed by particle bombardment with tungsten particles coated with either the pAHC25 (β-glucuronidase) or the pDP687 (anthocyanin) plasmid

construct. Plantlets were regenerated from callus cultures of the maize inbreds S0507W, D0940Y and K0315Y. The plantlets were regenerated on N6 or MS medium supplemented with 2,4-D (0.3 mg L^{-1}), kinetin (0.5 mg L^{-1}), sucrose (6% (m/v)) and agar (10 g L^{-1}); pH 5.8, at $27\pm1^{o}C$ under a 12 h light/12 h dark photoperiod. The results obtained provide a basis for future research on the improvement of maize by tissue culture and genetic engineering techniques.

INTRODUCTION

The plant family *Gramineae* (cereals and grasses) has world-wide importance for human and animal nutrition (Ozias-Akins & Lörz, 1984; Van Mellaert, 1992). Cereals constitute the most significant source of calories and protein for man since more than 52% of our food is derived from grains such as wheat, rice, maize, barley and millets (Vasil & Vasil, 1991; Van Mellaert, 1992). *Zea mays* L. (maize) is a large kernelled, highly domesticated, vigorous annual plant of tropical origin (Neuffer, 1994) and is one of the most widely grown agronomic crops in the world (Shillito, Carswell, Johnson, DiMaio & Harms, 1989).

It is as food for humans and livestock that maize is of greatest value to the world (Saunders, 1930; Jugenheimer, 1976; Kleese, Kirihara & Sandahl, 1991). Maize caryopses contain c.a. 77% starch, 2% sugar, 9% protein, 5% oil, 5% pentosan, and 2% ash on a dry mass basis. The ash of the caryopse contains salts of calcium, magnesium, phosphorus, aluminium, iron, sodium, potassium and chlorine (Jugenheimer, 1976). In South Africa, maize is commonly ground into a coarse meal for porridge or gruel making (Saunders, 1930). Corn on the cob, sweet corn (favoured as a green vegetable) and pop corn are also popular (Saunders, 1930; Jugenheimer, 1976). Maize flour (Jugenheimer, 1976), in combination with other flour types, may be used for the baking of bread, muffins, griddlecakes, waffles and pudding (Saunders, 1930). Other maize products for human consumption include maize starch, glucose, oil, syrup, sugar, hominy and breakfast cereals (Saunders, 1930; Jugenheimer, 1976) such as corn flakes (Saunders, 1930).

Maize is pre-eminently a fattening food for livestock, by virtue of its richness in carbohydrates and fats, but needs to be supplemented by other foods rich in nutrients, such as high quality protein, in which it is deficient (Saunders, 1930; Kleese, *et al.*, 1991). Prior to feeding, maize is often soaked,

cooked or ground (Saunders, 1930). Maize bran, gluten meal and feed, oil cake, distillers' and brewers' grains, and stover are used as fodder supplements (Saunders, 1930; Jugenheimer, 1976). Maize is an outstanding silage crop and is the main source of winter roughage on dairy farms in Natal (Ashby & Birch, 1988).

In industry, the distilling and fermentation industries manufacture ethyl and butyl alcohol, acetone and whisky (Jugenheimer, 1976). Maize pulp may be used as a source of cellulose for the manufacture of rayon, nitrocellulose and other similar products. Stalks may be used for lumber substitutes, such as wall and plaster boards. Dried maize plants, especially cobs, are used for fuel or charcoal production. Husks are used for filling cheap mattresses and pillows, as well as for braiding into mats, rugs, baskets and hats (Saunders, 1930).

Due to the importance of maize to the consumer and industry, its successful cultivation is essential. The tremendous increases in maize yields in the past 10 years (from 1.09 t ha^{-1} in the 1984/85 season to 2.48 t ha^{-1} in the 1993/94 season in the Republic of South Africa) (Anon, 1994) can be attributed to the persistent efforts of plant breeders in producing better varieties (Ozias-Akins & Lörz, 1984). As a natural cross-pollinator, maize is highly heterogenous and responsive to selection pressure. Humans have grown maize in all but the harshest of agricultural environments. Some cultivars of maize are able to grow as far north as southern Canada, while other cultivation areas range from the extremes of the desert oasis to tropical forest. Certain cultivars grow at sea level and others at 3000 m of elevation. Maize inbreds and cultivars mostly used for breeding and genetic purposes are much more restricted in adaptation and therefore require narrower limits on growth conditions in order to produce a useful crop. Cultivars that would be satisfactory for use under most plant cultivation conditions could no doubt be found or developed (Neuffer, 1994).

Maize inbreds have been a rich resource for fundamental and applied investigations. Inbreds constitute a sampling of the genetic diversity in maize that has been captured and partitioned into an array of uniform, reproducible genotypes. Maize inbreds, used as parents in hybrid seed production or in genetic studies, are highly homogenous homozygous inbreds, exhibiting a high degree of genetic stability and reproducibility. Inbreds typically have been developed through successive generations of self-pollination with artificial selection for desired attributes. The number of generations, and therefore the level of homozygosity, may vary considerably, although maize breeders in temperate climates usually release inbreds after seven or more generations of self-pollination and selection (Lee, 1994).

Cereals are an obvious and important target for genetic manipulation by modern biotechnological methods (Vasil & Vasil, 1991). Considerable interest is being directed towards the genetic improvement of maize both through conventional breeding and biotechnology (Shillito, *et al.*, 1989). Due to the potential for genetic engineering of maize via recombinant DNA and cell regeneration procedures, it would be advantageous if elite maize inbreds used in commercial hybrid production (Hodges, Kamo, Imbrie & Becwar, 1986; Shillito, *et al.*, 1989) could be transformed with isolated genes conferring agronomically desirable traits, thus bypassing lengthy back-crossing procedures. This would enable new genetic traits to be incorporated into ongoing breeding programs (Shillito, *et al.*, 1989) and simultaneously reduce the time required for the breeding of plants with disease resistance, improved nutritional quality or the ability to grow under conditions of great environmental stress (Ozias-Akins & Lörz, 1984). Therefore, the introduction of chimeric genes into elite inbreds could represent a significant saving in breeding time required to produce a commercial hybrid (Koziel, Beland, Bowman, Carozzi, Crenshaw, Crossland, Dawson, Desai, Hill, Kadwell, Launis, Lewis, Maddox, McPherson, Meghji, Merlin, Rhodes, Warren, Wright & Evola, 1993).

Genetic transformation and the production of transgenic plants is one of the most useful and powerful tools of plant biotechnology (Vasil, Vasil & Redway, 1990). Broadly speaking, transgenic plants refer to those plants in which functional foreign genes have been inserted into their genomes. Besides selectable marker genes (Uchimiya, Handa & Brar, 1989), a number of other foreign genes conferring insect tolerance (Koziel, *et al.*, 1993), virus protection (Murry, Elliott, Capitant, West, Hanson, Scarafia, Johnston, DeLuca-Flaherty, Nichols, Cunanan, Dietrich, Mettler, Dewald, Warnick, Rhodes, Sinibaldi & Brunke, 1993), herbicide tolerance (Fromm, Morrish, Armstrong, Williams, Thomas & Klein, 1990; Spencer, Gordon-Kamm, Daines, Start & Lemaux, 1990), increased tolerance to abiotic stresses (unfavourable soil, temperature and water conditions), improved quality characteristics, storage proteins and light regulated genes have been transferred to plants from a wide range of plants, bacterial and insect systems (Uchimiya, *et al.*, 1989). Unique sexual and somatic or cytoplasmic hybrids (cybrids), that combine desirable characters of sexually incompatible species, may be obtained by protoplast fusion (Vasil, 1988). In the majority of cases, foreign genes show expression in transgenic plants, are stably inherited in the progeny without detrimental effects on the host plant and show Mendelian monogenic segregation (Fromm, *et al.*, 1990; Walters, Vetsch, Potts & Lundquist, 1992). Insertion of one or a few foreign genes does not adversely affect the agronomic characteristics of host cultivars except for expression of the introduced gene (Uchimiya, *et al.*, 1989; Fromm, *et al.*, 1990). Therefore, genetic engineering methods complement conventional crop improvement programs by enabling the transformation of inbreds to be used in commercial hybrid production (Uchimiya, *et al.*, 1989). This approach could be helpful for improvement of the agronomic performance of a species by increasing the diversity of genes and germplasm available for incorporation into crop plants (Franks & Birch, 1991; Koziel, *et al.*, 1993). Alternatively the specific genetic deficiencies of otherwise high yielding and well adapted commercial cultivars could be overcome (Uchimiya, *et al.*, 1989;

Franks & Birch, 1991). However, there is a need for extensive laboratory and field testing of transgenic plants and their progenies before their usefulness can be realised on a commercial scale (Uchimiya, *et al.*, 1989).

Dramatic progress has been made in our understanding of and ability to alter the regulation of gene expression in plants and in techniques for the identification and isolation of genes of interest. Genetically engineered plants at present generally depend on the use of continuously expressed promoters driving dominant single gene traits (Gasser & Fraley, 1989). However, the isolation of useful genes governing economic characters such as disease resistance, salinity, drought and improved nutritional quality is a major bottleneck for producing transgenic crop varieties with desirable genetic properties (Uchimiya, *et al.*, 1989). Future plant genetic engineering will probably include alteration of traits that require subtle temporal and spatial regulation of gene expression and introduction and alteration of entire biosynthetic pathways (Gasser & Fraley, 1989). Transgenic plants would provide a wealth of information on gene expression in higher plants and possibly ways to switch genes on and off as and when required, thus making gene manipulation a more direct process for genetic improvement of crop plants (Uchimiya, *et al.*, 1989). The advances in crop improvement by genetic engineering have occurred so rapidly that the initial introduction of these crops in the marketplace will be influenced primarily by nontechnical issues, which include regulatory approval, proprietary protection and public perception (Gasser & Fraley, 1989).

Initial research has been focused on the engineering of traits that relate directly to the traditional roles of industry in farming, such as the control of insects, weeds and plant diseases. Progress has been rapid and genes conferring these traits have already been successfully introduced into several important crop species (Gasser & Fraley, 1989). In spite of great efforts, biotechnology research with the major cereals has lagged

behind research conducted with dicotyledonous plant species. New technology may solve many of the problems associated with cereal transformation (Van Mellaert, 1992).

Genetic engineering could have a great impact on the South African maize industry. Maize is potentially the highest yielding grain crop, and its ability to produce large amounts of green matter (80 to 100 t ha^{-1}) and dry matter (16 to 20 t ha^{-1}) within 120 to 160 d in the summer, makes it the most efficient in terms of irrigation water use. For maximum production, a medium maturity grain crop needs between 500 and 800 mm of water depending upon the climate (Mottram, 1988). However, in South Africa stress is normally associated with drought (Kaiser, 1988). The El Nino phenomenon in the Pacific Ocean has been responsible for recurring cycles of drought (Anon, 1995). Adequate water must be available at all times during the growing season if optimum yields are to be obtained (Manson, 1988). Other factors that can lead to sub-optimal growing conditions include diseases, nutritional factors, chemical factors that inhibit growth (e.g. aluminium), plant populations and wind damage. Diseases that mainly affect South African maize farmers include: leaf blight (*Helminthosporium turcicum*); grey leaf spot (*Cercospora zeae-maydis*); common rust (*Puccinia sorghi*); maize streak virus; root, stalk and cob rots (*Diplodia maydis*, *Gibberella zeae* and *Fusarium moniliforme*); tassel smut (*Sphacelotheca reiliana*); and boil smut (*Ustilago maydis*) (Kaiser, 1988). Insect pests cause significant damage to the maize crop, with the most important of these pests being cutworms, stalkborers, maize snout beetles, army worms, black maize beetles, spotted maize beetles and American bollworms (Barrow & Bell, 1988). Therefore, in South Africa commercial maize farmers and rural communities require cultivars that are robust, easy to cultivate, are tolerance to drought and insect pests, and have improved disease resistance. Additionally, the maize cultivars need to have improved characteristics so as to provide a more complete food which does not need protein supplementation.

The South African Maize Board, the Institute of the Agricultural Research Council and private breeding companies are involved in research to improve the consumption and quality of normal and high-lysine maize products. This research covers physical and chemical quality characteristics, fungal infestation and contamination with mycotoxins. Research which is presently being addressed in other areas includes: the production of Mexican dishes, such as tortillas, on an industrial level; the influence of fertilization on protein content, grain hardness (milling quality); the use biotechnology to engineer resistance to cobrot into maize; and resistance of storage pests to phosphine gas (Anon, 1994; Viljoen, 1995).

Internationally, plant biotechnologists have produced several products which are becoming accessible. For example, detasseling in hybrid seed production is a cost factor and accidental cross-pollination may be a threat to hybrid seed quality. Therefore, reliable male sterility in maize is of great interest to the whole maize breeding industry. Genetically engineered male sterility has been achieved in maize (Van Mellaert, 1992).

The development of crop plants that are tolerant to herbicides would provide more effective, less costly, and more environmentally appealing weed control (Gasser & Fraley, 1989; Somers, Marshall, Dotray, Parker, Wyse & Gengenbach, 1991). Glyphosate tolerance and resistance to sulfonylurea compounds has been engineered into various crops (Gasser & Fraley, 1989). Resistance to bialaphos, which is cleaved in plant cells to yield phosphinothricin (PPT), an inhibitor of glutamine synthase, has been achieved in stably transformed maize callus by expression of the *bar* gene (encoding phosphinothricin acetyltransferase) isolated from *Streptomyces hygroscopicus* (Fromm, *et al.*, 1990; Spencer, *et al.*, 1990).

The production of insect-resistant plants is another application of genetic engineering with important implications for crop improvement and for both the seed and agrochemical industries

(Gasser & Fraley, 1989). European corn borer (*Ostrinia nubilalis*), a major pest of maize in Europe and North America, is susceptible to various proteins produced by a number of strains of *Bacillus thuringiensis*. Maize plants, expressing high levels of a synthetic gene encoding a truncated version of one of these insecticidal proteins, exhibited excellent resistance to repeated heavy infestations of European corn borer (Koziel, *et al.*, 1993). In addition, transgenic tomato and tobacco plants containing the cloned *B. thuringiensis* gene have increased resistance to lepidopteran insects (Barton, Whiteley & Yang, 1987).

Significant resistance to viral infection may be achieved by expression of coat protein genes in transgenic plants (Gasser & Fraley, 1989; Uchimiya, *et al.*, 1989). For example, maize dwarf mosaic virus (MDMV) is the most widely distributed and important viral disease of maize. Transformed maize plants expressing the MDMV coat protein gene were resistant to inoculations with MDMV and mixed inoculations of MDMV and maize chlorotic mottle virus (Murry, *et al.*, 1993).

International successes with plant biotechnology have demonstrated techniques which may be used to improve the commercial and agronomic characteristics of maize by non-conventional means. As maize is the staple diet of many rural South Africans, the use of such techniques in a South African context would greatly supplement traditional breeding processes and be of great benefit to rural as well as commercial maize producers. For example, resistance to grey leaf spot of maize (*Cercospora zeae-maydis* - a new disease of maize in South Africa) could be achieved through transformation of South African maize genotypes in a shorter period of time than conventional breeding methods.

In South Africa, there is a need for a broad database for the tissue culturability of South African maize genotypes. Six South African maize inbred lines (M162W, 21A-6, F2834T, I137TN and

A441-5) have previously been examined for their suitability for tissue culture. 21A-6, from which plantlets were regenerated, exhibited the best tissue culture response out of the tested inbreds (Woodward & Furze, 1989). Reports on the successful regeneration of plantlets from callus induced from immature embryos of South African maize inbreds, are encouraging (Lennox, Palmer, Rybicki & Thomson, 1995). However, more extensive research needs to be undertaken to broaden the culturability database, and successfully regenerate transformed plants of South African elite maize germplasm.

Taking all of the above into consideration, a study was undertaken to determine whether selected South African elite maize inbreds were amenable to tissue culture and genetic transformation (Figure 1.1). Selected elite inbreds of maize with diverse backgrounds were grown under controlled environmental conditions in a glasshouse, or germinated under *in vitro* conditions. Various tissue types at different ages (leaf discs, immature embryos, leaf roll, pith and the bases of young seedlings) were excised from the maize plants and placed on tissue culture medium for the initiation of callus. Callus cultures were therefore initiated from various tissue sources (Chapter 2) and an attempt was made to establish suspension cell cultures from callus (Appendix E). The author was unable to conduct a full study on maize suspension cultures; therefore, the results are presented in an Appendix at the end of the thesis. It is hoped that this Appendix will aid future researchers who may wish to establish cell suspensions of maize. Subsequent to the establishment of callus cultures, conditions were established for the efficient isolation of protoplasts from leaf tissue and established callus cultures (Chapter 3). Suspension cultures were also treated with an enzyme solution in an endeavour to isolate protoplasts (Appendix E). Various culture conditions for maize protoplasts were investigated (Chapter 3). In preliminary transformation studies, isolated protoplasts were electroporated in the presence of plasmid DNA. As an alternative to the transformation of protoplasts by electroporation, maize callus

was electroporated or microprojectile bombarded. Transient expression of the β-glucuronidase and anthocyanin reporter gene systems were used to determine transformation events (Chapter 4). As a final conclusion to this study, investigations were undertaken into the regeneration of maize plantlets from callus cultures (Chapter 5). Regeneration is the *sine qua non* of stable transformation, and should have been presented in conjunction with the various approaches to tissue culture. However, even though the regeneration studies were undertaken in conjunction with the other *in vitro* culture techniques, the work is presented after the transformation studies in order to illustrate the logical progression from the establishment of a tissue culture, through transformation, to regeneration of transformed plantlets.

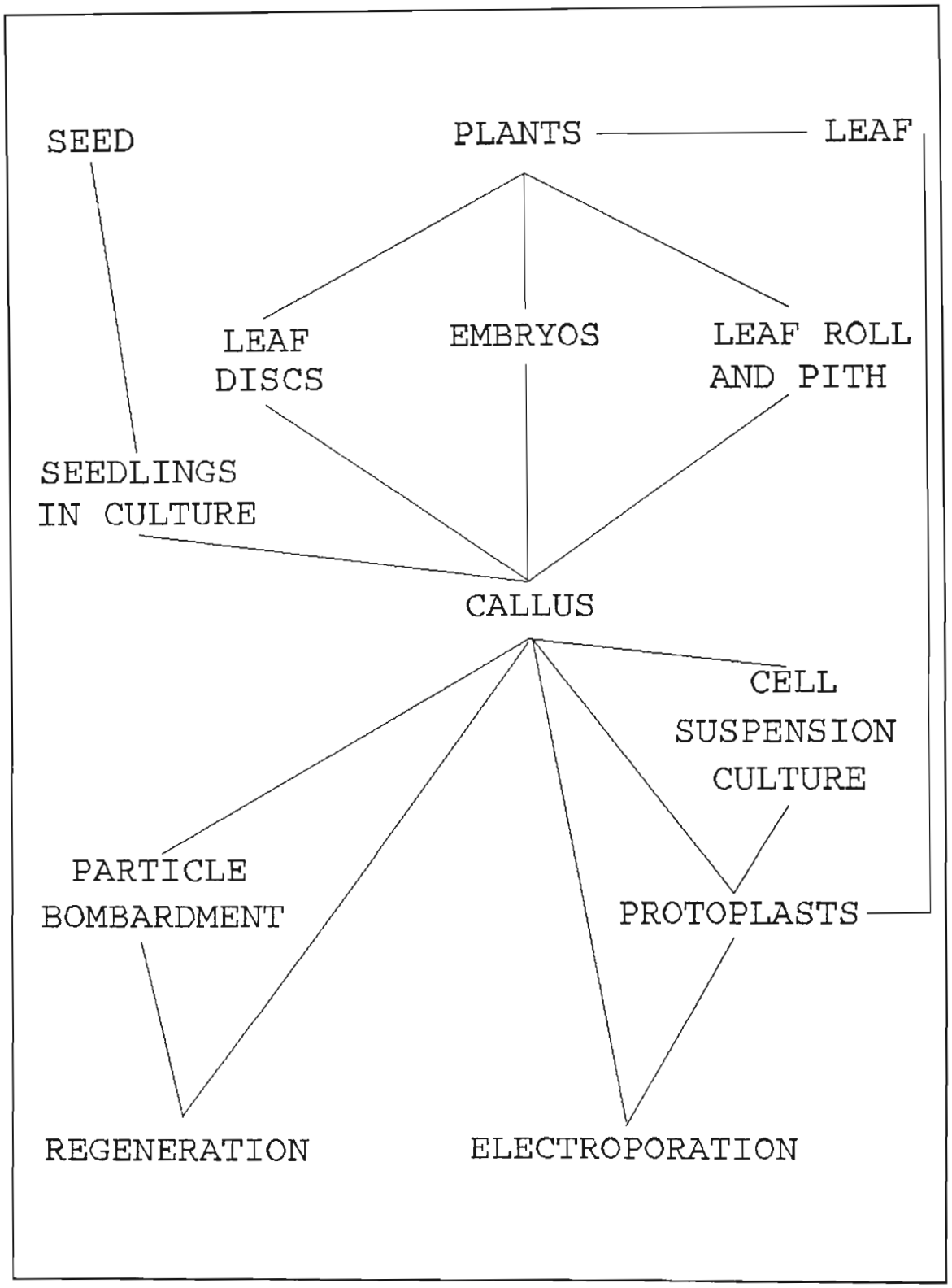


Figure 1.1 Schematic outline of the research strategy used in this study on the tissue culture and genetic transformation of South African elite maize inbreds.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Plant tissue culture is based on the premise that living cells, separated from the original plant, can develop into viable plants. This phenomenon is known as totipotency. The collective term, plant tissue culture, is used to describe the independent growth of excised plant organs, tissues or cells on a chemically defined medium. The various types of *in vitro* plant cultures that can be established include: callus; cell suspension; protoplast; anther; and organ cultures. The type of culture to be utilized in tissue culture is defined by the aim of the experiment (George & Sherrington, 1984). Once plant tissue culture techniques have been established for a particular plant species, transformation (Davey, Rech & Mulligan, 1989) and regeneration of transgenic plants (Harms, Lörz & Potrykus, 1976) may be undertaken.

Plant tissue culture has a long history, dating back to the end of the 19th century (Lindsey, 1993). Plant organ, tissue and cell culture procedures have developed rapidly in the half century since the pioneering efforts of Laibach, Gautheret, Nobecourt and White (Pierik, 1987). The associated concepts and techniques of plant tissue culture have reached a level of usefulness and application which has never been greater. Technical innovations have given new insights into fundamental aspects of plant differentiation and development, and have paved the way to the identification of strategies for the genetic manipulation of plants (Lindsey, 1993). If applied to agronomically important crop plants, plant tissue culture techniques are considered a powerful tool for plant breeding (Harms, *et al.*, 1976). As a consequence, a range of somatic cell and molecular techniques are now available to supplement conventional plant breeding (Davey, *et al.*, 1989).

1.2 Callus cultures of maize

A callus is defined as a continually dividing mass of generally poorly differentiated and disorganised plant cell aggregates. In morphological terms, callus can vary extensively, ranging from being very hard/compact, where the cells have extensive cell to cell contact, to being "friable" where the callus consists of small, disintegrating aggregates of poorly-associated cells and has a rather crumbly appearance. Callus morphology is often explant-dependent but can usually be altered by modification of the growth substance supplementation of the culture medium. Callus can grow indefinitely *in vitro* if it is provided with a constant supply of the appropriate nutrients and plant growth substances. This therefore requires that pieces of the growing cell mass be transferred (subcultured) to fresh medium at regular intervals. Callus cultures have an inherent degree of heterogeneity due to their size and nature. Chemical and physical gradients will be present within the callus mass, as there is a unidirectional supply of nutrients (from the medium below) and light and gases (predominantly from above). In some instances this heterogeneity is undesirable (e.g. in the production of uniform biomass). However, it may also be an important influential factor in the developmental response of the callus in, for example, plant regeneration (Hall, 1991).

1.2.1 Types of maize callus

Irrespective of the explant used, both embryogenic (capable of plant regeneration) and non-embryogenic (unable to undergo regeneration into plants) calluses are formed on callus initiation medium (Vasil, 1988; Vasil & Vasil, 1991). In maize tissue culture, two embryogenic and a non-embryogenic callus type can be distinguished. At the ultrastructural level, embryogenic cells are characterized by a relatively large nucleus, the presence of small vacuoles, and a dense cytoplasm, owing to the presence of many organelles. Many plasmodesmata occur between

adjacent embryogenic cells (Fransz & Schel, 1991). Embryogenic callus types are termed compact, nodular, or Type I callus (Vasil & Vasil, 1986; Fransz & Schel, 1991), and soft, friable, or Type II callus (Armstrong & Green, 1985; Vasil, Lu & Vasil, 1985; Fransz & Schel, 1991). Non-embryogenic callus is translucent and soft, grows more rapidly, and consists mostly of large and vacuolated cells (Vasil & Vasil, 1991). During the routine growth of embryogenic calli considerable numbers of non-embryogenic cells are formed. These must be removed during each subculture to ensure efficient and long-term regeneration. Maintenance of embryogenic calli is, therefore, technically demanding and labour intensive (Gnanapragasam & Vasil, 1992).

Embryogenic Type I callus is white, off-white, or pale yellow in colour, and is compact, organized and often nodular in appearance (Vasil & Vasil, 1986; Vasil, 1988; Vasil & Vasil, 1991; D'Halluin, Bonne, Bossut, De Beuckeleer, & Leemans, 1992). Type I callus consists of small, tightly packed, thin-walled, richly cytoplasmic cells containing a large nucleus, many amyloplasts, lipid bodies, and starch grains, but no large vacuoles (Vasil, 1988; Vasil & Vasil, 1991). Compact callus has a solid appearance with many scutellum-like bodies often in coalescence (Fransz & Schel, 1991). Such cultures form somatic embryos which can be germinated to obtain mature plants (Vasil & Vasil, 1986). Maize Type I callus cultures have slow growth habits (Armstrong & Green, 1985) and are difficult to maintain in culture beyond a few subcultures unless carefully selected for (Lu, Vasil & Ozias-Akins, 1982; Vasil & Vasil, 1991). Type I callus can be readily derived at high frequencies from cultured immature embryos in a wide variety of maize inbreds and hybrids (Lu, *et al.*, 1982; Lu, Vasil & Vasil, 1983; Novák, Dolezelová, Nesticky & Piovarci, 1983; Duncan, Williams, Zehr & Widholm, 1985; Tomes & Smith, 1985; Hodges, *et al.*, 1986; Imbrie-Milligan & Hodges, 1986; Vasil & Vasil, 1986).

Type II embryogenic callus is a soft, friable, rapidly growing callus of uniform type (Vasil, Vasil & Lu, 1984; Armstrong & Green, 1985; Tomes & Smith, 1985; Imbrie-Milligan & Hodges, 1986; Vasil & Vasil, 1986; Vasil, 1988; Hall, 1991). This callus type arises at a very low frequency either directly from the scutellum of cultured immature embryos as a primary callus or as spontaneously occurring sectors in established Type I callus cultures (Imbrie-Milligan & Hodges, 1986; Vasil & Vasil, 1986; Vasil & Vasil, 1991). Friable callus is characterized by a spongy, white or yellow-white, translucent, friable appearance and is correlated with a less differentiated callus state (Fransz & Schel, 1991; Vasil & Vasil, 1991). Microscopic examination of a squash preparation of Type II maize callus shows the typical embryogenic cells found in Type I callus (groups of small, isodiametric, thin-walled embryogenic cells with densely stained cytoplasm) dispersed with large, narrow, elongated, highly vacuolated, thick-walled non-embryogenic cells that are devoid of starch (Vasil & Vasil, 1986; Fransz & Schel, 1991; Vasil & Vasil, 1991). The highly vacuolated cells occupy the major part of friable callus, mainly because of their voluminous appearance (Fransz & Schel, 1991). Type II callus forms well-defined globular somatic embryos, with suspensor-like structures, in abundance (Vasil, *et al.*, 1984; Armstrong & Green, 1985; Tomes & Smith, 1985; Vasil & Vasil, 1986; Vasil, 1988) and can be maintained for long periods of time by subculture (Vasil & Vasil, 1991). Friable and embryogenic, but mucilaginous, callus is common in cereal cultures (Vasil & Vasil, 1986; Shillito, *et al.*, 1989). Non-mucilaginous friable callus is homogeneous with no clearly evident embryoids (Shillito, *et al.*, 1989).

Friable callus in cereal cultures was previously associated with non-embryogenic callus, which generates only shoots (Mott & Cure, 1978; Ozias-Akins & Vasil, 1982). However, since the discovery of friable callus with high embryogenic potential from embryo cultures of maize, Type II embryogenic callus became of more interest due to its high regenerative capacity over extended periods of time (Green and Rhodes, 1982).

1.2.2 Explants of maize used for callus initiation

Essentially all plant organs can be used as explant sources for callus initiation. The degree of success with different tissues can, however, vary extensively and calluses with differing morphologies are frequently obtained (Hall, 1991). Success in establishing embryogenic cultures depends on the careful selection of the initial explant based on its developmental stage and physiological condition. Only explants cultured at a specific developmental stage give optimum results (Vasil, 1988). Seeds and young, healthy aseptically-germinated seedlings or greenhouse-grown plants may be used for callus initiation (Hall, 1991). Meristematic regions (immature embryos, young leaf bases and immature inflorescences) have proven to be the most suitable explants with which to initiate embryogenic cultures from monocotyledonous plants (Hall, 1991; Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992). Friable and compact embryogenic callus can be obtained by the culture of immature embryos of many maize genotypes (Chang, 1983; Armstrong & Green, 1985; Duncan, *et al.*, 1985; Hodges, *et al.*, 1986; Prioli & Söndahl, 1989; Fransz & Schel, 1991).

There are indications that, in grasses, only immature tissues proliferate readily in culture, and that plants may only be regenerated when the explants derive from a source close to an embryogenic state, e.g. the scutellum of immature embryos (Wernicke & Milkovits, 1984). In contrast to dicotyledonous species where leaves are the most common source of tissue for *in vitro* culture and genetic manipulation (Gasser & Fraley, 1989), in monocotyledonous species, and particularly in cereals, leaf tissue has proved to have low proliferative and regenerative capacities and is therefore generally not used for genetic manipulation *in vitro* (Barcelo, Lazzeri, Martin & Lörz, 1992).

1.2.3 Applications of maize callus cultures

There is a wide range of applications for callus cultures. They may, for example, be used directly as a source of cell material for biochemical studies or secondary metabolite production (Hall, 1991). Plant regeneration in cereal tissue culture can be obtained from embryogenic callus (Mott & Cure, 1978; Ozias-Akins & Vasil, 1982). Somaclonal variants, often spontaneously produced in tissue cultures, can therefore be obtained (with or without the inclusion of a mutagenesis treatment) (Hall, 1991). Maize callus cultures may also be used in electroporation-mediated or microprojectile bombardment transformation experiments (D'Halluin, *et al.*, 1992). Callus cultures are, however, most commonly used as an intermediate step towards the initiation of cell suspension cultures for the production of plants from protoplasts (Shillito, *et al.*, 1989; Hall, 1991). Type I maize calli have proved to be unsuitable for the establishment of embryogenic suspension cultures, due to their compact nature (Vasil & Vasil, 1986; Vasil & Vasil, 1991). Friable Type II callus is, on the other hand, better suited for the initiation of embryogenic cell suspension cultures (Hall, 1991; Vasil & Vasil, 1991), for the isolation of protoplasts with regenerative capacity (Vasil & Vasil, 1986; Kamo, Chang, Lynn & Hodges, 1987; Rhodes, Lowe & Ruby, 1988a; Rhodes, Pierce, Mettler, Mascarenhas & Detmer, 1988b; Lyznik, Kamo, Grimes, Ryan, Chang & Hodges, 1989a; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989).

1.3 Embryogenic suspension cultures of maize

Cell suspension cultures are populations of single plant cells or small cell clumps cultured in an agitated liquid medium. Regenerable cell suspension cultures of maize are initiated exclusively from embryogenic calli (e.g. friable Type II callus originating from immature embryos) (Imbrie-Milligan & Hodges, 1986; Vasil, 1988; Hall, 1991).

Ideal cell suspensions typically used for protoplast isolation are fine and highly dispersed, consist of large numbers of small, densely cytoplasmic embryogenic cells (single or in small aggregates) (Vasil, 1988; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989; Mórocz, Donn, Németh & Dudits, 1990), are free of callus or organized meristems, and are capable of forming somatic embryos and plants (Vasil, 1988). Maize cell suspension cultures may contain a mixture of non-dividing elongated cells, dividing isodiametric cells, and globular, ovoid, and polar stages of somatic embryos, as well as small callus aggregates (Emons & Kieft, 1991). The formation of these small clusters of cells may be necessary for embryogenesis in maize (Kamo & Hodges, 1986). Visual selection, media manipulations, selective subculturing and filtration, and physical reduction of clump size are important for the development of ideal suspension cultures (Kamo, *et al.*, 1987; Rhodes, *et al.*, 1988a; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989; Sun, Prioli & Söndahl, 1989; Mórocz, *et al.*, 1990), yet only a few have yielded totipotent protoplasts (Prioli & Söndahl, 1989; Shillito, *et al.*, 1989; Mórocz, *et al.*, 1990). Faster-growing suspension cultures generally yielded more protoplasts per gram of suspension culture cells with higher plating efficiencies than slower-growing cultures (Rhodes, *et al.*, 1988a).

Well established cell suspension cultures of gramineous species (especially maize) are still rare and rather difficult to initiate and maintain (Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992; Krautwig, Lazzeri & Lörz, 1994). However, as maize mesophyll protoplasts are not competent for division and regeneration (Stirn, Hopstock & Lörz, 1994), embryogenic cell suspension cultures of maize that are capable of fertile plant regeneration (Rhodes, *et al.*, 1988a; Shillito, *et al.*, 1989; Horn, 1991a; Kyozuka & Shimamoto, 1991) are an excellent source of dividing and totipotent protoplasts for use in single cell manipulations such as gene transfer and somatic hybridization (Imbrie-Milligan & Hodges, 1986; Kamo & Hodges, 1986; Horn, 1991a; Vasil & Vasil, 1991; Taylor, Ko, Fraser, Masel & Adkins,

1994). The lack of common protoplast isolation and culture techniques indicates that the "quality" (genetic background and development) of embryogenic suspension cultures is more important for successful plant regeneration from protoplasts than the specific variables affecting protoplast isolation (Kyozuka & Shimamoto, 1991; Petersen, Sulc & Armstrong, 1992).

Advantages of suspension cultures include rapid culture growth, ease of protoplast preparation compared to intact plant tissues, and the uniform cell size and differentiation state of the suspension cultures compared to callus or plant tissues (Planckaert & Walbot, 1989). Embryogenic suspension cultures, capable of regeneration into plantlets, are the perfect material for freeze preservation and make ideal targets for transformation by microprojectile bombardment and electroporation as they can be returned to liquid for stringent selection (Horn, 1991a). Unfortunately, suspension cultures have been established for very few maize genotypes (Planckaert & Walbot, 1989). Furthermore, embryogenic callus as well as suspensions are labour intensive and highly susceptible to microbial contamination, and lose their morphogenetic potential during long-term culture (Gnanapragasam & Vasil, 1992).

1.4 The isolation of maize protoplasts

1.4.1 Introduction

Plant protoplasts have been in constant use for more than two decades, and have become one of the most versatile analytical tools in plant biology (Negrutiu, 1991). Isolated plant protoplasts are "naked" cells that have had their cell wall removed either by enzymatic digestion or by mechanical action. Plant regeneration via protoplasts is the epitome of plant cell totipotency as, depending upon culture conditions and species, they can be cultured as single cells that produce multicellular

colonies from which plants develop (Songstad, Somers & Griesbach, 1995). The free accessibility of the plasma membrane of protoplasts (Potrykus & Shillito, 1986) and the ability to regenerate plants from cultured protoplasts offers a useful tool for a wide range of genetic manipulations, such as somatic hybridization (Power & Cocking, 1970; Imbrie-Milligan & Hodges, 1986; Prioli & Söndahl, 1989; Krautwig, *et al.*, 1994) or direct gene transfer (Imbrie-Milligan & Hodges, 1986; Krautwig, *et al.*, 1994).

1.4.2 Sources of maize protoplasts

Maize protoplasts can be isolated in large quantities from a variety of plant organs or tissues (Imbrie-Milligan, Kamo & Hodges, 1987; Roest & Gilissen, 1989; Negrutiu, 1991). However, the tissue and genotype of the donor plant from which protoplasts are derived is important in determining whether cultured protoplasts will be able to divide and form callus (Imbrie-Milligan, *et al.*, 1987). In contrast to most dicotyledonous species, totipotent protoplasts of monocotyledonous plants cannot generally be isolated directly from plant tissues (Krautwig, *et al.*, 1994). Mesophyll protoplasts represent one of the tools best suited to genetic engineering of plants due to their morphological and genetic uniformity, their ease of isolation and availability in high numbers, and the relative absence of *in vitro* abnormalities when compared with cultured cells (Ozias-Akins & Lörz, 1984; Wang, Saleem, Fowke & Cutler, 1991). In cereals there is scant evidence that protoplasts isolated from leaves are capable of division (Roest & Gilissen, 1989; Hahne, Lörz & Hahne, 1990) and regeneration (Gupta & Pattanayak, 1993). The most frequently used sources for isolating large numbers of regenerable protoplasts in these species are, therefore, embryogenic cell suspensions (Vasil & Vasil, 1992) with high frequencies of densely cytoplasmic cells in aggregates (Shillito, *et al.*, 1989). Embryogenic callus cultures derived from immature embryos, root (Roest & Gilissen, 1989) and shoot tips, and immature embryos from a number of maize genotypes have also been

used for protoplast isolation (Imbrie-Milligan, *et al.*, 1987).

Cell suspensions are generally a better source of protoplasts than callus for a number of reasons. The faster growth rate of suspension cultures allows more rapid manipulation of material. The composition and thickness of the cell wall may be altered in rapidly dividing cells, and it becomes easier to isolate protoplasts from the resulting cultures. Additionally, as the small cell aggregates are more evenly exposed to the nutrient medium, hormonal gradients are less likely to occur within the cultures and a more homogenous population of protoplasts can be obtained. Rapidly subcultured cell suspensions in the exponential phase of growth usually provide the best source of totipotent protoplasts (Ozias-Akins & Lörz, 1984) for transformation experiments, as the resultant protoplasts are already programmed to divide (Horn, 1991b) and low levels of transcriptional activity exist in stationary phase cells (Fromm, Callis, Taylor & Walbot, 1987).

1.4.3 Methods used for the isolation of maize protoplasts

Prior to the introduction of cell wall-degrading enzymes, plant protoplasts were released by mechanical means. This involved tissue plasmolysis prior to slicing with a sharp blade. This procedure results in some of the tissue cell walls being cut open in a fortuitous orientation that allows plasmolysed protoplasts to escape. Although mechanical methods of protoplast release have been available for a long period of time, these methods have not gained wide acceptance as the protoplast yields have always been very low (Cocking, 1965). These low yields can be greatly improved through the use of a simple electrically-driven tissue slicer. This mechanical release procedure yields useful numbers of protoplasts without exposure to cell wall-degrading enzyme preparations that may have toxic or other adverse effects on the viability of protoplasts (Sun, Furtula & Nothnagel, 1992). This method of protoplast isolation is also useful when working with tissues that will not release protoplasts upon application of

standard cell wall degrading enzyme preparations (Nolte, Nothnagel & Coggins, 1990).

The isolation of plant protoplasts through the use of cell wall degrading enzymes was first reported by Cocking (1960), who used an enzyme mixture containing mostly cellulase to release protoplasts from roots of tomato seedlings. Since that pioneering work, enzyme-based techniques for preparing protoplasts have been developed and refined to enable routine isolation of protoplasts from a wide variety of plant tissues (Sun, *et al.*, 1992). Cell wall digestion enzymes are preparations from fungal mycelia and are not purified proteins. Enzyme extracts may therefore, unfortunately contain substances which inhibit other enzyme activities (Krautwig, *et al.*, 1994) and which can also alter or damage the protoplast (Pilet, 1972; Patnaik, Wilson & Cocking, 1982), leading to decreased viability (Hahne & Lörz, 1988). This viability effect may alternatively be an influence of a long incubation period (Krautwig, *et al.*, 1994).

Laser technology may additionally be used for protoplast isolation. A focused ultra violet light of a nitrogen laser may be used to perforate cell walls. Extrusion of the protoplast or parts thereof is controlled by a regulated decrease in osmolarity of the extracellular medium. Protoplasts have been isolated from various cells in this manner (De Boer, Van Duijn, Giesberg, Wegner, Obermeyer, Köhler & Linz, 1994).

1.4.4 Protoplast viability

The number of viable protoplasts in an isolate has to be determined in order for the protoplasts to be plated at the appropriate density for cell wall regeneration and division (Bengochea & Dodds, 1986). Although a number of methods are available for testing the survival of cells after tissues have been subjected to some form of environmental stress, each method has limitations in its field of application (Gaff & Okong'O-Ogola, 1971). Cytoplasmic streaming (Widholm, 1972), Calcofluor

White (Gahan, 1989), Evan's Blue dye (Gaff & Okong'O-Ogola, 1971; Kanai & Edwards, 1973b; Fromm, Taylor & Walbot, 1985), phenosafranine and fluorescein diacetate (FDA) (Widholm, 1972) can all be used as quick methods for viability determinations (Gahan, 1989). The best criterion for protoplast viability is cell wall resynthesis and subsequent cell division (Fromm, *et al.*, 1987). Calcofluor White is an excellent microfluorimetric stain for plant cell walls and can be used as an indicator of the onset of cell wall biosynthesis by cultured higher plant protoplasts (Nagata & Takebe, 1970; Galbraith, 1981).

1.4.5 The culture of maize protoplasts

Culture of protoplasts of cereals and grasses has proven to be very difficult. However, much progress has been made since the 1970's when sustained cell divisions were obtained in protoplasts isolated from cell suspension cultures (Vasil, 1983). Then, in 1980, it was shown that somatic embryos and plants can be obtained from protoplasts isolated from embryogenic suspension cultures (Vasil & Vasil, 1980). All reported instances of plant regeneration from protoplasts in the Gramineae are based on the use of embryogenic protoplasts from these cell lines (Vasil, 1987; Vasil, 1988; Roest & Gilissen, 1989). Similarly, the recovery of transgenic plants of maize (Rhodes, *et al.*, 1988b), rice (Shimamoto, Terada, Izawa & Fujimoto, 1989), and orchardgrass (Horn, Shillito, Conger & Harms, 1988) is also based on the use of embryogenic protoplasts (Klein, Roth & Fromm, 1980).

Specific culture conditions for the culture of maize protoplasts are varied. To achieve sustained growth of a maize protoplast culture the cells must be inoculated in a medium of correct osmolarity at a minimum plating density (Bengochea & Dodds, 1986). Previously, maize protoplasts were cultured in a thin layer of liquid medium in Petri dishes (Partanen, 1981; Prioli & Söndahl, 1989; Mórocz, *et al.*, 1990) without, or over an agar-solidified medium (Partanen, 1981; Mórocz, *et al.*, 1990), or

embedded in an agar medium. A microculture technique enables the culture of individual protoplasts in microdroplets (Schweiger, Dirk, Koop, Kranz, Neuhaus, Spangenberg & Wolff, 1987). Maize protoplasts may also be cultured in agarose droplets or beads in liquid culture medium (Shillito, Paszkowski & Potrykus, 1983; Roest & Gilissen, 1989). Apart from avoiding protoplast agglutination (which sometimes leads to browning and necrosis of the culture), plating in agarose stimulates colony formation from protoplasts of a wide range of species (Shillito, *et al.*, 1983; David, *et al.*, 1994).

Nurse cultures increase the efficiency of microcallus formation from maize protoplasts (Imbrie-Milligan & Hodges, 1986) and are advantageous for efficient plating of maize protoplasts at high and low densities (Kuang, Shamina & Butenko, 1984; Ludwig, Somers, Petersen, Pohlman, Zarowitz, Gengenbach & Messing, 1985; Kamo, *et al.*, 1987; Somers, Birnberg, Petersen & Brenner, 1987; Rhodes, *et al.*, 1988a; Rhodes, *et al.*, 1988b). The optimum nurse culture needs to be determined empirically for each protoplast source as significant interactions between the sources of protoplasts and feeder-cell lines have been observed (Petersen, *et al.*, 1992). X-irradiated protoplasts may be useful as feeder layers (Roest & Gilissen, 1989).

A solid feeder layer technique has been developed to improve callus formation and subsequent plant regeneration from maize suspension culture-derived protoplasts. This technique is now the preferred method of culture for maize protoplasts (Sun, *et al.*, 1989). In the solid feeder layer technique, protoplasts are plated in a small amount of media onto a cellulose nitrate (Ludwig, *et al.*, 1985; Prioli & Söndahl, 1989; Sun, *et al.*, 1989), Durapore® (polyvinylidene difluoride) (Shillito, *et al.*, 1989) or millipore (Lyznik, *et al.*, 1989a) filter over agarose-solidified media in which suspension feeder cells of an appropriate genotype are embedded. Callus colony formation frequencies increased with the use of this method (Ludwig, *et al.*, 1985; Rhodes, *et al.*, 1988a; Sun, *et al.*, 1989), and were

higher than frequencies obtained for conventional maize protoplast plating methods such as liquid culture or embedding in agarose media (Vasil & Vasil, 1987). Durapore® filters repeatably gave higher colony yields than millipore filters. Conditioning medium from the cells used for protoplast isolation was not found to increase colony formation, or to speed the growth of colonies derived from the protoplasts (Rhodes, *et al.*, 1988a; Shillito, *et al.*, 1989). The enhanced regenerative response of maize protoplasts cultured on filters may have been due to improved gaseous exchange or to a higher protoplast density in the smaller volume. The filter acts as a physical support, a barrier that prevents cross contamination of the cell culture layer from feeder cells and simplifies subculture of protoplast derived callus colonies (Partanen, 1981; Ludwig, *et al.*, 1985; Liu, 1988).

1.4.6 Regeneration of maize protoplasts

A prerequisite for the successful application of protoplast techniques to crop improvement is the regeneration of plants from maize protoplasts (Hall, 1988), which maintain the regeneration capacity of donor cells (Kuang, *et al.*, 1984; Prioli & Söndahl, 1989). Plant regeneration from protoplasts and especially from mesophyll cells has many advantages; these protoplasts constitute a stable material that is composed of cells originating from the same tissue and of the same developmental stage (Siminis, Kanellis & Roubelakis-Angelakis, 1994). Plant regeneration from protoplasts is a complex process which is dependent upon a number of parameters such as genotype (Chourey & Zurawski, 1981; Mórocz, *et al.*, 1990), culture media and age of the source suspension culture (Mórocz, *et al.*, 1990). Treatments such as protoplast fusion and direct DNA-uptake often diminish protoplast viability and reduce plating efficiency, therefore requiring that the basic culture conditions be as close to optimal as possible (Creemers-Molenaar & Van Oort, 1990).

Many important agricultural plant species such as maize exhibit recalcitrance during protoplast culture, i.e. the inability to express regenerating potential at the level of either cell division or morphogenesis *in vitro*. Oxidative stress, induced during protoplast isolation and subsequent culture, may be one of the factors contributing to recalcitrance of maize cultures (Siminis, *et al.*, 1994). The addition of natural or synthetic antioxidants to the protoplast isolation and culture medium significantly increases protoplast stability (Saleem & Cutler, 1987), the frequency of colony formation (Ishii, 1988) or plant regeneration frequency (Krens, Jamar, Rouwendal & Hall, 1990). Recalcitrance may additionally be related to the poor wound response of monocotyledonous plants and the difficulties encountered with their tissue culture (Hahne, Fleck & Hahne, 1989). The use of embryogenic cultures can overcome the recalcitrance of protoplasts derived from monocotyledons (Vasil & Vasil, 1980).

Despite intensive efforts for two decades, culture of mesophyll protoplasts of agriculturally important cereals has proven to be extremely difficult (Hahne, *et al.*, 1989; Wang, *et al.*, 1991). In maize, although there are some reports of regeneration of callus (Deka & Sen, 1976; Potrykus, Harms, Lörz & Thomas, 1977; Potrykus, Harms & Lörz, 1979) and fertile plants from suspension-derived protoplasts (Prioli & Söndahl, 1989; Shillito, *et al.*, 1989; Mórocz, *et al.*, 1990), protoplast regeneration is still not efficient and routine (Krautwig, *et al.*, 1994).

1.4.7 Transgenic protoplasts

As cell wall removal eliminates a major barrier to DNA delivery, protoplasts, in principle, are ideal cells for DNA delivery and selection of transgenic events, (Gad, Rosenberg & Altman, 1990; Songstad, *et al.*, 1995). To obtain transgenic plants through a protoplast system, an efficient transformation technique and effective selection and cell culture conditions are required. The basic advantage of the protoplast system may be that a large

amount of uniformly transformed material can be generated and screening can be done at very early stages to produce non-chimeric cell clones that exhibit proper integration and expression of the introduced genes (Dhir, Dhir, Savka, Belanger, Kriz, Farrand, & Widholm, 1992). High rates of cell regeneration, and subsequent callus formation, from protoplasts are required to enable efficient recovery of plant cell lines transformed by direct DNA uptake (Rhodes, *et al.*, 1988a). However, several economically important crops, including maize, are recalcitrant to regeneration and a high degree of genetic variation, termed somaclonal variation, is observed frequently among regenerants derived from cultured protoplasts (Rhodes, *et al.*, 1988b; Shillito, *et al.*, 1989). Although somaclonal variation might be of interest for crop improvement, it is undesirable for genetic manipulation directed towards the addition of desired genetic characters (Roest & Gilissen, 1989; Vasil, 1990).

1.5 Transformation of maize

1.5.1 Introduction

New insight into plant biology has been provided by the production of transgenic plants, which has become an important tool for the improvement of crop species (Kyozuka & Shimamoto, 1991). The genetic modification of major agronomic crops has undergone a revolution in the past few years. The development of novel transformation technologies and new *in vitro* culture systems has opened the way for the engineering of even the most recalcitrant of crops (Christou & Ford, 1995).

The first demonstration of the transfer of biologically active DNA into a plant was accomplished in *Petunia* through the use of *Agrobacterium tumefaciens* (Draper, Davey, Freeman, Cocking & Cox, 1982; Krens, Molendijk, Wullems & Schilperoort, 1982). *A. tumefaciens* can transfer DNA across the cell wall and into the

plant genome (Tomes, 1990; Larkin, Taylor, Gersmann & Brettell, 1990; Murray, 1991) and has been an extremely useful vector to routinely transfer foreign genes into dicotyledonous plants (Shen, Escudero, Schläppi, Ramos, Hohn & Koukalíková-Nicola, 1993; Smith & Hood, 1995). Unfortunately, *A. tumefaciens* has a limited natural host range primarily restricted to dicotyledonous plants. Major economic plant groups including the cereals, grasses and many legumes remain resistant to *A. tumefaciens*-mediated gene transfer (Larkin, *et al.*, 1990; Kyozuka & Shimamoto, 1991; Murray, 1991; Songstad, *et al.*, 1995) even though there has been some prospect of experimentally extending the range of species amenable to *A. tumefaciens* (Larkin, *et al.*, 1990; Smith & Hood, 1995). The only clearcut evidence for transferred DNA (T-DNA) transport from *A. tumefaciens* to maize comes from agroinfection (Shen, *et al.*, 1993). Efforts have therefore been made to develop direct gene transfer techniques to introduce foreign DNA into plant cells, without the need of an intermediate biological host (Fromm, *et al.*, 1985; Chen, Gartland, Davey, Sotak, Gartland, Mulligan, Power & Cocking, 1987; Fromm, *et al.*, 1987; Davey, *et al.*, 1989; Larkin, *et al.*, 1990; Murray, 1991; Rathus & Birch, 1991). Direct DNA uptake is applicable to both stable and transient gene expression studies and utilizes a range of vectors, including those employed for gene cloning. Unfortunately, the frequency of stable transformation is low (Davey, *et al.*, 1989).

The expression "direct gene transfer" appears to have been first used to refer to the uptake of "naked" (free) DNA into plant protoplasts without the mediation of *A. tumefaciens* or its Ti plasmid (Paszkowski, Shillito, Saul, Mandák, Hohn, Hohn & Potrykus, 1984). Many techniques developed for gene transfer into animal cells have been tested with some success on plant protoplasts (Rathus & Birch, 1991). Direct gene transfer has been achieved following chemical, physical, or electrical treatment of protoplasts, or even cells (Pollard & Walker, 1990). The chemical "helpers", e.g. polyethylene glycol (PEG), are known to act on both the DNA structure and the surface of plant

protoplasts. Chemical mediated procedures include co-cultivation of protoplasts with *A. tumefaciens* bacterial cells (Kosuge, Meredith & Hollaeder, 1983) or plasmid suspensions (Bengochea & Dodds, 1986), calcium phosphate co-precipitation (Bajaj, 1989; Sambrook, Fritsch & Maniatis, 1989; Rathus & Birch, 1991), DEAE-dextran (Goeddel, 1990), PEG induced uptake (Krens, *et al.*, 1982; Old & Primrose, 1985; Bajaj, 1989; Rathus & Birch, 1991), polybrene, poly-L-lysine and poly-L-ornithine complexed genes (Rathus & Birch, 1991), or fusion of plant cells with natural or synthetic liposomes containing DNA (Ahokas, 1987; Bajaj, 1989; Gad, *et al.*, 1990; Goeddel, 1990; Rathus & Birch, 1991). Physical methods of transformation include electric field-mediated DNA transfer (electroporation) (Fromm, *et al.*, 1985; Shillito, Saul, Paszkowski, Müller & Potrykus, 1985; Bajaj, 1989; Davey, *et al.*, 1989; Goeddel, 1990; Rathus & Birch, 1991), microinjection of DNA into nuclei (Reich, Iyer & Miki, 1986; Walker & Gingold, 1988; Davey, *et al.*, 1989; Goeddel, 1990; Neuhaus & Spangenberg, 1990; Rathus & Birch, 1991), macroinjection (De la Pena, Lörz & Schell, 1987), electroporation injection (electroinjection) (Morikawa, Iida, Matsui, Ikegami & Yamada, 1986; Lindsey & Jones, 1990), delivery of DNA to meristems via electrophoresis (Songstad, *et al.*, 1995), and microprojectile bombardment with DNA coated microprojectiles (Klein, Gradziel, Fromm & Sanford, 1988b; McCabe, Swain, Martinell & Christou, 1988; Davey, *et al.*, 1989; Goeddel, 1990; Tomes, 1990). DNA, RNA and gemini viral vectors (Brisson, Paszkowski, Penswick, Gronenborn, Potrykus & Hohn, 1984; Uchimiya, *et al.*, 1989; Pollard & Walker, 1990; Matzeit, Schaefer, Kammann, Schalk, Schell & Gronenborn, 1991) and microlazers are other vehicles being considered in the context of transformation (Pollard & Walker, 1990; Weber, Monajembashi, Wolfrum & Greulich, 1990).

Given the wide range and variability of plant cells, DNA constructs and experimental protocols, a "method of choice" for transformation does not seem to exist. Rather, each technique has its advantages and disadvantages (Songstad, *et al.*, 1995). Successful transformation depends on the optimal interaction and

cooperation between the target cells (accessibility to the DNA and good, stable, regeneration), the introduced nucleic acid (efficient gene constructs and stability during the delivery process), and the delivery procedure (efficient, simple and reproducible) (Gad, *et al.*, 1990). Overcoming the plasma and nuclear membrane barriers (in the case of protoplasts), plus the cell wall barrier (in the case of cells and tissues), is a technical problem common to all transformation methods (Gad, *et al.*, 1990; Rathus & Birch, 1991).

The ideal transformation system should be based on a DNA delivery technology that is efficient, simple and inexpensive. The process should enable efficient, high fidelity integration of transgenic sequence(s) into the recipient plant species genome(s) (nuclear, plastid or mitochondrial). Target cells for DNA delivery should be easy to isolate and exhibit totipotency from a wide range of genotypes within a species. The tissue culture process required for selection of transgenic cultures, and regeneration of plants therefrom, should be minimized to reduce the frequency of deleterious mutants (Songstad, *et al.*, 1995).

Transgenic monocotyledonous plants are desirable to study the expression of regulatory or structural genes derived from these species. In addition to basic studies using transgenic plants, transformation of monocotyledonous plants, particularly those in the Gramineae (the major cereals maize, rice, wheat and barley), is urgently needed for crop improvement (Kyozuka & Shimamoto, 1991). Maize transformation research is progressing rapidly. This progress is in part due to the development of direct DNA-delivery systems that are compatible with tissue culture techniques capable of regenerating fertile plants (Laursen, Krzyzek, Flick, Anderson & Spencer, 1994).

Prior to the development of microprojectile bombardment, maize transformation attempts were focused on direct DNA-delivery to protoplasts (Laursen, *et al.*, 1994). Maize mesophyll and root protoplasts were transformed through the use of polyethylene

glycol, with the introduced DNA being transiently expressed (Junker, Baker & Lörz, 1986; Junker, Zimny, Lührs & Lörz, 1987). The electroporation of maize protoplasts yielded stably transformed cells (Fromm, Taylor & Walbot, 1986) and sterile plants (Rhodes, *et al.*, 1988b). In 1990, microprojectile bombardment was the first direct DNA-delivery technique successfully used to produce fertile maize plants (Fromm, *et al.*, 1990; Gordon-Kamm, Spencer, Mangano, Adams, Daines, Start, O'Brien, Chambers, Adams, Willetts, Rice, Mackey, Krueger, Kausch & Lemaux, 1990; Aves, Genovesi, Willets, Zachweija, Mann, Spencer, Flick & Gordon-Kamm, 1992; Walters, *et al.*, 1992; Koziel, *et al.*, 1993). Since this breakthrough, polyethylene glycol (Omirulleh, Abrahám, Golovkin, Stefanov, Karabaev, Mustárdy, Mórocz & Dudits, 1993), electroporation (Rhodes, *et al.*, 1988b; Murry, *et al.*, 1993; Sukhapinda, Kozuch, Rubin-Wilson, Ainley & Merlo, 1993), electroinjection (D'Halluin, *et al.*, 1992) and microprojectile bombardment (Gordon-Kamm, *et al.* 1990; Koziel, *et al.*, 1993; Murry, *et al.*, 1993) have all been successfully used to obtain transgenic maize plants.

1.5.2 Transformation of maize protoplasts using electroporation

An electric shock method, termed electroporation, was described for the introduction of drugs into mammalian red blood cells (Langridge, Li & Szalay, 1987). As a consequence of this, an electroporation gene-transfer technique was investigated as an alternative strategy for protoplast transformation (Fromm, *et al.*, 1985). Experiments have been described in which *A. tumefaciens* Ti plasmid DNA (Langridge, Li & Szalay, 1986), a chimeric antibiotic resistance marker gene (Fromm, *et al.*, 1985) and plant genomic DNA were successfully introduced into plant protoplasts by electroporation (Langridge, *et al.*, 1987).

Electroporation involves the application of short high-voltage electrical pulses to a solution containing a mixture of protoplasts and foreign DNA. This electrical treatment reversibly

increases the permeability of mammalian, plant and microbial cell membranes, thereby enabling macromolecules such as DNA to enter the protoplasts (Fromm, *et al.*, 1985; Fromm, *et al.*, 1987; Jones, Tempelaar & Jones, 1987; Uchimiya, *et al.*, 1989; Rathus & Birch, 1991; Songstad, *et al.*, 1995) by diffusion (Van Wert & Saunders, 1992b) or an active process, termed electroosmosis (Dimitrov & Sowers, 1990). It has been suggested that pore formation during electroporation is a result of a 90^{o} block rotation of two nearest-neighbour lipids in the cell membrane bilayer (Sugar & Neumann, 1984). The application of electroporation for stable transformation has been extended to a number of plant species (Songstad, *et al.*, 1995) and is applicable to both monocotyledonous and dicotyledonous protoplasts (Fromm, *et al.*, 1985).

Electroporation is a fascinating cell membrane phenomenon with several existing biological applications. DNA introduction is the most common use for electroporation (Weaver, 1993). Electroporation-mediated transient assays allow rapid characterization of many aspects of transcription, RNA processing and translation, and protein stability and activity in several species (Callis, Fromm & Walbot, 1987; Fromm, *et al.*, 1987). Nucleic acids may be introduced by electroporation directly into isolated subcellular organelles such as chloroplasts. Therefore, electroporation may now be applied for the genetic modification of genes required for photosynthesis in economically important crop plants (Langridge, *et al.*, 1987).

Conditions for electroporation must be experimentally optimized for each cell type (Rathus & Birch, 1991) and should take into account the efficiency of uptake of DNA into protoplasts, and their survival rate after electroporation (Kyozuka & Shimamoto, 1991). DNA delivery procedures which result in optimum gene expression may be detrimental to the survival of protoplasts and thereby limit the number of surviving protoplasts available for the assessment of gene integration and heritability (Vasil, Hauptmann, Morrish & Vasil, 1988). Optimization can be

efficiently accomplished through the use of transient expression techniques (Rathus & Birch, 1991; Rathus & Birch, 1992). However, optimal conditions for recovery of stably transformed lines from electroporated protoplasts have sometimes proved to be different from those for optimal transient gene expression (Larkin, *et al.*, 1990).

The primary advantages of electroporation over polyethylene glycol or other chemically-mediated transformation treatments, are high frequencies of DNA transfer and avoidance of toxic chemicals such as PEG. Additionally, the electroporation treatment is reproducible, enables a great degree of control, is simple to apply and can be employed with a wide range of plant protoplasts (Fromm, *et al.*, 1985; Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Rathus & Birch, 1991). Transformation of protoplasts by electroporation permits the selection of single transformed cells. Regeneration via somatic embryogenesis will consequently result in transgenic embryos or plantlets derived from single cells. Therefore, large numbers of independently transformed plants can be obtained from a single protoplast electroporation experiment (Langridge, *et al.*, 1986). Electroporation of protoplasts, however, suffers from the drawbacks and limitations inherent with protoplast cultures (Songstad, *et al.*, 1995). Plasmalemma integrity is temporarily destroyed and thus electroporation often causes cytotoxicity (Fromm, *et al.*, 1986) and pronounced cell loss (Antonelli & Stadler, 1990). Electroporation of maize protoplasts leads to a decline in their viability (Kamo, *et al.*, 1987).

The uptake and expression of DNA in protoplasts from both monocotyledons (maize) and dicotyledons (carrots and tobacco) was shown after electroporation treatment (Table 1.1) (Fromm, *et al.*, 1985). Subsequent work provided transformed rice (fertile) (Shimamoto, *et al.*, 1989) and maize (sterile) (Rhodes, *et al.*, 1988b) plants through electroporation of protoplasts. Fertile transgenic maize plants expressing neomycin phosphotransferase (NPT II) (Rhodes, *et al.*, 1988b) and a maize dwarf virus coat

protein (Murry, *et al.*, 1993) were subsequently obtained.

Table 1.1 Examples of published work on transient and stable expression of DNA in electroporated maize protoplasts.

Expression at the protoplast, cell or plant level		References
Transient expression	Stable expression[1]	
Protoplasts		Fromm, *et al.*, 1985; Callis, *et al.*, 1987; Howard, Walker, Dennis & Peacock, 1987; Planckaert & Walbot, 1989
Cells (from protoplasts derived from suspension culture)		Kamo, *et al.*, 1987
	Cells (from protoplasts derived from suspension culture)	Fromm, *et al.*, 1986; Huang & Dennis, 1989; Lyznik, *et al.*, 1989a
	Plants (from protoplasts derived from suspension culture)	Rhodes, *et al.*, 1988b; Murry, *et al.*, 1993
	Haploid plants (from protoplasts derived from suspension cultures derived from microspore-derived callus)	Sukhapinda, *et al.*, 1993

[1]Stable transformation refers to integration of foreign DNA into the nuclear genome.

Although most transformation studies have used isolated protoplasts, genetic material has been electroporated into whole or complete plant cells. Reports have demonstrated that it is possible to electroporate DNA through thin plant cell walls (Van Wert & Saunders, 1992b). For example, the uptake and expression of DNA by intact leaf segments of rice, wheat, maize and barley has been reported (Dekeyser, Claes, De Rycke, Habets, Van Montagu

& Caplan, 1990).

1.5.3 Electroporation of intact maize tissues

Due to the problems associated with electroporation of protoplasts, electroporation-mediated DNA delivery has been extended to intact plant tissues. For example, the electroporation of intact immature embryos is a breakthrough that is both easier and less genotype-dependent than other methods of obtaining fertile transgenic maize plants (Songstad, *et al.*, 1995). It is clearly advantageous to use morphogenetic cell aggregates rather than protoplasts for those species, varieties and/or cultivars (particularly monocotyledons), where plant regeneration from protoplasts is difficult or yet to be achieved. The electroporation of tissues, of small cell number and high morphogenetic capacity such as proembryonic structures, may become a feasible alternative to the electroporation of protoplasts (Lindsey & Jones, 1990). Cell suspension cultures which can be induced to regenerate plants via somatic embryogenesis are therefore ideal targets. Cell walls which are not permeable enough for electroinjection may be partially digested with cellulase before application of the electric pulse (Jain, Newton & Tuleen, 1988).

The plant cell wall is a barrier to DNA uptake by intact cells. However, under certain circumstances, expression of DNA electroporated into intact cells can be demonstrated. The plasma membrane of intact or partially digested cells within cell aggregates can be reversibly permeabilized by electroporation. Stably transformed cell lines can thus be produced. This transformation technique is referred to as electroinjection. The level of expression obtained by electroinjection is, however, lower than that obtained by electroporation of protoplasts (Lindsey & Jones, 1990). Additionally, leakage of nucleic acids and metabolites from the cell membrane pores may result, as has been reported in protoplast electroporation experiments (Jain, *et al.*, 1988). Since organised tissues are used as the target for

transformation, there exists the possibility that selected tissues are chimeric and consequently give rise to chimeric transformed plants (Sukhapinda, *et al.*, 1993). Once again, therefore, electroporation and regeneration conditions have to optimized for the specific experimental system (Lindsey & Jones, 1990).

The wide range of cells that can express DNA after electroporation, and the observation that one electrical pulse can introduce nucleic acids simultaneously into more than six cell layers, has lead to the conclusion that the potential of electroinjection might match the potential of microprojectile bombardment. Electroinjection, despite utilizing more DNA than microprojectile bombardment, can be performed with a simple electroporation apparatus, has a high penetration power into adjacent cell layers, and DNA-binding tungsten particles do not need to be constructed (Dekeyser, *et al.*, 1990). In addition, electroinjection offers a means to deliver DNA to plant organs that may be sensitive to damage associated with biolistics (including heavy metal toxicity) and offers an alternative to the expense of microprojectile bombardment expendables (e.g. gold and tungsten microprojectiles) (Songstad, Halaka, DeBoer, Armstrong, Hinchee, Ford-Santino, Brown, Fromm & Horsch, 1993).

Partial enzymatic digestion of sugarbeet suspension cell walls with pectin-digesting enzymes permitted plasmid DNA introduction by electroporation (Lindsey & Jones, 1987; Lindsey & Jones, 1990). Subsequently, transient expression of NPT II reporter genes was achieved in electroporated leaf bases of several monocotyledonous species including maize, without the need for cell wall-degrading enzymes (Dekeyser, *et al.*, 1990). Stable transformation of maize via electroporation (with NPT II reporter gene constructs) of enzymatically treated immature embryos, mechanically wounded Type I callus (D'Halluin, *et al.*, 1992) and pectolyase treated suspension cultures (Laursen, *et al.*, 1994) have been reported (Table 1.2). Most importantly, the transformation of maize tissue by electroinjection does not

require the establishment of genotype-dependent embryogenic Type II callus or cell suspension cultures and facilitates the engineering of new traits into agronomically relevant maize inbreds. Additionally, the electroinjection technique only requires a short tissue culture period (D'Halluin, *et al.*, 1992), thus diminishing the chance of phenotypic abnormalities and reduced fertility that are often encountered in transformation experiments requiring long tissue culture periods (Fromm, *et al.*, 1990; Gordon-Kamm, *et al.*, 1990; Walters, *et al.*, 1992).

Table 1.2 Examples of published work on transient and stable expression of DNA in electroinjected maize tissues.

Expression at the protoplast, cell or plant level		References
Transient expression	Stable expression[1]	
Leaf bases		Dekeyser, *et al.*, 1990
	Plants (from suspension culture)	Laursen, *et al.*, 1994
	Plants (from immature embryos and callus)	D'Halluin, *et al.*, 1992

[1]Stable transformation refers to integration of foreign DNA into the nuclear genome.

1.5.4 Transformation of maize via microprojectile bombardment

Key steps to develop a gene transfer technique were taken by researchers at Cornell University who developed a range of devices to accelerate tungsten microprojectile to velocities sufficient to penetrate onion epidermal cells (Sanford, Klein, Wold & Allen, 1987). It was therefore recognized that particle bombardment (dubbed the "biolistic" (biological ballistics) process by its inventors) (Sanford, 1988) could be a nearly universal mechanism for transporting substances such as biological stains, synthetic macromolecules, proteins (enzymes or antibodies) and genetic material into any living cell, with particular significance for genetic transformation (Sanford, *et*

al., 1987).

Microprojectile bombardment is a technique whereby DNA is delivered into cells of intact plant organs or cultured tissues via a particle bombardment process. Small DNA-coated, high density particles (tungsten or gold microprojectiles) are accelerated to high velocities by a particle gun apparatus. These dense particles thus acquire sufficient kinetic energy to penetrate living plant cells and membranes and carry foreign DNA into the interior of bombarded cells (Klein, Wolf, Wu & Sanford, 1987; Uchimiya, *et al.*, 1989; Klein, Goff, Roth & Fromm, 1990; Birch & Bower, 1994; Fromm, 1994) which survive to express and sometimes incorporate the introduced genes (Birch & Bower, 1994). This method of DNA delivery is considered crude, but effective (Franks & Birch, 1991), is suitable for a wide range of dicotyledonous and monocotyledonous species (Songstad, *et al.*, 1995), is unlimited by the *A. tumefaciens* host range (Larkin, *et al.*, 1990) and circumvents the use of protoplasts in transient assay systems (Klein, Knowlton & Arentzen, 1991). Microprojectile bombardment can introduce genes into a wide range of tissues including suspension cultures (Klein, *et al.*, 1990; Taylor, Vasil & Vasil, 1993), callus cultures (Klein, *et al.*, 1990; Wan, Widholm & Lemaux, 1995), tissues isolated directly from plants or even tissues of whole seedlings (Klein, *et al.*, 1990).

A number of advantages make microprojectile bombardment the method of choice for engineering major agronomic crops (Christou & Ford, 1995). Firstly, transformation of organized and potentially regenerable tissue is possible therefore permitting the introduction of foreign genes into elite germplasm (Klein, *et al.*, 1980; Christou & Ford, 1995) by a less genotype-dependent maize transformation procedure (Wan, *et al.*, 1995). Transient gene expression following bombardment has been demonstrated in numerous tissues representing many different species. Therefore, microprojectile bombardment enables the transformation of recalcitrant species and allows for the examination of basic plant developmental processes (Christou & Ford, 1995).

Microprojectile bombardment is the only procedure capable of delivering DNA into cells in virtually any tissue of any organism. This technique is therefore widely used for transformation of species that are not susceptible to *A. tumefaciens*-infection or not amenable to protoplast culture subsequent to electroporation (Songstad, *et al.*, 1995).

Any microprojectile bombardment system needs to be tailored to suit a particular species and tissue target. Firstly, it is important to achieve maximal DNA delivery rates to cells, and secondly to consider the best possible strategy for generating stable transformants (Franks & Birch, 1991). The frequency of transformation by microprojectiles is comparable to that observed for other methods used for the transformation of plant protoplasts such as electroporation or PEG-mediated delivery (Klein, Kornstein, Sanford & Fromm, 1989a).

Microprojectile bombardment has a number of applications. It may be used for efficient inoculation with infectious nucleic acids and studies of gene regulation based on transient expression of introduced DNA in target cells (Birch & Bower, 1994). This application is valuable for the study of how gene expression is controlled by environmental (Bruce, Christensen, Klein, Fromm & Quail, 1989; Ludwig, Bowen, Beach & Wessler, 1990), tissue specific (Twell, Klein, Fromm & McCormick, 1990; Klein, Roth & Fromm, 1989b), developmental stage (Franks & Birch, 1991) and genetic factors (Klein, *et al.*, 1989b). Particle bombardment may also be used for: cell lineage analysis using chimeric transformants expressing visible marker genes; reproducible transformation of cellular organelles (Birch & Bower, 1994) (e.g. for engineering organelle encoded herbicide resistances into crop plants; for the study of basic processes such as photosynthesis) (Sanford, 1988; Daniell, Vivekananda, Nielsen, Ye, Tewari & Sanford, 1990); and regeneration of transgenic organisms expressing useful new genes, following the selection of stably transformed target cells (Birch & Bower, 1994).

Several plant species have been transformed by particle bombardment (Tomes, 1990), which has been shown to deliver DNA and result in the transient expression (Table 1.3) of chloramphenicol acetyltransferase (CAT) (Klein, Fromm, Weissinger, Tomes, Schaaf, Sletten & Sanford, 1988a), and β-glucuronidase (Oard, Paige, Simmonds & Gradziel, 1990) genes in cultured maize cells (Reggiardo, Arana, Orsaria, Permingeat, Spitteler & Vallejos, 1991). Furthermore, stable transformants (Klein, *et al.*, 1989a; Spencer, *et al.*, 1990) and fertile transgenic maize plants (Fromm, *et al.*, 1990; Gordon-Kamm, *et al.*, 1990), expressing resistance to either chlorsulfuron or phosphinothricin, have been produced by this method (Klein, *et al.*, 1989a; Fromm, *et al.*, 1990; Spencer, *et al.*, 1990; Gordon-Kamm, *et al.*, 1990). Stably transformed maize plants expressing an insecticidal protein from *B. thuringiensis*, conferring resistance to European corn borer, have additionally been produced by particle bombardment (Koziel, *et al.*, 1993).

Beyond the need for improved tissue culture systems for efficient regeneration of transgenic organisms from transformed cells, there are some important limitations on particle bombardment for gene transfer. A major technical limitation to microprojectile-mediated gene transfer is the inconsistency of results between successive, replicated treatments, even though results are repeatable (Franks & Birch, 1991). This experimental variability will be overcome through experimental design. A second limitation is legal, regarding various patents (Birch & Bower, 1994). Additionally, a high proportion of transformed regenerants is, unfortunately, likely to be chimeric when using meristematic target tissue. As direct selection (on medium supplemented with antibiotics or herbicides) would be lethal to many chimeric plants, numerous regenerating plants must be screened, using an assay for reporter gene expression (Franks & Birch, 1991). By contrast, regeneration from bombarded dedifferentiated cells permits early selection for reporter genes and increases the possibility of uniformly transformed plants as observed with maize (Gordon-Kamm, *et al.*, 1990). However, depending upon the

selectable marker gene and the plant species used, cross-protection between cells may still result in chimeric primary regenerants (Franks & Birch, 1991).

Table 1.3 Examples of published work on transient and stable expression of microprojectile-delivered DNA in maize tissues.

Expression at the protoplast, cell or plant level		References
Transient expression	Stable expression[1]	
Aleurone, embryos		Klein, *et al.*, 1988b; Klein, *et al.*, 1989b; Goff, Klein, Roth, Fromm, Cone, Radicella & Chandler, 1990
Callus		Goff, *et al.*, 1990
Seedlings		Ludwig, *et al.*, 1990
Suspension cell culture		Klein, *et al.*, 1988a; Klein, *et al.*, 1988b; Oard, *et al.*, 1990; Finer, Vain, Jones & McMullen, 1992; Taylor, *et al.*, 1993
	Callus (from suspension culture)	Klein, *et al.*, 1989a; Spencer, *et al.*, 1990
	Plants (from Type II callus)	Aves, *et al.*, 1992; Walters, *et al.*, 1992
	Plants (from suspension culture from highly embryogenic, friable Type II callus)	Fromm, *et al.*, 1990; Gordon-Kamm, *et al.*, 1990; Murry, *et al.*, 1993; Vain, McMullen & Finer, 1993b
	Plants (from embryos)	Koziel, *et al.*, 1993

[1]Stable transformation refers to integration of foreign DNA into the nuclear genome.

1.5.5 Patterns of integration and expression of exogenous DNA in recipient cells

The incorporation of foreign DNA by direct gene transfer requires many different steps. The DNA must pass the cell membrane, be transferred to the nucleus, pass the nuclear membrane, be integrated into the chromosomal DNA and finally, be expressed (Benediktsson, Köhler & Schieder, 1990). In tobacco, transient expression of a transferred gene is usually detected between 1 to 96 h after transformation (Pröls, Töpfer, Schell & Steinbiβ, 1988), with the peak usually occurring between 24 and 48 h after gene transfer (Fromm, *et al.*, 1985). This expression is dependent on the plasmid, marker gene and plant material used (Pröls, *et al.*, 1988).

A number of criteria must be fulfilled in order to verify the transgenic nature of a plant. Firstly, the marker gene used to select or identify transgenic plants has to be biochemically assayed. Secondly, the marker gene (and the gene of interest) have to be physically detected in the genome of the plant by Southern blotting. In addition, it has to be shown that the foreign DNA is covalently linked to the nuclear DNA of the recipient. Finally, the gene has to be inherited in Mendelian fashion by the progeny of the primary transformant. There is a growing awareness that these tests are crucial in accepting a plant as being transgenic (Heberle-Bors, Moreno, Alwen, Stöger & Vicente, 1990). In cereals in particular, it appears that foreign DNA can be detected in the primary transformants, but fails to be detected in the next generation (Gordon-Kamm, *et al.*, 1990; Heberle-Bors, *et al.*, 1990).

In regenerated transformants, integration patterns vary markedly from one individual to the next. Most transformed plant tissues may contain one or numerous intact copies of the marker gene inserted at a single site or multiple sites within the genome. Multiple rearranged forms of the introduced DNA at various sites in the genome may also occur (Klein, Harper, Svab, Sanford, Fromm

& Malinga, 1988c; Klein, *et al.*, 1989a; Gordon-Kamm, *et al.*, 1990). A transgenic expression cassette is more likely to be rearranged if expression of that gene is not selected for during callus growth (Register, Peterson, Bell, Bullock, Evans, Frame, Greenland, Higgs, Jepson, Jiao, Lewnau, Sillick & Wilson, 1994). If, after repeated subculture, integration patterns remain unchanged, it can be inferred that no genomic rearrangements have occurred subsequent to transformation (Gordon-Kamm, *et al.*, 1990). This implies that the concatenation and rearrangement of foreign DNA occur prior to integration (Franks & Birch, 1991).

1.5.6 Co-transformation using exogenous DNA

Improvement of the agronomic value of major crops such as maize is likely to involve the introduction of multiple genes, many of which will not provide selectable or directly screenable phenotypes among the initial products of transformation (Christou & Swain, 1990). Co-transformation involves the simultaneous uptake of two different plasmids, one with a non-selectable gene of interest and the other with a selectable marker (as contrasted to transformation of two linked genes on the same plasmid). This technique is an important application for genetic engineering of plant cells (Schocher, Shillito, Saul, Paszkowski & Potrykus, 1986; Lyznik, Ryan, Ritchie & Hodges, 1989b; Dhir, *et al.*, 1992) as multiple genes can thus be introduced into cells. Attempts to over-express plant genes by the introduction of additional copies have, in several cases, resulted in a dramatic co-ordinate suppression of the exogenous transgene and its endogenous homologue (Jorgensen, 1990; Napoli, Lemieux & Jorgensen, 1990). This phenomenon, termed antisense, is not clearly understood, but could clearly be important in plant genetic manipulation work involving multiple gene copies (Franks & Birch, 1991).

1.5.7 Biochemical assays for determination of transformation

The two types of biochemical assays used in transformation studies are transient and stable assays. After gene transfer, the majority of introduced plasmid DNA persists as free plasmid DNA in the nucleus for a period of c.a. 7 d. During its transient existence in the cell, the introduced DNA is transcribed into mRNA in the nucleus and, given the appropriate regulatory signals, is translated into protein in the cytoplasm. Transient gene expression is very useful to measure gene transfer efficiency as results can be obtained in 2 d and the efficiency can be quantified (Fromm, 1994). A prime requisite for using transient assays of gene expression is that the cell type of interest be competent to take up exogenous DNA (Klein, *et al.*, 1990). The transient expression of introduced genes is monitored by measuring the production of RNA or protein. Enzymatic activity is a convenient and sensitive measure (Fromm, *et al.*, 1987). Furthermore, the actual cells expressing the introduced DNA can be visualized with e.g. the β-glucuronidase (GUS) (Jefferson, Kavanagh & Bevan, 1987) or anthocyanin genes (Goff, *et al.*, 1990). A reliable transient expression system is therefore immediately applicable for studies of foreign promoter strength (Rathus & Birch, 1992).

In a small percentage of transiently expressing cells, the exogenous plasmid DNA will integrate into the chromosomes (Franks & Birch, 1991; Fromm, 1994). If these cells continue to divide, they grow into stably transformed calli containing the exogenous plasmid DNA. While transiently expressing cells are a good measure of the efficiency of gene transfer, they are not entirely predictive of stable transformation frequencies between different cultures. However, higher transient frequencies in a particular culture seem to correlate with better stable transformation frequencies in that culture system (Fromm, 1994).

Genes whose products are conveniently assayed are termed reporter genes as they are used to demonstrate expression of introduced genes. Reporter genes must have the following properties: stability of the expressed protein both *in vivo* and *in vitro* under a range of conditions; low background from endogenous enzyme activity; and a sensitive, quick, simple, clean and quantifiable assay (Rathus & Birch, 1991). Possibly the most versatile of the commonly used reporter genes is the GUS gene isolated from *Escherichia coli* (Jefferson, *et al.*, 1987). Anthocyanin reporter genes have also proved particularly useful (Fromm, 1994). Luciferase reporter genes allow sensitive non-toxic *in situ* assays, but require very expensive low-light camera equipment (Planckaert & Walbot, 1989; Rathus & Birch, 1991). Other assayable gene products include chloramphenicol acetyltransferase (CAT), β-galactosidase, neomycin phosphotransferase II (NPT II), and hygromycin phosphotransferase (Fromm, *et al.*, 1987).

Due to the relatively low transformation rates in most plant species, gene transfer experiments are usually performed with selectable markers such as antibiotic resistance genes (Benediktsson, *et al.*, 1990). There are a number of selectable markers available. An ideal selectable marker allows transgenic maize cells to grow rapidly in the presence of a metabolic inhibitor which prevents the growth of all non-transgenic neighbouring cells. The more efficient this process, the quicker the growth of the transgenic calli can be observed (Fromm, 1994). Unfortunately, grass species tend to have high natural resistance to aminoglycoside antibiotics, such as kanamycin, which are commonly used for the selection of transformed plants (Hauptmann, Vasil, Ozias-Akins, Tabaeizadeh, Rogers, Fraley, Horsch & Vasil, 1988). The various selectable markers used successfully in monocotyledons are genes encoding the NPT II, hygromycin phosphotransferase, acetolactate synthase (ALS), and phosphinothricin acetyltransferase (PAT) activities (Fromm, 1994).

1.6 Plant regeneration from maize tissue cultures

1.6.1 Introduction

An integral part of plant biotechnology research for crop improvement is the *in vitro* regeneration of mature, fertile and genetically stable plants. This is accomplished via somatic embryogenesis or organogenesis (Kamo, Becwar & Hodges, 1985; Malik & Saxena, 1992). Somatic embryos, resembling zygotic embryos, can be induced to form in cultured plant tissues from embryonic cells (Primrose, 1991). These embryoids can develop into fully functional plants without the need to induce shoot and root formation on artificial media (Bengochea & Dodds, 1986). Regeneration of monocotyledonous plants has been shown to be predominantly by the process of somatic embryogenesis (Vasil, 1987). The sequential formation of roots and shoots on callus is known as indirect organogenesis. The cultural conditions required to achieve organogenesis vary from species to species, and have not been determined for every type of callus (Primrose, 1991). An elevation in the level of cytokinin in callus culture medium induces shoot formation, while auxin increases promote root formation. During organogenesis, therefore, manipulation of hormonal factors stimulates plantlet formation by the development of adventitious roots on the shoot buds that have formed. The plant tissue used, regeneration medium and environmental factors all contribute to the induction of plant regeneration (Bengochea & Dodds, 1986).

Detailed morphological observations by Komamine and co-workers (1990) revealed that four phases (Phase 0, 1, 2 and 3) were recognised in the early stages of embryogenesis. In Phase 0, competent single cells (termed State 0 cells, which are small, isodiametric and densely cytoplasmic form embryogenic cell clusters (State 1) in the presence of auxin. During this phase, the cell clusters arising from single cells gain the ability to develop into embryos when auxin is removed from the medium. These

cell aggregates are termed State 1 cell clusters. Phase 1 is induced by transfer of State 1 cell clusters to auxin-free medium. During this phase, cell clusters proliferate slowly and apparently an undifferentiated manner. After Phase 1, rapid cell division occurs in certain parts of the cell clusters, leading to the formation of globular embryos. This phase is designated Phase 2. In the following phase (Phase 3) plantlets develop from globular embryos via heart and torpedo shaped embryos. Besides phytohormones, cell-to-cell interaction plays a role in somatic embryogenesis (Komamine, Matsumoto, Tsukahara, Fujiwara, Kawahara, Ito, Smith, Nomuro and Fujimura, 1990). A rather high cell density (10^5 cells mL^{-1}) is required for the formation of embryogenic cell clusters from single cells (Nomura & Komamine, 1986), whereas a lower cell density (2×10^4 cells mL^{-1}) favours the development of embryos from embryogenic cells (Fujimura & Komamine, 1979).

1.6.2 Genetics of maize regeneration

Plant regeneration by somatic embryogenesis has been reported in tissue cultures of most cereal and grass species (Gnanapragasam & Vasil, 1992), including maize (Vasil & Vasil, 1986). In some crops, including maize (Green & Phillips, 1975), alfalfa (Keyes & Bingham, 1979), oats (Cummings, Green & Stuthman, 1976), barley (Hanzel, Miller, Brinkman & Fendos, 1985) and petunia (Izhar & Power, 1977), regeneration from callus has been found to be genotype specific. Commercially important inbreds and hybrids from several different maize groups are capable of being regenerated via somatic embryos (Hodges, *et al.*, 1986). For example, the inbred A188 (Minnesota Crop Improvement Association) (Kamo, *et al.*, 1985) has a high frequency of plant regeneration and when crossed into recalcitrant inbreds the resulting F1 hybrids are regenerable. It is thought that nuclear genes exhibiting dominance (or possibly incomplete dominance) are important in the formation of somatic embryos and regeneration of maize plants (Hodges, *et al.*, 1986). Further studies on the genetic control of somatic embryogenesis are still needed to

better understand and manipulate this trait (Prioli, da Silva & Söndahl, 1990). Therefore, through backcrossing with recalcitrant commercial inbreds (combined with tissue culture selection in each generation), hybrids of agronomic importance and tissue culture properties (e.g. competence for genetic transformation) can be developed (D'Halluin, *et al.*, 1992). For example, a key to obtaining maize protoplasts that are capable of rapidly forming an embryogenic callus may be a combination of the cell-division traits of one genotype (e.g. Black Mexican Sweet) with the somatic-embryo formation traits of callus from another genotype (e.g. A188) into an F1 hybrid (Kamo, *et al.*, 1987). These genotypes would not necessarily be genotypes with good field characteristics; however, through backcrossing the agronomical characteristics of the hybrid can be improved (D'Halluin, *et al.*, 1992).

Somaclonal variation has been observed in progeny of maize plants regenerated from callus. Since the donor plants were highly inbred, they were considered homozygous inbreds. Variants observed include an apparent nuclear male sterility, alterations for plant morphology (brachytic, erect leaves, and wrinkled leaves), chlorophyll deficiency (albino and striped), and changes in the endosperm colour (light yellow and lemon), in some cases associated with chlorophyll deficiency. Additionally, alteration of one band of zein polypeptide and a cytoplasmic male sterile mutant have been observed. Abnormalities noticed among plants regenerated from suspension cultures and protoplasts of maize include the absence of tassels and/or ears, terminal ears, development of female flowers in the tassel, and short internodes. Heterosis observed among several hybrids obtained in crosses between somaclones and the donor inbred, suggests the possibility of exploring somaclonal variation to obtain sister lines for hybrid production. Although the heterosis in these hybrids was obvious, further studies are necessary to analyze this phenomenon and its possible agronomic uses. The genetic variation observed among progenies of regenerated plants substantiates the usefulness of somaclonal variation in rapidly

providing small and stable changes in maize inbreds (Prioli, *et al.*, 1990).

Demethylation and methylation occurs at a high frequency during regeneration and could be an important cause of tissue culture-induced variation (Phillips, Somers & Hibberd, 1988). Occurrence of the frequent homozygous alterations in original regenerated plants implies a non-random mutational mechanism (Kaeppler & Phillips, 1993). The mutagenic nature of tissue culture in maize is manifested as qualitative mutations (Armstrong & Phillips, 1988; Zehr, Williams, Duncan & Widholm, 1987), quantitative trait variation (Zehr, *et al.*, 1987), cytological abnormalities usually resulting from chromosome breakage (Armstrong & Phillips, 1988), and the activation of transposable elements (Peschke, Phillips & Gengenbach, 1987). Methylation changes might effect variation in several ways, including chromatic structural changes, and changes in gene expression (Kaeppler & Phillips, 1993).

1.6.3 Regeneration and field testing of transgenic maize plants

In the past 15 to 20 years considerable progress has been made in plant regeneration from tissues, cells, and protoplasts of various Gramineae species. The regeneration of plants from tissue cultures of maize was first reported in 1975, utilizing immature embryos as the tissue source (Green & Phillips, 1975). It was demonstrated that plant regeneration occurred by organogenesis (Springer, Green & Kohn, 1979). Subsequently, regeneration of maize was reported via organogenesis in callus derived from a variety of explants, such as leaf bases (Chang, 1983) and mesocotyls (Harms, *et al.*, 1976; Torné, Santos, Pons & Blanco, 1980). These explants do not efficiently produce plants, whereas immature embryos produce numerous plants from organogenic and embryogenic callus (Kamo, *et al.*, 1985). It was consequently found that plant regeneration in maize could also occur by somatic embryogenesis (Lu, *et al.*, 1982). Both types of regeneration arise from hard, irregularly shaped, nodular, white

or yellow callus that appears distinctly different from the soft, granular, grey/yellow and translucent callus incapable of plant regeneration (Duncan, *et al.*, 1985). Consequently, visual selection of regeneration-competent calli has become the method of choice for the detection and maintenance of plant regeneration capability in maize tissue cultures (Torné, *et al.*, 1980; Lu, *et al.*, 1982; Lu, *et al.*, 1983).

To avoid any kind of risk, certain guidelines and regulations have been proposed concerning the performance of research with recombinant DNA technology. Field tests must be conducted on transgenic maize before it can be released to the grower. To evaluate transgenic plants in field trials, provision for a containment facility is essential. For field testing of genetically engineered plants prior approval of the concerned government is essential (Uchimiya, *et al.*, 1989).

In conclusion, it is evident that the successful regeneration of transgenic maize plants has enormous potential for crop improvement. Field tests have been performed on both elite transgenic maize plants, and hybrids resulting from crosses of these transgenic inbreds with commercial inbreds. Characteristics introduced into transgenic maize plants have been successfully inherited by subsequent generations (Fromm, *et al.*, 1990; Gordon-Kamm, *et al.*, 1990; Walters, *et al.*, 1992; Koziel, *et al.*, 1993). Therefore, through supplementation of traditional plant breeding programmes, transformation and regeneration of maize plants by biotechnological means could have an enormous impact on the improvement of this important cereal crop.

CHAPTER 2
THE INITIATION OF MAIZE CALLUS CULTURES

2.1 Introduction

A callus culture is a continually-dividing mass of generally poorly differentiated and disorganised plant cell aggregates (Hall, 1991). Success in establishing embryogenic callus cultures (Type I or II) of maize depends on the careful selection of the initial explant based on its genotype (Chourey & Zurawski, 1981), developmental stage and physiological condition (Vasil, 1988). It is therefore important to screen a wide range of genotypes to select promising ones which will perform well under tissue culture conditions (Chourey & Zurawski, 1981).

2.1.1 Plant tissue culture media

A significant factor in cell, tissue or organ culture is the choice of nutritional components and growth regulators. In the past two to three decades, a large number of reports have appeared on modifications of approximately two dozen basic components. Literature reports on particular media have given some reproducible, as well as non-reproducible, results. An appreciation and knowledge of the nutritional needs and metabolic requirements of cultured cells and tissues is invaluable in deciding on the type of media to use and its preparation (Gamborg, 1991). The choice of a suitable medium is therefore dictated by the species or type of plant and the purpose of the tissue culture which will be employed (Kyle, 1987).

Nutrient media for tissue culture must contain the inorganic salts required by any growing plant, a carbon or energy source, growth regulators and vitamins. Other components which may be added for specific purposes include organic nitrogen compounds, tricarboxylic acid compounds and plant extracts (Gamborg, 1991).

The Murashige-Skoog (MS) (Murashige & Skoog, 1962) or Linsmaier and Skoog (LS) (Linsmaier & Skoog, 1965) salt compositions are the most widely used, especially in plant regeneration procedures. The N6 medium and its derivatives have also had wide applications for different plant species and culture objectives (Gamborg, 1991).

The four classes of compounds with growth regulator activity are auxins, cytokinins, gibberellins and abscisic acid. Auxins are required in cultured tissues for the induction of cell division. Some auxins may be used for root formation and are often used in combination with cytokinins, which have an essential role in differentiation and regeneration of most plant species (Gamborg, 1991). 2,4-Dichlorophenoxyacetic (2,4-D) acid, the most commonly used auxin in maize tissue culture (Close & Ludeman, 1987), must be maintained at high levels to preserve the embryogenic nature of cultures (Vasil, 1988). The addition of amino acids may enhance cell growth and facilitate differentiation towards plant regeneration. Complex organic additives (protein hydrolyzates; coconut milk) (Gamborg, 1991), yeast extract and malt extract, have been used in tissue culture media to increase growth rates (Green, Phillips & Kleese, 1974).

2.1.2 Greenhouse and *in vitro* cultivated plant material for maize callus initiation

Maize can be cultivated in the greenhouse by following good cultural practices that provide conditions falling within the crop's natural requirements. With careful management, moderately vigorous maize plants can be grown in pots. Consideration should be given to soil fertility, pH, lighting and watering. Soil fertility can be maintained throughout the growing season by "time-release" fertilizers and spot fertilization. Generally, a lowering of soil pH is required, with iron sulphate being a good acidifier (Neuffer, 1994).

Greenhouse-grown plant material for callus initiation must be entirely free from infection (including viruses), devoid of any signs of insect attack (which can lead to the internal contamination of the explant material) and be in a well maintained state. Unlike *in vitro* grown plants, for which constant conditions are maintained, the physiological state of greenhouse-grown plants can, without care, vary considerably and this can have a profound influence upon the response of cultured explants (Hall, 1991).

Immature embryos of maize are a good source of totipotent cells with which to initiate embryogenic cultures (Hall, 1991; Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992). In maize, fertilization occurs between 16 and 24 h after pollination, depending on temperature and silk length. The zygote does not divide until c.a. 10 or 12 h after fertilization. Approximately 14 d after fertilization, the embryo possesses a prominent shoot apical meristem surrounded by the coleoptilar ring on its anterior surface and is backed by a scutellum. This is termed the coleoptilar stage embryo. By this stage the embryo is c.a. 1 mm or more in length. The first leaf primordium arises c.a. 16 d after pollination on the lower side of the shoot apical meristem as a crescent-shaped bulge (Figure 2.1). At this stage the embryo proper is 1 mm or more in length, and is referred to as stage 1 (Randolph, 1936). Embryos of this stage have been utilized in many tissue culture experiments to initiate embryogenic callus (Sheridan & Clark, 1994). The developmental stage of the embryo is extremely important and should be determined by embryo length (base to tip of scutellum) rather than by days post-pollination (Armstrong, 1994).

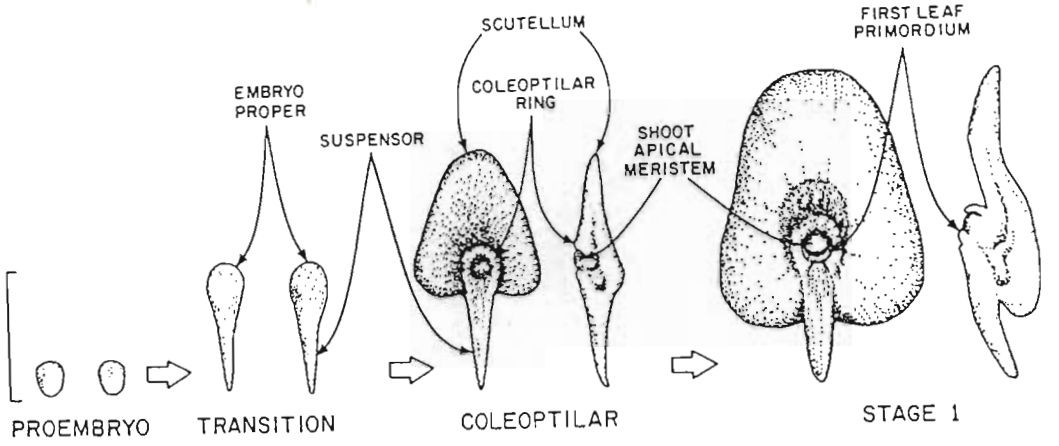


Figure 2.1 Early embryogenesis in maize. For each stage the drawing on the left shows the external features of the embryo in face view while the drawing on the right shows the embryo in longitudinal radial section with the face of the embryo at the left (bar = 0,5 mm) (Sheridan & Clark, 1994).

Young unexpanded maize leaf tissue (leaf rolls) for callus initiation can be obtained either from seedlings or young shoots (Chang, 1983; Vasil & Vasil, 1991). It has, however, been hypothesised that in some cereal crops the ability of leaf cells to express morphogenic capability decreases as the leaf matures (Chang, 1983; Wernicke & Milkovits, 1984). Grass leaves grow from a basal meristem, and therefore, the youngest part of the leaf is at the base and the oldest at the tip (Vasil & Vasil, 1991). Immature basal leaf sections taken from regions that are temporally and spatially closest to the meristem are consequently a better source of leaf material with a high embryogenic response (Wernicke & Milkovits, 1984).

Sterilisation of greenhouse grown plant material can prove difficult both due to sensitivity of the explant to the chemicals used and to the external morphology of the plant (e.g. the presence of waxy scale or many hairs which can prevent good contact with the sterilisation solution). Seed can generally withstand more severe sterilisation conditions than plant tissues. Therefore, sterilised seeds, germinated *in vitro*, consequently enable easier isolation of sterile explants for callus initiation (Hall, 1991). Aseptic cultures are recommended as the donor tissue is grown under controlled conditions, ensuring a relatively large physiological uniformity (Roest &

Gilissen, 1989). Additionally, as all the plant material is at a very young stage, there is a high potential for cell division within the explants. Low germination frequencies can however, be problematic (Hall, 1991).

2.1.3 Conditions required for the initiation and maintenance of maize callus

Explants of maize have been cultured on many media formulations with mixed results. Callus from maize leaf explants and immature embryos has generally been initiated on the same formulation of nutrient medium (Vasil & Vasil, 1991). Generally MS medium is better than N6 medium in the production of embryogenic callus from immature embryos of maize (Hodges, *et al.*, 1986). Maize immature embryos placed on N6 medium produce more friable callus than those on MS medium (Kamo, *et al.*, 1985). The concentration of sucrose in callus initiation medium has a marked effect on the nature and efficiency of callus formation (Lu, *et al.*, 1983; Vasil, *et al.*, 1985). High sucrose (6% m/v) in both MS and N6 media induces a higher percentage of Type I embryogenic callus from embryos. This callus grown on higher levels of sucrose, however, has a reduced ability to regenerate (Lu, *et al.*, 1983; Hodges, *et al.*, 1986; Swedlund & Locy, 1993). It is possible to generate friable embryogenic Type II callus from compact embryogenic Type I cultures by reducing the sucrose concentration in the medium, provided that the compact callus contains some less differentiated regions (Vasil, *et al.*, 1984; Fransz & Schel, 1991). Addition of silver nitrate (0 to 20 mg L^{-1}), an inhibitor of the physiological action of ethylene (Beyer, 1976), to the culture medium of maize immature embryos can dramatically improve the frequency of initiation of embryogenic Type II cultures (Vain, Yean & Flament, 1989), and yet is detrimental to maintenance of these cultures (Sellmer, Ritchie, Kim & Hodges, 1994). Myo-inositol (0 to 1000 mg L^{-1}) was not found to be a crucial requirement for maize callus growth (Green, *et al.*, 1974) or embryogenesis (Vasil & Vasil, 1986). Substantial increases in growth of embryo-derived maize callus cultures are obtained by

an increase in the casein hydrolysate concentration (0.5 g L^{-1}) (increase in available nitrogen) in the medium and/or by the addition of molybdate (Duncan, *et al.*, 1985).

In most monocotyledonous species, the embryogenic response of leaf tissue is induced by exposure to relatively high levels of auxin (Horn, 1991a). However, when 2,4-D is used as the sole growth regulator in the induction of callus from immature embryos, there is a pronounced genotypic effect on culture induction (Close & Ludeman, 1987). The number of genotypes which form regenerable callus cultures is increased with the use of dicamba (3,6-dichloro-*o*-anisic acid), substituted in place of 2,4-D. This also results in a decrease in the amount of shoot formation and callus browning which is usually produced by 20 to 30 d old callus grown on media containing 2,4-D (Duncan, *et al.*, 1985). The addition of amino acids, which are precursors of polyamine synthesis (especially arginine but also ornithine), may be used to improve the rate of embryogenic callus production in an auxin-established maize culture system (Santos, Claparols & Torné, 1993). L-proline, a source of reduced nitrogen, stimulates friable callus initiation and somatic embryogenesis from maize immature embryos cultured on N6 medium (Armstrong & Green, 1985; Vasil & Vasil, 1986; Vasil & Vasil, 1991). On the other hand, L-proline has no effect on friable callus formation and somatic embryoid formation in MS grown cultures (Armstrong & Green, 1985). Other exogenous amino acids (asparagine, glycine and serine) have a positive effect on the improvement of somatic embryogenesis in an established maize embryogenic system (Claparols, Santos & Torné, 1993).

Maize explants for callus initiation and maintenance can be cultured in the dark (Duncan, *et al.*, 1985; Imbrie-Milligan & Hodges, 1986; D'Halluin, *et al.*, 1992) or under a 16 h light/8 h dark photoperiod (2 to 20 μmol m^{-2} s^{-1}) (Green & Phillips, 1975; Armstrong & Green, 1985; Emons & Kieft, 1991), although even a 13 h light/11 h dark regime has been successfully used (Prioli & Söndahl, 1989). Temperatures at which maize callus cultures are

incubated range from 23°C (D'Halluin, *et al.*, 1992) to 30°C (Green & Phillips, 1975; Armstrong & Green, 1985; Fransz & Schel, 1991), with an average of 27 to 28°C commonly being used (Lu, *et al.*, 1982; Duncan, *et al.*, 1985; Petersen, *et al.*, 1992). A subculture period of 2 wk is frequently used (Duncan, *et al.*, 1985; Fransz & Schel, 1991).

2.1.4 The initiation and maintenance of callus cultures of selected South African maize germplasm

Limited research has been conducted on the tissue culture of South African maize germplasm. In a study conducted by Woodward & Furze (1989), six South African inbreds (M162W, M37W, 21A-6, F2834T, I137TN and A441-5) were examined for their suitability for tissue culture. The best tissue culture response was obtained for 21A-6, from which plantlets were regenerated. M162W, M37W and F2834T, however, also exhibited potential for tissue culture (Woodward & Furze, 1989).

The aim of the experiments presented in this chapter was to initiate callus from various explants of South African maize inbreds. Initially explants were taken from plants of 4 wk old plants cultivated in the greenhouse. Leaf discs and stem pieces, as well as the more immature leaf rolls and pith sections, of these greenhouse-grown maize plants were placed on callus initiation medium. In subsequent experiments, more immature maize tissue was used for callus initiation investigations. Explants which included the very base of the youngest leaves from young maize seedlings germinated *in vitro*, as well as stage 1 immature embryos (Figure 2.1), were therefore utilized for callus initiation. The callus initiated in this study was utilized in investigations into the establishment of a protocol for the efficient isolation of maize protoplasts (Chapter 3). Initiated callus was also electroporated or microprojectile bombarded in preliminary transformation studies (Chapter 4).

Ten South African developed elite maize inbreds of diverse backgrounds were used in this study (Table 2.1). These highly elite inbreds have been developed over the last thirty years at Ukulinga Research Farm (University of Natal, Pietermaritzburg). The elite inbreds were all bred for specific characteristics (including high yield, drought tolerance, insect tolerance, and resistance to disease) and all are successfully used in commercial hybrid production.

2.2 Materials and methods

2.2.1 Greenhouse cultivation of maize plants

Plants of 10 South African developed maize inbreds of diverse backgrounds (Table 2.1) were cultivated under controlled environmental conditions in a greenhouse. Prior to planting, the greenhouse was sprayed with Redspider-Cide® (2 mL L^{-1}; a.i. tetradifon) and Malathion® (2.5 mL L^{-1}; a.i. mercaptothion). Each pot was fertilized with 10 g 2N:3P:2K. Six caryopses were planted per pot. One week post-emergence, the seedlings were fertilized with 3N:2P:1K and Superphosphate. The seedlings were watered daily and given nutrients in the following order (seven day cycle):

Day 1: Chemicult® (hydroponic nutrient powder);

Day 2: Trelmix® (trace element solution) (5 mL per 3 L water); and

Day 3: Nitrogen in the form of liquid NH_4NO_3, at a rate of 200 kg ha^{-1}.

Shortly before maturity, the plants were given a booster of 2N:3P:2K.

The insecticidal sprays Redspider-Cide® and Malathion® were applied to the growing maize plants to control redspider and aphids. Benlate® (a.i. benomyl) was applied to prevent the plants from withering and dying (damping off) at a young age. All

insecticidal sprays, fertilizers and compost were supplied by Greenfingers Nursery, Hayfields, Pietermaritzburg, South Africa. Maize caryopses were initially planted in silica sand (Eggo sand; dry graded). Three replications were planted and the pots arranged at random on the greenhouse benches, one replication per bench. Soil and compost (Gromor®) in a ratio of 1:1 were used as an alternative potting medium. One replication was planted and arranged at random on a greenhouse bench.

Table 2.1 Characteristics of 10 South African elite inbreds (Gewers & Whythe, 1986).

Inbred	Type[1]	Heterotic group	Source
F2834T	YN	(F)	F2834T/Teko Yellow
I137TN	YN	(I)	I137TN (TYxNYHT)
D0940Y	YN	(M)	M37W/21A/Jellicorse
M28Y	YN	(R)	
M162W	WN	(K)	M162W/K64R
M37W	WN	(M)	M37W/21A/Jellicorse
K054W	WMOD	(F)	
S0507W	WMOD	(M)	
K0315Y	YMOD	(M)	
B0394Y	YMOD	(F)	F2834T/Teko Yellow

[1]YN = yellow normal
WN = white normal
WMOD = white modified *opaque-2*
YMOD = yellow modified *opaque-2*

2.2.2 Callus initiation from leaf discs and stem pieces of greenhouse-grown maize plants

Using an adapted technique from Green, *et al.* (1974), leaf discs and stem pieces of maize plants were placed on callus initiation medium to determine whether callus would form. Young healthy, unblemished leaves were excised from 4 wk old maize plants that had been cultivated in a greenhouse. Leaf explants of the youngest leaves were cut as close to the leaf whirl as possible (Figure 2.2; e.g. leaf 4). Stem explants were obtained from the coleoptilar region of the young plants. The plant material was cut into manageable pieces and washed under a tap to remove any surface dirt. All further manipulations were performed on a laminar flow bench.

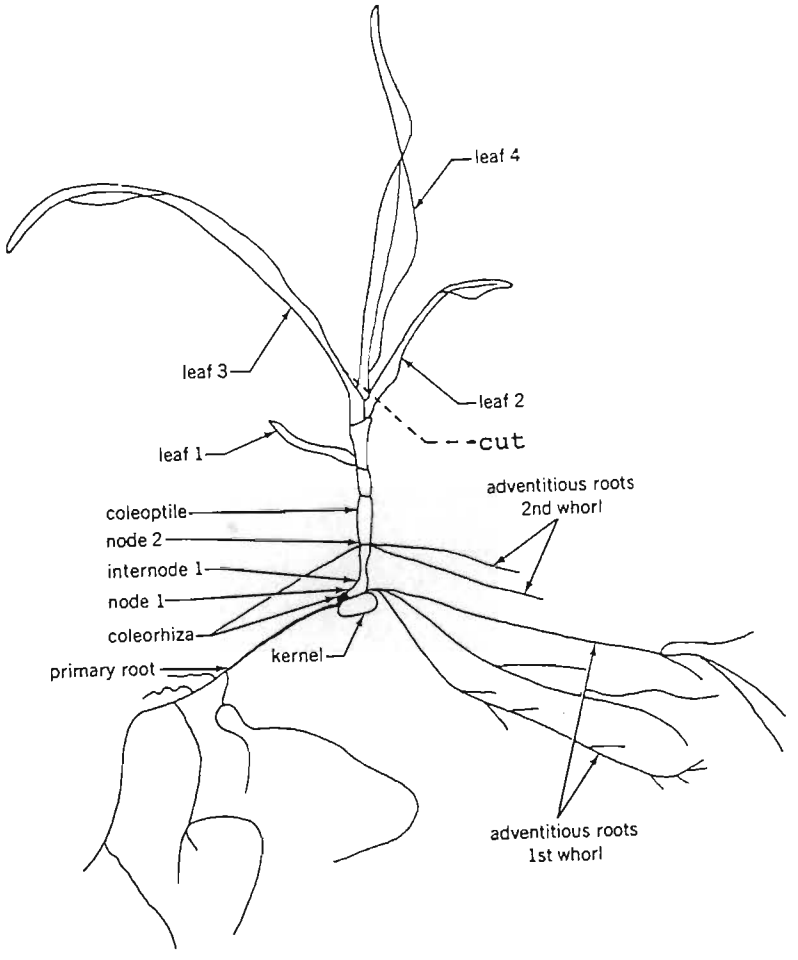


Figure 2.2 Diagrammatic representation of seedling of maize (*Zea mays* L.). Details: primary root - root that originated within the coleorhiza; first node - scutellar node; first internode - internode between scutellum and coleoptile; second node - coleoptilar node; first leaf - first leaf above the coleoptile (Esau, 1977).

The leaf and stem tissue was immersed in 95% ethanol for 60 s to remove any surface bacterial contamination and rinsed once in sterile distilled H_2O. For more stringent surface sterilisation, the plant material was immersed in hypochlorite-detergent solution (1.75% (v/v) commercial bleach (Jik) and 1% (v/v) detergent or wetting agent (Tween 20)) for 5 min. The sterilised plant material was rinsed once in sterile distilled H_2O and placed in a sterile glass Petri dish (90 mm diameter; used throughout this study) containing sterile distilled H_2O. Using sterile instruments, the leaf material was cut into squares

suitable for culture (c.a. 1 cm^2). Stem pieces were cut transversely into explants c.a. 1 cm in length. Where the hypochlorite-detergent solution had killed the cells on the edge of the explants, turning the tissue brown, the dead tissue was removed. Any pieces showing damage were discarded. Cut explants were immersed in 70% (v/v) ethanol for 60 s and rinsed twice in sterile distilled H_2O. Excess liquid was shaken off of the explants, which were then placed on callus initiation medium (N6 (according to Rhodes & Gray, 1991) + 2% (m/v) sucrose + 1 to 2 mg L^{-1} 2,4-D + 10 g L^{-1} agar, pH 5.8 (Appendix A.1) or MS modified (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984) + 2% (m/v) sucrose + 1 to 2 mg L^{-1} 2,4-D + 10 g L^{-1} agar, pH 5.8 (Appendix A.2)) in Petri dishes (six discs per plate), or glass culture tubes (70 cm^3; 15 mL medium), and placed in an incubator in the dark at 25°C. Petri dishes and culture tubes were sealed with Parafilm "M" when all excess liquid had evaporated and there was no visible sign of microbial contamination. Alternatively, Petri dishes were placed in stacks of five, sealed in transparent plastic bags, and incubated in a Conviron® (controlled environment chamber) at 25°C. The light regime in the Conviron® consisted of 12 h light/12 h (19.5 μmol m^{-2} s^{-1}) dark under half incandescent and full inflorescent lighting.

Plant material cultured in this manner was monitored twice weekly for the first signs of callus growth or microbial contamination. Tissue was transferred to fresh medium every 2 wk. Any contaminated cultures were discarded once uncontaminated leaf and stem explants had been aseptically transferred to fresh medium. Slightly contaminated leaf and stem explants (no callus yet initiated), or tissue on medium which had turned brown, was removed from the medium and rinsed once in sterile distilled H_2O. The tissue was then immersed in 70% (v/v) ethanol for 30 s, rinsed once in sterile distilled H_2O, placed on fresh medium and returned to the incubator.

2.2.3 Callus initiation from leaf rolls and pith sections of greenhouse grown maize plants

Leaf rolls and pith pieces of maize plants were placed onto callus initiation medium to observe whether callus formation would occur. The method used was adapted from Green, *et al.* (1974). Healthy unblemished maize plants (6 to 7 wk old), grown in a greenhouse, were cut off close to the soil. A section of leaf roll and pith tissue c.a. 10 cm in length (Figure 2.3), was used as the explant. The outer leaves were removed from the plant material and the remaining inner leaf material washed under a tap to remove any surface dirt. The tissue was cut into manageable pieces (5 to 6 cm in length). The rest of the procedure was performed on a laminar flow bench.

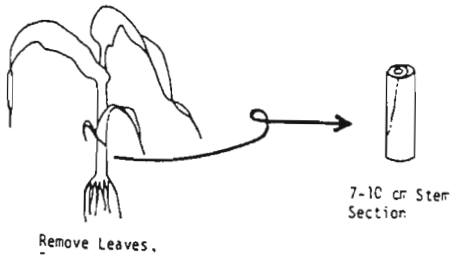


Figure 2.3 Leaf rolls and pith sections used for callus initiation (Green, *et al.*, 1974).

The tissue was sterilised according to the procedure for leaf discs and stem pieces (Section 2.2.2) and placed in a sterile glass Petri dish containing sterile distilled H_2O. Using sterile implements, the tissue ends that had been damaged by the hypochlorite-detergent solution were removed. Leaf roll pieces were sliced into sections c.a. 1 to 4 cm in length. The hard pith tissue (situated at the base of the plant, close to the soil) was transversely sliced into pieces c.a. 1 to 2 mm in width. The tissue pieces were immersed in 70% (v/v) ethanol for 60 s and rinsed briefly in two rinses of sterile distilled H_2O. Excess liquid was shaken off of the explants. The entire unfurled leaf tissue was unrolled and the innermost two leaves (referred to as leaf roll) placed on callus initiation medium (MS modified

(according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984) + 2% (m/v) sucrose + 10 g L^{-1} agar; pH 5.8 (Appendix A.2); supplemented with 3, 5, 10 or 15 mg L^{-1} 2,4-D) in sterile Petri dishes. Pith tissue pieces were placed on the same callus initiation medium with a cut surface in contact with the medium. The Petri dishes were sealed with Parafilm "M", inverted (to decrease condensation on the tissue) and incubated in a custom-built controlled environment chamber (Appendix A.4) at $27\pm1^{o}C$ with a 12 h light/12 h dark photoperiod (19.1 μmol m^{-1} s^{-1}). The cultures were observed every second day, and subcultured to fresh medium every 2 wk.

2.2.4 Initiation of callus from maize seedlings germinated *in vitro*

The technique of Harms, *et al.* (1976) was adapted for the initiation of callus from young maize seedlings. Mature maize caryopses of the 10 inbreds were placed in 95% ethanol for 10 s for surface sterilisation. The caryopses were transferred to sterile distilled H_2O containing 1% (v/v) Tween 20 and shaken gently for 1 min, after which each caryopse was examined. Any caryopses with translucent patches beneath the pericarp, indicating the penetration of water via a fractured seed coat, were discarded. The remaining caryopses were immersed in hypochlorite-detergent solution (two thirds strength (2.3% (v/v)) commercial bleach (Jik) and 1% (v/v) detergent or wetting agent (Tween 20)) for 15 min for further sterilisation. This, and all further manipulations were performed aseptically on a laminar flow bench.

Sterilised caryopses were rinsed for 10 min each in three sterile distilled H_2O washes. The maize caryopses were removed from the last rinse and placed on MS Seedling medium + 1% (m/v) sucrose + 10 g L^{-1} agar; pH 5.8 (according to Wernicke & Brettell, 1982; Appendix A.3) in glass culture tubes (70 cm^3; 15 mL medium). The caryopses were orientated with the embryo-bearing side facing upwards as this orientation avoided emergence of the shoot into

the agar and resulted in a much more evenly sized population of plants. The culture tubes were sealed with Parafilm "M" and incubated at 27±1°C in the custom-built controlled environment chamber with a 12 h light/12 h dark photoperiod (19.1 μmol m^{-2} s^{-1}).

When the germinated seedlings were 10 d old (Plate 1), medium sized plantlets were selected. These were removed from culture with the aid of sterile forceps, for callus initiation. The seedlings were placed in sterile glass Petri dishes. Using a sterile scalpel, the caryopse, roots and adventitious roots were removed leaving an explant consisting of the mesocotyl, coleoptile and first plumular leaf (Figure 2.4). The explant (consisting mainly of tightly rolled leaves) was cut into sections c.a. 1.5 mm in length. These tissue sections, consisting of the lower third of the leaf blade and the leaf sheath (hereafter referred to as the leaf base), were placed on callus initiation medium (MS modified medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) supplemented with either 2% or 6% (m/v) sucrose, 10 g L^{-1} agar and 3 mg L^{-1} 2,4-D; pH 5.8). Explants from the mesocotyl region were placed with one cut surface in contact with the medium. All tissue pieces were placed on the medium in the same order as they were excised from the shoot, i.e. the first section was cut from the base of the mesocotyl region, while the last section placed on the medium was cut from the lower third of the first plumular leaf. Sections consisting of rolled up leaf tissue were separated into the respective leaf pieces and placed on the callus initiation medium in the order of: outer layer; first plumular leaf; and innermost leaf. This was followed by the next section in the same order. Sections were placed on the medium in this specific order so as to determine precisely which regions of the young maize seedlings best produced callus. The Petri dishes, each containing explants cut from one seedling, were sealed with Parafilm "M", inverted and incubated at 27±1°C in the custom-built controlled environment chamber with a photoperiod of 12 h light/12 h dark (19.1 μmol m^{-2} s^{-1}). Every 14 d the tissue

was subcultured to the same medium for callus maintenance. Any dead or brown explant tissue was excised from the callus and discarded. Macroscopic callus detail was examined and photographed with a Zeiss Stemi SV 6° dissecting microscope (equipped with a Pentax K1000 camera using Fuji colour film (200 ASA)).

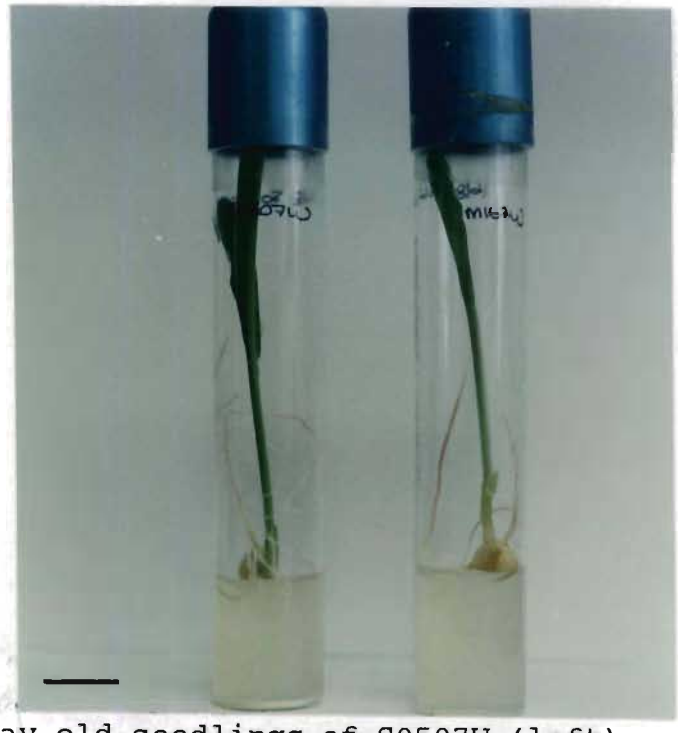


Plate 1 Ten day old seedlings of S0507W (left) and M162W (right) germinated *in vitro* (bar = 16.6 mm).

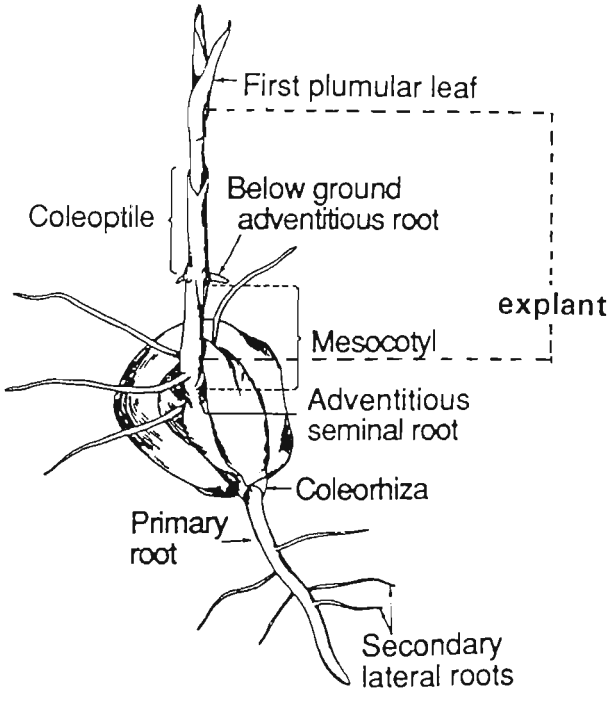


Figure 2.4 Young maize seedling showing early stages in development (Feldman, 1994).

2.2.5 Callus initiation from immature embryos of maize

2.2.5.1 Hand pollination of maize plants

The maize genotypes used for immature embryo isolation and culture included the 10 inbreds utilized in preceding experiments, as well as a hybrid PAN 473 (parents: M162W and NPP PNR). Mature maize plants, that had been cultivated in the greenhouse, were self-pollinated to obtain selfed immature embryos. When the first tassels appeared, ear shoots were covered with plastic ear shoot bags (20.5 x 10 cm) before the silks emerged so that they were protected from all but the desired pollen. Once the first day's silks were visible, the tip of the husks and silks was cut off squarely as far down the husks as possible to avoid cutting off the tip of the cob inside (cutting back). This was done to ensure that fully set ears were obtained from hand pollination. The following day the silks had emerged to form a thick brush and were ready for pollination. Viable pollen was collected in brown paper bags. To collect pollen, the plant was carefully bent so that the top open end of the brown bag, covering the tassel, was higher than the bottom. The bag and tassel were sharply shaken and the tassel then carefully withdrawn. The tassel bag was carried (open end folded) to the desired protected silks. A small amount of pollen was dusted onto the silks and the ear re-covered. After 2 wk, embryo size was determined by peeling back the husks of an ear still attached to the plant. The tips of several caryopses in the centre portion of the ear were sliced off using a scalpel and the developing endosperm and embryo scooped out with a spatula. The embryo, located on the side of the caryopse facing the tip of the ear, was removed and measured with a ruler.

2.2.5.2 Isolation of immature maize embryos and initiation of callus

The method of Vasil and Vasil (1991) was modified for the initiation of callus from immature maize embryos. Developing ears were removed 9 to 15 d after pollination. The outermost husks of the ear were stripped off and the remaining portion (ear surrounded by three to four husks) was sterilised with 70% (v/v) ethanol. The rest of the procedure was performed on a laminar flow bench. The remaining husks and silks were aseptically removed from the sterilised ear. The top third of the developing caryopse was sliced off with a scalpel. The embryos (0.5 to 3.0 mm in length) were removed using a sterile modified spatula and placed with their embryo-axis in contact with callus initiation medium (MS modified medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 2 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (i.e. with the rounded scutellar surface exposed) in sterile Petri dishes. This orientation retards germination of the embryo and induces proliferation of the scutellar cells to produce callus (Green & Phillips, 1975). The Petri dishes were sealed with Parafilm "M" and inverted. The embryos were either placed in the custom-built controlled environment chamber at $27\pm1^{o}C$ with a 12 h light/12 h dark photoperiod (17.4 μmol m^{-2} s^{-1}), or in an incubator at $25^{o}C$ in the dark.

After 2 to 3 wk of incubation, developing callus was subcultured onto fresh MS modified medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8 (hereafter termed MS Callus maintenance medium (MS-CM medium)). Subculture was performed either by transferring whole calli to fresh medium, or by sectioning large calli into smaller pea sized (c.a. 1 cm^3) pieces before transfer to fresh medium. Subsequently, callus pieces were subcultured to fresh MS-CM medium (modified according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose

+ 2 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8 every 14 d, at which time any unhealthy or dead tissue was removed.

Callus samples, stained with Safranin (1% (m/v)), were squashed under cover slips on microscope slides. The squashed callus was examined with a Zeiss Axiophot® microscope with a built-in camera. The callus types observed with the microscope were photographed with Kodak Ektachrome 160 tungsten film. Macroscopic callus detail was examined and photographed with a Zeiss dissecting microscope.

2.3 Results and discussion

2.3.1 Greenhouse cultivation of maize plants

The conditions prevailing during the different periods of growth of maize plants (fertilizer, irrigation water, rainfall, temperature and photoperiod) affect the physiological state of immature embryos and plants used for callus induction. It would be advantageous, therefore, to grow the experimental plants under controlled greenhouse conditions (Lu, *et al.*, 1983). At the time the experiments were conducted, however, the greenhouse was being refurbished and there was no wet-wall to effectively control the greenhouse temperature. In order to control the nutrient/fertility levels available to the maize plants, cultivation was initially performed in silica sand. The seedlings potted in silica sand emerged after 8 d. The water retention capacity of the silica was, however, low and, coupled with the high evaporative demand in the greenhouse, led to the majority of the plants dying at a very young age from drought stress. Therefore, plants for all further experimentation were grown in a mixture of soil and compost which had a higher water retention capacity. Consequently, the nutrient levels could not be controlled to the same extent as with the silica sand. The seedlings potted in soil and compost were more vigorous than the

ones cultivated in silica sand, although some still suffered from heat stress. Subsequent plantings, of either one or two replications, were performed in a soil and compost mix.

2.3.2 Callus initiation from leaf discs and stem pieces of greenhouse-grown maize plants

Eight days after maize leaf and stem explants of 4 wk old maize plants had been placed onto callus initiation medium in the dark, leaf explants that were not discarded due to microbial contamination were still mainly green. A few of the leaf disc explants had developed small brown areas, while the stem pieces had already turned completely brown. After 3 wk, S0507W, M28Y and I137TN leaf explants were green with slightly brown areas, as were M162W and F2834T. M37W, B0394Y and D0940Y explants were green, with brown, yellow or translucent patches. K054W explants were brown and translucent. After 5 wk in culture the leaf explants had still not undergone any noticeable change apart from progressive browning. No callus initiation had taken place.

Two weeks after leaf discs in Petri plates were placed in stacks of five under light in the Conviron®, leaf explants in the top two Petri dishes of the stacks had started to turn brown. The leaf explants in the bottom three dishes were still green in colour. It therefore appeared as if the leaf discs were sensitive to light as the ones closer to the light source turned brown a lot quicker than the ones shaded from the light. It was concluded that the top Petri dish was receiving too much light. The light regime was consequently altered to half incandescent and half inflorescent light (13.9 μmol m^{-2} s^{-1}). Thirty three days after culture initiation, the light regime was changed so that only half incandescent light was used. After 47 d the light was again changed to only half fluorescent light (12.2 μmol m^{-2} s^{-1}). Nine days later the light was changed for the last time to full fluorescence (16.3 μmol m^{-2} s^{-1}). These alterations to the light regime did not, however, improve matters. The leaf explants progressively turned brown, and no callus initiation was observed.

Throughout the culture of maize leaf discs and stem explants, therefore, no callus was initiated from leaf discs or stem explants of 4 wk old maize plants which had been cultured on callus initiation medium. This result was obtained for all 10 inbreds, for the two different media tested (N6 medium (according to Rhodes & Gray, 1991; Appendix A.1) + 2% (m/v) sucrose + 1 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8; and MS modified medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 2 mg L^{-1} 2,4-D + 10 g L^{-1} agar, pH 5.8) and for the different culture conditions of light and dark. The explants remained inert in culture and slowly turned from green to yellow to brown, the longer they were kept in culture. After c.a. 30 d, the majority of the explants were brown and had died. Leaf and stem pieces were continually transferred to fresh medium, until c.a. 80 d had elapsed, when it was evident that under the prevailing conditions no callus formation was going to take place. The lack of callus initiation may have been due to a loss of sensitivity to 2,4-D, an otherwise most potent growth regulator in tissue culture (Wernicke & Brettell, 1982). Other workers have found that callus could be initiated from growing point tissues of maize plants at the six to 10 leaf stage, using medium supplemented with 2 to 5 mg L^{-1} 2,4-D (Green, *et al.*, 1974). Perhaps a higher 2,4-D concentration of 5 to 10 mg L^{-1} may have initiated callus from the leaf disc or stem explants. This higher 2,4-D concentration was applied in subsequent investigations on the initiation of callus from leaf roll and pith sections of greenhouse-grown plants.

2.3.3 Callus initiation from leaf rolls and pith sections of greenhouse grown maize plants

In an attempt to initiate callus from leaf roll and pith explants of maize plants, a 2,4-D concentration range of 3 to 15 mg L^{-1} was used (Table 2.2). The different excised leaves of the maize leaf roll differed in appearance due to leaf age (Figure 2.3). The leaf roll tissue used for callus initiation ranged in colour from almost transparent pale yellow green at the lowest part of the

leaf roll (near the base of the plant), to green at the furthermost section used (c.a. 10 cm from the base of the plant). The pale yellow green leaf roll tissue was soft, fragile and easily torn. The pith pieces were derived from the much harder, compact tissue at the base of the maize plant.

Table 2.2 Number of leaf roll and pith explants incubated on callus initiation medium (MS modified + 2% (m/v) sucrose + 10 g L^{-1} agar; pH 5.8; supplemented with 3, 5, 10 or 15 mg L^{-1} 2,4-D).

Inbred	Tissue type	Number of tissue pieces placed on callus initiation medium			
		3mg L^{-1} 2,4-D	5mg L^{-1} 2,4-D	10mg L^{-1} 2,4-D	15mg L^{-1} 2,4-D
I137TN	Leaf roll	12	0	0	0
M162W	Leaf roll	14	2	2	2
M37W	Leaf roll	16	3	3	7
M37W	Pith	6	0	0	0
M28Y	Leaf roll	2	2	2	2
M28Y	Pith	0	6	0	0
S0507W	Leaf roll	16	6	6	7
S0507W	Pith	14	0	0	0
K054W	Leaf roll	14	3	5	6
K054W	Pith	15	0	0	0
D0940Y	Leaf roll	12	0	0	0
D0940Y	Pith	8	0	0	0

One day after the initiation of the experiment, the youngest green leaf roll sections, which had been unrolled prior to placement on the medium, had faded to a cream colour. A few of the more darker green unrolled sections had become translucent in patches. The leaf roll pieces which were still tightly rolled remained green. After 48 h most of the leaf roll pieces were still losing their colour. Leaf roll explants were placed in partial contact with the callus initiation medium. The regions in contact with the medium turned brown and subsequently died. Some puckering of the explant tissue was noticed, especially in regions that were raised off of the medium. This puckering occurred on the leaf roll segments derived from the section near the base of the plant (Plate 2). The leaf roll segments derived

from the region furtherest from the base of the plant retained their green colour for a longer time period than the other sections, which had turned cream.

Raised portions of the leaf roll explants turned bright green and after 14 d on callus initiation medium, a small amount of callus was observed on leaf rolls of S0507W and D0940Y which had been incubated on medium supplemented with 5 mg L^{-1} 2,4-D (Plate 3). Twenty days after culture initiation, a number of the Petri dishes had been discarded due to microbial contamination. In all of the remaining Petri dishes, the leaf roll pieces had gone cream or light brown and had become puckered and wrinkled. No callus had been initiated on any of the leaf rolls, other than one leaf roll each of S0507W and D0940Y on medium supplemented with 5 mg L^{-1} 2,4-D. This callus was initiated on very green and wrinkled leaf rolls that had been broken up and damaged during isolation, thus confirming that callus formation is a wound response under the correct endogenous and exogenous conditions. The initiated callus had a white, sugary (friable) appearance. Some hard, compact white callus was also formed. The S0507W and D0940Y calli were however non-vigorous and it was not long before the calli had become brown. The leaf roll tissue was continually transferred to fresh medium every 2 wk until either the explants had died, or it became evident that no callus was going to form.

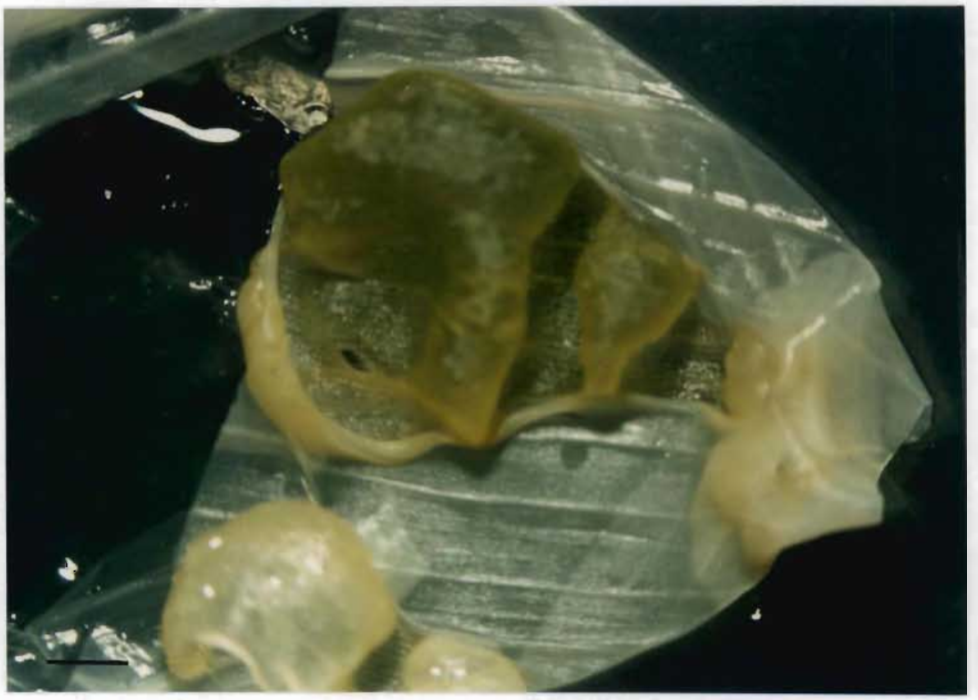


Plate 2 Leaf roll of D0940Y, excised from the very basal portion of the leaf explant, after 2 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 5 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Colour has faded to cream, and raised leaf section is becoming puckered (bar = 1.25 mm).

Plate 3 Leaf roll of D0940Y, incubated for 2 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 5 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8), showing slight callus formation at cut end of leaf roll explant (a) (bar = 1 mm).

Three days after culture initiation, the pith explants were still a pale to dark green colour. The central areas of the pith pieces had become either concave or raised in the middle (convex). After 8 d, the majority of the pith pieces were showing a convex appearance. The upper surface of the pith tissue had a sugary, friable appearance of callus growth (Plate 4). The callus cells were compact and round in shape. The whole pith explant swelled outwards with the callus growth. Around the edges of the pith pieces, a more compact cream callus appeared. The bottom surface of the pith tissue tended to turn brown or black; this brown colour extended into the medium surrounding the pith explants. The pith tissue was therefore subcultured to fresh medium whenever brown medium was visible. The pith pieces were kept in culture for two months longer than the leaf roll pieces to observe whether callus that had been initiated would grow. After 10 wk, the pith pieces appeared the same as at 8 d, and had started to turn brown. They were therefore discarded as the callus was not going to develop any further.

Plate 4 Pith explant of S0507W showing callus initiation (on upper surface) after 2 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

The higher 2,4-D concentration range of 3 to 15 mg L^{-1} used in this investigation had little or no effect on the initiation of callus from the leaf roll and pith tissue of maize plants. In fact, the higher 2,4-D concentration appeared to cause the leaf roll tissue to turn brown at a faster rate. Callus initiation medium supplemented with 3 mg L^{-1} 2,4-D, and to a certain degree medium supplemented with 5 mg L^{-1} 2,4-D, therefore appears to be the most suitable medium for callus initiation. Medium with higher concentrations of 2,4-D caused explant tissue to brown at a rate too high for callus initiation to take place.

The orientation of the leaf roll and pith pieces on the callus initiation medium seemed to be unimportant (results not shown). The convex or concave appearance of the pith pieces after incubation on medium may have been due to their orientation. This response may have been associated with the direction of solute flow in phloem tissue from the base to the top of the plant. The amount of callus initiated from maize leaf roll and pith explants was too small, and its viability declined too rapidly, for this callus to be of any use for protoplast isolations or transformation experiments.

Surface sterilisation may be potentially hazardous to delicate plant material such as young leaf tissue and leaf rolls. The surface sterilisation of the fragile leaf roll, combined with the 2,4-D concentration effect, may have together caused the leaf roll explants to become brown and die prematurely in culture. However, the lack of callus initiation from leaf rolls, leaf discs and stem pieces, is more likely due to the age of the tissue, as it has been hypothesised that in some cereal crops the ability of leaf cells to express morphogenic capability decreases as the leaf matures (Chang, 1983). Even though the leaf roll consists of immature tissue, the plants were 6 to 7 wk old. The leaf roll explants were therefore not very totipotent or undifferentiated, and only a very small amount of callus was initiated. Therefore, in subsequent experiments, immature leaf tissue was excised for callus initiation investigations.

2.3.4 Initiation of callus from maize seedlings germinated *in vitro*

As grass leaves grow from a basal meristem (Vasil & Vasil, 1991), the young leaf bases of young *in vitro* grown seedlings (germinated on culture medium in test tubes) were used for the initiation of callus. Explants were taken from regions close to the meristem, and the very bases of the youngest leaves (the lower third of the leaf blade and the leaf sheath). Due to the seedlings being germinated in a sterile environment, any detrimental effect of sterilising agents on delicate material was eliminated. Maize caryopses can generally withstand more severe sterilisation conditions than plant tissues (Hall, 1991). Sterile explants for callus initiation were therefore easier to obtain. Additionally, plant material of each inbred used for callus initiation is standardized (ensuring a relatively large physiological uniformity) when the seedlings are cultivated in a controlled environment. Field and greenhouse conditions prevailing during the different periods of growth (fertilizer, irrigation water, rainfall, temperature and photoperiod), which affect the physiological state of plants used for callus induction, are also therefore avoided. In addition, as all of the plant material is at a very young stage, there is a high potential for cell division within the explants.

The caryopses placed on MS Seedling medium germinated as expected. However, some of the caryopses developed fungal infections (*Fusarium* spp and *Diplodia* spp) that appeared to be from an endogenous source, and were therefore discarded. *Fusarium* spp and *Diplodia* spp are common fungal infections of maize caryopses. The inbreds infected by these fungi were: K054W; M37W; M28Y; D0940Y; I137TN; F2834T and B0394Y. B0394Y was very vulnerable to fungal infection; therefore, all B0394Y caryopses were discarded prior to callus initiation experiments, whereas the other inbreds all produced a few seedlings that were uncontaminated. These seedlings were used for callus initiation.

A more concentrated sterilisation solution (perhaps full strength commercial bleach (3,5% (m/v) sodium hypochlorite in Jik)), or a longer sterilisation period may have lessened the fungal contamination. However, this more stringent sterilisation procedure may have had a detrimental effect on the germination percentage of the maize caryopses. The procedure of Berjak, Whittake & Mycock (1992), for the eradication of fungal contaminants, was brought to the attention of the author by an examiner of the thesis. Slight improvement of this procedure can be applied to maize caryopses: eradication of fungal contaminants (without affecting the embryo) is achieved by the immersion of the material in water at 57^{o}C for 20 min (time may depend on size of the material). The fungi imbibe water and become physiologically active, and are consequently killed by the hot water long before the embryo (Watt, 1996; personal communication). Application of this technique to the maize caryopses used in this study (in particular B0394Y) may have eliminated the fungal contamination.

On callus initiation medium supplemented with 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D, the innermost, rolled-up leaves of the shoot, excised from the coleoptilar region, changed colour from a pale to darker green, while the outer leaves turned brown progressively. Two days after the tissue had been placed onto callus initiation medium, this innermost green leaf tissue had swollen considerably. The following day, callus formation was visible to the naked eye, especially on explants of S0507W. The callus initiation started at the edges of the tissue (Plate 5) and could be seen in the central regions at first as the enlarging of cells. In most cases, the callus was initiated from cells at or close to the midrib of the leaf section. Apical meristem regions also produced callus (Plate 6). Tissue sections from the mesocotyl region of the seedlings, however, only produced a small amount of white callus that had a soft, friable appearance (Plate 7). After 6 d on callus initiation medium, anthocyanin production was observed as purple streaks in tissue pieces of M162W (Plate 8).

Plate 5 Callus initiation, on a young leaf base explant of a M28Y seedling germinated *in vitro*, after 1 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Callus becoming evident at the edges of the tissue (a) (bar = 0.83 mm).

Plate 6 Nodular, cream embryogenic callus initiated from an apical meristem of D0940Y, after 4 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1 mm).

Plate 7 Soft, friable non-embryogenic callus initiated from the mesocotyl region of a S0507W seedling after 1 wk on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 0.83 mm).

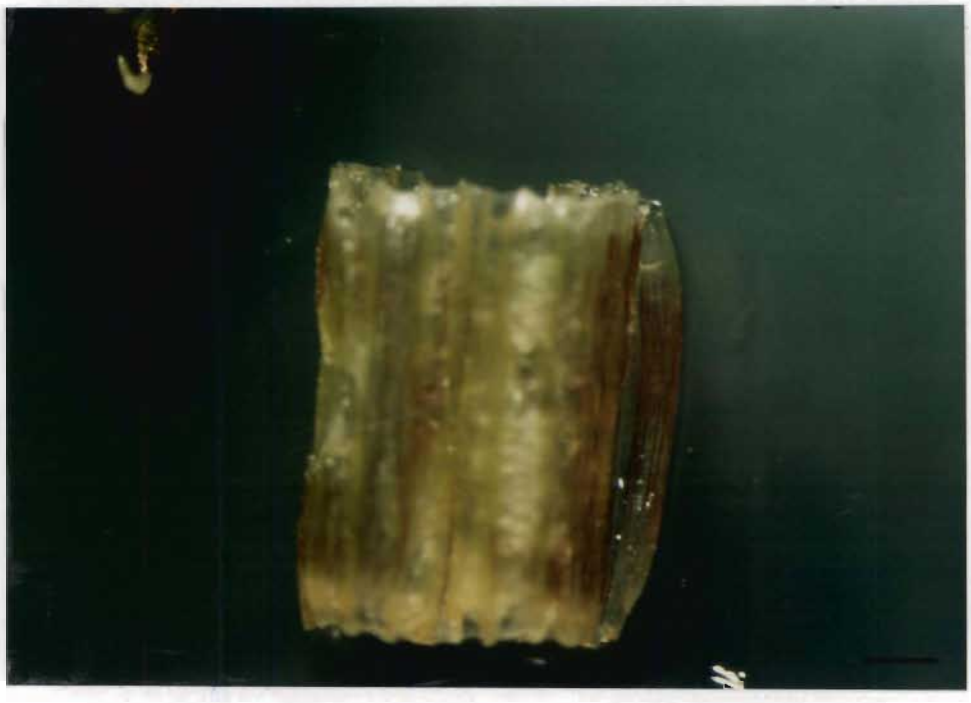


Plate 8 Anthocyanin production in a leaf tissue explant of M162W after 6 d on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 0.63 mm).

Callus initiation, on medium supplemented with 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D, occurred on tissue sections of the 10 inbreds used, except B0394Y which was discarded before callus initiation due to fungal contamination. S0507W was the first to show signs of callusing, while M28Y and M162W produced the most callus. The callus of these two inbreds was vigorous and could be maintained in culture for long periods of time. In the initial stages of culture the callus of all inbreds (except B0394Y) grew considerably and was reasonably fast growing. However, callus of some inbreds, namely S0507W, I137TN and M37W, started to brown and die after c.a. 4 wk in culture. Callus of other inbreds, namely M162W and M28Y, continued to thrive. The callus that was initiated was white, soft and healthy looking and grew fairly rapidly. Most callus was compact and fairly moist. Under the specific culture conditions used, each inbred formed callus with typical characteristics (Table 2.3). D0940Y, M162W and M28Y produced a harder more compact callus than the other inbreds. This callus also had some green regions where photosynthesis was occurring (Plate 9).

Plate 9 Five week old green and white soft callus of M28Y initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Table 2.3 Summary of observed typical characteristics of callus formed from young leaf bases of each inbred placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) for 5 wk. Nodular = compact, hard callus.

Inbred	Typical characteristics of callus
M28Y	Green and white hard compact callus. Some differentiation forming strange elongated shapes. Moist soft white callus as well as nodular cream callus. Fast growing (Plate 5 and Plate 9).
M162W	Cream coloured, moist, soft friable callus. Fast growing (Plate 10).
S0507W	Hard, compact, white to yellow callus that turns brown and dies. Some soft, moist, brown or grey callus (Plate 7 and Plate 11).
M37W	Soft, moist, cream callus. Moist and slimy. Turns brown or grey and dies (Plate 12).
I137TN	Some nodular cream callus. Parts turning grey or brown. Mostly moist, hard, slimy callus (Plate 13).
K0315Y	Callus tended to turn grey and brown before dying. Some creamy yellow nodular callus. Other soft white callus which is very moist and slimy (Plate 14).
D0940Y	Nodular cream callus in some parts. Otherwise soft, moist callus. Most turning brown (Plate 6 and Plate 15).
K054W	Small nodules in nodular cream callus (Plate 16).
F2834T	Cream to brown to grey soft, moist callus. Tended to brown early in culture (Plate 17).

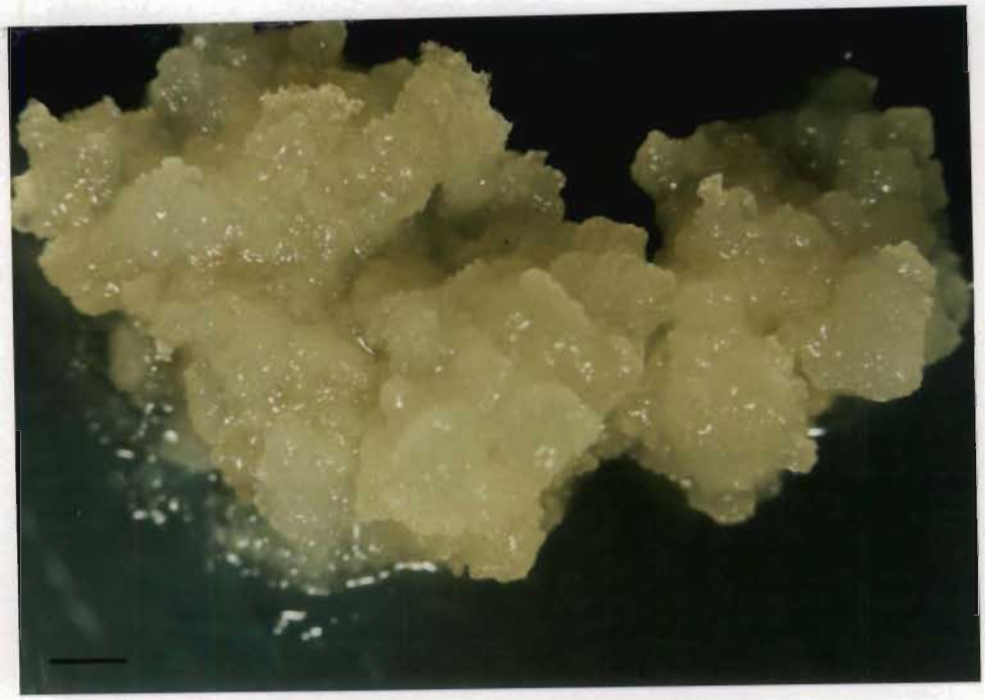


Plate 10 Four month old soft, moist callus of M162W initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 11 Five week old hard, compact, white embryogenic callus (a) and soft, moist non-embryogenic callus (b) of S0507W initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 12 Five week old nodular, cream callus of M37W initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

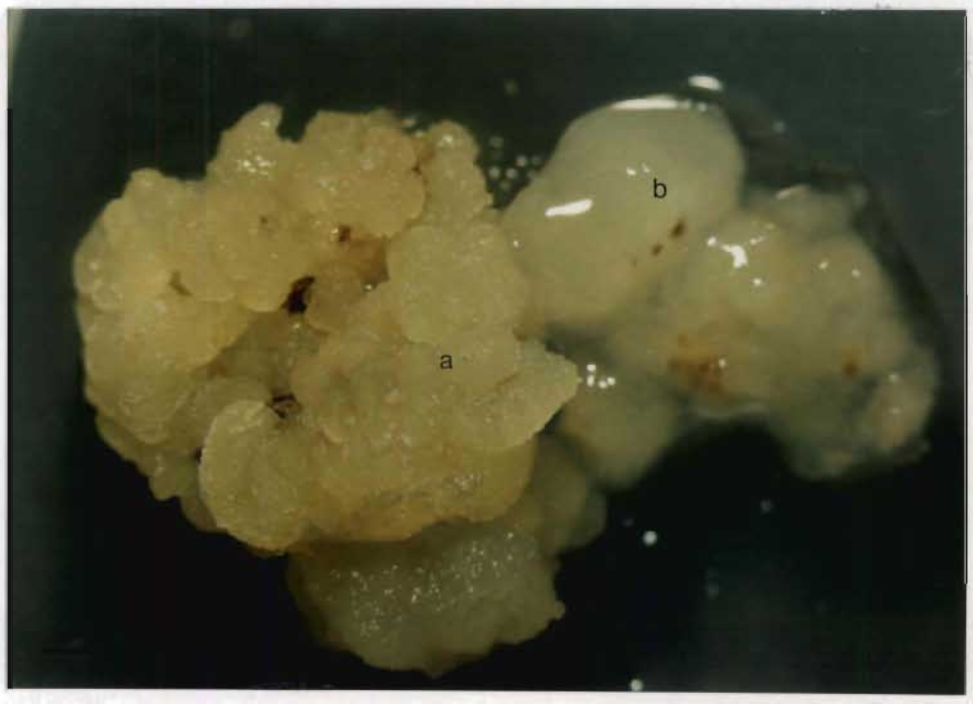


Plate 13 Four month old nodular, cream embryogenic callus (a) and soft, moist non-embryogenic callus (b) of I137TN initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 14 Five week old creamy yellow, nodular callus of K0315Y initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 15 Five week old nodular, cream callus of D0940Y initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 16 Five week old nodular, cream callus of K054W initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 17 Five week old soft, moist callus of F2834T initiated from a young leaf base placed on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Young leaf bases placed on callus initiation medium supplemented with 6% (m/v) sucrose and 3 mg L^{-1} 2,4-D formed callus in a similar fashion to that observed on medium supplemented with 2% (m/v) sucrose. The callus initiated for most inbreds had a soft, friable morphology and was cream in colour. All of the inbreds produced prolific rhizogenic tissue on this callus initiation medium. The roots varied in morphology from long, thin roots with a normal appearance, to short, thick, abnormal root-like structures. During subculture, the roots were excised from the callus; however, new roots were formed in the subsequent 2 wk before the next subculture. As observed on callus initiation medium supplemented with 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D, each inbred produced callus with typical characteristics under these culture conditions (Table 2.4; Plates 18 to 25). Callus initiated on this medium had a much drier appearance than the moister callus produced on medium supplemented with 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D. The frequency of callus formation was determined for both callus initiation media utilized (Table 2.5).

Plate 18 Six week old soft, cream callus (a) of M28Y initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Many roots formed (b) (bar = 1.25 mm).

Table 2.4 Summary of observed typical characteristics of callus formed from young leaf bases of each inbred placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) for 6 wk. Nodular = compact, hard callus.

Inbred	Typical characteristics of callus
M28Y	Hard, compact, nodular green and yellow callus. Small amount of soft, cream callus. Many roots formed; some long and thin, others short and thick with lots of root hairs (Plate 18).
M162W	Soft, cream friable callus with a few green regions. Soft, nodular appearance. Many normal long, thin roots with lots of root hairs. A few short, thick abnormal roots (Plate 19).
S0507W	Soft, cream to grey callus with some long, thin roots of normal appearance (Plate 20).
I137TN	Soft, cream to white callus. Many short, thick, roots of abnormal appearance with callus initiation occurring near the root tip. Many root hairs (Plate 21).
K0315Y	Soft, cream to yellow callus. Many long, thin cream roots (Plate 22).
D0940Y	Soft, cream to grey callus. Lots of short, thick roots (Plate 23).
K054W	Compact, cream to white, nodular callus. A few normal roots with many root hairs (Plate 24).
F2834T	Soft, cream to grey friable callus. Small amount of compact, yellow callus. A few normal roots and root hairs (Plate 25).

Plate 19 Six week old soft, cream callus of M162W initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 20 Six week old soft, cream callus (a) of S0507W initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 21 Six week old soft, cream callus (a) of I137TN initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Many roots formed (b) (bar = 1.25 mm).

Plate 22 Six week old soft, cream callus (a) of K0315Y initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Many roots formed (b) (bar = 1.25 mm).

Plate 23 Six week old soft, cream callus (a) of D0940Y initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8). Many roots formed (b) (bar = 1.25 mm).

Plate 24 Six week old cream, nodular callus of K054W initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Plate 25 Six week old soft, cream callus of F2834T initiated from a young leaf base placed on callus initiation medium (MS modified + 6% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

Table 2.5 Frequency of callus formation from young leaf bases of maize seedlings placed on callus initiation medium supplemented with 2% (m/v) or 6% (m/v) sucrose and 3 mg L^{-1} 2,4-D.

Inbred	Total no. of leaf bases plated on medium with 2% (m/v) sucrose	Frequency of callus initiation on 2% (m/v) sucrose (% leaf bases forming callus)	Total no. of leaf bases plated on medium with 6% (m/v) sucrose	Frequency of callus initiation on 6% (m/v) sucrose (% leaf bases forming callus)
K0315Y	77	34	89	26
S0507W	117	30	71	20
M37W	40	25	0	0
M28Y	174	24	67	12
D0940Y	61	20	12	58
F2834T	31	16	76	8
M162W	176	14	81	20
I137TN	125	14	98	6
K054W	54	6	21	14

The nine inbreds which germinated *in vitro*, produced callus at different frequencies on the two different callus initiation media (Table 2.5). The polarity of the explants on the callus initiation medium did not seem to be important, as was discovered previously (Vasil & Vasil, 1991). On 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D supplemented medium, K0315Y produced callus at the highest frequency (34%), while on medium supplemented with 6% (m/v) sucrose and 3 mg L^{-1} 2,4-D, D0940Y produced callus at the highest frequency (58%). The callus initiation frequencies are low as an explant of c.a. 8 cm in length was sliced into pieces for callus initiation; however, only a small region of this entire explant produced callus. This region was c.a. 1 to 3 cm in length and consisted of the very basal portion of the youngest leaf in the coleoptilar region of the seedling (above the ligule). Previous results have shown that the capacity for initiating callus was indeed limited to a 4 cm zone at the base of young maize leaves (including the leaf base) (Wenzler & Meins, 1986; Vasil & Vasil, 1991), with the youngest 2 to 5 leaves being the best for culture (Vasil & Vasil, 1991). It would therefore appear that the innermost, youngest leaf roll tissue situated close to the apical meristem is the best explant for callus initiation from young maize seedlings. These results support the finding that immature basal leaf sections taken from regions that are temporally and spatially closest to the meristem, are a good source of leaf material with a high embryogenic response for callus initiation (Wernicke & Milkovits, 1984).

Under the callus initiation conditions used, on the two different callus initiation media, each inbred (apart from B0394Y) produced callus with typical characteristics (Table 2.3; Table 2.4). The two inbreds that produced the most vigorous callus growth on both initiation media, were M28Y and M162W. Calluses of these inbreds could be sustained in culture for three to four months longer than any of the other inbreds. On initiation medium supplemented with 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D, embryogenic Type I compact, nodular embryogenic callus was initiated in the inbreds M28Y, S0507W, I137TN, K0315Y, D0940Y and K054W. Type II, friable

embryogenic callus was produced by M28Y, M162W, M37W and F2834T. All of the inbreds also produced non-embryogenic callus interspersed amongst the embryogenic callus. Type I embryogenic callus was produced on callus initiation medium supplemented with 6% (m/v) sucrose and 3 mg L^{-1} 2,4-D, in M28Y and K054W. Friable, embryogenic Type II callus was produced in the inbreds M28Y, M162W, S0507W, I137TN, K0315Y, D0940Y and F2834T. Non-embryogenic callus was also observed interspersed amongst the embryogenic callus. On 6% (m/v) sucrose, however, less callus was produced, with this callus being drier than the moist callus observed on 2% (m/v) sucrose. The callus on 6% (m/v) sucrose was more embryogenic (large globular cells protruding outwards from the callus surface) than that observed on 2% (m/v) sucrose. However, the rhizogenic nature of the callus maintained on 6% (m/v) sucrose made subculture of these callus cultures extremely time consuming. This result follows the observation in the literature, that the selection and maintenance of embryogenic callus, on a high concentration of sucrose, is difficult (Swedlund & Locy, 1993).

2.3.5 Callus initiation from immature embryos of maize

Immature embryos of maize (0.5 to 3.0 mm in length) are an excellent source material for the initiation of embryogenic callus (Hall, 1991; Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992) as the tissue is totipotent and the embryos are amenable to regeneration (Hall, 1991; Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992). Embryos at stage 1 are utilized to initiate embryogenic callus (Sheridan & Clark, 1994). As the embryo at this stage (16 d after pollination; 1 mm or more in length) is very immature and undergoing differentiation, it is an excellent source for callus initiation. Immature embryos used in this study resembled those of stage 1 (Figure 2.1), with the embryo proper and suspensor each comprising approximately equal parts (c.a. 0.25 to 1.5 mm in length).

The initiation of callus from the isolated maize embryos progressed as observed in the literature, i.e. a small zone of epidermal cells proliferate to form friable callus while the adaxial scutellum cells enlarge and degenerate (Vasil, *et al.*, 1985). Therefore, tissue cultures initiated from maize embryos have calli formation predominantly scutellar in origin (Springer, *et al.*, 1979). Within the first 3 d of culture, the immature embryos (light cream in colour) became enlarged, resulting in a dome-shaped scutellum. The surface of the embryos glistened. By the third or fourth day of culture, callus proliferation became visible on the scutellar surface. Subsequently, callus developed on the periphery of the immature embryo and appeared to originate from the scutellar region. Proliferating callus could be seen on the coleorhizal end of the embryo 1 wk after the initiation of cultures (Plate 26). The coleoptile end of the scutellum and the embryo axis were not involved in callus formation. After 8 d of culture, opaque cream, compact embryogenic callus with prominent ridges and furrows became evident. Small green spots and leafy areas were observed on the surface of compact callus initiated under the 12 h light/12 h dark photoperiod. The initiated callus continued to multiply and was subcultured after 2 wk at which time the frequency of callus initiation was calculated (Table 2.6). At the time of subculture, a number of the calli had developed root hairs. These, and the scutellar tissue which had not grown, were excised and the calli were subcultured to fresh medium containing 3 mg L^{-1} 2,4-D (MS-CM medium).

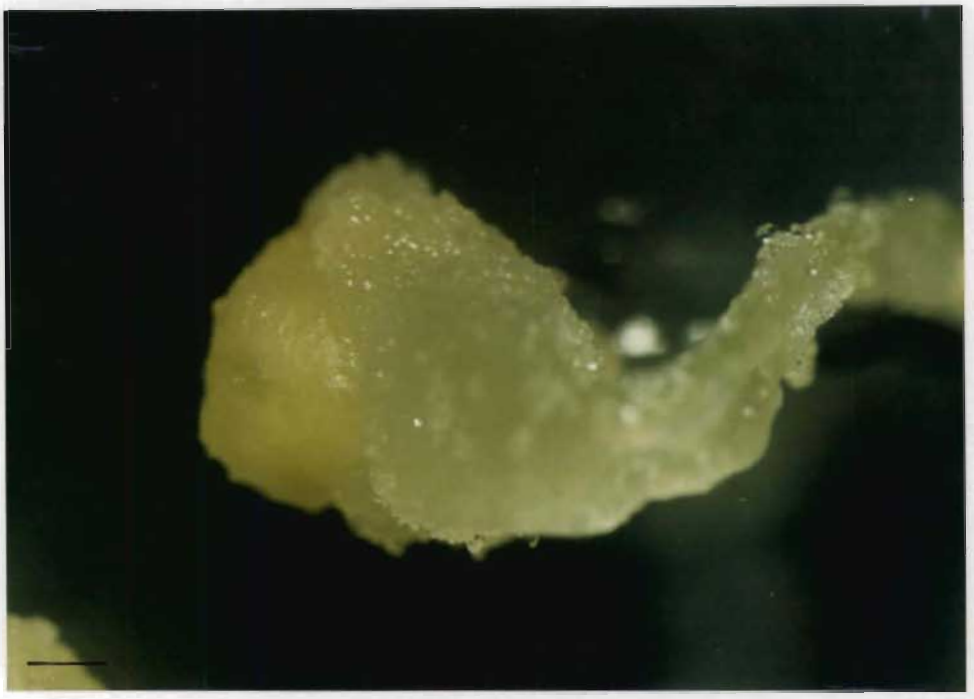


Plate 26 Callus initiation at the coleorhizal end of an F2834T embryo, 1 wk after plating on callus initiation medium (MS modified + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8) (bar = 0.83 mm).

Table 2.6 Frequency of callus initiation from 9 to 15 d old immature embryos of 10 maize inbreds and one hybrid.

Genotype	Total no. of immature embryos isolated	Frequency of callus initiation (% embryos forming callus)
M37W	2	100
I137TN	19	100
M28Y	92	99
D0940Y	74	86
M162W	104	84
PAN 473	537	82
S0507W	93	82
K0315Y	149	74
K054W	270	56
F2834T	146	42
B0394Y	82	37

The 11 genotypes used for the initiation of callus from immature embryos produced callus on initiation medium at varying rates (Table 2.6). As observed in the literature, some genotypes produced callus far more readily than others (Lu, *et al.*, 1982). The inbreds I137TN and M37W appeared to produce callus with the

highest frequency. This result, however, may not be accurate as only a few embryos were isolated from each of the two inbreds (both having low fertility rates), all of which initiated callus. Considerably more embryos would need to be isolated to make the results more comparable. Immature embryos of M28Y initiated callus at almost the same frequency (99%) as I137TN (100%) and M37W (100%). F2834T and B0394Y produced the least amount of callus from immature embryos. The callus formed from these two inbreds was not all that viable and tended to be of a type that could not be used for further experimentation. The frequency of callus formation observed may not be an accurate assessment due to the fact that the frequency of callus formation is dependent upon the size of the embryo. The larger embryos that were isolated tended to germinate and not produce callus, i.e. embryos which were too mature were used from some of the genotypes. Therefore, callus initiation frequencies observed may be an underestimate for some of the genotypes.

The immature embryos used for callus initiation were chosen by size, rather than age, as considerable variability was found in material selected on the basis of age of the embryos. This was also found by other workers (Lu, *et al.*, 1982). Initiation of callus from immature embryos was most effective if the embryo was between 0.5 and 3 mm in length. No embryogenic callus was formed if the embryos were not at the right stage of development (when the embryo axis is about half the length of the scutellum which is rather opaque and containing some starch). Embryos smaller than 0.5 mm were translucent owing to the absence of storage starch, and too small to be seen with the naked eye for isolation. Larger embryos (2 mm) mainly produced compact callus. Alternatively, embryos any larger than 3 mm in length tended to undergo germination rather than callus proliferation. Embryos placed with their scutellar surface in contact with the medium (i.e. embryo axis uppermost) also tended to germinate. Additionally, on these embryos, more callus initiation was initially observed, but this callus development was limited and eventually ceased.

The developmental stage and physiological state of the embryos at the time of excision and culture is extremely important (Lu, *et al.*, 1983). Developmental stage should be determined by embryo length (base to tip of scutellum) rather than by days post-pollination (Armstrong, 1994). In this study it was indeed found that the age of the embryo could not be used effectively as an indication of embryo length. The rate of embryo development has been found to be dependent upon temperature (Sheridan & Clark, 1994). In this investigation, the prevailing season had a profound effect on the size of the embryos. In the spring or summer months the embryos were the right size (c.a. 1 to 1.5 mm) for callus initiation after 15 d. However, in the colder autumn and winter months the embryos took much longer to develop and after 15 d were not even visible to the naked eye. During these months the embryos had to be left on the plant to develop for 10 to 15 d longer. It also became apparent that the size of the embryo was affected by genotype. Inbred maize plants, self-fertilized on the same day as the hybrid PAN 473, tended to have smaller embryos than those of the hybrid PAN 473, i.e. the hybrid embryos developed at a faster rate. The inbreds, however, all had embryos of similar size to one another. It would therefore appear as if an inbred embryo matures at a slower rate than a hybrid embryo.

Under the callus initiation conditions used, callus of typical morphology for each genotype was initiated (Table 2.7). Two types of callus were observed to be initiated from maize immature embryos: a soft, friable yellow to white callus; and a compact, nodular cream to white, more differentiated callus. These two callus types were examined and photographed with a Zeiss Axiophot® microscope. Squashes of soft, friable yellow to white callus showed a loose arrangement of elongated, irregularly shaped, vacuolated non-embryogenic cells (Plate 27) surrounding clusters of small, regularly shaped (isodiametric), densely cytoplasmic embryogenic cells. The cream to white, compact, nodular callus was composed of relatively small, densely cytoplasmic, isodiametric embryogenic cells (Plate 28). Dispersed

amongst the nodules of this second callus type were small leaf primordia which were green if the callus was incubated under the 12 h light/12 h dark photoperiod.

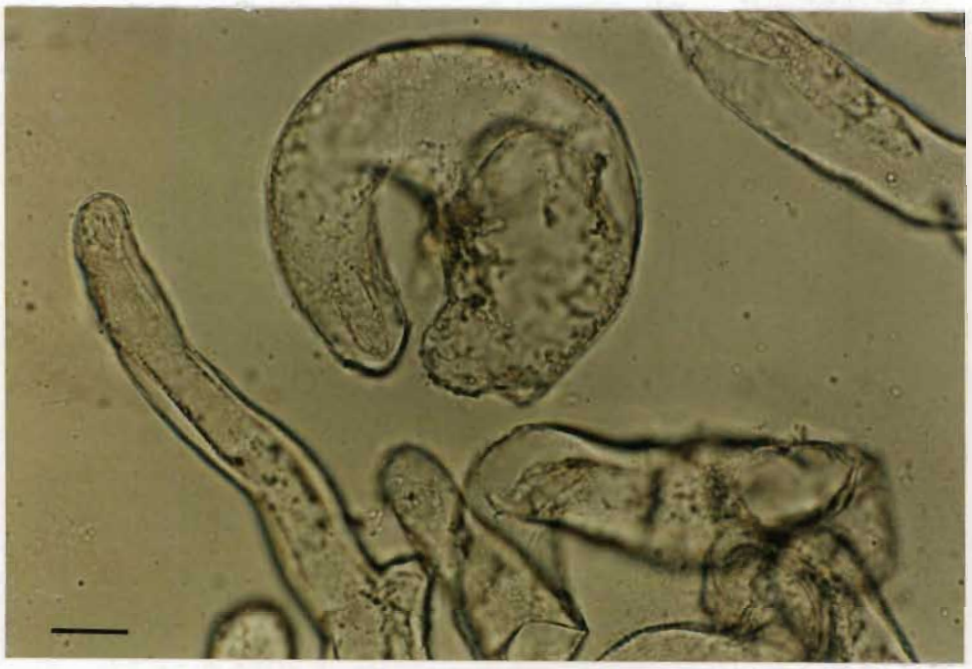


Plate 27 Large, irregularly shaped and elongated, vacuolated non-embryogenic callus cells (bar = 24.6 μm).

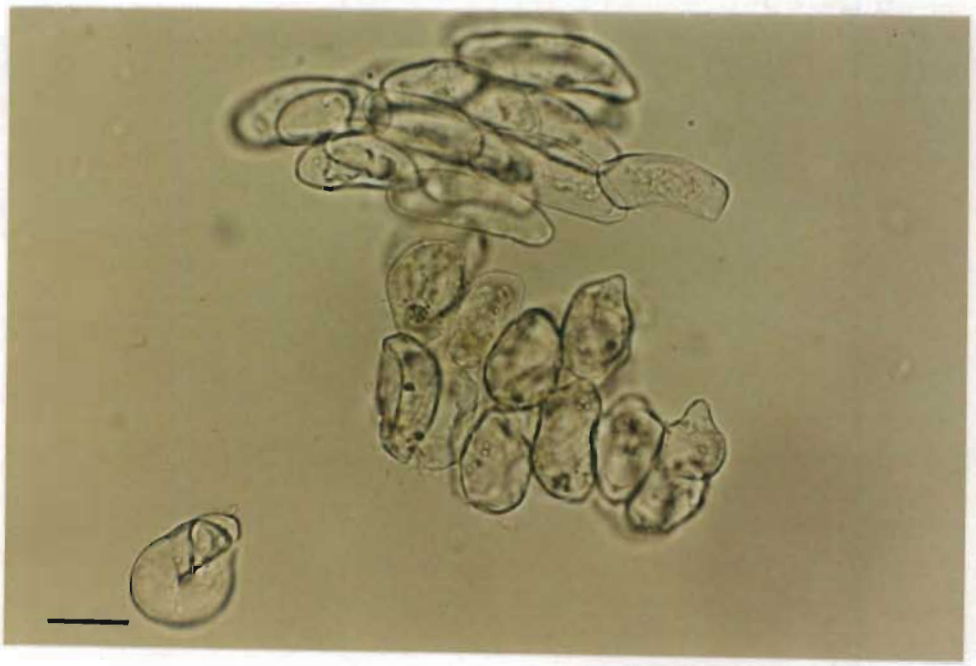


Plate 28 Isodiametric, densely cytoplasmic embryogenic callus cells (bar = 24.6 μm).

Table 2.7 Summary of observed typical characteristics of callus initiated from 9 to 15 d old immature embryos of 10 maize inbreds and one hybrid.

Genotype	Typical characteristics of callus
M28Y	Some compact, hard, nodular yellow callus; some cream moist callus; sugary white friable callus (fastest growing callus) (Plate 29).
M162W	Some cream to yellow compact callus; some soft loose cream to grey callus; some compact moist cream to white callus; cream or white sugary friable callus with some green regions.
F2834T	Not much callus formation; soft, moist cream to white friable callus; tended to go grey and brown (Plate 30).
M37W	Soft moist cream callus that tended to turn grey and then brown; not much callus formation.
I137TN	Sugary white, friable to compact moist callus.
B0394Y	Not much callus formation; soft, loosish cream callus that tended to turn brown and die (Plate 31).
K054W	Compact, nodular, fairly dry cream to white callus with some green areas; scutellum tended to turn brown (Plate 32).
K0315Y	Hard, compact, nodular white to yellow/cream callus; other soft moist cream and grey callus; scutellum tended to turn brown (Plate 33).
D0940Y	Callus formed is very similar to that for S0507W; cream, compact, slimy callus with lots of root formation; some green leaf initials.
S0507W	Some white, moist loosish callus with a few green areas; some compact, very hard, nodular white to yellowish callus; some friable white callus; scutellum tended to turn green (Plate 34).
PAN 473	Some nodular white callus; mostly compact yellowish callus.

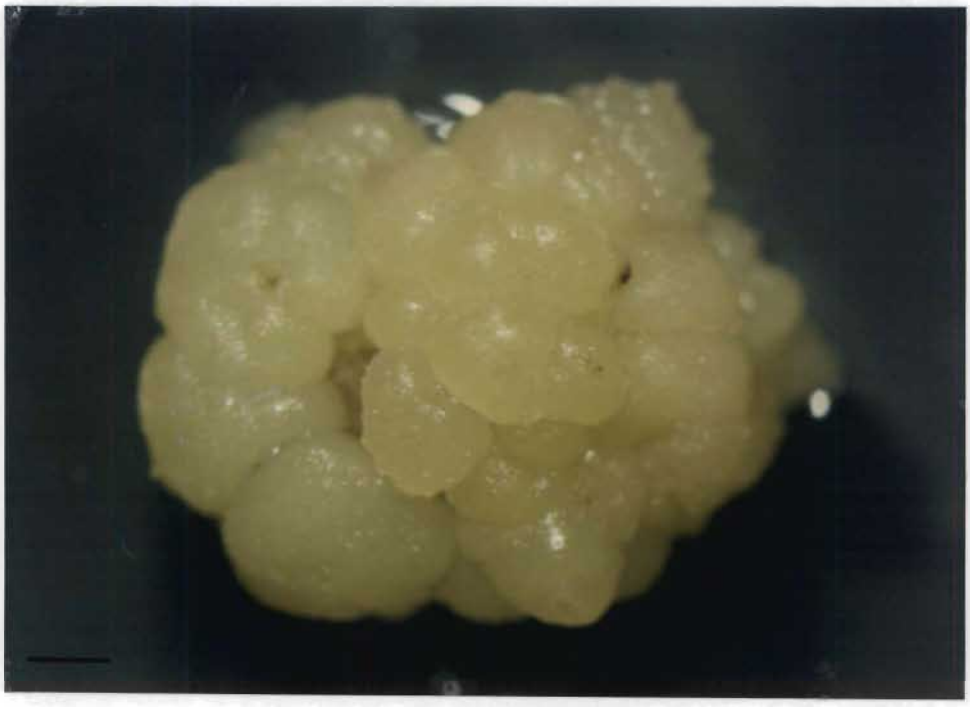


Plate 29 Eight month old compact, nodular cream coloured callus initiated from an immature embryo of M28Y (bar = 1 mm).

Plate 30 Three month old soft, friable white callus initiated from an immature embryo of F2834T (bar = 1.25 mm).

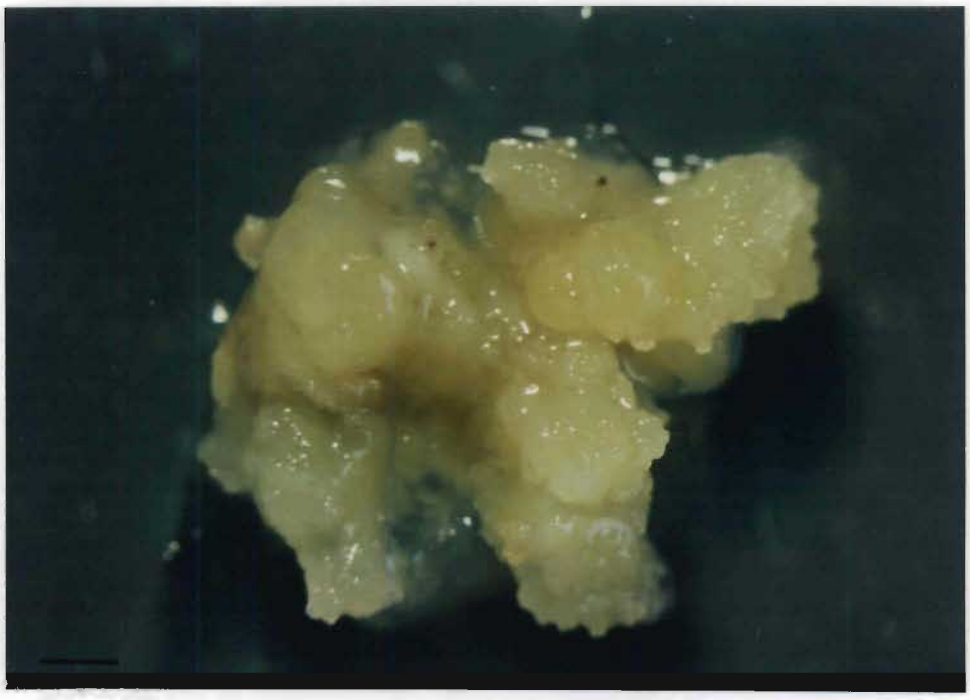


Plate 31 Two month old soft, moist, cream callus initiated from an immature embryo of B0394Y (bar = 1.25 mm).

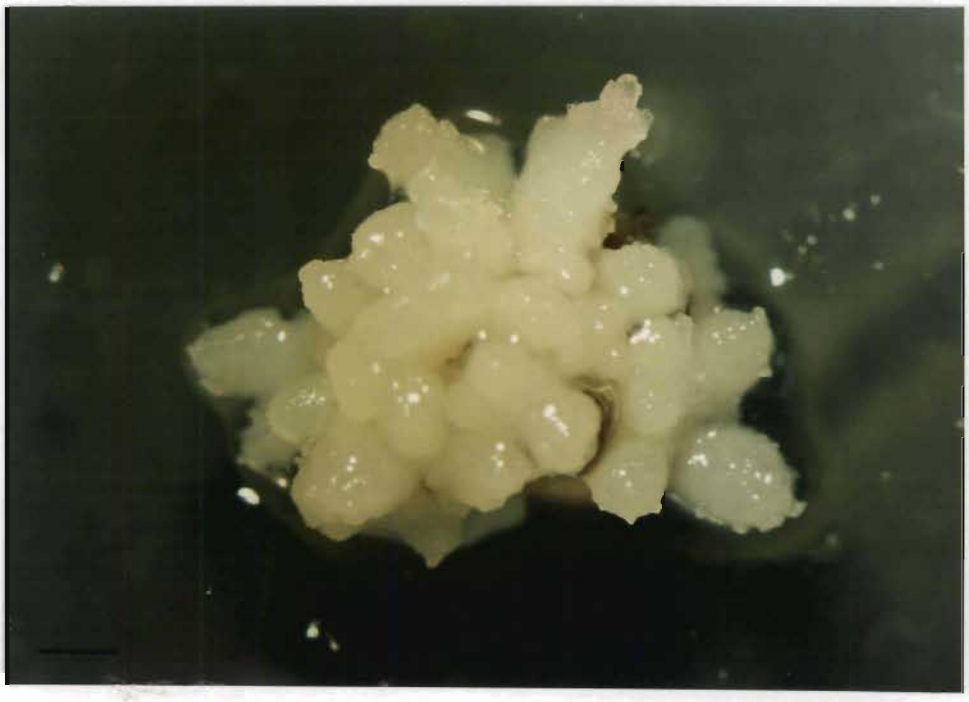


Plate 32 Two month old compact, nodular cream to white callus initiated from an immature embryo of K054W (bar = 1 mm).

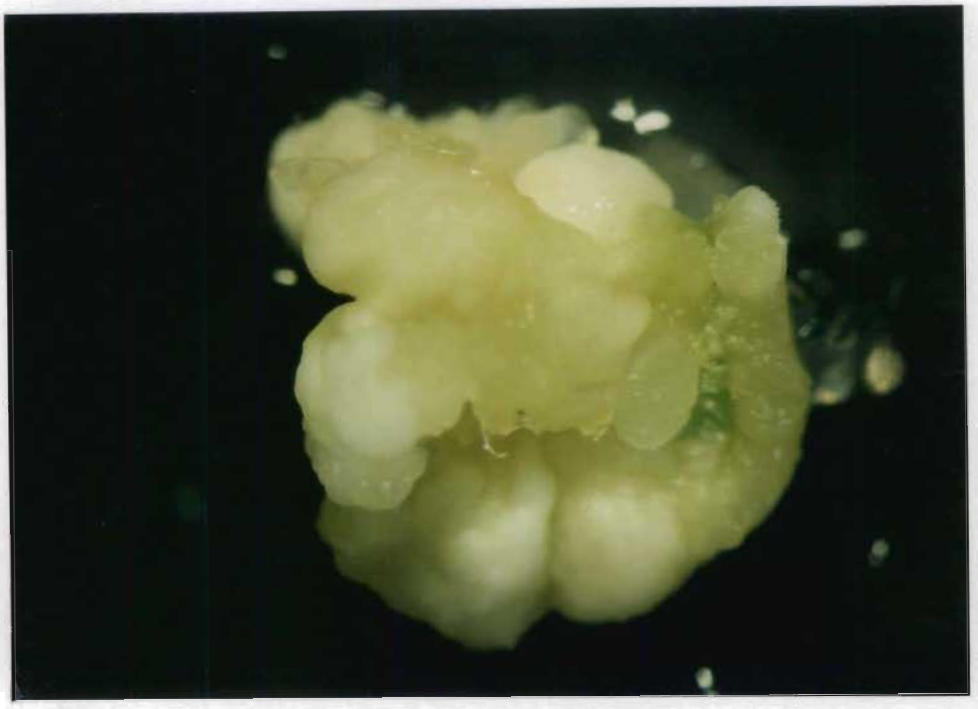


Plate 33 Two month old compact, white to cream callus initiated from an immature embryo of K0315Y (bar = 1 mm).

Plate 34 Two month old friable, white to cream callus (a) (with some nodular white callus (b)) initiated from an immature embryo of S0507W (bar = 1 mm).

Embryogenic Type I callus was initiated in the genotypes M28Y, M162W, I137TN, K054W, K0315Y, D0940Y, S0507W and PAN 473. M28Y, I137TN, K0315Y and S0507W also produced friable embryogenic callus of Type II. All of the genotypes produced non-embryogenic callus interspersed amongst the embryogenic callus. F2834T, M37W, and B0394Y only produced friable embryogenic Type II callus interspersed with non-embryogenic callus.

Embryos incubated in the absence of light gave rise to similar callus to those incubated under a 12 h light/12 h dark photoperiod. The principal difference observed was that the embryos incubated in the dark gave rise to callus lacking in chlorophyll, whereas callus initiated in the light developed green differentiated shoots. Due to the vigorous growth of the callus, frequent subculturing was necessary to refresh the culture medium and prevent necrosis. The calluses maintained in the dark developed at a slightly slower rate than those incubated under light. These calluses were maintained in culture for some time, but the growth rate tended to gradually slow down and eventually stop. Therefore, the embryos were subsequently incubated in the light/dark photoperiod as opposed to dark incubation. During callus maintenance, substantial differentiation was limited by increasing the 2,4-D concentration to 3 mg L^{-1}.

It is known that callus of monocotyledonous plants, in particular maize, does not survive in culture more than a couple of months, unless careful subculture regimes are maintained (Lu, *et al.*, 1982; Vasil & Vasil, 1991; Armstrong, Romero-Severson & Hodges, 1992). The callus initiated from maize immature embryos in this study was maintained on MS-CM medium for a number of months. Gradually the callus started to turn brown and die, possibly due to the exposure to 2,4-D. Some genotypes browned at an earlier stage than others and were discarded when it became evident that the callus was dead. The inbreds kept in culture for the longest time included M28Y and K054W. Callus of M28Y has been maintained in culture for two years. B0394Y, F2834T, M37W and I137TN were

inbreds that tended to lose viability at an early stage in culture. D0940Y, K0315Y, M162W, PAN 473 and S0507W were maintained in culture for longer, but died before M28Y and K054W.

CHAPTER 3
ISOLATION AND CULTURE OF MAIZE PROTOPLASTS

3.1 Introduction

Success in maize protoplast isolation, culture and regeneration is dependent on a wide range of variables. In maize tissue culture, the genotype of the original explant is known to critically affect the success rate in protoplast culture (Potrykus, 1979; cited by Chourey & Zurawski, 1981). It is therefore important to screen a wide source of genotypes to select promising cultivars (Chourey & Zurawski, 1981). The physiological state of donor tissue and its pre-treatment (season, day-length, light intensity, temperature and relative humidity, type of medium, transfer schedule, age) can be a key factor in protoplast isolations (Imbrie-Milligan & Hodges, 1986; Imbrie-Milligan, *et al.*, 1987; Prioli & Söndahl, 1989; Roest & Gilissen, 1989; Sun, *et al.*, 1989). For example, plants may be kept in darkness, prior to enzymatic digestion, to significantly reduce starch content and thus increase the yield of intact protoplasts (Mehrle, Naton & Hampp, 1990). A substantially higher yield and viability of protoplasts is obtained from maize callus (Type I and II) maintained on MS medium rather than N6 medium (Imbrie-Milligan & Hodges, 1986; Lyznik, *et al.*, 1989a). The presence of non-embryogenic cells should be avoided when conducting protoplast isolation experiments (Horn, 1991b).

Many factors need to be considered in the isolation of protoplasts. The first of these is the use of appropriate cell wall degrading enzymes which do not affect protoplast viability. The enzyme type used and its optimum concentration may vary with the plant tissue used as explant source (Kanai & Edwards, 1973b). The isolation conditions (amount of time in enzyme, amount of tissue per volume of enzyme incubation medium, agitation, preplasmolysis of source tissue, tissue type), as well as

purification procedures, affect both protoplast yield and viability (Imbrie-Milligan & Hodges, 1986). Variation in the conditions required for isolation of protoplasts from maize leaves, callus and cell suspension cultures (Table 3.1), emphasises the need to optimize isolation conditions for the specific experimental system utilized. A method for efficient purification of protoplasts, by filtration and/or flotation, from the mixture of undigested tissue, broken protoplasts, chloroplasts and other organelles has to be established for each experimental system (Kanai & Edwards, 1973b).

The quality and age of the cell wall degrading enzyme(s) can have significant effects on the yield and subsequent regrowth of protoplasts (Horn, 1991b; Krautwig, *et al.*, 1994), and on the transient expression activity of transformed maize protoplasts (Krautwig, *et al.*, 1994). Protoplasts are even damaged by highly purified enzyme preparations (Ishii, 1988). The source of the cellulase used in cell wall digestion largely determines subsequent protoplast cell wall synthesis and cell division in culture (Imbrie-Milligan & Hodges, 1986; Dugas, Li, Khan & Nothnagel, 1989). Studies suggest that plasma membrane receptors for certain lectins (Walko, Furtula & Nothnagel, 1987) and carbohydrate-binding antibodies (Knox & Roberts, 1989) are altered by cell wall-degrading enzyme preparations. For the isolation of protoplasts from maize, Onozuka Cellulase R-10, Pectolyase Y23 and Macerozyme® R-10, are the most common ingredients of enzyme solutions (Prioli & Söndahl, 1989; Mitchell & Petolino, 1991), although other enzyme combinations have proven successful (Table 3.2). Unfortunately, however, it has been found that the addition of Macerozyme® R-10 to an enzyme mixture has a negative effect on protoplast yield, plating efficiency and transient expression activity in transformed protoplasts (Krautwig, *et al.*, 1994).

Table 3.1 Conditions utilized by other workers for protoplast isolation from maize leaves, callus and cell suspension cultures[1].

Digestion period	Shaking (rpm)	T (°C)	Centrifugation	Reference[2]
3 h		30	100 g, 10 min	1
4 h	40	30	500 rpm, 2 to 4 min	2
1 h		30	100 g, 5 min	3
17 h		25	100 g, 5 min	4
4 to 12 h		25	100 g	5
2 to 2.5 h	40	26	200 g	6
4.5 to 5 h	50	26	100 g, 5 min	7
4 h	40	21	50 g, 15 min	8
4 h		25	50 g, 15 min	9
3 to 5 h	60	25	400 g, 3 min	10, 11
4 h	70	25	50 g, 15 min	12
3.5 h	60	37	70 g, 5 min	13
1.5 h	50	20	100 g, 8 min	14
3 h	40	25	200 g	15
4 to 5 h	60 to 70	26	40 g, 5 min	16
2 to 3 h	50 to 90	23	600 rpm, 5 min	17
4 h	25	28	70 g	18
5 to 6 h	60 to 70	26	50 g, 5 min	19
5 to 6 h	50 to 60	25	100 g, 3 min	20

[1]Tissue type digested: Leaf: References 1, 3, 4, 10, 11.
Callus: References 8, 13, 15.
Cell suspension cultures: References 2, 5-7, 9, 12, 14, 16-20.

[2]References

1. Bates, Gaynor & Shekhawat, 1983.
2. Chourey & Zurawski, 1981.
3. Devi & Raghavendra, 1992.
4. Evans, Keates & Cocking, 1972.
5. Fennell & Hauptmann, 1992.
6. Fromm, *et al.*, 1987.
7. Huang & Dennis, 1989.
8. Imbrie-Milligan & Hodges, 1986.
9. Kamo, *et al.*, 1987.
10. Kanai & Edwards, 1973a.
11. Kanai & Edwards, 1973b.
12. Lyznik, *et al.*, 1989b.
13. Motoyoshi, 1971.
14. Petersen, *et al.*, 1992.
15. Planckaert & Walbot, 1989.
16. Prioli & Söndahl, 1989.
17. Rhodes, *et al.*, 1988a.
18. Somers, *et al.*, 1987.
19. Sun, *et al.*, 1989.
20. Vasil & Vasil, 1987b.

Table 3.2 Enzymes used by other workers for the isolation of protoplasts from maize.

Enzymes	Concentration range (% (m/v))	Source of protoplasts	Reference[1]
Cellulases			
Onozuka R-10	1.0-5.0	Leaf,callus, suspension	3,4,13,14, 16,20.
Onozuka RS	0.5-3.0	Leaf,callus, suspension	2,6,11,17, 19,21-23, 25-27.
Meicelase	4.0	Leaf	5.
Cellulysin	1.0-2.0	Leaf,suspension	1,7,8.
Cellulase(CEL)	1.0	Callus, suspension	9,10,12, 18,19,24.
Driselase	0.1	Suspension	22.
Cellulase TC	0.66	Suspension	15.
Hemicellulases			
Rhozyme	0.5-1.0	Suspension	2,7,8,19, 23.
Hemicellulase	0.5	Suspension	3,9,20.
Pectinases			
Macerozyme® R-10	0.1-2.0	Leaf,callus, suspension	4-6,11,13-16,25.
Pectolyase Y23	0.02-1.5	Leaf,callus, suspension	6-8,10,11, 15,17-19, 21,22,26.
Pectinol R10	1.0	Leaf	5.
Pectinase	0.25-1.0	Suspension	9,24,27.
Macerase®	1.0	Suspension	26.
Pectinol fest	0.5	Suspension	3,20.

[1]References

1. Bates, *et al.*, 1983.
2. Carswell, Johnson, Shillito & Harms, 1989.
3. Chourey & Zurawski, 1981.
4. Devi & Raghavendra, 1992.
5. Evans, *et al.*, 1972.
6. Fennell & Hauptmann, 1992.
7. Fromm, *et al.*, 1987.
8. Fromm, *et al.*, 1986.
9. Huang & Dennis, 1989.
10. Imbrie-Milligan & Hodges, 1986.
11. Junker, *et al.*, 1987.
12. Kamo, *et al.*, 1987.
13. Kanai & Edwards, 1973a.
14. Kanai & Edwards, 1973b.
15. Maas & Werr, 1989.
16. Motoyoshi, 1971.
17. Neuhaus-Url, Lusardi, Imoberdorf & Neuhaus, 1994.
18. Petersen, *et al.*, 1992.
19. Planckaert & Walbot, 1989.
20. Potrykus, *et al.*, 1979.
21. Prioli & Söndahl, 1989.
22. Rhodes, *et al.*, 1988a.
23. Shillito, *et al.*, 1989.
24. Somers, *et al.*, 1987.
25. Sukhapinda, *et al.*, 1993.
26. Sun, *et al.*, 1989.
27. Vasil & Vasil, 1987b.

Osmolarity and pH of protoplast isolation solutions need to be optimized (Imbrie-Milligan & Hodges, 1986; Roest & Gilissen, 1989). Plant cells have an internal pressure which is contained by the cell wall. To prevent lysis, protoplast isolation is conducted in hypertonic conditions. This leads to plasmolysis and frees the cell wall from its structural role. Both the osmotic agent and its concentration can be critical (Potrykus & Shillito, 1986). An osmoticum such as sorbitol, mannitol or sucrose must be used at levels in which the protoplasts are stable (Kanai & Edwards, 1973b). The osmoticum commonly used for maize protoplasts is mannitol, at concentrations ranging from 0.2 M (4% (m/v)) (Chourey & Zurawski, 1981; Kamo, *et al.*, 1987) to 0.6 M (11% (m/v)) (Motoyoshi, 1971; Imbrie-Milligan & Hodges, 1986). An average concentration of 0.4 M (7% (m/v)) is, however, more commonly used (Huang & Dennis, 1989; Prioli & Söndahl, 1989; Fennell & Hauptmann, 1992). Sorbitol is rarely used as an osmotic agent for maize protoplasts (Evans, *et al.*, 1972; Kanai & Edwards, 1973a; Kanai & Edwards, 1973b; Devi & Raghavendra, 1992).

Competence is described as the ability of a cell to respond in desired ways to specific stimuli during development. This phenomenon is a key factor determining protoplast division, proliferation and regeneration. *In vitro* competence probably has a genetic, developmental and physiological basis, and can apparently be lost during the isolation and culture of protoplasts. Experience suggests that totipotency may not be a general character of plant cells, but is restricted to competent cells (Potrykus & Shillito, 1986).

The method of protoplast culture (discussed in Chapter 1), the culture medium, and the physical incubation environment are additional factors which need to be considered for the successful culture and regeneration of maize protoplasts (Prioli & Söndahl, 1989; Roest & Gilissen, 1989; Sun, *et al.*, 1989). Cell population density affects the protoplasts' division response more than any other experimental variable applied (Potrykus, *et al.*, 1979). A

number of media have been used for the culture of maize protoplasts, and are based on the basic medium on which donor cells were reared (Kuang, *et al.*, 1984). The more commonly used media including: MS based media (Fromm, *et al.*, 1986; Kamo, *et al.*, 1987; Huang & Dennis, 1989); N6 based media (Rhodes, *et al.*, 1988a; Rhodes, *et al.*, 1988b; Sun, *et al.*, 1989; Petersen, *et al.*, 1992; Rhodes & Gray, 1991); modified Nitsch and Nitsch medium (Imbrie-Milligan, *et al.*, 1987); and modified Kao and Michayluk (KM) medium (Vasil & Vasil, 1987b; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989). The N6 based medium, N6ap, has a large positive effect on protoplast growth both during the first and second week of culture (Rhodes, *et al.*, 1988a).

For maize, a pH of 6.0 appears to be best for both protoplast isolation and culture. Calcium concentration affects the formation of cell clusters in maize, and sucrose is the preferred carbon source. Combinations of napthalene-1-acetic acid (NAA), 2,4-D and N^6-benzylamino-purine (BAP) give the best results (Imbrie-Milligan, *et al.*, 1987). The presence of 2,4-D was found to be essential for the induction of division in cultured protoplasts of maize (Vasil & Vasil, 1987b), while the salicylic acid derivative, acetylsalicylic acid (ASA), has been found to promote colony formation from protoplasts (Carswell, *et al.*, 1989; Shillito, *et al.*, 1989). Casein hydrolysate and coconut water are both essential for a high efficiency of microcallus formation from maize protoplasts. The use of amino acids gives mixed results, with most inhibiting cluster formation. Alanine, proline and glutamine, however, increase the production of cell clusters from maize protoplasts, as does the addition of potential cell wall precursors (Imbrie-Milligan, *et al.*, 1987). Most culture media contain myo-inositol, which is beneficial as a component of cell wall metabolism (Gamborg, 1991). Silver nitrate increases the viability of maize protoplasts by providing protection against the action of ethylene (Taylor, *et al.*, 1994), and experimental results have suggested that extracellular calmodulin plays an important role in promoting cell wall regeneration of protoplasts and cell division (Sun, Bian, Zhao,

Zhao, Yu, & Shengjun, 1995). Additionally, electrostimulation (e.g. electroporation) has been used to enhance protoplast division, colony formation and plant regeneration (Ochatt, Chand, Rech, Davey & Power, 1988; Rech, Ochatt, Chand, Davey, Mulligan & Power, 1988; Roest & Gilissen, 1989).

Attempts have been made to substantially increase maize protoplast division frequency and colony formation by modifying the culture conditions (pH, combinations of culture media and osmoticum, light quality and intensity, temperature, hormone variations) (Potrykus, *et al.*, 1979). Protoplast cultures of maize are usually incubated at temperatures of between 26°C (Fromm, *et al.*, 1986; Imbrie-Milligan, *et al.*, 1987; Huang & Dennis, 1989; Sukhapinda, *et al.*, 1993) and 27°C (Vasil & Vasil, 1987b; Shillito, *et al.*, 1989). Cultures are either maintained in the dark (Fromm, *et al.*, 1986; Imbrie-Milligan, *et al.*, 1987; Huang & Dennis, 1989; Sukhapinda, *et al.*, 1993) or under a light regime of 16 h light/8 h dark (Sun, *et al.*, 1989).

Maize protoplasts obtained from Type I and Type II calli from several genotypes were shown to be capable of synthesizing cell walls and forming small clusters of cells (Imbrie-Milligan & Hodges, 1986; Imbrie-Milligan, *et al.*, 1987). Protoplasts isolated from embryogenic callus cultures derived from immature embryos of maize are suitable for analysis of transient gene expression using electroporation-mediated DNA transfer (Planckaert & Walbot, 1989). As derivation of suspension cultures is difficult compared to the establishment of callus cultures from maize embryos (Vasil & Vasil, 1987b), preparation of protoplasts directly from callus would simplify the use of new genotypes for transient gene expression. Results suggest that the time-consuming and often unsuccessful step of deriving cell suspension cultures suitable for protoplast preparation prior to transient expression studies can thus be avoided (Planckaert & Walbot, 1989). Plating efficiency, however, is always extremely poor with embryogenic callus-derived protoplasts (Horn, 1991b). In addition, it is difficult to use compact morphogenic callus

directly for protoplast isolation due to the size and density of the meristematic regions (Ozias-Akins & Lörz, 1984).

3.1.1 Isolation and culture of protoplasts from tissue cultures of South African maize inbreds

The aim of experiments presented in this chapter was to isolate high yields of viable maize protoplasts for use in transformation experiments. During initial experiments, protoplasts were isolated from maize leaf tissue to determine the optimum concentration of mannitol to be used as osmotic agent in subsequent experiments (Section 3.2.1.1). Leaf tissue was used for this optimization as this tissue was readily available in large quantity for protoplast isolation. Conditions for the isolation of protoplasts from maize callus were subsequently established. These conditions included developing the best enzyme combination and concentrations for the isolation of viable protoplasts (Section 3.2.2). Protoplasts isolated from leaf and callus were cultured. Protoplasts isolated from callus cultures were also used in preliminary transformation investigations (Chapter 4).

3.2 Materials and methods

3.2.1 Protoplast isolation from maize leaf tissue for optimization of mannitol concentration and a protoplast culture investigation

3.2.1.1 Optimization of mannitol concentration

Experimental material

Two maize genotypes were used in experiments to determine the optimum concentration of mannitol to be used as osmoticum in subsequent protoplast isolation experiments. The two genotypes

were chosen at random from greenhouse grown plants. These were the inbred M162W and a hybrid PAN 473 (parents: M162W and NPP PNR). Leaf protoplasts were initially isolated from PAN 473 plants (Replicate 1). Subsequently, leaf protoplasts were isolated from PAN 473 (Replicate 2) and M162W plants which had been cultivated two months after the PAN 473 plants used for Replicate 1. Maize protoplasts were isolated from leaf tissue using adaptations of the method used by Dodds and Roberts (1985).

Sterilisation and preconditions

Young healthy, maize leaves were excised from plants that had been grown in soil in the greenhouse (Section 2.2.1). All maize plants were approximately one month old at the time of protoplast isolation, and the second visible leaf (sixth or seventh leaf from the base of the plant) was used for each of the protoplast isolations. Excess dirt was removed from the leaf tissue by brief washing with tap water. The rest of the procedure was carried out on a laminar flow bench.

The leaf tissue was sterilised in hypochlorite-detergent solution (2/3 (v/v) commercial bleach (Jik) and 1% (v/v) detergent or wetting agent (Tween 20)) for 15 to 20 min. The tissue was rinsed three times in sterile distilled H_2O, each rinse lasting 10 min. The bottoms of sterile glass Petri dishes were well covered with CPW salt solution (Appendix B.1) containing mannitol in a range of concentrations (3, 5, 7, 9, 11, 13, 15% (m/v)). Each Petri dish was covered and its weight recorded. In a separate sterile glass Petri dish the leaf midribs and damaged tissue ends (caused by the hypochlorite-detergent solution) were removed with a sterile scalpel. The abaxial surfaces of the leaves were scored in four different directions so as to allow the enzyme solution easier access to the interior tissue for digestion. The leaf tissue was cut into pieces c.a. 1.5 cm in length. In the various Petri dishes, an equal amount of leaf tissue was floated on the CPW salt solution, with the abaxial surface downwards. Each Petri dish was covered and weighed. The mass of leaf tissue digested

in each Petri dish was hence calculated. Leaf tissue was plasmolysed for 1 h in this manner.

Protoplast isolation conditions

After 1 h, the CPW salt solutions (range of mannitol concentrations) were removed from each Petri dish with sterile Pasteur pipettes. The CPW salt solutions were replaced with the enzyme solutions (Appendix B.1), substituted with the required concentrations of mannitol. Ten millilitres of enzyme solution were used per 1.5 g leaf tissue. The Petri dishes were covered, wrapped in aluminium foil and left for 16 h in the dark at room temperature.

After 16 h of digestion, the enzyme solutions were removed with sterile Pasteur pipettes. The tissue pieces were not disturbed during this process. Ten millilitres of CPW salt solution (containing the correct concentration of mannitol) was pipetted into each Petri dish, which in turn was inclined at a slight angle with a piece of cotton wool soaked in 70% ethanol. The leaf pieces were teased apart and the protoplasts gently squeezed out with the aid of two sterile Pasteur pipettes. The resultant solutions, each containing protoplasts and tissue debris, were carefully sieved through pieces of sterile 60 μm Nybolt® mesh (Swiss Silk Bolting Cloth Manufacturing Company Limited, Zurich) into sterile containers. The protoplast solutions were then gently transferred to sterile gradated 15 mL conical centrifuge tubes with the aid of sterile Pasteur pipettes. The tubes were balanced and the protoplast solutions centrifuged for 10 min (50 g) in a Hettich Universal swinging bucket benchtop centrifuge.

Purification and viability determination

The supernatants were removed from each centrifuge tube with sterile Pasteur pipettes, and discarded. The protoplast pellets were gently resuspended (by gentle tilting) in 10 mL CPW salt

solution (with the required mannitol concentration). The suspensions were recentrifuged for 10 min at 50 g. The supernatants were discarded and the protoplast pellets carefully resuspended in 10 mL N6 Protoplast culture medium (N6-PC medium; according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D; pH 5.6. The suspensions were centrifuged for 10 min at 50 g, the supernatants discarded and the protoplast pellets gently resuspended in 5 mL N6-PC medium (Appendix B.1). A drop of each protoplast suspension was placed on a Fuchs-Rosenthal Haemocytometer and examined with an Olympus (CK2) inverted microscope. The number of protoplasts mL^{-1} and the number of protoplasts yielded g^{-1} digested tissue were determined.

The protoplast suspensions were centrifuged for a further 10 min at 50 g. The supernatants were removed and N6-PC medium (Appendix B.1) was added to bring the protoplast suspensions to the required density for culture (1 x 10^6 protoplasts mL^{-1}; Rhodes & Gray, 1991). The viability of each protoplast preparation was determined with Evan's Blue dye. A drop of Evan's Blue dye solution (Appendix B.1) was added to a drop of protoplast suspension on a Fuchs-Rosenthal Haemocytometer and examined with an Olympus inverted microscope. Viable protoplasts excluded the dye and remained green in colour. Dead or damaged protoplasts were not able to exclude the dye and thus appeared blue. The percentage viability of each protoplast solution was determined.

3.2.1.2 Protoplast culture investigation

Subsequent to the optimization of mannitol concentration, leaf protoplasts were isolated and cultured to determine if leaf protoplasts of South African inbreds resynthesize cell walls and divide in culture. Leaves of the 10 inbreds (Table 2.1) were digested according to the procedure outlined in Section 3.2.1.1, with a mannitol concentration of 11% (m/v) as osmoticum. Protoplast suspensions (c.a. 2 mL), at the desired density (1 x 10^6 protoplasts mL^{-1}), were gently poured onto N6 Protoplast

culture medium (N6-PC medium (according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 6 g L^{-1} agar (Oxoid) and supplemented with 11% (m/v) mannitol; pH 5.8; hereafter this medium is referred to as N6(0.6) medium) in Petri dishes. The Petri dishes were covered and sealed with Parafilm "M". Protoplasts cultures were incubated on this medium in the dark (Fromm, *et al.*, 1986; Imbrie-Milligan, *et al.*, 1987; Huang & Dennis, 1989; Sukhapinda, *et al.*, 1993) at 25^{o}C and monitored every second day for signs of cell wall synthesis and division. If necessary (i.e. after c.a. 1 wk in culture), the protoplasts were diluted with a suitable amount of N6-PC medium (according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D; pH 5.8) without mannitol. This would give any dividing cells added nutrients, as well as dilute the mannitol concentration of the medium.

3.2.2 Protoplast isolation from maize callus

3.2.2.1 Pilot study to establish protocol

Experimental material

A pilot study was initially undertaken to establish an efficient protocol for protoplast isolation from maize callus. A number of enzyme combinations and digestion conditions were examined (Table 3.4). Some digests were kept stationary, while others were placed on a rotary shaker operating at a speed of 60 rpm. After digestion, the callus was either immediately observed with the inverted microscope, or teased apart using modified drawn out Pasteur pipettes prior to observation. A vacuum infiltration step was also included, and an alteration in pH of the digestion buffer tested. The various enzymes tested in this experiment were: Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd, Tokyo); Cellulase (Sigma® Chemical Co., St Louis); Macerozyme® R-10 (Yakult Honsha Co., Ltd, Tokyo); Macerase® Pectinase (Calbiochem-Novabiochem Corporation, USA); and Pectinase (Sigma® Chemical

Co., St Louis).

Callus of M28Y was used to establish the enzyme concentration and digestion conditions required for the isolation of protoplasts from maize callus. This callus had been initiated from young leaf bases of seedlings germinated in glass culture tubes (Section 2.2.4). M28Y callus was chosen as it was the most vigorous of all the inbreds, therefore producing a large amount of callus for use in experiments. The callus was green and white, friable and fairly fast growing (Plate 9). Pieces of callus (stained with 1% (m/v) Safranin) were gently squashed under a coverslip on a microscope slide and observed with a Zeiss Axiophot® microscope to ascertain general callus morphology. For protoplast isolation, a more compact callus, with smaller, more regularly shaped cells (i.e. embryogenic in nature), was chosen for enzymatic digestion.

Protoplast isolation conditions

The method of Motoyoshi (1971) was adapted for the isolation of maize protoplasts from callus. Callus used in the isolation of protoplasts was subcultured 1 to 2 wk prior to experimentation. All procedures were carried out on a laminar flow bench. Callus pieces were transferred from MS Callus maintenance (MS-CM) medium (MS modified (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar (Oxoid); pH 5.8) to a sterile disposable Petri dish and sliced up into small pieces (1 to 2 mm thick) with the aid of a sterile scalpel. Callus (0.75 g) was transferred to sterile 50 mm glass Petri dishes. The callus pieces were immersed in 5 mL callus digestion buffer (range of enzyme types at varying concentrations (Table 3.4) dissolved in Wash buffer (Appendix B.2) and filter sterilised through a 0.45 μm Millipore filter). The Petri dishes were covered and wrapped in aluminium foil (Dodds & Roberts, 1985). The callus in digestion buffer was statically incubated for 16 h in the dark at 25^{o}C.

Purification and viability determination

After 16 h, the digested callus pieces were gently teased apart using modified drawn out Pasteur pipettes. In this manner the protoplasts were released. The resultant protoplast/ cell debris suspensions were gently sieved through pieces of sterile Nybolt® mesh (60 μm pore size). The filtrates were collected in sterile 50 mL round-bottom centrifuge tubes. The Petri dishes, in which digestion had taken place, were rinsed out with 5 mL of Wash buffer (Appendix B.2). This rinse was followed by filtration through Nybolt® (60 μm pore size), and the filtrates were added to the protoplast/cell debris suspensions already obtained, bringing their volumes up to 10 mL. The tubes were balanced and centrifuged at 50 g for 10 min in a Hettich Universal swinging bucket benchtop centrifuge. The supernatants were discarded and the protoplast pellets gently resuspended in 10 mL Wash buffer (Appendix B.2). The suspensions was recentrifuged for 10 min at 50 g. This step was repeated. The protoplast pellets were gently resuspended in 2 mL N6-PC medium (according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D; pH 5.8) containing 11% (m/v) mannitol as osmoticum. A Fuchs-Rosenthal Haemocytometer was used to determine the number of protoplasts mL^{-1} and the number of protoplasts yielded g^{-1} digested callus. The protoplast suspensions were centrifuged for 10 min at 50 g and the required number of millilitres of N6-PC medium (11% (m/v) mannitol) (Appendix B.1) was added to dilute the protoplasts to the required density for culture (1 x 10^6 to 2 x 10^6 protoplasts mL^{-1}; Rhodes & Gray, 1991). Evan's Blue dye (Appendix B.1) or Fluorescein diacetate (FDA) (Appendix B.2) were used to determine protoplast viability. For Fluorescein staining, 50 μL Fluorescein diacetate (FDA) solution (Appendix B.2) was mixed with an equal volume of protoplast suspension and viewed within 10 to 15 min of staining. Viewing was done with a Zeiss Axiophot® microscope equipped with an Osram HBO 50 high pressure mercury lamp. The protoplasts were viewed using the Fluorescein isothiocyanate (FITC) filter combination, consisting of the excitation filter BP 450-490 nm,

the barrier filter LP 530 nm and the chromatic beam splitter FT 510 nm. With FDA, viable protoplasts fluoresced apple green when viewed under ultraviolet light. Dead protoplasts did not fluoresce. The percentage viability of each protoplast solution (as determined by FDA) was calculated.

3.2.2.2 Establishment of enzyme concentrations for the isolation of protoplasts from maize callus

Using the established protocol for the isolation of protoplasts from callus (Section 3.2.2.1), experiments were conducted to determine an optimum concentration of enzymes to be used for the isolation of maize protoplasts from callus. M28Y callus (used in Section 3.2.2.1) initiated from young leaf bases of *in vitro* germinated seedlings was used for the experiments. Results obtained from the pilot study (Table 3.4) led to the use of stationary digestion with the enzymes Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd) and Macerase® Pectinase (Calbiochem), followed by teasing apart of digested callus with drawn out Pasteur pipettes (Table 3.3; Enzyme combinations 1 to 6). As plant cells are also known to contain hemicellulose, Hemicellulase (Sigma® Chemical Co., St Louis) was included in the enzyme buffer at the same concentration as the pectinase. Callus was digested with the optimum concentration of Onozuka Macerozyme® R-10 (Yakult Honsha Co., Ltd) to compare this pectinase to Macerase® Pectinase (Table 3.3; Enzyme combination 7). Additionally, Cellulase and Pectinase (Sigma® Chemical Co.) were used at the optimum concentrations, for comparison (Table 3.3; Enzyme combination 8). A purification step using 21% (m/v) sucrose was also included (Enzyme combination 9). For this sucrose purification, the protoplasts were gently resuspended in a 21% (m/v) sucrose solution after the first centrifugation. This suspension was then centrifuged and the top 1 ml pipetted into a clean, sterile centrifuge tube. The remainder or the isolation protocol was then performed.

After 16 h digestion, callus was teased apart in the digestion buffer, with the aid of drawn out Pasteur pipettes. All treatments were teased apart for 10 min, in order for results to be as consistent as possible. The number of protoplast yielded g^{-1} digested callus and protoplast viability were determined for each of the experiments.

Two controls were used for these experiments. The first control involved the incubation of callus in Wash buffer (Appendix B.2) without enzymes, followed by teasing apart with modified Pasteur pipettes. This determined if any protoplasts were isolated by the mechanical treatment. For the second control, callus was incubated in digestion buffer, without any teasing apart of the callus following digestion. This control determined if protoplasts were released by the enzymatic incubation.

Table 3.3 Experimental design for establishment of enzyme type/source and concentration for the isolation of protoplasts from maize callus.

Enzyme combination	Enzyme type and concentration[1]			
	Cellulase	% (m/v)	Pectinase	% (m/v)
1	Onozuka R-10	1.5	Macerase®	0.3
2	Onozuka R-10	2.0	Macerase®	0.4
3	Onozuka R-10	2.5	Macerase®	0.5
4	Onozuka R-10	3.0	Macerase®	0.6
5	Onozuka R-10	3.5	Macerase®	0.7
6	Onozuka R-10	4.0	Macerase®	0.8
7	Onozuka R-10	2.5	Macerozyme® R-10	0.5
8	Cellulase	2.5	Pectinase	0.5
9	Onozuka R-10	2.5	Macerase®	0.5

[1]Hemicellulase (Sigma® Chemical Co.) was used throughout this study at the same concentration as the pectinase used.

3.2.2.3 Culture of protoplasts isolated from maize callus

Protoplasts were isolated from callus of nine inbreds and cultured according to the technique of Rhodes and Gray (1991). The established protocol (Section 3.2.2.1) and the optimum concentration of enzymes (2.5% (m/v) Onozuka R-10; 0.5% (m/v) Macerase®; 0.5% (m/v) Hemicellulase) was used. Protoplast suspensions at the required density for culture (1 x 10^6 protoplasts mL^{-1}) were gently pipetted onto Millipore filters (AABG 047 SO; 0.8 μm; 600 μL protoplast suspension per filter). These filters had been placed on top of a layer of N6(0.6) protoplast culture medium (N6-PC medium (according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D) containing 11% mannitol and solidified with 6 g L^{-1} agar; pH 5.8) in Petri dishes. The Petri dishes were covered and sealed with Parafilm "M". The protoplast cultures were incubated in the dark at 25°C and monitored every second day for evidence of cell wall synthesis and division. After 1 wk of incubation, the filters containing protoplasts were transferred to fresh N6(0.6) medium without mannitol. This would give any dividing cells added nutrients, as well as dilute the mannitol concentration of the medium.

3.3 Results and discussion

3.3.1 Protoplast isolation from maize leaf tissue for optimization of mannitol concentration and a protoplast culture investigation

3.3.1.1 Optimization of mannitol concentration

As the osmoticum commonly used for maize protoplasts is mannitol (Chourey & Zurawski, 1981; Imbrie-Milligan & Hodges, 1986; Kamo, *et al.*, 1987), a range of mannitol concentrations (3 to 15% (m/v)) was used in the leaf protoplast isolation solutions

to determine the optimum mannitol concentration to be used as osmotic agent in subsequent experiments. After isolation and purification, the protoplast suspensions were examined with an Olympus inverted microscope. A very small number of large protoplasts were visible in the solutions containing 3 and 5% (m/v) mannitol. However, these protoplasts appeared stressed due to the low concentration of mannitol. Very few protoplasts were intact, and cellular debris was present in abundance due to lysis of the protoplasts. The majority of the protoplasts appeared to be non-viable (all blue when mixed with Evan's Blue dye), and there were not enough whole protoplasts to determine the number of protoplasts yielded g^{-1} digested leaf tissue and viability percentages. The protoplast solutions containing 7% (m/v) mannitol contained a number of round protoplasts. Cellular debris was also present, but was not as abundant as in the preparations containing 3 and 5% (m/v) mannitol. In solutions containing 9, 11, 13 and 15% (m/v) mannitol, enzymatic digestion yielded many protoplasts. The solutions containing 11 and 13% (m/v) mannitol yielded round, green protoplasts in suspensions with the least cell debris. In solutions containing 15% (m/v) mannitol, cellular debris was apparent amongst green protoplasts. The number of protoplasts isolated g^{-1} digested leaf tissue (Figure 3.1), and the respective viabilities of each protoplast preparation (Figure 3.2), were determined.

Cellular debris, observed in leaf protoplast preparations, was not removed by either filtration or centrifugation. After centrifugation, cellular debris should have remained in solution and been removed in the supernatant. However, this was not the case and an alternative purification procedure for maize leaf protoplasts, e.g. a two-step Percoll (polymer of silica) (Kanai & Edwards, 1973) or a discontinuous (layered) gradient (Bengochea & Dodds, 1986) may have been used. Other South African workers have used a sucrose gradient of 40% for the flotation of maize protoplasts (Watt, 1996; personal communication).

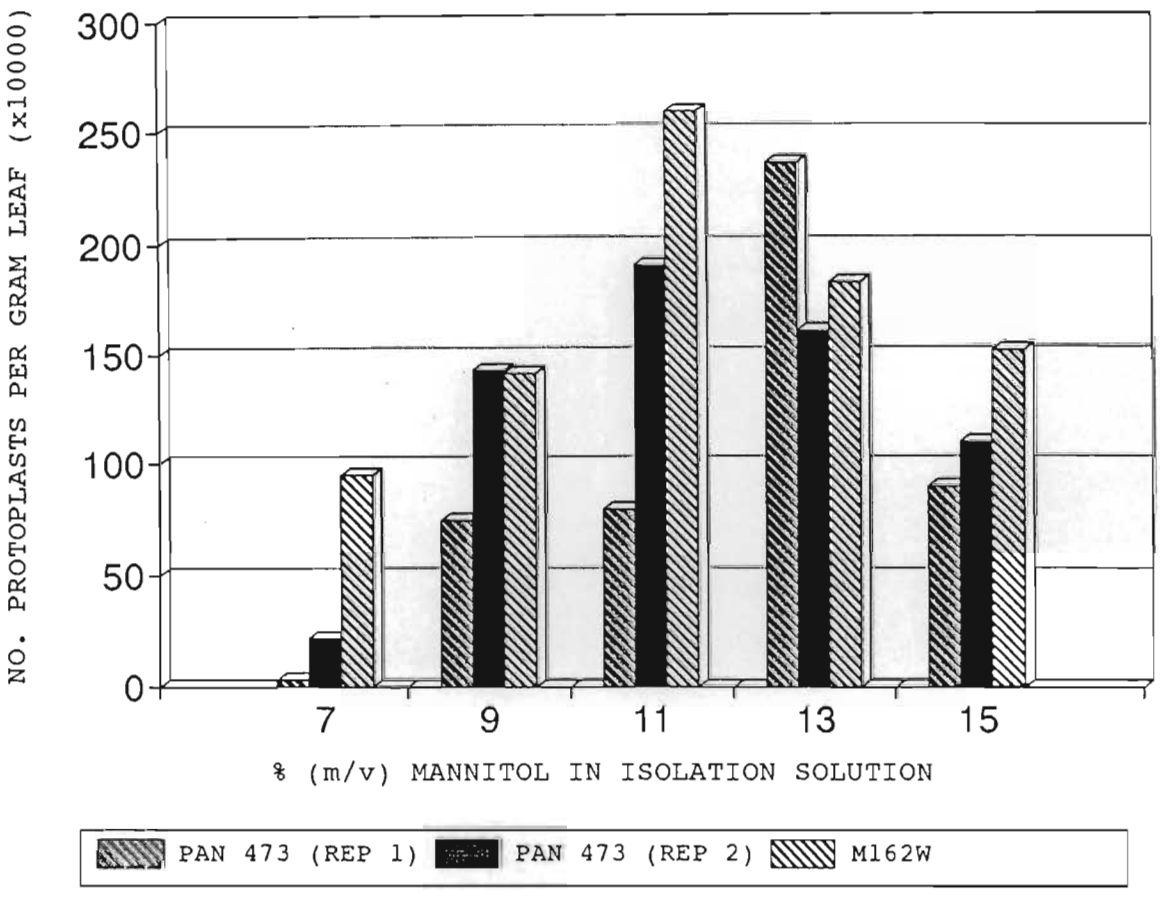


Figure 3.1 Number of protoplast isolated g^{-1} leaf tissue digested for PAN 473 (Replicates 1 and 2) and M162W.

The number of protoplasts isolated g^{-1} leaf tissue digested increased with increasing mannitol concentration, reached a maximum, and then decreased in a hyperbolic fashion (Figure 3.1). For M162W and PAN 473 (Replicate 2), the highest yield of protoplasts was obtained when the mannitol concentration was 11% (m/v). The highest yield of protoplasts for PAN 473 (Replicate 1) was obtained when the mannitol concentration was 13% (m/v). Protoplast yield is affected by osmotic concentrations, as at non-optimum concentrations, protoplasts take up or lose water via osmosis, and consequently lyse (Dodds & Roberts, 1985). Therefore, less protoplasts are obtained.

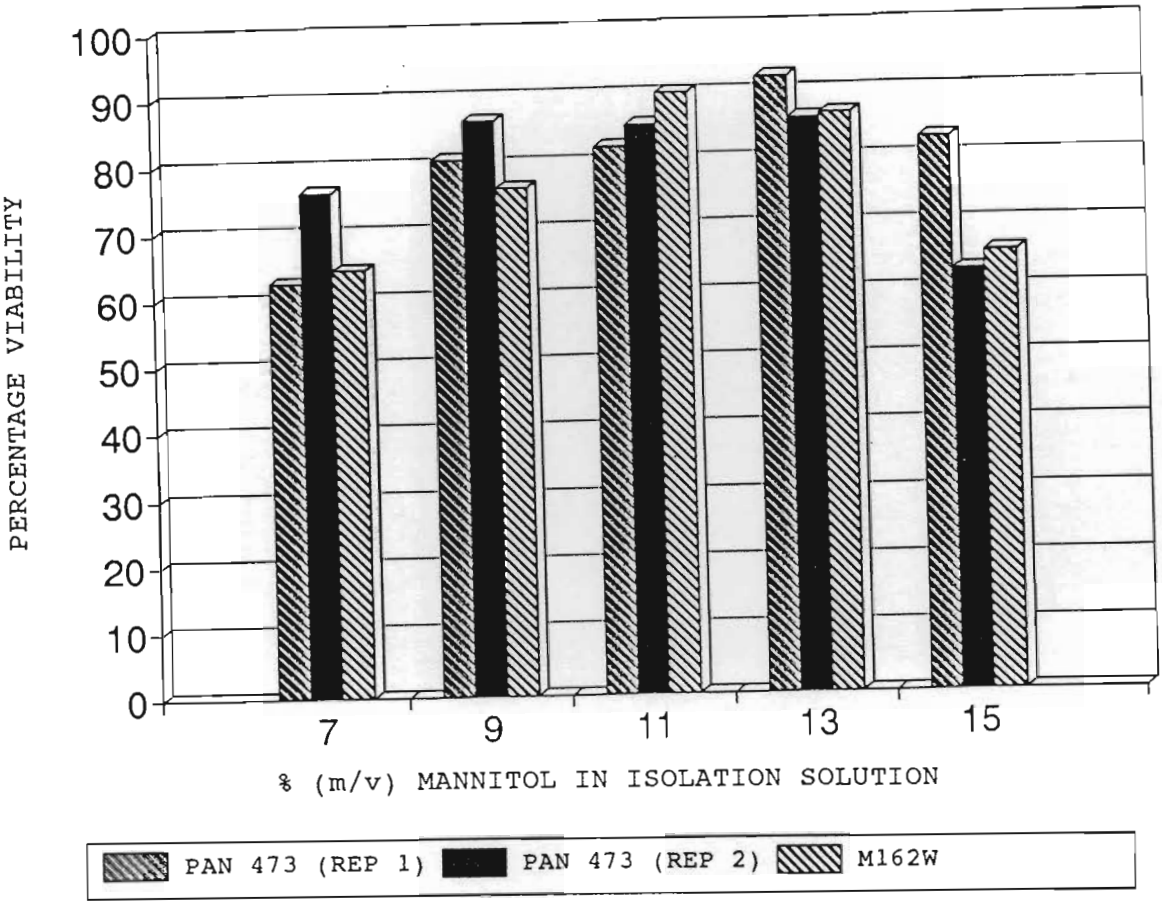


Figure 3.2 Percentage viability of PAN 473 (Replicates 1 and 2) and M162W protoplast preparations as determined by Evan's Blue staining immediately after isolation.

Protoplast viability was also affected by mannitol concentration. A peak in viability was observed when a concentration of between 11 and 13% (m/v) mannitol was used (Figure 3.2). The different results obtained for PAN 473 from Replicates 1 and 2 may be due to the fact that the plants were cultivated two months apart (Replicate 1 in early spring and Replicate 2 in mid-summer), and were consequently exposed to different environmental conditions. The results obtained for the two genotypes (Pan 473 and M162W) do not differ significantly, with both genotypes requiring an optimum of between 11 and 13% (m/v) mannitol as osmoticum. However, optimal mannitol concentration may be genotype specific,

with M162W being one of the parents used to produce the hybrid PAN 473. Additionally, the hybrid was more vigorous than the inbred. However, due to time constraints, and the fact that both genotypes produced similar results, no further optimization experiments for mannitol concentration were conducted.

The results obtained differ from experiments conducted on overseas maize cultivars, which reported 7% (m/v) as the optimum mannitol concentration for the isolation of maize protoplasts (Huang & Dennis, 1989; Prioli & Söndahl, 1989; Fennell & Hauptmann, 1992). South African maize plants have been bred for harsh environmental conditions in particular drought tolerance, and as a consequence have thicker cell walls to prevent the loss of water. Also, they undergo a process of osmoregulation whereby they accumulate solutes in the cytosol thereby maintaining cell turgor despite high evaporative demand under drought conditions. So, when exposed to water, the protoplasts with their high solute concentrations would take up an excess of water leading perhaps to lysis. Consequently, protoplasts of South African maize inbreds require a much higher osmotic concentration to prevent the movement of water into the protoplast, and subsequent lysis. From the results obtained, it would appear as though 11% (m/v) mannitol is the optimum concentration of mannitol for use as an osmotic agent for protoplasts of South African maize inbreds. The fluctuation between 11% (m/v) on the one hand and 13% (m/v) on the other (Replicates 1 and 2 of PAN 473) may, however, point to the fact that the optimum concentration of mannitol is in fact 12%. In conclusion, subsequent protoplast isolation experiments were conducted with 11% (m/v) mannitol as osmoticum.

3.3.1.2 Culture of leaf-derived maize protoplasts

Protoplasts of the 10 inbreds (Table 2.1) were isolated from leaf tissue (11% (m/v) mannitol as osmoticum) and cultured in liquid medium over a layer of solidified protoplast medium. An N6 based protoplast culture medium was used for the culture of maize protoplasts, as this medium has a large positive effect on

protoplast growth, both during the first and second week of culture (Rhodes, *et al.*, 1988a). After 4 d of incubation, a film of white material was observed in the protoplast cultures of the 10 inbreds. This white film occurred at the interface between the solidified medium (bottom layer) and the liquid medium above. When examined with an Olympus inverted microscope, the cultures had a very dark appearance. Many small background particles (chloroplasts and other cellular debris) were observed. In some areas of the cultures the liquid medium appeared to have become dry. After 8 d had elapsed, there was no change in the protoplast cultures. A further 6 d later (2 wk after isolation), still no change was observed. Cell wall formation or cell division had still not occurred. At this time, the protoplast cultures were diluted with 1 mL N6-PC medium (Appendix B.1) without any mannitol. This addition of medium diluted the mannitol concentration, replenished nutrients and maintained the cultures in a liquid state. However, no further changes were noted in any of the cultures, which were eventually discarded.

The white film observed at the interface between the two media phases of the protoplast cultures may be due to precipitation of either the protoplasts and cellular debris, or solutes in the culture medium. Liquid protoplast culture medium which was stored for any length of time tended to develop a precipitate. It is therefore possible that both of these factors contributed to the filmy appearance of the protoplast cultures.

In all of the protoplast cultures, for the 10 inbreds, no cell wall regeneration or cell division occurred. Rather, the protoplasts became aggregated, sank to the bottom of the liquid medium and seemed to become stuck to the upper surface of the agar solidified medium. Protoplasts tend to sink in osmotic solutions containing mannitol (Eriksson, 1985), and it is therefore likely that the protoplasts were unable to respire in the liquid medium. The lack of cell division of leaf protoplasts is not uncommon in maize (Stirn, *et al.*, 1994). The more extended incubation periods which are necessary for the isolation of

cereal-leaf protoplasts may result in the lack of cell division of the protoplasts in culture (Evans, *et al.*, 1972). This may also apply to maize callus protoplasts isolated in this study, which also required a digestion period of 16 h.

3.3.2 Protoplast isolation from maize callus

3.3.2.1 Pilot study to establish protocol for the isolation of protoplasts from maize callus

A pilot study was initially undertaken to establish an efficient protocol for the isolation of protoplasts from maize callus. Immediately after digestion, the callus was either examined with an Olympus inverted microscope, or teased apart using modified drawn out Pasteur pipettes prior to observation (Table 3.4).

Teasing apart of digested callus with drawn out Pasteur pipettes was monitored with the aid of an Olympus inverted microscope. Most protoplasts were released from the friable embryogenic callus consisting of small, regularly shaped cells. Non-embryogenic callus consisting of larger, sausage-shaped cells did not release many protoplasts.

Initially, 4 wk old callus was used for protoplast isolation experiments. No protoplasts were released from this callus for essentially two reasons: due to the age of the callus, the cell walls may have been too thick for the enzymes to digest; and the enzyme concentration may not have been high enough. Consequently, for subsequent protoplast isolations, callus which had been subcultured 1 to 2 wk prior to experimentation was used.

Table 3.4 Summary of observations of experiments conducted in a pilot study to establish the conditions required for the isolation of protoplasts from maize callus.

Expt. condition	Enzymes	Enzyme Conc. %(m/v)	pH	State	Observation
1	Onozuka R-10 Macerase®	1.0 0.2	5.8	Rotating	No protoplasts isolated as seen with the inverted microscope.
2	Onozuka R-10 Macerase®	1.5 0.3	5.8	Rotating	A few protoplasts of variable size isolated - 30-80 μm in diameter.
3	Onozuka R-10 Macerozyme® R-10	1.5 0.3	5.8	Rotating; Teased apart	Teasing released slightly more protoplasts.
4	Onozuka R-10 Macerozyme® R-10 or Macerase®	4.0 1.0	5.8	Rotating; Teased apart	Slightly more protoplasts released.
5	Onozuka R-10 Macerozyme® R-10	4.0 1.0	5.8	Rotating; Teased apart	Vacuum infiltrated. Small number of protoplasts.
6	Onozuka R-10 Macerase®	4.0 1.0	5.8	Stationary; Teased apart	Many protoplasts released after thorough teasing.
7	Onozuka R-10 Macerozyme® R-10	4.0 1.0	5.8	Stationary; Teased apart	Very few protoplasts released.
8	Onozuka R-10 Macerozyme® R-10	4.0 1.0	5.4	Stationary; Teased apart	Very few protoplasts released.
9	Cellulase Pectinase	4.0 1.0	5.8	Rotating	Damage to tissue observed - cells collapsed and lysed.

Low concentrations of enzymes (1.0% Onozuka R-10; 0.2% Macerase®), combined with shaking on a rotary shaker, did not release protoplasts from the callus (Experimental condition 1; Table 3.4). However, the use of a slightly higher enzyme concentration (1.5% Onozuka R-10; 0.3% Macerase®) led to the isolation of a few protoplasts (Experimental condition 2; Table 3.4). The protoplasts isolated ranged in size from 30 to 80 μm in diameter, were densely cytoplasmic and mitochondrial activity was observed in these protoplasts with a Zeiss Axiophot® microscope, providing additional proof of their viability. This technique was, however, very inefficient as many undigested callus cells were present in the protoplast preparation. Therefore, it was decided to gently tease apart the callus after digestion, using modified drawn out pipettes, to release the protoplasts. A different pectinase (Macerozyme® R-10 rather than Macerase®; Experimental condition 3; Table 3.4), and a higher enzyme concentration (4.0% Onozuka R-10; 1.0% Macerozyme® R-10 or Macerase®; Experimental condition 4) were used in conjunction with this teasing. These digestion conditions released a few more protoplasts from the callus. Therefore, the altered enzyme combination and concentration improve the protoplast yield, although slight teasing released slightly more protoplasts.

Due to the poor yields of isolated protoplasts, and the observation that protoplasts were released through gentle teasing of digested callus, it was decided that the enzyme solution was not coming into adequate contact with the cells in the interior of the callus. Therefore, the enzyme solution was vacuum infiltrated into the callus, prior to incubation, to force the enzyme solution into the intercellular spaces of the callus (Experimental condition 5; Table 3.4). The enzyme combination was 4.0% (m/v) Onozuka R-10 and 1.0% (m/v) Macerozyme® R-10. However, vacuum infiltration did not increase protoplast yields, with only a small number of protoplasts being released after the callus was teased apart. The rotating action of the rotary shaker did not lead to foaming of the enzyme solution, which would have

destroyed the enzymes. The rotating motion may have reduced the contact time between the enzyme molecules and the walls of the cells. Therefore, the poor digestion of the callus may be attributed to the rotating motion of the rotary shaker. Consequently, enzyme digestions were kept stationary with two types of pectinase being used at the higher concentration (4.0% (m/v) Onozuka R-10 and 1.0% (m/v) Macerase® in Experimental condition 6; 4.0% (m/v) Onozuka R-10 and 1.0% (m/v) Macerozyme® R-10 in Experimental condition 7). Stationary digestion with Onozuka R-10 and Macerase® (Experimental condition 6) yielded a considerable number of protoplasts after teasing with drawn out pasteur pipettes. However, very few protoplasts were released by stationary digestion with Onozuka R-10 and Macerozyme® R-10 (Experimental condition 7). As the pH of the enzyme solution may have been too high for Macerozyme® R-10, causing it to be inefficient, the pH was dropped to 5.4 in Experimental condition 8. This change in conditions did not yield any more protoplasts. From these results it was concluded that Macerase® was a more efficient pectinase enzyme than Macerozyme® R-10, with a combination of stationary digestion and teasing apart of the callus being efficient treatments to use for the isolation of maize callus protoplasts.

Cellulase (4.0% (m/v)) and Pectinase (1.0% (m/v)) (Sigma® Chemical Co.) were also used for the digestion of callus, to test the effectiveness of these enzymes (Experimental condition 9; Table 3.4). However, digestion with these enzymes resulted in damage to the callus and lysis of the protoplasts. This damage may be due to the use of a very high enzyme concentration, as Cellulase and Pectinase are of a higher purity than the other enzymes used in this study.

In conclusion, various protoplast isolation conditions were chosen for subsequent isolation of protoplasts from maize callus. These conditions included a 16 h static digestion with Cellulase Onozuka R-10 and Macerase® Pectinase in the dark at 25°C, followed by teasing of the callus with drawn out Pasteur

pipettes. Additionally, a mannitol concentration of 11% (m/v) was used as an osmotic agent in subsequent experiments.

3.3.2.2 Establishment of enzyme concentrations for the isolation of protoplasts from maize callus

Using the established protocol obtained from the pilot study (Section 3.2.2.1), experiments were conducted to determine the optimum concentration of enzymes to be used for the isolation of maize protoplasts from callus. Protoplast yields and viability were determined for each of the enzyme combinations (Figure 3.3 and Figure 3.4).

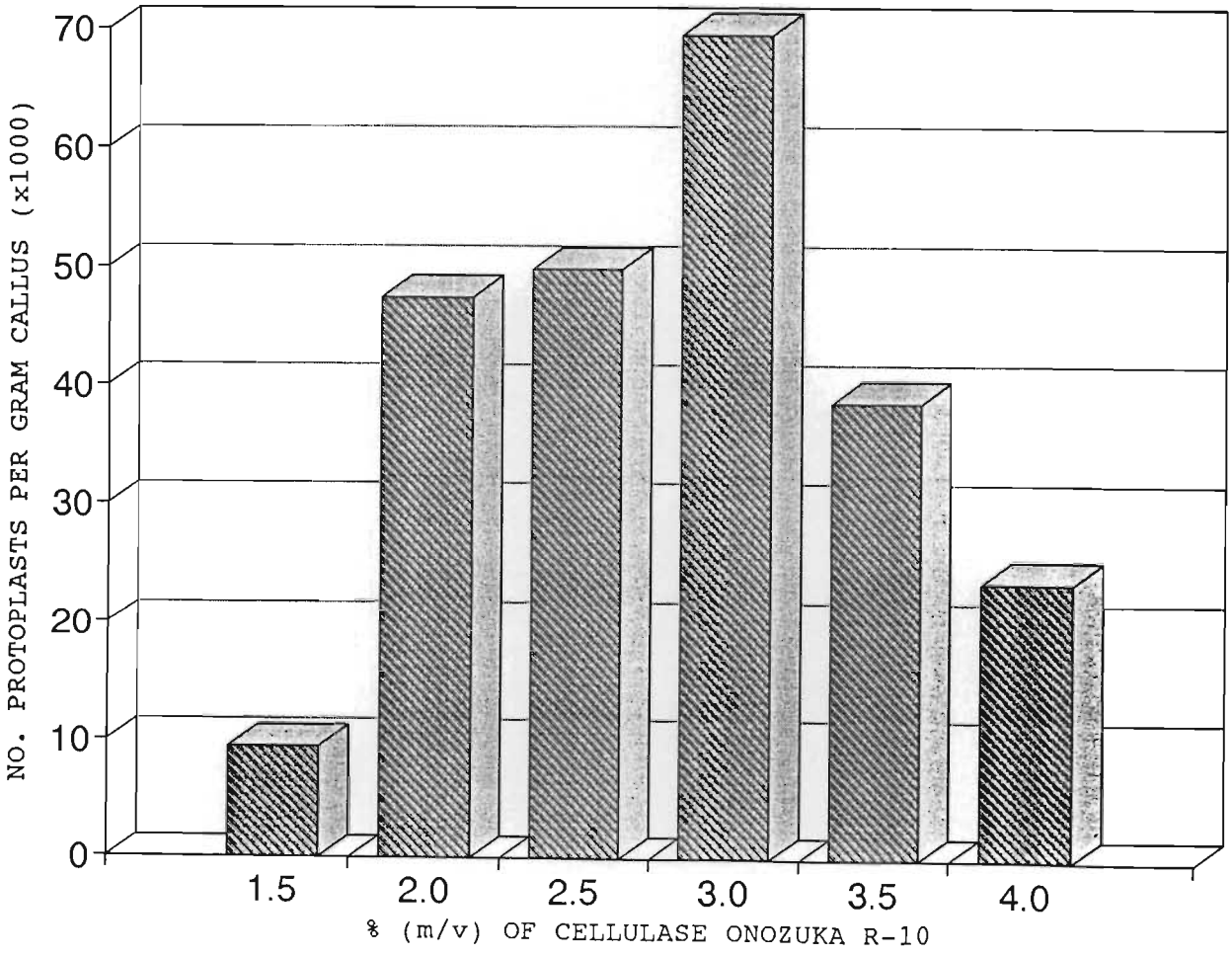


Figure 3.3 Protoplast yield g^{-1} M28Y callus digested by various concentrations of Onozuka R-10 and Macerase® pectinase enzymes.

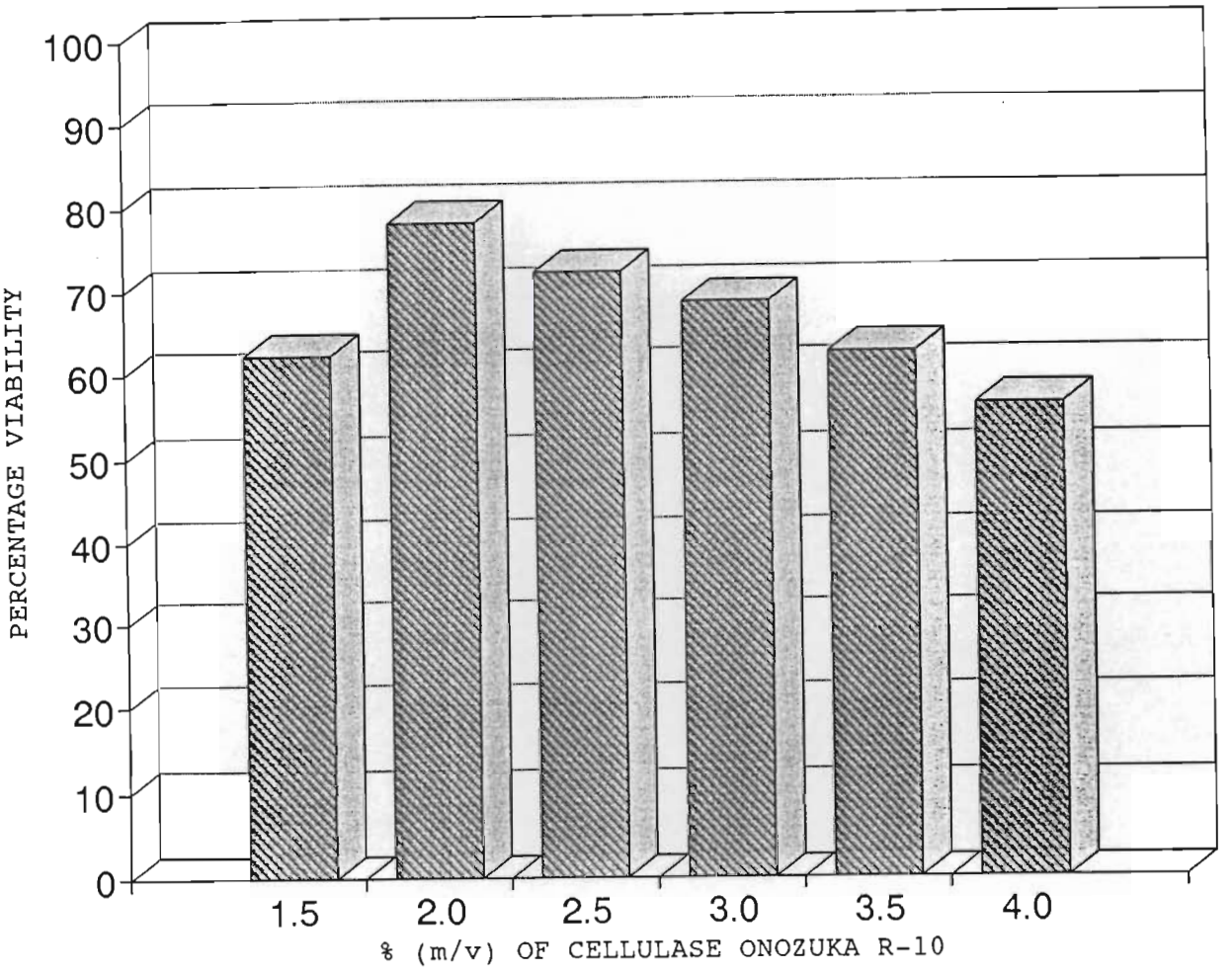


Figure 3.4 Percentage viability of protoplasts (as determined by fluorescein diacetate staining) isolated from M28Y maize callus digested by various concentrations of Onozuka R-10 and Macerase® pectinase enzymes.

Two control treatments (incubation of callus in Wash buffer without enzymes, followed by teasing with Pasteur pipettes; and incubation in digestion buffer without subsequent teasing) were used during this experiment. As expected, no protoplasts were isolated by either of the two controls. However, protoplasts were released when the enzymatic digestion was combined with the mechanical teasing apart of the callus (Enzyme combination 1 to 6; Table 3.3). It was therefore concluded that both enzymatic and mechanical procedures were required for the isolation of maize protoplasts from callus of South African maize inbreds. Cells of South African maize plants may perhaps have thick cell walls as the plants have been bred to withstand harsh

environmental conditions, in particular drought. The thicker cell walls would be more lignified and bound to one another more strongly, and are therefore more difficult for the enzyme solution to digest. Therefore, some form of mechanical action (in this case teasing apart with drawn out Pasteur pipettes) would aid the release of protoplasts from partially digested callus.

After 16 h digestion, 10 min teasing, and purification by centrifugation, all protoplast preparations were examined with an Olympus inverted microscope. Enzyme combinations 1 to 6 yielded differing numbers of protoplasts, with the highest yield (6.94×10^4 protoplasts g^{-1} digested callus) obtained when 3.0% (m/v) Cellulase Onozuka R-10 was used (Figure 3.3). However, protoplast viability was highest (78.1%) when a concentration of 2.0% (m/v) Onozuka R-10 was used (Figure 3.4). As the aim of this experiment was to obtain the highest yield of viable protoplasts, a compromise was made, and subsequent experiments were conducted with the enzyme concentrations used in Enzyme combination 3 (2.5% (m/v) cellulase; 0.5% (m/v) pectinase). In this experiment it was also observed that the higher the enzyme concentration, the more easy the callus was to tease apart. However, these high enzyme concentrations are not economically viable due to the expense of the enzymes. In addition, protoplast viability declined as a more concentrated enzyme solution was used (Figure 3.4).

In each protoplast preparation, a proportion of aggregates of undigested cells was observed amongst the isolated protoplasts (Plate 35). It therefore appeared as if the cell walls were not being digested properly by the pectinase enzyme. The thicker cell wall of South African maize cells could also explain these aggregates of undigested cells. Other researches have also found that washing via sedimentation results in the pelleting of some small undigested cell aggregates (Horn, 1991b). Dead cells, cell fragments with a cell wall, and living protoplasts surrounded by a cell wall could not be removed from the protoplast preparation by the washing procedure, as they sedimented along with the

protoplasts during centrifugation (Motoyoshi, 1971). Consequently the two pectinases, Macerase® and Onozuka Macerozyme® R-10 were compared to determine whether a change in pectinase would yield more protoplasts (Enzyme combination 7; Table 3.3). Both pectinases did not completely digest the cell walls, and both produced similar yields of protoplasts with similar viability percentages (Macerase®: 4.98×10^4 protoplasts isolated g^{-1} digested callus; 72.1% viability; Onozuka Macerozyme® R-10: 3.23×10^4 protoplasts isolated g^{-1} digested callus; 70.5% viability). Therefore, the two pectinases may have been used interchangeably for the isolation of protoplasts from maize callus. However, it has been found that the addition of Macerozyme® R-10 to the enzyme mixture has a negative effect on protoplast plating efficiency and transient expression activity in transformed protoplasts (Krautwig, Lazzeri & Lörz, 1994). Hence, further use of this enzyme was avoided. Other workers used Macerozyme® R-10 for the isolation of protoplasts from maize callus (Motoyoshi, 1971). However, as no yields were published, no comparison of protoplast numbers could be done. The pectinase Pectolyase Y23 has been used for the isolation of protoplasts from maize callus (Imbrie-Milligan & Hodges, 1986; Neuhaus-Url, *et al.*, 1994). Use of this enzyme in further optimization experiments may increase the yield of protoplasts from callus.

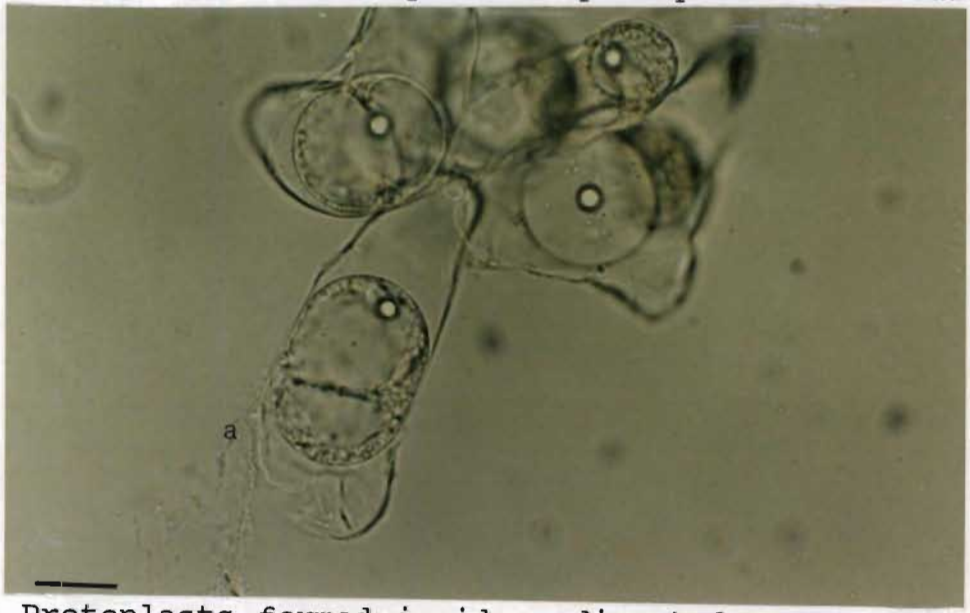


Plate 35 Protoplasts formed inside undigested callus cells of M28Y, with one beginning to squeeze out of the damaged portion of one cell (a) (bar = 24.6 μm).

Due to the inefficient digestion of the cell wall by Macerase® and Onozuka Macerozyme® R-10, a sucrose purification step was introduced in an attempt to eliminate undigested cell aggregates. Centrifugation in a sucrose solution sediments cellular debris, while viable protoplasts float as a layer on top of the sucrose solution. The protoplasts can therefore be gently pipetted from the top of the solution. However, sucrose purification of the protoplast suspensions led to the subsequent loss of all callus-derived protoplasts. It was therefore concluded that the sucrose density used (21% (m/v)) may not have been high enough for the protoplasts to float as a layer on top of the solution. The protoplasts, therefore, remained in suspension after centrifugation, and were subsequently lost. Other researches have also found that attempts to collect maize protoplasts by floating on sucrose did not provide adequate quantitative preparations (Kanai & Edwards, 1973b). This problem may be due to the very high density of embryogenic cells/protoplasts (Horn, 1991b). However, flotation of maize protoplasts on a sucrose gradient of 40% has been used successfully for purification (Watt, 1996; personal communication).

When Cellulase (2.5% (m/v)) and Pectinase (0.5% (m/v)) (Sigma® Chemical Co.) enzymes were used for callus digestion (Enzyme combination 8; Table 3.3), many protoplasts were isolated (1.01×10^5 protoplasts isolated g^{-1} digested callus) with a viability percentage (82.2%) comparable to that observed with the other enzymes used in this study (Figure 3.3 and Figure 3.4). Examination of this digest with an Olympus inverted microscope showed no evidence of undigested cellular material as seen in previous experiments. Instead, a large number of protoplasts were visible amongst considerable cellular debris (Plate 36). Cellulase and Pectinase, therefore, digested the callus far more efficiently than the other enzymes used. Additionally, the callus was very easy to tease apart with the modified Pasteur pipettes. However, during protoplast yield determinations, the large amount of cellular debris present obscured the protoplasts, making yield and viability estimates extremely difficult. The large amount of

cellular debris observed for this enzyme combination may have been due to either over-digestion by the enzymes, or to an inadequate purification protocol. Cell wall degrading enzymes can affect plasma membrane properties (Murray, 1991). Therefore, damage of the cell membrane after 16 h digestion by the highly purified Sigma® enzymes may have led to lysis of the protoplasts. A further experiment was conducted with the same enzyme combination, but using a digestion period of 4 h. However, this digestion period was not long enough, and no protoplasts were isolated. However, due to time constraints, this experiment could not be repeated. It is possible that a digestion period of 6 to 8 h (with fresh enzymes after 4 h digestion) may have improved the protoplast isolation procedure.

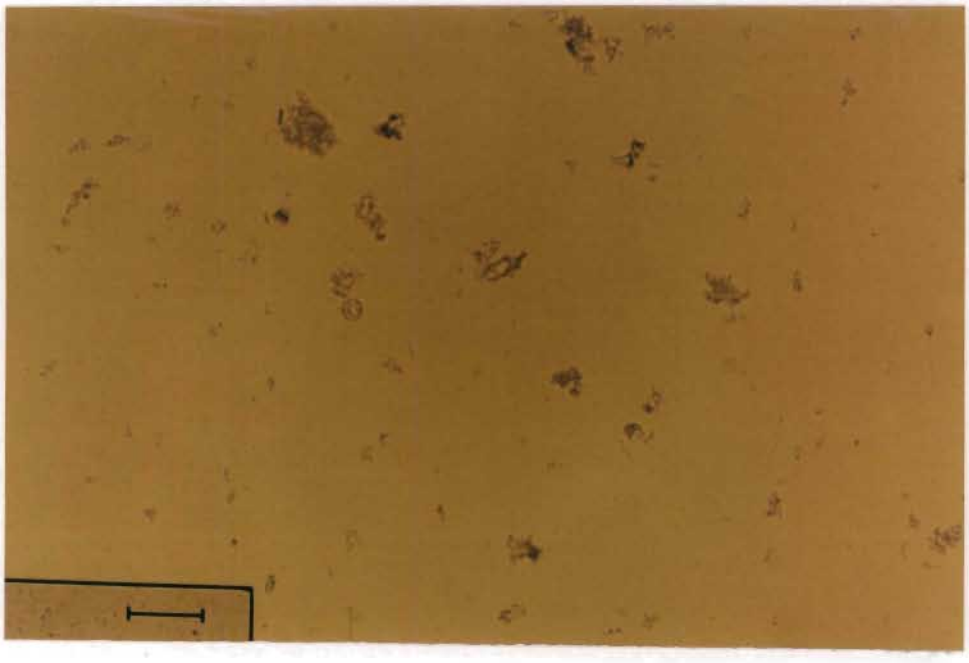


Plate 36 Considerable cellular debris observed in M28Y protoplast preparation after digestion by Cellulase and Pectinase (Sigma®) (bar = 100 μm).

Maize protoplasts isolated from callus ranged in size from 30 to 80 μm in diameter. This is fairly consistent with other investigators, who found protoplast sizes ranging from 20 to 50 μm in diameter (Imbrie-Milligan & Hodges, 1986;

Planckaert & Walbot, 1989). However, isolation conditions differed considerably (Table 3.1), in that a longer digestion period of between 4 and 16 h was required. Protoplast yields were also considerably lower than those reported in the literature, which are of the order of 10^6 (Imbrie-Milligan & Hodges, 1986; Junker, *et al.*, 1987; Planckaert & Walbot, 1989; Neuhaus-Url, *et al.*, 1994). As a 60 μm mesh was used for the purification of the maize protoplasts, any protoplasts larger than this (i.e. up to 80 μm in diameter) would not have passed through the mesh and were hence excluded from the protoplast preparations. This may have contributed to the low protoplast yields experienced. In addition, other researchers have worked with genotypes which provide high protoplast yields (Imbrie-Milligan & Hodges, 1986; Junker, *et al.*, 1987). The yield discrepancy encountered in these investigations may in part also be attributed to the thickness of the cell wall of South African maize tissue. Therefore further optimization of the protoplast isolation protocol is still required.

In conclusion, an efficient protocol for the isolation of protoplasts from maize callus was established. Static digestion of callus with 2.5% (m/v) Callulase Onozuka R-10, 0.5% (m/v) Macerase® Pectinase and 0.5% (m/v) Hemicellulase (Sigma®) for 16 h in the dark at 25°C was followed by teasing of the digested callus with Pasteur pipettes. A mannitol concentration of 11% (m/v) was used as an osmoticum.

3.3.2.3 Culture of protoplasts from maize callus

The established protoplast isolation protocol was applied to callus of a number of inbreds. Nodular, compact callus (e.g. K0315Y and I137TN) was more difficult to digest and was harder to tease apart with drawn out Pasteur pipettes than the softer, more friable callus types (e.g. M162W). Callus of M28Y yielded the most protoplasts g^{-1} callus digested, with callus of F2834T, S0507W and B0394Y yielding the least (Table 3.5). However, yields of protoplasts from the other inbreds used were

generally not much lower than M28Y. Overall, callus derived from immature maize embryos yielded less protoplasts g^{-1} digested callus than callus initiated from young leaf bases of maize seedlings germinated *in vitro*. For example, callus of D0940Y derived from immature embryos yielded 0.99 x 10^4 protoplasts g^{-1} digested callus, while D0940Y callus derived from young leaf bases yielded 1.22 x 10^4 protoplasts g^{-1} digested callus. Protoplast viabilities were generally quite high and ranged from 60 to 80%. However, protoplasts of B0394Y and I137TN had very low viabilities. Callus of these inbreds, used for the isolation of protoplasts, had begun to turn brown (indicating the presence of necrotic cells) after two months of incubation. This could account for the lower viability percentages. M37W callus was not digested, as only a very small amount of callus was obtained during callus initiation experiments. In addition, this callus tended to turn brown in culture, and as such was unsuitable for protoplast isolation.

Table 3.5 Protoplast yield (number of protoplasts g^{-1} fresh mass of callus digested) and viability of protoplasts digested from callus of various maize inbreds. Three replicates were used.

Inbred	Source of callus[1]	Yield (no. of protoplasts (x 10^4) g^{-1} fresh mass callus digested)	Percentage viability
M28Y	Leaf base	4.04	69.4
M162W	Leaf Base	1.64	78.3
K0315Y	Leaf base	1.53	71.1
I137TN	Leaf base	1.22	32.8
D0940Y	Leaf base	1.22	60.0
D0940Y	Embryo	0.99	66.7
K054W	Embryo	2.53	80.4
F2834T	Embryo	0.37	80.0
S0507W	Embryo	0.39	62.2
B0394Y	Embryo	0.68	33.3

[1] Source of callus refers to the source of the explant used for callus initiation (Leaf base = explants taken from *in vitro* germinated caryopses; Embryo = immature embryos used for callus initiation).

Isolated callus protoplasts (at a density of 10^4 protoplasts mL^{-1}; Table 3.5) were cultured on filters over solidified protoplast culture medium. The cultures were monitored every second day, and after 1 wk the filters were transferred to medium without mannitol. Five days after the protoplasts had been isolated, the surface of the filters appeared to have a glittery, grainy appearance (Plate 37). Every second week, the filters were transferred to fresh medium.

Plate 37 Glittery, grainy appearance of M28Y protoplast culture after 1 wk on a Millipore filter (bar = 1.25 mm).

No callus colonies were regenerated from callus-derived protoplasts cultured on filters over solidified medium. The black colour of the filters themselves made microscopic examination extremely difficult. These filters were used to prevent the protoplasts from adhering to the surface of the solidified medium, and to provide better aeration.

Time permitting, various other protoplast culture media and supplements and physical conditions may have resulted in some success with the culture of protoplast isolated from leaf and callus tissue. For example, Nitsch and Nitsch medium (Imbrie-Milligan, *et al.*, 1987), or KM medium (Vasil & Vasil, 1987b; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989) may have been

used with supplements such as coconut water, NAA, BAP and cell wall precursors (Imbrie-Milligan, *et al.*, 1987). In addition, the physical growth conditions under which the maize protoplasts were cultured may not have been ideal. For example, high humidity is usually essential. Some workers place sealed Petri dishes (3 cm in diameter) inside 90 cm Petri dishes containing a layer of wet filter paper. This is then sealed and placed on top of wet cotton wool inside a sealed plastic container (Watt, 1996; personal communication). This increase in humidity may also have contributed to the successful culture of the maize protoplasts, especially those isolated from leaf tissue, as these cultures became dried out.

CHAPTER 4
TRANSFORMATION OF MAIZE TISSUE

4.1 Introduction

Transformed monocotyledonous plants, particularly those in the Gramineae, are urgently required for crop improvement (Kyozuka & Shimamoto, 1991). The inability to accomplish *Agrobacterium*-mediated genetic transformation of the majority of monocotyledonous plants, including maize (Larkin, *et al.*, 1990; Murray, 1991), led to the development of direct gene transfer techniques. Transgenic maize has been successfully produced thorough the use of direct gene transfer techniques such as particle bombardment (Gordon-Kamm, *et al.*, 1990; Aves, *et al.*, 1992; Walters, *et al.*, 1992; Murry, *et al.*, 1993), electroporation of protoplasts (Rhodes, *et al.*, 1988b; Murry, *et al.*, 1993), and electroporation of intact maize tissues including cell suspension culture cells and callus (D'Halluin, *et al.*, 1992; Laursen, *et al.*, 1994).

4.1.1 Electroporation of maize protoplasts and callus

The application of short high-voltage electrical pulses (electroporation), reversibly increases the permeability of cells and protoplasts (Fromm, *et al.*, 1985; Fromm, *et al.*, 1987; Jones, *et al.*, 1987). The creation of pores permits exchange of intracellular and extracellular components, thus enabling transformation with exogenous DNA (Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Rathus & Birch, 1991; Songstad, *et al.*, 1995). Electroporation equipment is based on the discharge of a capacitor (Joersbo & Brunstedt, 1990). A high-capacitance discharge, coupled with a timer circuit, results in an approximately rectangular (square) pulse of microsecond duration, whereas a low-capacitance discharge gives an exponentially

decaying pulse of millisecond duration (Fromm, *et al.*, 1987; Langridge, *et al.*, 1987; Murray, 1991; Rathus & Birch, 1991). Plant protoplasts have been successfully electroporated using both exponential decay and square wave pulse types (Langridge, *et al.*, 1987; Larkin, *et al.*, 1990).

Various variables need to be optimized for efficient electroporation of different cell types (Murray, 1991; Rathus & Birch, 1991). The size and origin of the recipient cells (plant or tissue culture) (Langridge, *et al.*, 1987; Murray, 1991), electrical conditions, and the buffer used for electroporation all need to be optimized (Fromm, *et al.*, 1987; Langridge, *et al.*, 1987; Murray, 1991). Considerable work may, however, be required to define optimal conditions for the particular cell line under study. Furthermore, conditions established in one laboratory do not necessarily work well in another (Sambrook, *et al.*, 1989). The "optimum" conditions determined for any new system will depend on plant genotype, the electroporation equipment, and the order in which parameters are optimized (Rathus & Birch, 1992).

The electrical conditions which need to be optimized include: the type and strength of the electric field used for electroporation; and the number and duration of electric pulses (Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Langridge, *et al.*, 1987; Sambrook, *et al.*, 1989; Rathus & Birch, 1992; Van Wert & Saunders, 1992b). Capacitance is perhaps most important in determining successful DNA delivery by electroporation into intact plant tissues (Songstad, *et al.*, 1995). Other factors for consideration in electroporation experiments are the temperature at which electroporation is conducted (on ice or at room temperature) (Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Langridge, *et al.*, 1987); and the increase in temperature caused by the electrical pulse which may significantly decrease cell viability (Fromm, *et al.*, 1985; Pollard & Walker, 1990). A heat shock treatment at 45^{o}C, followed by cooling on ice, prior to electroporation may increase transformation frequency (Shillito, *et al.*, 1985; Rhodes, *et al.*, 1988b; Rathus & Birch, 1992). The pH and ionic

strength of the electroporation buffer need to be optimized (Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Langridge, *et al.*, 1987; Larkin, *et al.*, 1990; Rathus & Birch, 1992), as gene transfer efficiency increases with pH over the range from pH 5.0 to pH 9.0 (Fromm, *et al.*, 1987). A pH of 8.0 has been extensively used in electroporation buffers (Rhodes, *et al.*, 1988b). The presence of the divalent cations Ca^{2+} (Fromm, *et al.*, 1985) and Mg^{2+} in the electroporation buffer can increase electroporation efficiency (Langridge, *et al.*, 1987; Rathus & Birch, 1992). An electroporation buffer which enhances binding of the foreign DNA to the protoplast membrane could be used. For example, polyethylene glycol, known to facilitate membrane fusion, may increase transformation levels when included in the buffer (Langridge, *et al.*, 1987). The concentration and nature (single- or double-stranded, linear, closed circular or supercoiled) of the nucleic acid to be used for electroporation, as well as the presence or absence of carrier DNA, are additional factors which must be considered (Fromm, *et al.*, 1985; Ecker & Davis, 1986; Fromm, *et al.*, 1987; Jones, *et al.*, 1987; Langridge, *et al.*, 1987; Shillito & Potrykus, 1987; Rathus & Birch, 1992; Van Wert & Saunders, 1992b). Typical DNA concentrations used for electroporation are 10 to 100 $\mu g\ mL^{-1}$ (Fromm, *et al.*, 1987).

Once DNA is introduced by electroporation into the cytoplasm, it may be degraded by endonucleases. Carrier DNA may be helpful in saturating endonuclease activity, thereby increasing the chances of transient and stable expression (Jones, *et al.*, 1987), or may act as a homologous intermediate for recombination of the plasmid with genomic DNA (Shillito, *et al.*, 1985). The use of species specific repetitive DNA sequences as carriers or included in the plasmid vector itself, may increase gene transformation and expression, as well as direct DNA integration to particular loci in the host chromosomes (Jones, *et al.*, 1987).

During protoplast electroporation experiments, protoplast survival generally becomes limiting as other parameters are optimized. Maize protoplasts are more sensitive to high-voltage

electric pulses than, for example, tobacco protoplasts (Huang & Dennis, 1989). "Optimum" conditions are therefore a compromise between increasing the transfer of DNA across the plasma membrane and decreasing protoplast viability (Huang & Dennis, 1989; Murray, 1991). It is difficult to compare transformation frequencies obtained by different authors as calculations are based on different parameters: some on the number of protoplasts treated; others on the number of surviving cells that divide; and still others on the number of dividing colonies that form callus (Huang & Dennis, 1989).

4.1.2 Particle bombardment of maize tissue

Nucleic acids can be delivered directly into intact tissues, cells or protoplasts through the use of high-velocity microprojectiles. Spherical microprojectiles carrying RNA, plasmids, or DNA, are literally shot into the cell by a particle gun apparatus (Primrose, 1991). A variety of devices have been constructed for particle bombardment. Particle gun designs are based on the air rifle (Oard, *et al.*, 1990; Oard, 1993), electric discharge (McCabe, *et al.*, 1988; Christou, *et al.*, 1989; McCabe & Christou, 1993), gunpowder detonation (Klein, *et al.*, 1988a; Klein, *et al.*, 1988b; Klein, *et al.*, 1988c; Klein, *et al.*, 1989a; Gordon-Kamm, *et al.*, 1990), high-voltage electric discharge through a water droplet (Birch & Bower, 1994), helium designs (Sanford, 1991), as well as a micro-targeting gun (Sautter, 1993). Most devices currently utilized use high pressure helium together with consumable material, such as rupture disks, macrocarriers and stopping screens. These devices are expensive and setting up of each bombardment can be complicated and time consuming, especially when many samples need to be bombarded (Brown, Tian, Buckley, Lefebvre, McGrath & Webb, 1994). A simple particle bombardment device (Vain, *et al.*, 1993a) accelerates DNA-coated tungsten particles directly through a partial vacuum by pressurized helium. This new device delivers DNA efficiently, quickly and cost effectively into plant cells (Brown, *et al.*, 1994). Particle bombardment is usually undertaken in a partial

vacuum, as this allows microprojectiles to maintain their velocity over a longer distance due to decreased air resistance (Klein, *et al.*, 1988a).

Numerous variables can be optimised to increase the efficiency of gene delivery by the particle bombardment process. Repeated bombardment of the same sample increases efficiency. Modifications in the number and spatial distribution of particles that impact with the target tissue may increase the number of cells that are penetrated and receive DNA (Klein, *et al.*, 1988a; Klein, *et al.* 1988b). The optimal size and composition of microprojectiles has to be determined empirically and may differ for specific cell types (Klein, *et al.*, 1988a; Oard, *et al.*, 1990; Franks & Birch, 1991; Birch & Bower, 1994; Brown, *et al.*, 1994). Particles that are too small may fail to penetrate certain cells, while particles that are too large may be lethal to others (Klein, *et al.*, 1988a). Microprojectiles with a diameter of 0.8 to 1.2 μm are generally used for particle bombardment of large plant cells (Franks & Birch, 1991). Additional parameters which need to be optimized for efficient particle bombardment include: the distance travelled by DNA-carrying microprojectiles (Klein, *et al.*, 1988a; Brown, *et al.*, 1994), the velocity at which the microprojectiles impact with the cells (Klein, *et al.*, 1988a; Klein, *et al.* 1988b; Franks & Birch, 1991; Birch & Bower, 1994), the helium pressure used to accelerate the particles (Brown, *et al.*, 1994), the vacuum pressure within the sample chamber during bombardment, and the shape of the microprojectiles (Klein, *et al.*, 1988a). The concentration and form (linear or supercoiled) of the plasmid DNA (Klein, *et al.*, 1989a), the optimal method of adsorption of DNA onto the microprojectiles (Klein, *et al.*, 1988a; Klein, *et al.* 1988b; Franks & Birch, 1991; Birch & Bower, 1994; Fromm, 1994), and better ways to optimally distribute and anchor target cells before bombardment, also need to be determined (Klein, *et al.*, 1988a). The potential for expression of the bombarded DNA in the recipient tissue is also important (Franks & Birch, 1991).

Characteristics of target tissue to be used for particle bombardment experiments (e.g. size of target cells and their stage of development) (Klein, *et al.*, 1991) substantially affect both transient expression and stable transformation frequencies (Birch & Bower, 1994; Brown, *et al.*, 1994). For example, organized tissues with thicker cell walls require higher particle velocities for penetration than thin-walled cells from suspension culture. Cells with large vacuoles may be more subject to damage by microprojectiles due to disruption of cellular compartmentation (Birch & Bower, 1994). To reduce or limit the loss of cell cytoplasm through lesions formed by microprojectiles, the turgor pressure of cells may be reduced prior to bombardment (Klein, *et al.*, 1988a).

4.1.3 Reporter genes for transformation studies

Genes which may be conveniently assayed to demonstrate transformation events, are termed reporter genes, and are cloned into suitable vectors for transformation. Almost any small, high-copy-number *E. coli* cloning vector containing an *E. coli* origin of replication, appropriate control sequences and a bacterial selectable marker gene, can be used for transformation. The most common promoters used to drive constitutive expression in plant cells, are nopaline synthase (NOS) (Rathus & Birch, 1991) and the cauliflower mosaic virus fragment (CaMV 35S) containing 400 to 1000 bp of 35S upstream sequence (Omirulleh, *et al.*, 1993). The CaMV 35S promoter gives higher transient expression in monocotyledonous plant cells than the NOS promoter (Fromm, *et al.*, 1985; Howard, *et al.*, 1987; Junker, *et al.*, 1987) or the maize alcohol dehydrogenase (ADH1) promoter (Howard, *et al.*, 1987; Junker, *et al.*, 1987; Planckaert & Walbot, 1989), although the difference between CaMV 35S and ADH1 is not all that marked (Luehrsen & Walbot, 1991; Taylor, *et al.*, 1993). A construct based on the maize ubiquitin gene (Ubi1) increases gene expression levels above that of CaMV 35S and ADH1 (Christensen, Sharrock & Quail, 1992). Therefore, the use of an ubiquitin based plasmid may improve expression in multiple cell types as well as

in several different species of the Gramineae (Taylor, *et al.*, 1993).

Two particularly useful reporter genes for transient assays are the *E. coli* β- glucuronidase (GUS) gene (encoded by the *uidA* locus) and the anthocyanin regulatory genes *C1* and *R* (*Lc* or *B*) (Fromm, 1994). β-Glucuronidase is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides, many of which are available commercially as spectophotometric (p-nitrophenyl glucuronide), fluorometric (4-methyl umbelliferyl-glucuronide i.e. MUG) and histochemical (5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid i.e. X-Glu) substrates. GUS has several useful features which make it a superior reporter gene system for plant studies. Firstly, many plants which were assayed for inherent GUS levels exhibited no or insignificant GUS activity, providing a null background in which to assay chimeric gene expression. Secondly, the presence of GUS is easily, sensitively and cheaply assayed in target tissue. A histochemical assay enables easy and rapid detection of localized GUS activity in different cells and tissues. This assay is, however, destructive, as the product is toxic to plant cells (Jefferson, *et al.*, 1987). The *R* and *C1* anthocyanin regulatory genes induce endogenous maize anthocyanin structural genes, if present, resulting in purple anthocyanin pigmented cells. This anthocyanin response is genotype-dependent, in that all of the anthocyanin structural genes must be present. Most non-pigmented maize genotypes will show anthocyanin pigmentation after transformation (Fromm, 1994). While the GUS reaction product diffuses out and colours multiple cells, the purple anthocyanin pigment is vacuolar and therefore should be restricted to a single cell. In either case, these two reporter genes allow the efficiency of gene transfer to be measured as the number of pigmented cells 2 d after transformation. A dissecting microscope is required to observe anthocyanin pigmented cells. The number of foci obtained reflects both the gene transfer efficiency and the expression of the transferred plasmid (Fromm, 1994).

4.1.4 Transformation of maize tissue of elite South African maize inbreds

The aim of experiments presented in this chapter was to determine whether South African maize inbreds of diverse backgrounds would be amenable to direct gene transfer by electroporation or particle bombardment. Embryogenic callus, and protoplasts isolated from maize callus, were electroporated in the presence of a plasmid encoding the GUS gene and assayed for transient GUS activity using a histochemical GUS assay. Callus was additionally subjected to particle bombardment with tungsten particles bearing a plasmid encoding the GUS gene. Subsequent particle bombardment of callus employed plasmids encoding the *R* and *C* anthocyanin genes. Callus transformed by microprojectile bombardment with the anthocyanin reporter gene construct was placed onto regeneration medium (Chapter 5).

4.2 Materials and methods

4.2.1 Electroporation instruments

Two types of electroporation instrument were used in electroporation experiments. The first electroporator was the Pro-Genetor™ (Hoefer Scientific Instruments, San Francisco; Plate 38). This electroporator used a PG105 ring electrode with a diameter of 10 mm. Samples for electroporation were placed in the wells of a Costar® Tissue Culture Cluster^{24} Dish (Plate 39). The second type of electroporator used was the BIO-RAD Gene Pulser® (Bio-Rad Laboratories, CA; Plate 40) with the BIO-RAD Pulse Controller set on a resistance of ∞ ohms, and the BIO-RAD Capacitance Extender set to 960 μF. This capacitance was used to obtain pore formation as a capacitance of 900 μF has been successfully used for the transformation of maize callus (D'Halluin, *et al.*, 1992). The BIO-RAD electroporator used disposable BIO-RAD Gene Pulser® cuvettes with a gap of 4 mm

between each electrode, and delivered exponentially decaying pulses to the electroporated samples. The distance between the electrodes in the electroporation chamber determines the voltage required to obtain pore formation in the cell membrane according to the formula $E = V/d$, where E is the electric field strength (V cm^{-1}), V is the voltage applied (V), and d the distance between the electrodes in the electroporation chamber (cm) (Langridge, *et al.*, 1987).

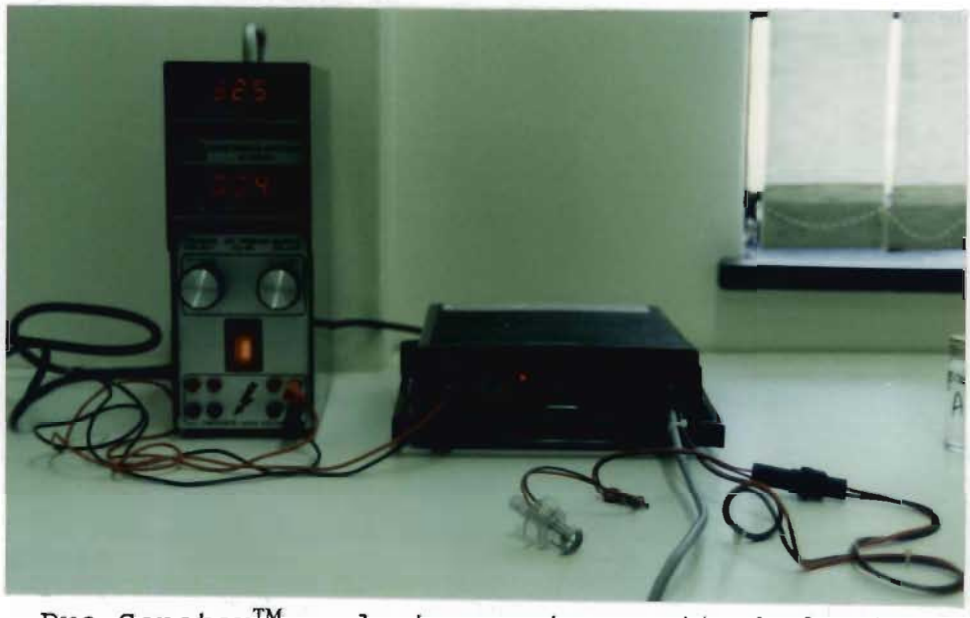


Plate 38 Pro-Genetor™ electroporator attached to DC electrophoresis power supply (PS500X; Hoefer Scientific Instruments, San Francisco) with PG105 ring electrode (foreground).

Plate 39 Samples of maize callus placed in wells of four Costar® dishes in readiness for electroporation with the Pro-Genetor™ electroporator.

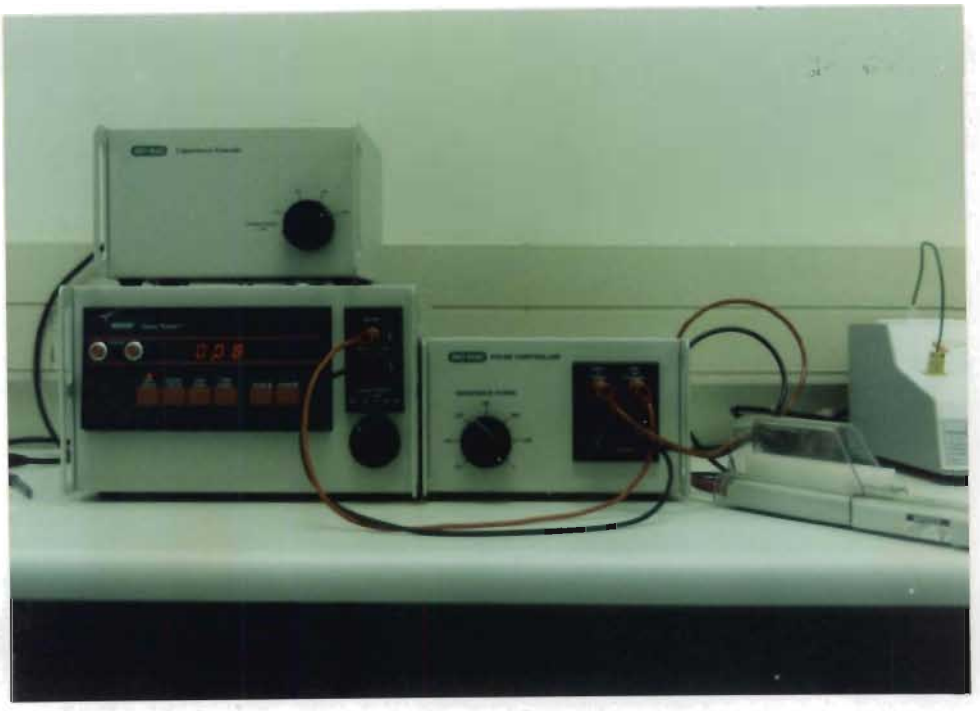
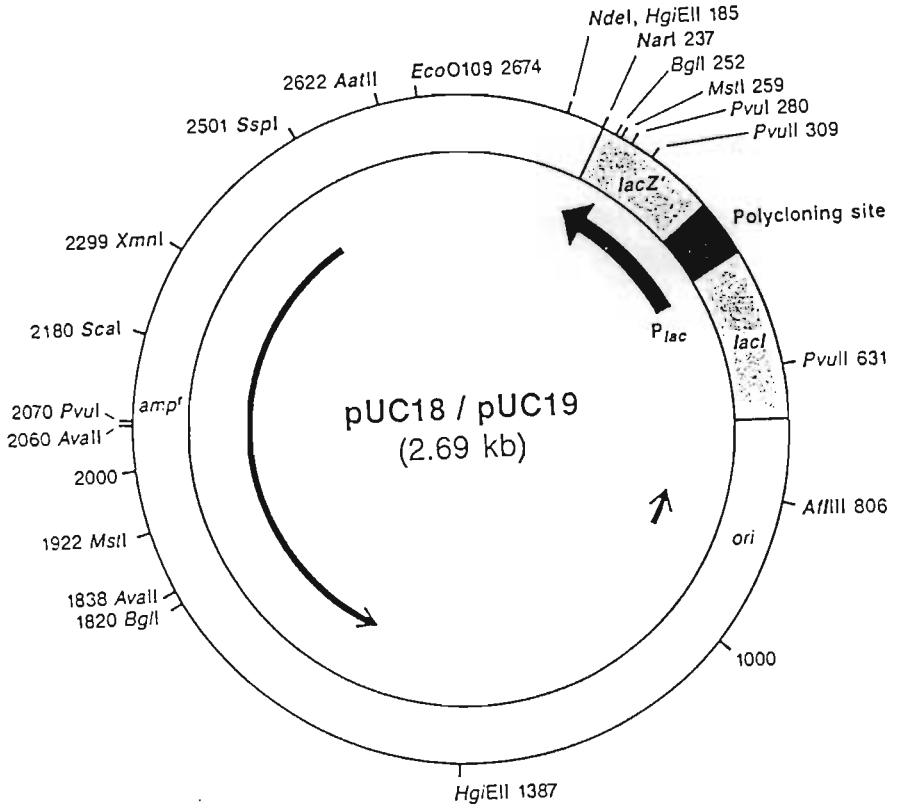


Plate 40 BIO-RAD Gene Pulser® used in electroporation experiments.

4.2.2 Preparation of plasmid DNA for electroporation studies

4.2.2.1 pBI221 plasmid construct

Gene transfer, by electroporation of maize protoplasts and callus, was attempted with the GUS plasmid pBI221 (Clontech Laboratories, Inc., CA). The pBI221 plasmid construct (5.63 kB) is a vector in which the 3.0 kb Hind III to Eco RI fragment of pBI121, containing the CaMV 35S promoter-GUS-NOS poly A site, has been cloned into the corresponding sites of pUC19 (Figure 4.1). This vector therefore facilitates the production of high-yield plasmid preparations for direct DNA expression techniques such as electroporation and protoplast fusion. The pBI221 plasmid construct encodes the GUS gene as well as resistance to the antibiotic ampicillin (Jefferson, 1987).



Polycloning Sites

pUC19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

HindIII *SphI* *PstI* *SalI* *AccI* *HincII* *XbaI* *BamHI* *SmaI* *XmaI* *KpnI* *SacI* *EcoRI*

GUS Gene Fusion Plasmids : pBI121

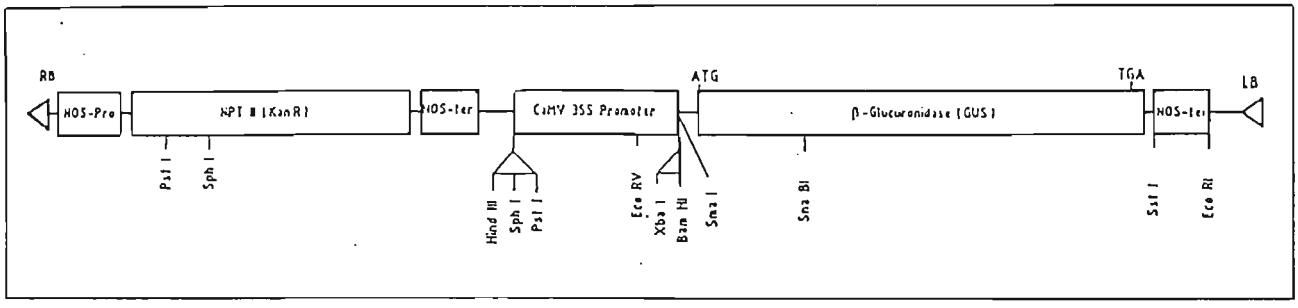


Figure 4.1 Plasmids pUC19 and pBI121, used for the construction of the pBI221 plasmid construct (Sambrook, *et al.*, 1989).

4.2.2.2 Transformation of *E. coli* HB 101 with pBI221 plasmid DNA

To obtain pBI221 plasmid DNA in sufficient quantities for electroporation experiments, the plasmid was transformed (using the method outlined in Sambrook *et al.*, 1989) into the bacterial host *E. coli* HB 101 for amplification. A volume of 5 mL of an overnight culture of *E. coli* HB 101 was pipetted into 50 mL of Luria broth (Appendix C.1) in a 250 mL Erlenmeyer flask. The flask was shaken at 37°C at 150 rpm. The absorbance (A_{540}) of the rapidly growing bacterial culture was measured until an optical density of 0.5 was reached, i.e. late log phase (Sambrook, *et al.*, 1989). The bacterial culture was then chilled on ice. Ice-cold solutions were used for the following procedures. One millilitre of the bacterial culture was aseptically pipetted into each of two separate, sterile 1.5 mL Eppendorf tubes on ice. The Eppendorf tubes were centrifuged in a Beckman minifuge for 2 min at 10 000 rpm to pellet the cells. The supernatants were removed. To each of the two Eppendorf tubes, 400 µL of 0.1 M $MgCl_2$ (Appendix C.1) was added to resuspend the bacterial cells. The Eppendorf tubes were centrifuged for 2 min to pellet the bacterial cells, and the supernatants were removed. The bacterial pellets were resuspended with 200 µL of 0.1 M $CaCl_2$ solution (Appendix C.1). The tubes were left on ice for at least 30 min, to enable the bacterial cells to become competent, i.e. temporarily permeable to small DNA molecules. After 30 min, plasmid pBI221 DNA (3 µL or 1.5 µg) was added to one Eppendorf tube. The other Eppendorf tube served as a control. Both Eppendorf tubes were incubated on ice for 30 min, during which time the plasmid DNA was taken up by the competent cells. Hereafter, pre-warmed solutions were used. Luria Broth (5 mL; Appendix C.1) was pre-warmed at 37°C. The bacterial cells in both Eppendorf tubes were heat shocked at 37°C for 2 min, after which 1 mL of pre-warmed Luria Broth was added. The Eppendorf tubes were then incubated at 37°C for 30 min, allowing the bacteria to recover and express the antibiotic resistance markers encoded by the plasmid.

For the experimental tube (i.e. bacteria + plasmid pBI221 DNA), two Luria Agar plates were required, each supplemented with ampicillin at a concentration of 100 μg mL^{-1} (Appendix C.1). For the control tube, two unsupplemented Luria Agar plates were required to check the viability of the bacterial cells. An additional Luria Agar plate, containing ampicillin at a concentration of 100 μg mL^{-1}, was also required (to check that no control bacteria were initially ampicillin resistant due to the presence of a resistance factor such as is present in the pBI221 plasmid). The transformed bacterial cell suspension (300 μL) was pipetted onto each of the appropriate plates and spread with a flamed, cooled "hockey stick". Similarly, the control bacterial suspension was spread onto its appropriate plates. After all liquid had been absorbed by the plates, the plates were inverted and incubated overnight at 37^{o}C. After 24 h, all plates were examined to determine which plates contained bacterial growth. As the pBI221 plasmid confers resistance to ampicillin, transformed bacterial colonies were picked from their respective Luria Agar plate and subsequently grown at 37^{o}C on Luria Agar supplemented with ampicillin (60 μg mL^{-1}; Appendix C.1). During growth on this medium, the plasmid numbers were amplified.

4.2.2.3 Isolation of amplified pBI221 plasmid DNA from *E. coli* HB 101

Amplified pBI221 plasmid DNA was isolated from *E. coli* bacterial cells using the Magic™ Maxiprep DNA Purification kit (Promega Corp., USA). A transformed *E. coli* HB 101 colony was inoculated into 500 mL Luria Broth supplemented with ampicillin (60 μg mL^{-1}; Appendix C.1) in a 2 L Erlenmeyer flask. The flask was shaken at 37^{o}C overnight at 150 rpm. The bacterial cells were pelleted by centrifugation at 11 000 rpm for 10 min in a Beckman centrifuge (J2-21M; JA 21 rotor). The cell pellet was completely resuspended in 15 mL of Cell resuspension solution (Appendix C.1). Cell lysis solution (15 mL; Appendix C.1) was added. The solution was mixed gently, but thoroughly, by inversion. Cell lysis was complete when the solution had become clear and viscous (c.a. 20 min).

Neutralization solution (15 mL; Appendix C.1) was added and the solution immediately mixed by inverting the centrifuge bottle several times. The solution was centrifuged at 11 000 rpm for 15 min. The cleared supernatant was carefully decanted to a new centrifuge bottle (avoiding the white precipitate). Concentrated Isopropanol (0.6 volumes) was added and the solution mixed by inversion. The solution was recentrifuged at 11 000 rpm for 15 min. The supernatant was discarded and the DNA pellet resuspended in 2 mL TE buffer (Appendix C.1). The Magic™ Maxiprep DNA purification resin was thoroughly mixed and an aliquot of 10 mL added to the DNA solution. The solution was mixed by gentle swirling. For each Maxiprep, one Magic™ Maxicolumn was used. The Maxicolumn tip was inserted into a vacuum manifold. The resin/DNA mix was gently transferred to the Maxicolumn. A vacuum was applied to pull the resin/DNA mix into the Maxicolumn. Column wash solution (10 mL; Appendix C.1) was added to the centrifuge bottle that had contained the DNA/resin mix. The solution was swirled and immediately poured into the Maxicolumn. A vacuum was applied to draw the wash solution through the Maxicolumn. Another 12 mL of Column wash solution was added to the Maxicolumn and a vacuum applied to draw the solution through the Maxicolumn. To rinse the resin, 5 mL of 80% (v/v) ethanol was added to the Maxicolumn and a vacuum applied to draw the ethanol through the Maxicolumn. The resin was dried using a benchtop swinging bucket centrifuge. The Maxicolumn was placed into its reservoir and centrifuged at 2 500 rpm for 5 min. The Maxicolumn was removed and the liquid decanted from the reservoir. The Maxicolumn was replaced in the reservoir and 1.5 mL of preheated (65 to 70^{o}C) sterile distilled H_2O was added. After 1 min, the DNA was eluted from the Maxicolumn by centrifugation at 2 500 rpm for 5 min. The DNA preparation was removed from the reservoir and stored in a 1.5 mL Eppendorf tube at -20^{o}C.

The concentration and purity of the prepared plasmid pBI221 DNA samples was assessed spectrophotometrically using a Beckman Spectrophotometer. Readings were taken at wavelengths of 260 nm

and 280 nm. Using the 260 nm reading, the concentration of the plasmid was determined (an optical density of 1 corresponds to c.a. 50 $\mu g\ mL^{-1}$ for double-stranded DNA, 40 $\mu g\ mL^{-1}$ for single-stranded DNA and RNA, and c.a. 20 $\mu g\ mL^{-1}$ for single-stranded oligonucleotides). The ratio between the readings at 260 nm and 280 nm provided an estimation of the purity of the nucleic acid. Pure DNA and RNA preparations have ratios of 1.8 and 2.0 respectively.

The structure of the pBI221 plasmid preparations was checked by means of agarose gel electrophoresis (Sambrook, *et al.*, 1989). Gel electrophoresis units were placed in a deep freeze 1 h before use. A 0.8% agarose gel solution was prepared (Appendix C.1). The casting tray was levelled, the running plate placed in the casting tray and the well former placed in position. The agarose solution was poured into the casting tray when it had cooled to c.a. 50°C. TBE Running buffer (1X TBE buffer; Appendix C.1) was poured into the gel unit. Once the gel had set, the well former was removed and the gel (in the running plate) placed in the gel unit. Running buffer was added until the gel was covered. The plasmid DNA samples (400 ng) and bacteriophage Lambda molecular weight markers III were loaded into the gel. The electrophoresis gel was run at 100 V for 90 min. As the gel contained ethidium bromide (Appendix C.1), it could be inspected for DNA bands using an ultra violet transilluminator.

4.2.3 Electroporation of maize protoplasts

Maize protoplasts were isolated from M28Y and M162W callus and electroporated using the technique of Rhodes and Gray (1991). Callus of these inbreds had a vigorous growth habit and was therefore used extensively for a number of different manipulations, including electroporation, as large amounts of callus was available. M28Y and M162W callus was initiated from young leaf bases of *in vitro* germinated seedlings (Section 2.2.4). Protoplasts were isolated from callus according to the procedure outlined in Section 3.2.2. After the last

centrifugation step, the protoplasts were gently resuspended in 2 mL N6-PC medium (according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 11% (m/v) mannitol, with the pH adjusted to 8.0. Protoplast yield was determined and the protoplast density adjusted to 3 to 4 x 10^6 protoplasts mL^{-1} for transformation. The percentage of viable protoplasts was determined by staining with fluorescein diacetate (FDA) solution (Appendix B.2).

Aliquots of protoplasts (0.5 mL) were transferred into the wells of a Costar® dish. The number of wells loaded was determined by the number of protoplasts obtained and the size of the experiment. The Costar® dish was placed on ice for 5 min. Simultaneously, a mixture of 75 µL 2 M KCL (Appendix C.2), 0.4 mL N6-PC medium ((according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 11% (m/v) mannitol, with the pH adjusted to 8.0) and 30 to 40 µg pBI221 plasmid DNA, was precooled on ice for 5 min in an Eppendorf tube. A plasmid preparation was prepared in this way for each loaded well of the Costar® dish. After 5 min, the entire contents of an Eppendorf tube was added to an aliquot of protoplasts in one well of the Costar® dish. Immediately after the two solutions had been gently mixed, each well was electroporated on ice. Maize protoplasts were electroporated with the Pro-Genetor™ electroporator, using a range of voltages (100, 200, 300, or 400 V), a pulse of standard duration (50 ms) and a standard amount of pBI221 plasmid DNA (40 µg). Two controls were used for each inbred: incubation of protoplasts with pBI221 plasmid DNA (40 µg), but no electroporation (Control 1); and electroporation (250 V) in the absence of pBI221 plasmid (Control 2). The Costar® dish was left on ice for 10 min, and then transferred to room temperature for a further 10 min. The percentage of viable protoplasts was determined by staining with FDA solution (Appendix B.2). The protoplasts were gently transferred to sterile glass Petri dishes (50 mm diameter) and 2 mL N6-PC medium ((according to Shillito, *et al.*, 1989; Rhodes & Gray, 1991; Appendix B.1) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D

+ 11% (m/v) mannitol; pH 5.8) was added. The electroporated protoplasts were incubated in the dark at 25°C for 24 h (Electroporation strategy 1) or 48 h (Electroporation strategy 2).

After 24 or 48 h incubation, the protoplasts were assayed for transient expression of the GUS gene (histochemical GUS assay modified from Jefferson, *et al.*, 1987). The substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid stock (X-Glu; Sigma® Chemical Co.), is hydrolysed by the β-glucuronidase enzyme expressed by transgenic cells. Samples were kept in the dark at all times during the assay, as the blue coloration obtained for a positive assay is light sensitive. The protoplasts were gently placed into sterile 1.5 mL Eppendorf tubes and centrifuged at 300 rpm for 6 min in a Beckman centrifuge (J2-21M; JA 18.1 rotor). The supernatant was gently removed and the protoplast pellet gently resuspended in 200 µL Fixer buffer (Appendix C.3). The Eppendorf tubes were placed on ice for 3 min and recentrifuged. The supernatant, containing the Fixer buffer, was gently removed. The protoplasts were twice centrifuge-washed with 200 µL sodium phosphate buffer (Appendix C.3) via centrifugation.

After the second wash, the protoplasts were gently resuspended in 160 µL incubation buffer (Appendix C.3) and 40 µL 10 mM X-Glu (Appendix C.3). The Eppendorf tubes were laid flat in an incubator at 37°C, and incubated in the dark for 24 h. After 24 h incubation, the protoplasts were immediately observed with a Zeiss Axiophot® microscope for the presence of blue coloration (an indication of the transient expression of the GUS gene).

4.2.4 Electroporation of maize callus

Embryogenic callus (Type I and II), derived from maize immature embryos (Section 2.2.5) and young leaf bases of *in vitro* germinated seedlings (Section 2.2.4), was used in callus electroporation experiments. Maize callus was electroporated using the technique of D'Halluin, *et al.* (1992). The callus was

immersed in EPM buffer (without KCl; Appendix C.2) in a sterile glass Petri dish (50 mm diameter) and sliced into pieces c.a. 1.5 mm thick with a sterile scalpel. After 3 h preplasmolysis in this buffer at room temperature (25°C), the callus pieces were transferred to electroporation cuvettes, or the wells of a Costar® dish, each cuvette or well containing 600 µL EPM buffer (with KCl; Appendix C.2). Approximately 150 mg of callus fragments was transferred to each cuvette or well. pBI221 Plasmid DNA (10 to 25 µg) was added to each cuvette, or well of a Costar® dish, and co-incubated with the preplasmolysed callus fragments for 1 h.

After 1 h co-incubation, the cuvettes or Costar® dishes, were transferred to an ice bath for 10 min. After 10 min, the callus pieces were electroporated on ice with either the Pro-Genetor™ electroporator, or the BIO-RAD Gene Pulser® electroporator (Table 4.1). Two controls were used for each experiment: electroporation without pBI221 plasmid DNA; and incubation with pBI221 plasmid DNA, but no electroporation. Immediately after electroporation, 300 µL of fresh liquid N6aph Solution (Appendix C.2) was added to each sample. The cuvettes, or Costar® dishes, were incubated on ice for 10 min prior to transfer of the electroporated callus pieces to MS-CM medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8. Electroporated callus was incubated under normal callus maintenance conditions for 24 h, to allow expression of the GUS gene.

After 24 h, a histochemical GUS assay, modified from Jefferson, *et al.* (1987), was used to identify positive transformants. Electroporated callus was removed from MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8.) and placed in the wells of a Costar® dish. The callus pieces were overlaid with 400 µL X-Glu Substrate buffer (Appendix C.3). The Costar® dish was placed inside a vacuum

desiccator and a vacuum of 580 mm Hg was pulled for 15 min. The vacuum was released and the Costar® dish removed from the vacuum desiccator. Triton X-100 (3 μL mL^{-1}) was added to each sample. The Costar® dish was incubated at 37°C in the dark for 24 h, after which the number of developing blue spots was counted. An additional 400 μL substrate (plus Triton X-100) was added. The callus was incubated for a further 24 h to detect any spots developing at a slower rate. Any transformed callus pieces were placed into 95% ethanol, in Eppendorf tubes, for storage at room temperature.

A number of electroporation experiments were conducted on maize callus (Table 4.1). During initial experiments (Electroporation strategies 3 to 5), two different voltages (375 and 450 V), two different concentrations of pBI221 plasmid DNA (10 and 20 μg), a different number of pulses delivered to each sample (one to four pulses), and the two different electroporators (Pro-Genetor™ and Gene Pulser®) were compared. Electroporation strategy 6 employed a range of voltages (200 to 450 V), an increased pBI221 plasmid DNA concentration (25 μg), one pulse of twice the duration of that used in Electroporation strategies 3 to 5 (50 ms), and callus of the inbreds M28Y and M162W. The Pro-Genetor™ electroporator was used for this experiment. This experiment was repeated with the BIO-RAD Gene Pulser® in Electroporation strategy 7. Due to the differing gap size between the electrodes of the two electroporators, different voltages were applied. The field strength applied to each sample, however, was identical for both electroporators. The pulse lengths used for Electroporation strategy 7 were set by the BIO-RAD Gene Pulser®, and thus could not be preset as for the Pro-Genetor™. The pulse lengths applied in Electroporation strategy 7 therefore ranged from 44 ms to 60 ms in length. In addition to these experiments, callus was pretreated to soften the tissue prior to electroporation. Therefore, Electroporation strategies 6 and 7 were repeated with callus pretreated in one of the following ways: callus was incubated in the dark for 24 h before commencement of the electroporation experiment; callus underwent

slight digestion with enzymes prior to preplasmolysis; or callus was incubated in the dark for 24 h prior to enzymatic digestion. For enzymatic digestion, callus pieces were incubated in Enzyme solution (Appendix C.2) for 5 min after which the Enzyme solution was removed with sterile plugged Pasteur pipettes. The enzymatically treated callus pieces were washed in N6aph Solution and then preplasmolysed according to the procedure for undigested callus.

Table 4.1 Experimental design for the electroporation of maize callus.

Electroporation strategy	Inbred	Electroporator	DNA (μg)	Pulse length (ms)	Pulse no.	Volt. (V)
3	K054W	Pro-Genetor™	10	25	1	375
	M28Y	Pro-Genetor™	10	25	1	375
4	M28Y	Pro-Genetor™	10	25	2	375
	M28Y	Pro-Genetor™	10	25	2	450
	M28Y	Pro-Genetor™	20	25	2	375
	M28Y	Gene Pulser®	10	67	2	375
5	M28Y	Pro-Genetor™	10	25	2	375
	M28Y	Pro-Genetor™	10	25	3	375
	M28Y	Pro-Genetor™	10	25	4	375
	M28Y	Pro-Genetor™	10	25	3	450
6	M28Y	Pro-Genetor™	25	50	1	200
		Pro-Genetor™	25	50	1	250
	and	Pro-Genetor™	25	50	1	300
		Pro-Genetor™	25	50	1	350
	M162W	Pro-Genetor™	25	50	1	400
		Pro-Genetor™	25	50	1	450
7	M28Y	Gene Pulser®	25		1	80
		Gene Pulser®	25		1	100
	and	Gene Pulser®	25		1	120
		Gene Pulser®	25		1	140
	M162W	Gene Pulser®	25		1	160
		Gene Pulser®	25		1	180

4.2.5 The Particle Inflow Gun (PIG)

The Particle Inflow Gun (PIG) (Finer, *et al.*, 1992), used throughout this study, was based on helium propulsion of microprojectiles in a vacuum chamber (Figure 4.2). The PIG was constructed locally at the South African Sugar Association Experiment Station (SASEX), Mount Edgecombe.

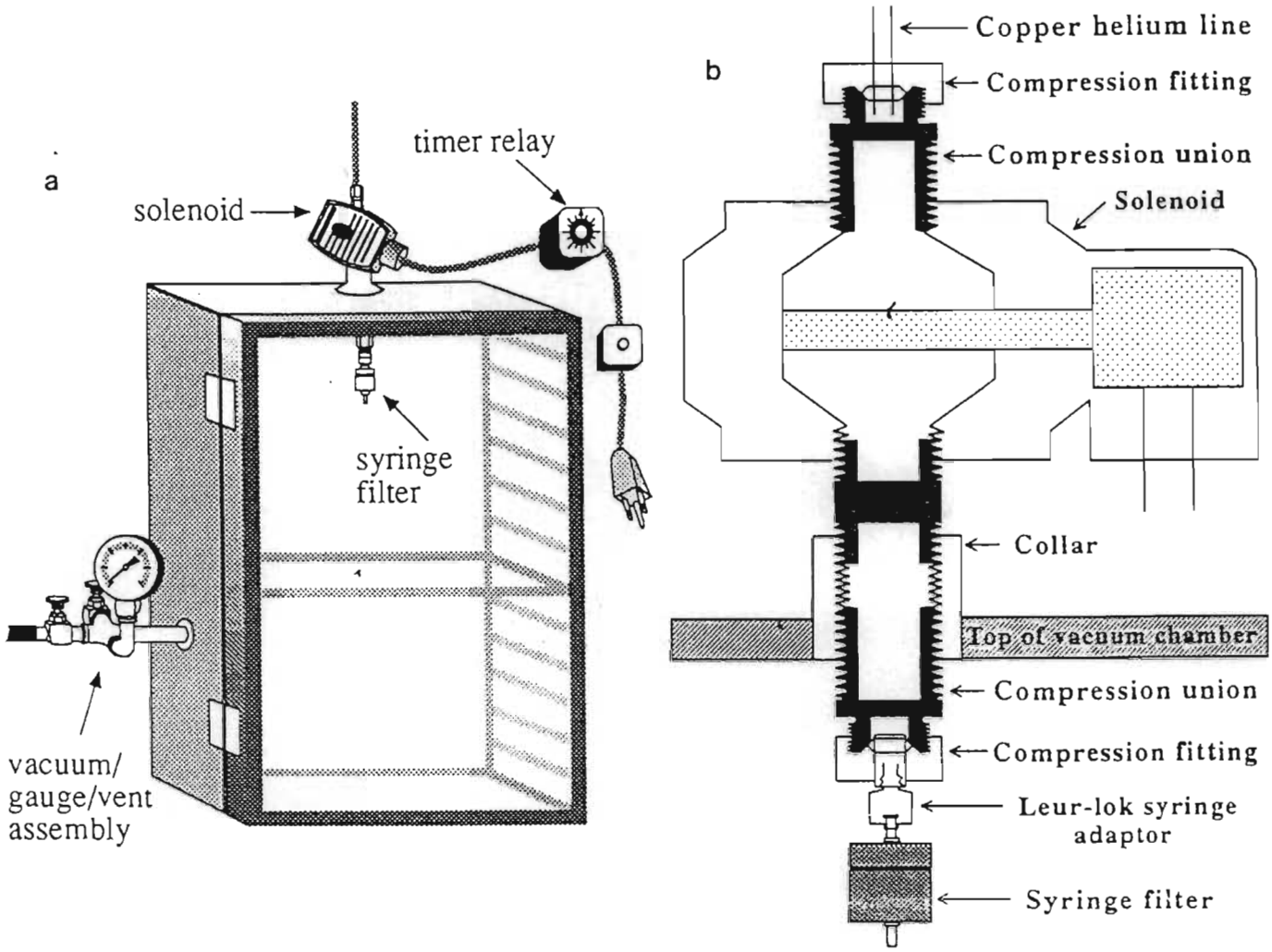


Figure 4.2 Particle Inflow Gun used for particle bombardment of maize callus. (a) Graphic illustration of the Particle Inflow Gun. (b) Schematic diagram showing connections from the helium line through the syringe filter (Finer, *et al.*, 1992).

4.2.6 Preparation of plasmid DNA for particle bombardment studies

Two different plasmid constructs were used for particle bombardment experiments, both of which were generously donated by the Biotechnology Department of the South African Sugar Association Experiment Station (Mt Edgecombe, South Africa). The first plasmid, pAHC25 (BAR-GUS construct; 9881 bp; Figure 4.3), incorporates the GUS gene, as well as the *bar* gene, which expresses resistance to the herbicide Basta (a.i. phosphinothricin). The ubiquitin promoter drives expression in this plasmid construct. The second plasmid used for particle bombardment experiments was the plasmid pDP687 (Pioneer Hi-Bred International Inc.; Figure 4.4). This plasmid construct is 9 kB in size and contains two anthocyanin transcriptional activators, *R* and *C*, each under the control of a double CaMV 35S promoter.

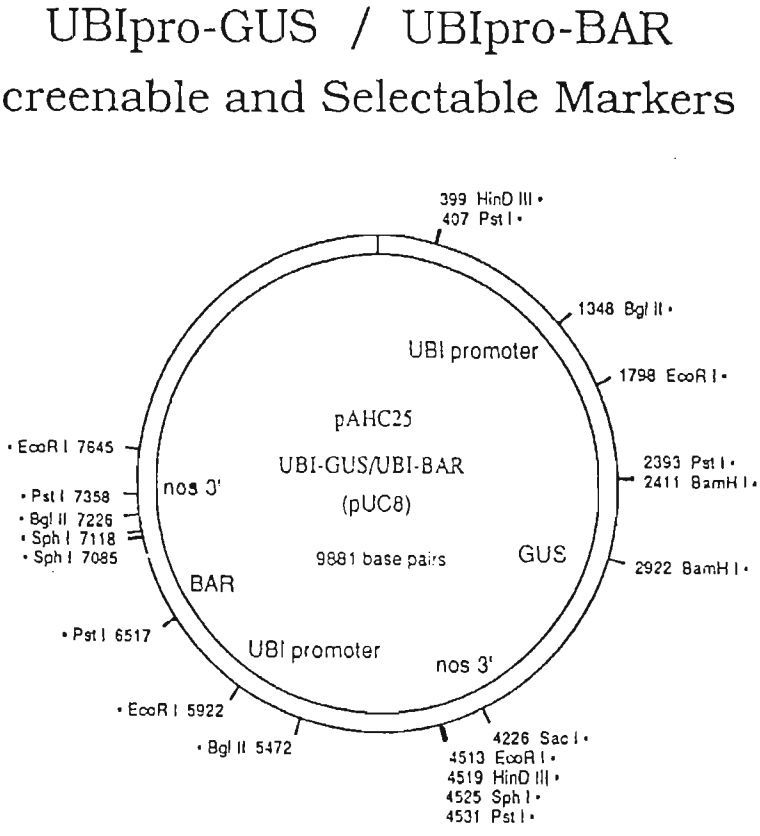


Figure 4.3 pAHC25 Plasmid construct incorporating the β-glucoronidase gene and the *bar* gene, which expresses resistance to the herbicide Basta.

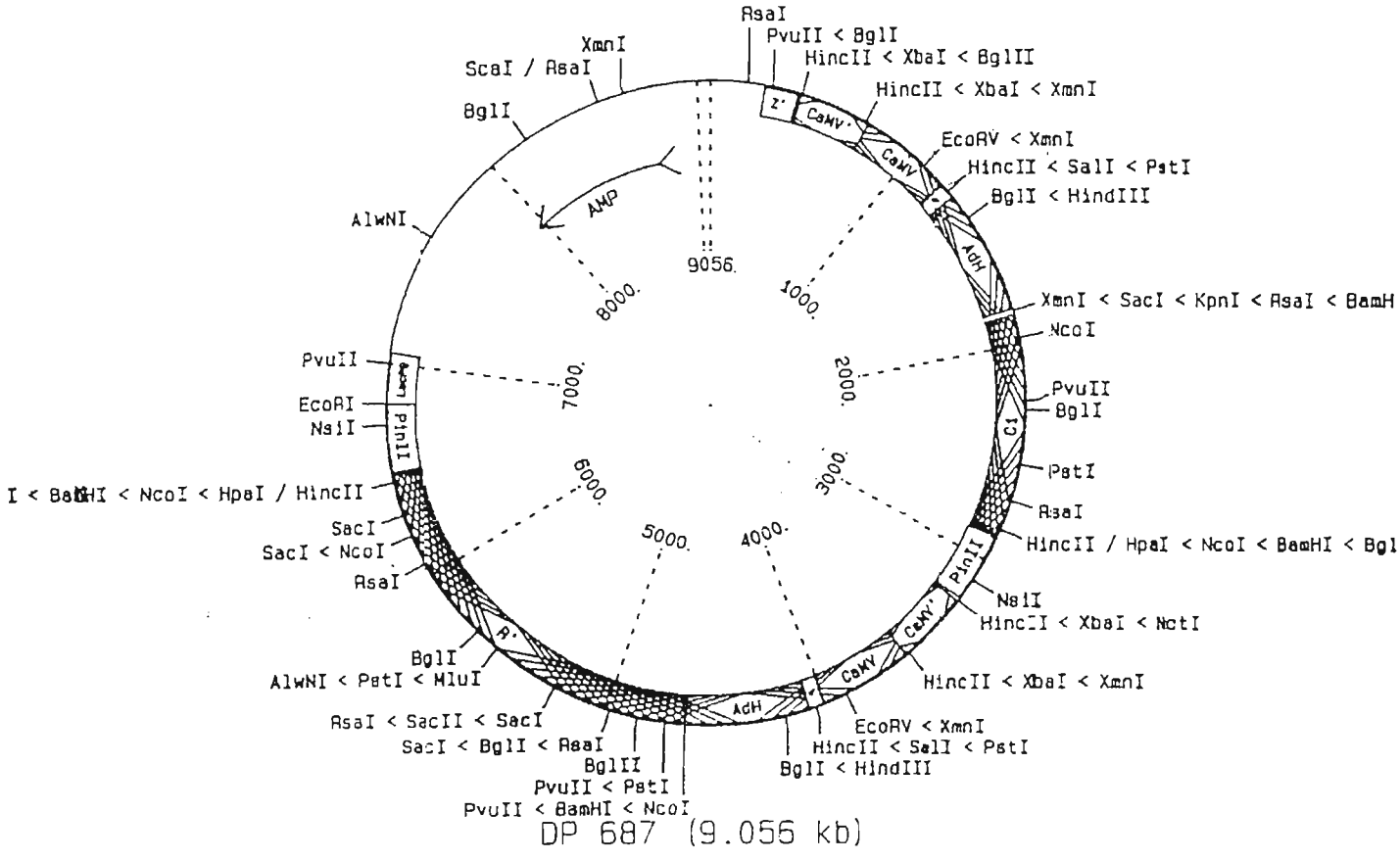


Figure 4.4 pDP687 Plasmid construct containing the two anthocyanin transcriptional activators, *R* and *C*.

Plasmid DNA was precipitated onto tungsten particles prior to particle bombardment experiments using a modified technique of Finer, *et al.* (1992). Tungsten particles (1.1 μm in diameter; M17; Bio-Rad Laboratories, CA) were sterilised in 70% (v/v) ethanol and washed twice with sterile distilled H_2O. The particles were resuspended in sterile distilled H_2O at a concentration of 100 mg mL^{-1}. At room temperature this suspension (25 μL) was mixed with the plasmid DNA solution (20 μL; 1 μg μL), sterile distilled H_2O (25 μL) and 10 μL of 0.1 M spermidine (filter sterilised through a 0.45 μm Millipore filter). The mixture was vortexed and placed on ice for 5 min before being briefly centrifuged. The supernatant was removed and the coated particles were resuspended in ethanol (30 μL; 100% (v/v)). The tungsten suspension was vortexed prior to each bombardment.

4.2.7 Particle bombardment of maize callus

Type I and Type II embryogenic callus was used for particle bombardment experiments using a modified method of Finer, *et al.* (1992). This callus had been initiated from young leaf bases of *in vitro* germinated seedlings (Section 2.2.4) and immature embryos of maize (Section 2.2.5). The plasmid construct, pAHC25, was used in an initial experiment to determine whether callus of randomly chosen South African maize genotypes (M28Y, PAN 473 and K054W) was amenable to transformation by particle bombardment. Callus for this initial experiment was pretreated 3 d prior to the particle bombardment study, so as to reduce the turgor pressure of the cells. For callus pretreatment, callus (c.a. 1 g) was transferred to MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8.) containing 0.2 M mannitol and 0.2 M sorbitol. Subsequent experiments were undertaken with the pDP687 plasmid construct. Three days before particle bombardment with pDP687, the callus to be used was divided in half, with the first half being pretreated to reduce turgor pressure. The remaining half of the callus was left on MS-CM medium (without sorbitol or mannitol) ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8.). Both untreated and pretreated callus was bombarded under the same conditions. A further experiment was conducted using untreated M28Y callus and the pDP687 plasmid construct. In this experiment the callus was placed at three different distances (9.0, 10.5 and 14.0 cm) from the syringe filter unit containing the pDP687 plasmid DNA-coated tungsten particles to determine the effect of distance travelled (Table 4.5). Additionally, two bombardments of this callus was compared to one bombardment.

All particle bombardment manipulations were carried out on a laminar flow bench. The particle inflow gun (PIG) was placed on the laminar flow bench overnight, with the ultraviolet light left

on for sterilisation. The vacuum chamber inside the PIG was wiped down with 95% (v/v) ethanol. Callus for bombardment was placed in a sterile disposable Petri dish. For experiments conducted with the pAHC25 plasmid construct, two nuts with a mesh (gauze) glued between them, were placed over the callus and held down on the Petri dish with a piece of prestik (Bostik). A layer of nylon mesh (200 μm^2) was placed over callus which was to be bombarded with the pDP687 plasmid construct. The Petri dish was placed on an adjustable shelf in the top position within the chamber ($\pm$ 9 cm below the syringe filter unit containing the DNA-coated tungsten particles).

For bombardment, the DNA particle suspension (6 μL of pAHC25 plasmid DNA or 2 μL of pDP687 plasmid DNA) was placed in the centre of a 1 mm metal screen in a disassembled 13 mm Swinney filter holder (Millipore, Germany). The filter unit was reassembled and screwed into a needle adaptor above the callus. The chamber was evacuated to a pressure of 90 kPa and the particles were discharged when the helium (1000 kPa) was released by a solenoid linked to a timer relay (0.05 s). The vacuum was removed from the chamber. Three blasts were applied to callus bombarded with tungsten particles coated with pAHC25 plasmid DNA. Only one blast was applied to callus bombarded with tungsten particles coated with pDP687 plasmid DNA. Controls consisted of bombardment of callus with uncoated microprojectiles. Bombarded callus was placed on MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8.) and incubated for 24 h under normal callus maintenance conditions to allow for transient expression of the plasmid genes.

Callus bombarded with tungsten particles coated with the plasmid pAHC25 (GUS) construct were assayed for expression of the GUS gene using the histochemical GUS assay modified from Jefferson, *et al.* (1987). The callus was removed from MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D

+ 10 g L^{-1} agar; pH 5.8.) after 24 h and placed in a sterile McCartney bottle. The callus was overlaid with 1 mL X-Glu Substrate buffer (Appendix C.3; without Triton X-100 as this causes excessive bubbling during vacuum infiltration). The callus was vacuum infiltrated until all bubbling had ceased. The callus pieces were removed from the McCartney bottle with tweezers and placed in a small disposable plastic Petri dish. One millilitre of X-Glu Substrate buffer (containing Triton X-100) was added. The dishes were incubated at 37^{o}C in the dark for 24 h. After 24 h the number of developing blue spots was counted. An additional 1 mL X-Glu Substrate buffer (with Triton X-100) was added. The callus was incubated for a further 24 h to detect any slower developing spots. Any callus pieces expressing the GUS gene were placed into 95% ethanol in Eppendorf tubes for storage at room temperature.

For experiments conducted with the pDP687 plasmid (anthocyanin) construct, callus as incubated in the dark at 27$\pm$1^{o}C for 3 d after bombardment. Transient expression of the anthocyanin gene was assessed by counting the number of purple foci with a Zeiss dissecting microscope. Transformed callus was maintained in the dark on MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} agar; pH 5.8.) for 1 wk, after which it was incubated under a 16 h light/8 h dark photoperiod. After 1 wk the callus was transferred to regeneration medium (Chapter 5).

4.3 Results and discussion

4.3.1 Preparation of plasmid DNA for electroporation

E. coli HB 101 cells were successfully transformed with the pBI221 plasmid DNA as the bacteria were able to grow on medium supplemented with ampicillin. The Magic™ Maxiprep kit, used to isolate plasmid pBI221 DNA from the transformed bacterial cells, yielded c.a. 0.614 $\mu g\ \mu L^{-1}$ plasmid DNA from 250 mL of bacterial culture. The pBI221 plasmid preparations were determined to be relatively pure as the ratio between the readings at 260 nm and 280 nm was c.a. 1.8. The structure of the plasmid was checked by means of agarose gel electrophoresis. Three bands of DNA were observed for each DNA preparation. These bands corresponded to covalently closed circular plasmid DNA, open circular plasmid DNA, and linear plasmid DNA. A band of c.a. 5.6 kB was observed, confirming the integrity of the plasmid.

4.3.2 Electroporation of maize protoplasts

Maize protoplasts were electroporated in an attempt to obtain transformation. The effect of electroporation on protoplast viability was investigated (Figure 4.5). Protoplast viability percentages, determined by FDA staining, were all similar prior to electroporation as one isolate of protoplasts was divided into aliquots for one electroporation experiment. The applied electrical voltage had a detrimental effect on viability of both M162W and M28Y protoplasts. The higher the electroporation voltage, the lower the percentage viability as determined by FDA staining, i.e. after electroporation, protoplast viability decreased in an exponential fashion with increasing voltage (Figure 4.5). These results are consistent with those of other researchers, who found that higher electroporation voltages produced more transient gene expression, but resulted in a concomitant reduction in protoplast viability (Fromm, *et al.*, 1985; Rhodes, *et al.*, 1988b; Huang & Dennis, 1989). No positively

transformed protoplasts were obtained, i.e. no protoplasts exhibiting the characteristic blue coloration of GUS gene expression were observed when the assayed protoplasts were examined with a Zeiss Axiophot® microscope.

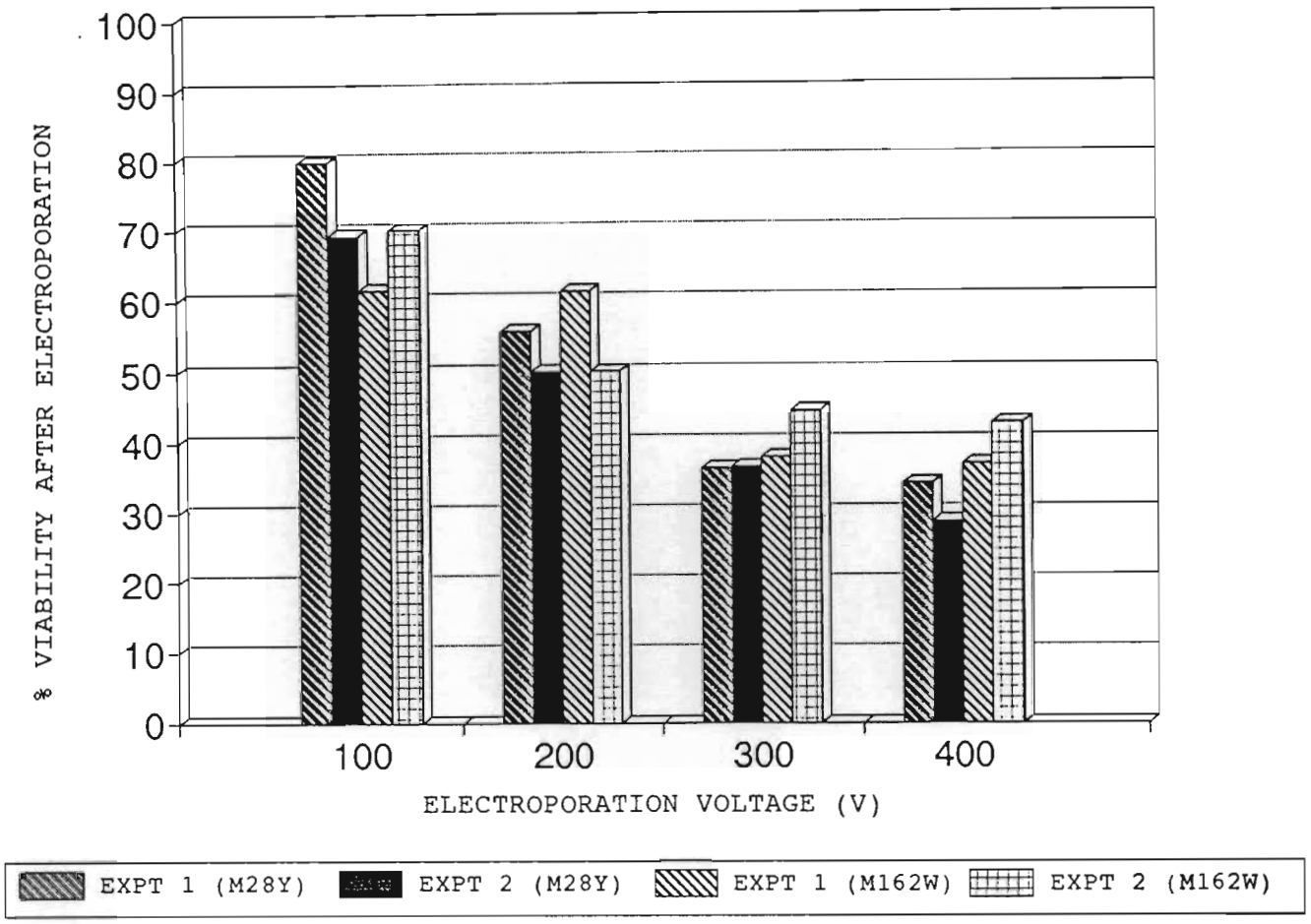


Figure 4.5 The effect of a range of electroporation voltages (100 to 400 V) on the viability of M28Y and M162W maize protoplasts (viability determined using FDA staining).

It has been found that transient gene expression from DNA templates is maximal between 24 and 48 h after electroporation (Fromm, *et al.*, 1987). Transient expression of introduced genes can be detected as early as 3 h and as late as 2 wk after electroporation (Fromm, *et al.*, 1987), and may be detected 1 to 3 d following electroporation even for plant species where protoplasts fail to divide (Rathus & Birch, 1991). Therefore, subsequent to electroporation of maize protoplasts, two different incubation times of 24 and 48 h were used. However, no positive

transformants were detected by the GUS histochemical assay, even after 48 h incubation. There may be several reasons for this. Firstly, no transformed protoplasts may have been obtained. The GUS assay may not be stringent enough to detect transformed protoplasts from amongst untransformed protoplasts, or the incubation period may have been too short. Finally, transformed protoplasts may either not have expressed the GUS gene, or may not have expressed it at a high enough rate for the expression to be detected (Fromm, *et al.*, 1987).

4.3.3 Electroporation of maize callus

During initial experiments on the electroporation of maize callus (Electroporation strategies 3, 4 and 5; Table 4.1), no positively transformed GUS expressing cells were observed after the histochemical assay. The GUS gene was not expressed in any callus electroporated under any of the conditions used. An increase in voltage (from 375 V to 450 V), differing numbers of pulses (1 to 4) delivered to each sample, an increase in the concentration of pBI221 plasmid DNA (from 10 μg to 20 μg) and the use of the Gene Pulser® were all ineffective at transformation of the callus. This may be due to a number of reasons including: a concentration of plasmid DNA which is below that required for transformation; a pulse length of too short duration; or a voltage which is not optimum for callus. However, electroporation of callus with the Pro-Genetor™ electroporator in the presence of a slightly higher pBI221 plasmid DNA concentration (25 μg), with a pulse of double the length used before (50 ms), and a range of voltages, produced a number of positively transformed callus pieces (Electroporation strategy 6; Table 4.2). Transformed cells appeared as diffuse blue spots in the callus (Plate 41A; Plate 41B).

Table 4.2 Number of GUS expressing callus foci yielded after electroporation of maize callus with the Pro-Genetor™ electroporator (in the presence of pBI221 plasmid DNA).

Voltage (V)	Number of blue foci	
	M28Y	M162W
200	0	64
250	0	0
300	0	214
350	731	687
400	690	0
450	117	0

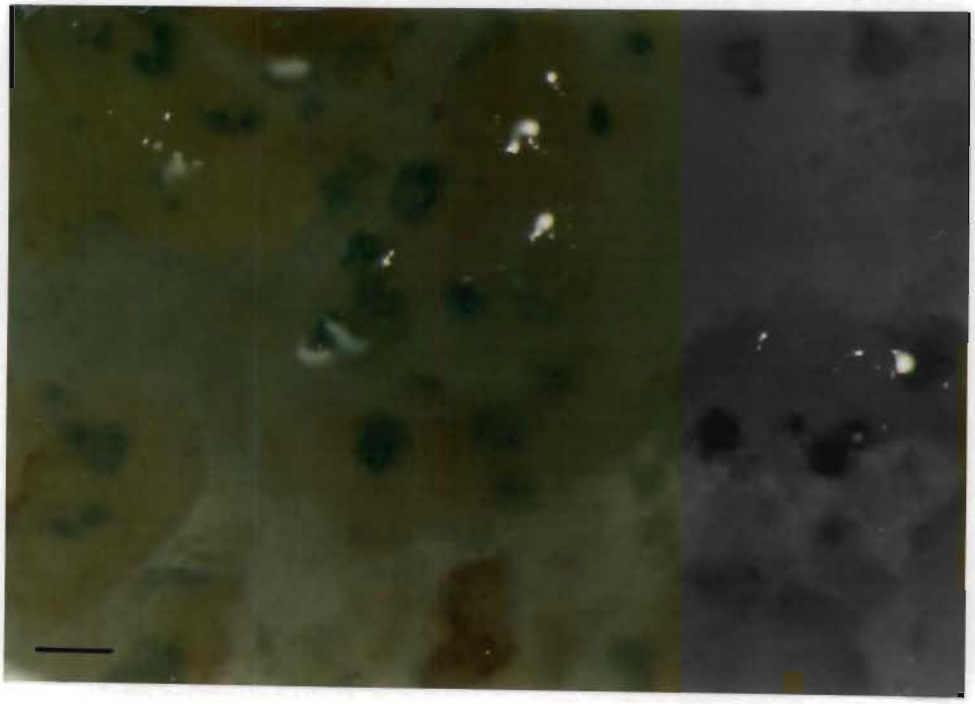


Plate 41A Blue GUS expressing areas of M162W callus after electroporation in the presence of pBI221 plasmid DNA (bar = 0.5 mm).

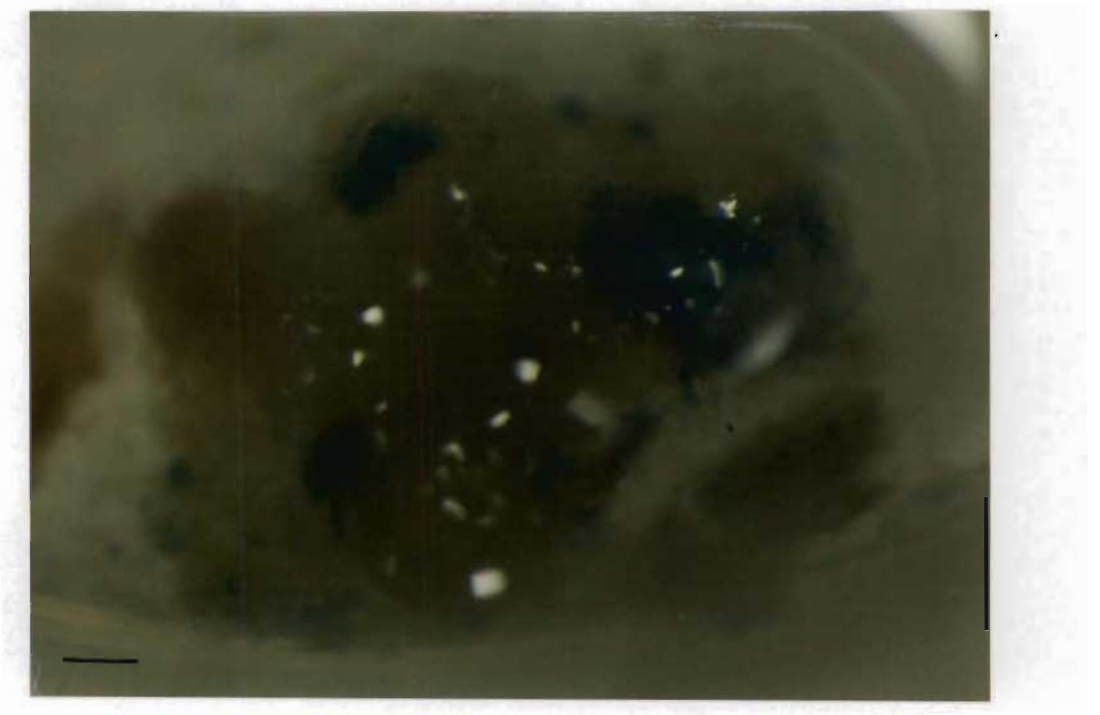


Plate 41B Blue GUS expressing areas of M162W callus after electroporation in the presence of pBI221 plasmid DNA (bar = 0.4 mm).

Both the Pro-Genetor™ and the BIO-RAD Gene Pulser® electroporators were compared. It would appear that the Gene Pulser® electroporator is ineffective for the transformation of maize callus, as no positive transformants were obtained in any of the experiments in which this electroporator was used. On the other hand, the Pro-Genetor™ produced a number of transformed callus pieces, for both M28Y and M162W, over the range of voltages used. The transformed callus may be produced due to the higher DNA concentration or to the longer pulse time used. The GUS expressing foci are diffuse as the callus was left in the assay solution for 72 h. Therefore, the blue dye diffused out from transformed cells into neighbouring tissue. The GUS reaction product has been known to diffuse out and colour multiple cells (Fromm, 1994). The greatest number of transformed callus cells was obtained when a voltage of 350 V was used. This result was comparable to experiments conducted in the past by other researchers. Maize callus has previously been transformed with a voltage of 375 V (D'Halluin, *et al.*, 1992). Lack of

transformation of maize callus at some of the applied voltages may be due to the plasmid DNA preparations used. Due to the large amounts of DNA required for each electroporation experiment, and the small amount of DNA purified each time from the bacterial cells, different plasmid preparations had to be used in the experiments. Some of these DNA preparations had been in storage for nearly two years. It was therefore theorized that some of the DNA preparations had deteriorated during storage, and therefore were inadequate for transformation experiments.

For subsequent electroporation experiments, callus was incubated in the dark for 24 h, and/or was enzymatically digested, prior to the start of the electroporation experiment so as to make the callus softer and more porous. Treatment of suspension culture cells of sugarbeet with pectin-digesting enzymes resulted in increased uptake and expression of DNA after electroporation (Lindsey & Jones, 1990). However, pretreatment of maize callus did not yield any positive transformants. It is probable that the deteriorated state of the DNA preparations used was responsible for this negative result. However, due to time constraints, this experiment could not be repeated.

4.3.4 Particle bombardment of maize callus

During initial experiments with the pAHC25 plasmid construct, two nuts with a mesh (gauze) glued between them were placed over the callus and held down with a piece of prestik (Bostik). This anchoring of callus was necessary, as the force of the tungsten particles impacting with the callus was enough to shoot the tissue out of the Petri dish when not held down. However, the prestik used to hold the nuts in place over the callus was a potential source of contamination. As a consequence, in subsequent experiments with the pDP687 plasmid construct use was made of a layer of nylon mesh (200 μm^2) placed over the callus. This nylon mesh could be sterilised and additionally may have increased transient expression in the target tissue. Researchers have found that a mesh screen, although blocking some

microprojectiles, increases transient expression frequencies by deflecting particles. Therefore, cells are penetrated over a wider target tissue area (Franks & Birch, 1991).

As the GUS histochemical assay is destructive to cell viability (Jefferson, *et al.* 1987), the initial particle bombardment experiment using the pAHC25 plasmid (GUS construct) was conducted to determine whether any transient gene expression could be obtained. As an alternative, the bombarded callus could have been placed on medium supplemented with 1 mg L^{-1} Basta for selection of transformed callus cells. The *bar* gene from *Streptomyces hygroscopicus* (present in the pAHC25 plasmid construct) encodes PAT, an enzyme which detoxifies the herbicide phosphinothricin (PPT; the active ingredient in the herbicide Basta) via acetylation (Kramer, DiMaio, Carswell & Shillito, 1993). However, as only a small amount of callus was bombarded, and the aim was to detect positive transient expression, selection was not performed. Subsequent experiments, performed with the pDP687 plasmid construct, did not require any assay to be performed. The purple anthocyanin was produced by the transgenic cells and was observed with a Zeiss dissecting microscope. Consequently, transgenic callus could be subcultured to regeneration medium.

Initial particle bombardment of maize callus with the pAHC25 (GUS) plasmid construct, led to the presence of a number of blue GUS expressing foci on the callus (Plate 42). The blue spots on the bombarded callus represented transient gene expression in transformed callus. The blue foci were counted for each of the three genotypes used in the initial experiment (Table 4.3) after 24 h and 48 h in the GUS assay solution. Some spots were more intensely blue than others. Callus pieces with no transformed spots were discarded.

Plate 42 Blue GUS expressing spots on callus of PAN 473 bombarded with the pAHC25 plasmid construct (bar = 1 mm).

Table 4.3 Number of blue GUS expressing callus areas produced by particle bombardment with tungsten particles coated with the pAHC25 plasmid construct.

Genotype	No. of blue spots after 24 h	No. of blue spots after 48 h
M28Y	14	22
K054W	18	18
PAN 473	39	42

The three genotypes used for the initial particle bombardment experiment were M28Y, K054W and PAN 473. M28Y had the softest callus, while callus of K054W was harder and more embryogenic in nature (due to its nodular appearance). PAN 473 had the hardest callus in this initial experiment and it was additionally far more nodular and faster growing than callus of the two inbreds. The more compact nodular callus of PAN 473 yielded the most transiently expressing cells after bombardment.

The number of blue GUS expressing foci detected increased after a further 24 h incubation in GUS assay solution. This may be due to either the expression of GUS in callus cells situated below the surface layers of the callus, or to callus cells which metabolised at a slower rate. This slower metabolic rate may also be responsible for the range in intensity seen in the blue foci. In some areas, the callus appeared grey due to the presence of tungsten (Plate 43). These areas of callus had been heavily bombarded, which resulted in a hard crust of tungsten. The cells in these areas appeared to be dead as no transient expression was observed. More transformation appeared to occur at the perimeter of the grey areas (i.e. from scatter). It was therefore concluded that three bombardments used with tungsten particles coated with the pAHC25 plasmid construct led to overkill in some areas of the callus.

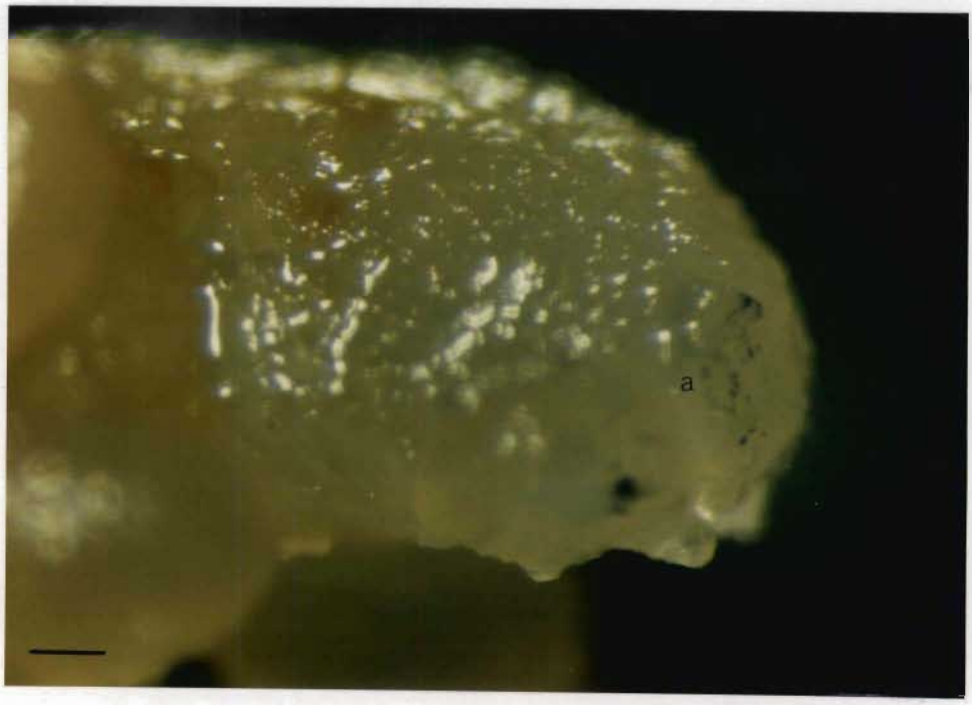


Plate 43 Callus of K054W after bombardment with tungsten particles coated with the pAHC25 plasmid. Area of callus appears grey due to presence of tungsten (a) (bar = 0.25 mm).

Mannitol and sorbitol were used to pretreat callus prior to particle bombardment. The high osmotic potential reduces the turgor of cells in the callus and may therefore potentially limit damage to cells. However, it has been found that osmotically

adjusted cells exhibit a substantially lower level of transient expression after particle bombardment than cells maintained in normal medium (Klein, *et al.*, 1988a). Consequently, in further particle bombardment experiments, half of the callus for bombardment was pretreated, while the other half remained untreated.

The pDP687 (anthocyanin) plasmid construct was used in subsequent particle bombardment experiments conducted on maize callus. After 3 d of maintenance following bombardment, the callus was observed with a Zeiss dissecting microscope. A number of small purple anthocyanin expressing foci were observed (Plate 44). The number of foci was enumerated (Table 4.4) for both pretreated and untreated callus. The purple colour of the anthocyanin expressing foci did not diffuse into the surrounding callus (Plate 45) as the purple anthocyanin pigment is vacuolar and should be restricted within a single cell (Fromm, 1994).

Plate 44 Purple anthocyanin expressing foci observed on M28Y callus after bombardment with tungsten particles coated with the pDP687 plasmid construct (bar = 0.25 mm).

Table 4.4 Number of purple anthocyanin expressing callus areas produced by particle bombardment with tungsten particles coated with the pDP687 plasmid construct.

Inbred	Source of Callus[1]	No. of purple anthocyanin producing spots	
		Pretreated callus[2]	Untreated callus[3]
M28Y	Leaf base	44	2142
M28Y	Leaf base	47	49
S0507W	Embryo	14	35
K054W	Leaf base	8	13
F2834T	Embryo	4	4
K0315Y	Leaf base	126	3
D0940Y	Embryo	59	15
M162W	Leaf base	29	10
I137TN	Leaf base	28	5
M28Y	Embryo	26	6
B0394Y	Embryo	18	4

[1]Source of callus refers to the tissue which was used to initiate callus (Leaf base = explants from *in vitro* germinated seedlings; Embryo = immature embryos).

[2]Pretreated callus was placed on MS-CM medium containing 0.2 M mannitol and 0.2 M sorbitol, 3 d before bombardment, to reduce turgor pressure.

[3]Untreated callus was not placed on medium containing mannitol or sorbitol 3 d before bombardment.

Plate 45 Anthocyanin expressing foci of M28Y callus (bar = 0.2 mm).

The number of foci observed varied for each inbred used (Table 4.4). Callus which was treated to reduce turgor pressure, was more amenable to transformation in some inbreds than in others. Of all the inbreds, untreated callus of M28Y yielded the most foci after bombardment. Pretreated callus of K0315Y also yielded a similar number of foci. It therefore became apparent that reduction of turgor pressure is beneficial in some inbreds, but not in others. These results indicate a significant effect of genotype and suggest that the bombardment protocol should be optimized for each inbred under investigation. Both M28Y and K0315Y callus was compact, nodular Type I embryogenic callus tissue. Callus of inbreds which did not produce many foci after bombardment (e.g. B0394Y and F2834T), tended to be softer callus. It would therefore appear that Type I embryogenic maize callus is the best callus for use in particle bombardment experiments.

A further particle bombardment experiment was conducted to investigate the effect of the distance travelled by the projectiles on the number of transiently expressing foci. After particle bombardment with microprojectiles placed at various distances from the callus, the number of foci observed with the Zeiss dissecting microscope was counted (Table 4.5). The number of foci observed after two bombardments, of the same callus, was also counted.

Table 4.5 The effect of distance travelled by microprojectiles coated with pDP687 plasmid DNA, and the number of bombardments performed, on the number of purple foci observed in untreated M28Y callus.

Inbred	Source of callus[1]	Number of bombardments	Distance travelled by projectiles	Number of purple foci
M28Y	Leaf base	1	9.0 cm	167
M28Y	Leaf base	1	10.5 cm	40
M28Y	Leaf base	1	14.0 cm	35
M28Y	Leaf base	2	9.0 cm	150

[1]Source of callus refers to the tissue which was used to initiate callus (Leaf base = *in vitro* germinated seedlings).

Results of this experiment indicate that the further the particles have to travel before impact with the callus, the less transiently expressing callus cells are produced. Additionally, two bombardments of callus placed on the top adjustable shelf in the PIG vacuum chamber resulted in slightly less anthocyanin expressing foci than one bombardment. However, as this experiment was not repeated, and due to the fact that there is a large amount of variation between successive bombardments (even though results are repeatable) (Franks & Birch, 1991), the lower number of foci observed may be due to a number of factors, including cell death by repeated bombardment.

The large number of foci observed after bombardment with tungsten particles coated with the pDP687 plasmid construct, may be attributed to the promoters used to drive the construct. The pAHC25 plasmid construct did not produce as many blue foci as the number of purple foci produced by the pDP687 plasmid construct. It was therefore concluded that the maize ubiquitin promoter used to drive the pAHC25 plasmid construct was not as effective as the double CaMV 35S promoter used to drive the anthocyanin genes of the pDP687 plasmid construct.

In conclusion, therefore, maize callus can be successfully transformed through the use of particle bombardment or electroporation. In this particular study it was found that an electroporation voltage of 350 V (50 ms duration), delivered by a Pro-Genetor™ electroporator, was adequate for successful transformation of maize callus with the pBI221 (β-glucuronidase) plasmid construct. On the other hand, particle bombardment, with the microprojectiles placed at a distance of 9 cm above the callus, also produced transiently expressing callus cells. Along with a successful regeneration protocol, further optimisation of these two transformation procedures may (in the future) enable the successful production of transgenic maize plants in South Africa.

CHAPTER 5
PLANTLET REGENERATION FROM MAIZE TISSUE CULTURES

5.1 Introduction

An integral part of plant biotechnology research for crop improvement is the *in vitro* regeneration of mature, fertile and genetically stable plants (Kamo, *et al.*, 1985; Malik & Saxena, 1992). In order to regenerate transformed plants, the mechanisms of regeneration need to be known (Emons, Samallo-Droppers & Van Der Toorn, 1993). In the Gramineae, plant regeneration from leaf tissue has been reported; however, attempts in maize have in the past had limited success (Chang, 1983). Plant regeneration from maize callus has been found to be genotype specific and has a heritable basis (Green & Phillips, 1975; Duncan, *et al.*, 1985; Hodges, *et al.*, 1986). Regeneration of plantlets from maize callus occurs by organogenesis (Springer, *et al.*, 1979) and somatic embryogenesis (Lu, *et al.*, 1982). Both types of regeneration occur in compact, nodular, irregularly shaped, opaque white or yellow callus with prominent ridges and furrows (Lu, *et al.*, 1982; Duncan, *et al.*, 1985). Initially, regenerating callus forms compact white scutellar bodies, followed by morphogenesis and the development of root- and shoot-like structures (Green & Phillips, 1975; Kamo, *et al.*, 1987).

Frequently, published regeneration procedures are non-reproducible (Gamborg, 1991). Therefore, for most genotypes of various plant species, regeneration procedures have been developed by trial and error (Roest & Gilissen, 1989). Plant regeneration by somatic embryogenesis from maize tissue cultures (protoplasts, compact callus or embryogenic cell suspensions) can often be accomplished by simply reducing or eliminating 2,4-D from the medium onto which the tissue is transferred (Green & Phillips, 1975; Harms, *et al.*, 1976; Ozias-Akins & Lörz, 1984; Vasil, *et al.*, 1984; Duncan, *et al.*, 1985; Kamo & Hodges, 1986;

Kamo, *et al.*, 1987; Rhodes, *et al.*, 1988a). The addition of phytohormones to regeneration medium of maize can have significant effects on the regeneration potential of the tissue. Auxin is the most important factor for the regulation of induction and development of embryogenesis in maize and has different effects in different phases of embryogenesis. Cytokinins are also involved in embryogenesis (Komamine, *et al.*, 1990). Selected concentrations of both kinetin and BAP (N^6-benzylamino-purine) increase maize plantlet formation over controls without cytokinins, especially when added in combination with 0.25 mg L^{-1} 2,4-D. Zeatin generally has a negative effect on regeneration frequencies (Shillito, *et al.*, 1989).

Researchers have found that regeneration of maize plants can be accomplished in a three-step process. Tissue is incubated on each regeneration medium for about 2 wk. Regeneration medium 1, consisting of MS salts and vitamins, 100 mg L^{-1} myo-inositol, 0.1 mg L^{-1} 2,4-D, 2.64 mg L^{-1} ABA (abscisic acid) and 2% (m/v) sucrose, promotes somatic embryo differentiation (Armstrong, 1994). Regeneration medium 2 (N6 salts and vitamins, 6% (m/v) sucrose, no phytohormones) promotes embryo enlargement and maturation (Kamo, *et al.*, 1985; Armstrong, 1994). Tissue transferred to Regeneration medium 2 containing 0.25 μg mL^{-1} 2,4-D, does not produce more plants than tissue transferred to medium without 2,4-D (Kamo, *et al.*, 1985). Germination of somatic embryos is promoted by Regeneration medium 3 (MS salts and vitamins, 100 mg L^{-1} myo-inositol, 20 g L^{-1} sucrose) (Vasil & Vasil, 1986; Armstrong, 1994). The first two steps are carried out in the dark at 28°C, and the final step under a 16 h light/8 h dark photoperiod (Armstrong, 1994). Maize plants have also been regenerated from embryogenic maize callus by a reduction in the level of dicamba in the medium and the addition of kinetin (Swedlund & Locy, 1993). Dicamba, however, increases the time required for plantlet formation on regeneration medium by approximately 7 d (Duncan, *et al.*, 1985).

Manipulating the balance of ABA, L-proline, mannitol and sucrose in the external medium can regulate somatic embryo development of maize to a large extent. Abscisic acid is thought to be involved in the control of many events during zygotic embryogenesis. It is an important factor in the maturation of embryos and in the inhibition of precocious germination. Incubation on medium of high osmotic potential (i.e. the use of mannitol) can also be used to inhibit precocious germination, while simultaneously maintaining the culture in an embryogenic, rather than rhizogenic, state (Emons, *et al.*, 1993). The concentration of sucrose in the regeneration medium has a marked effect on the number of embryoids which differentiate. High levels of exogenous sucrose have been found to increase the frequency of somatic embryogenesis in maize (Lu, *et al.*, 1983). With 6% (m/w) sucrose in the maturation medium (Regeneration medium 2), somatic embryos ripen by continued accumulation of dry matter in the scutellum (Emons & Kieft, 1991). Medium containing L-proline, an osmoprotectant (Rhodes, Handa & Bressan, 1986) and source of nitrogen (Armstrong & Green, 1985), stimulates more plant regeneration than medium lacking L-proline (Kamo, *et al.*, 1985). Thiamine hydrochloride and casein hydrolysate increase the number of somatic embryos in maize immature embryo-derived callus cultures (Vasil & Vasil, 1986). Silver nitrate, an inhibitor of the physiological action of ethylene (Beyer, 1976), has been shown to increase somatic embryogenesis and shoot regeneration in maize, carrot, sunflower and wheat tissue culture (Songstad, Duncan & Widholm, 1988; Taylor, *et al.*, 1994). Additionally, researchers have found that mechanically dispersed embryogenic maize callus, produced by forcing callus through an 800 μm screen, developed the greatest number of shoots g^{-1} fresh mass of embryogenic callus (Swedlund & Locy, 1993).

Embryogenic callus aggregates consist of a number of globular stage somatic embryos attached to a loose callus. This callus seems to act as a tissue, stimulating scutellum formation and suppressing germination and shoot meristem formation, a role that may be attributed to the suspensor (Emons & Kieft, 1991). The

attachment of somatic embryos to undifferentiated callus is a prerequisite for somatic embryogenesis (Emons & Kieft, 1991). It has been shown that somatic embryos of maize develop (directly or indirectly) from single cells (Vasil & Vasil, 1987a; Jones & Rost, 1989). Consequently, plants derived from embryogenic cultures are neither chimeras nor variants, but true clones. The single cell origin, non-chimeral nature and genetic fidelity of plants derived from somatic embryos are very attractive features for any genetic transformation system (Vasil, *et al.*, 1990).

Mature and fully developed maize somatic embryos show the typical organization of grass zygotic embryos, having a compact and white scutellum, coleoptile and coleorhiza (Lu, *et al.*, 1982; Novák, *et al.*, 1983; Vasil & Vasil, 1986). Somatic embryos of maize are always formed in the surface layers of embryogenic callus, with the shoot end of the embryo invariably directed outwards and the root end embedded in the callus. This demonstration of strictly polarized development may be a response to gravity or be regulated by exogenous or endogenous gradients of plant growth regulators and/or nutrients (Vasil, 1988).

The influence of callus age on embryogenesis must be considered. Maize embryogenic callus that is a few years old has a lower regeneration potential than that of younger embryogenic callus (Santos, *et al.*, 1993). Additionally, the percentage of abnormal plants regenerated increases with longer callus culture phases (Kamo, *et al.*, 1985).

Once a good root system has been developed by regenerated plants, they should be carefully removed from the regeneration medium. The root system must be thoroughly washed, and the plants then placed into pots containing growth medium. It is critical to maintain the freshly transplanted plants in a high humidity environment for several days. Placing well-watered plants into large, sealed, transparent plastic bags is a simple, economical way to accomplish this. Condensation should become visible on the inside of the plastic bags. After several days, the humidity

should be gradually reduced to harden off the plants (e.g. by cutting a few holes into the plastic bag). Several days later, the plants should be hardened off well enough to survive typical growth chamber or greenhouse conditions for maize. During the hardening off stage, temperature and light intensity should be lower than for normal maize greenhouse conditions (Armstrong, 1994).

5.1.1 Investigations into the regeneration of plantlets from tissue cultures of South African maize inbreds

The aim of experiments conducted in this chapter was to establish a protocol for the regeneration of maize plantlets from callus cultures. Regeneration of plantlets from callus is the essence of stable transformation, and should therefore have been presented in conjunction with the various approaches to tissue culture presented in Chapter 2. However, even though the regeneration studies were undertaken in conjunction with the other *in vitro* culture techniques, the work is presented after the transformation studies in order to illustrate the logical progression from the establishment of a tissue culture, through transformation, to regeneration of transformed plantlets. Various different regeneration media were tested in the regeneration studies. Callus initiated from young leaf bases of *in vitro* germinated seedlings (Section 2.2.4) and immature embryos (Section 2.2.5) was used to establish regeneration conditions. The established protocol was then used in an attempt to regenerate plantlets from maize cell suspension cultures (Appendix E) and callus which had been subjected to particle bombardment with tungsten particles coated with the pDP687 (anthocyanin) plasmid construct (Section 4.2.7).

5.2 Materials and methods

5.2.1 Establishment of a regeneration protocol for maize callus

All regeneration procedures were conducted on a laminar flow bench. Small pieces of callus (c.a. 1 cm^3) were subcultured from MS-CM medium ((according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D + 10 g L^{-1} 2,4-D; pH 5.8.) to Regeneration medium 1 in Petri dishes (Table 5.1). Callus of maize inbreds, initiated from immature embryos (Section 2.2.5) and young leaf bases of *in vitro* germinated seedlings (Section 2.2.4), was used. The fresh mass of the callus subcultured to regeneration medium was determined (Table 5.3) so that the number of plantlets regenerated per mg fresh mass of callus could be calculated. The callus was incubated for 10 d on Regeneration medium 1, either in the dark at 25°C, or under a 12 h light/12 h dark photoperiod at 27±1°C (Table 5.1). The regeneration cultures were not inverted, and were placed on top of white paper when incubated under the 12 h light/12 h dark photoperiod. After 10 d, the callus was subcultured to Regeneration medium 2 and incubated at 27±1°C under a 12 h light/12 h dark photoperiod. Every 14 d hereafter, the callus was subcultured to fresh Regeneration medium 2. Various regeneration media and culture conditions were tested in Experimental conditions 1 to 14 (Table 5.1) in order to investigate the effect of regeneration medium, light, genotype and explant type on the regeneration of maize plantlets from selected inbreds.

Table 5.1 Regeneration media and incubation conditions used to establish a regeneration protocol for maize callus.

Experimental condition	First 10 d of incubation		Regeneration medium 2[b]
	Light or dark	Regeneration medium 1[a]	
1	Light	MS - 2,4-D + 6% (m/v) sucrose	MS - 2,4-D + 2% (m/v) sucrose
2	Dark		
3	Light	N6 - 2,4-D + 6% (m/v) sucrose	N6 - 2,4-D + 2% (m/v) sucrose
4	Dark		
5	Light	MS + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	MS - 2,4-D + 2% (m/v) sucrose
6	Dark		
7	Light	N6 + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	N6 - 2,4-D + 2% (m/v) sucrose
8	Dark		
9	Light	MS + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	MS - 2,4-D + 2% (m/v) sucrose
10	Dark		
11	Light	N6 + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	N6 - 2,4-D + 2% (m/v) sucrose
12	Dark		
13	Light	MS + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin[c] + 6% (m/v) sucrose	MS + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin + 2% (m/v) sucrose
14	Light	N6 + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin + 6% (m/v) sucrose	N6 + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin + 2% (m/v) sucrose

[a]Regeneration media were made up according to Appendix A.1 (N6) and Appendix A.2 (MS), with either 6% (m/v) or 2% (m/v) sucrose.

[b]Maize callus on Regeneration medium 2 was always incubated at $27\pm1^{o}C$ under a 12 h light/12 h dark photoperiod.

[c]Kinetin was added from a stock solution (Appendix D).

The callus cultures were monitored every second day. Any developing plantlets were photographed using a Zeiss dissecting microscope equipped with a Pentax K1000 camera using Fuji colour film (200 ASA). The plantlets were then carefully excised from the callus and placed on Plantlet medium ((according to Wernicke

& Brettell, 1982; Appendix D) + 1% (m/v) sucrose; pH 5.8.), either in glass culture tubes (70 cm^3; 15 mL medium), or in 500 mL Schott (Duran) glass bottles (100 mL medium). To allow the plantlets to exchange gases with the external environment, the glass culture tubes were sealed with Parafilm "M", while a sterile glass jar was placed over the mouth of a Schott bottle and sealed in placed with Parafilm "M". The plantlets were incubated at 27±1°C under a 12 h light/12 h dark photoperiod to allow a good root system to be established. Once the plantlets were c.a. 6 cm in length, they were carefully removed from their glass culture tubes or Schott bottles. The medium was rinsed from the roots with sterile distilled H_2O. The plantlets were then planted in sterile Potting medium (Appendix D) in 10 cm plastic pots. Each pot was placed into a sterile modified 2 L Coca-Cola® bottle (Coca-Cola Southern Africa (PTY) LTD.). The Coca-Cola® bottles were modified in the following manner: the transparent bottle portion was removed from the black plastic base; the top 10 cm of the bottle was removed with scissors and discarded; and finally, the remaining portion of the bottle was inverted and placed inside the black base to form a clear dome over the potted plant. The Coca-Cola® bottles and pots were sterilised by a rinse in 3.5% (m/v) sodium hypochlorite (full strength Jik), followed by a rinse with sterile distilled H_2O. The Coca-Cola® bottles with the potted maize plants, were placed into the custom-made controlled environment chamber (Appendix A.4) at 27±1°C under a 12 h light/12 h dark photoperiod. The plants were monitored every second day, and watered with Hoogland nutrient solution (Appendix D).

5.2.2 Application of established regeneration protocol to maize callus which had been subjected to particle bombardment

Callus which had been subjected to particle bombardment with tungsten particles coated with the pDP687 (anthocyanin) plasmid construct (Section 4.2.7) was analyzed (Section 4.3.3) and then subcultured to MS regeneration medium supplemented with 0.3 mg L^{-1}

2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8. The fresh mass of the callus subcultured to regeneration medium was determined (Table 5.2). The callus cultures were monitored every second day for signs of regeneration. After 10 d, the callus was subcultured to the second regeneration medium (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8). The regeneration cultures were examined every second day and subcultured to fresh medium every 2 wk.

Table 5.2 Mass of particle bombarded callus subcultured to regeneration medium.

Inbred	Source of callus[1]	Fresh mass of callus (g)	
		Pretreated[2]	Untreated[3]
M28Y	Leaf base	0.69	1.19
M28Y	Embryo	0.15	0.06
S0507W	Embryo	0.07	0.12
D0940Y	Embryo	0.11	0.16
I137TN	Leaf base	0.38	0.16
B0394Y	Embryo	0.09	0.12
F2834T	Embryo	0.09	0.05
K054W	Leaf base	0.28	0.33
K0315Y	Leaf base	0.41	0.35
M162W	Leaf base	0.38	0.49

[1]Source of callus refers to the tissue which was used to initiate callus (Leaf base = explants of *in vitro* germinated seedlings; Embryo = immature embryos).

[2]Pretreated callus refers to callus which was placed onto MS-CM medium containing 0.2 M mannitol and 0.2 M sorbitol, 3 d before bombardment, to reduce turgor pressure.

[3]Untreated callus was not placed on medium containing mannitol or sorbitol 3 d before bombardment.

5.3 Results and discussion

5.3.1 Establishment of a regeneration protocol for maize callus

Callus regeneration experiments were conducted in two steps, and not three (Section 5.1), as somatic embryos were often formed on medium used for callus maintenance. The presence of embryoids was confirmed by observation with a dissection microscope. Consequently, the first regeneration step, normally utilized for somatic embryo differentiation (Armstrong, 1994), was omitted. The fresh mass of callus placed on regeneration medium was determined for each Experimental condition used (Table 5.3). After 10 d on Regeneration medium 1, the callus regeneration cultures were examined and subcultured to their respective second media, Regeneration medium 2. This second medium was either N6 or MS based, was auxin free, and contained 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8. After 14 d on Regeneration medium 2, the callus cultures were examined with a Zeiss dissecting microscope. The various regeneration media tested to develop a regeneration protocol for maize callus all induced different responses in the callus cultures.

Table 5.3 Fresh mass of maize callus subcultured from callus maintenance medium to Regeneration medium 1 in each regeneration experiment.

Experimental condition	Regeneration medium 1	Inbred	Source of callus[1]	Fresh mass of callus (g)
1	MS - 2,4-D + 6% (m/v) sucrose	M28Y	Embryo	0.21
		K054W	Embryo	0.10
		M28Y	Leaf base	0.32
		M162W	Leaf base	0.23
		D0940Y	Embryo	0.28
		F2834T	Embryo	0.10
2	MS - 2,4-D + 6% (m/v) sucrose	K054W	Embryo	0.07
		M28Y	Leaf base	0.10
		M162W	Leaf base	0.19
		D0940Y	Embryo	0.09
3	N6 - 2,4-D + 6% (m/v) sucrose	M28Y	Embryo	0.34
		K054W	Embryo	0.11
		M28Y	Leaf base	0.26
		M162W	Leaf base	0.16
		D0940Y	Embryo	0.38
		F2834T	Embryo	0.31
		S0507W	Embryo	0.12
4	N6 - 2,4-D + 6% (m/v) sucrose	M28Y	Leaf base	0.18
		M162W	Leaf base	0.05
		D0940Y	Embryo	0.08
5	MS + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	M28Y	Embryo	0.21
		K054W	Embryo	0.07
		M28Y	Leaf base	0.38
		M162W	Leaf base	0.26
		D0940Y	Embryo	0.09
		K0315Y	Embryo	0.31
6	MS + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	K054W	Embryo	0.05
		M28Y	Leaf base	0.09
		M162W	Leaf base	0.05
		D0940Y	Embryo	0.09
7	N6 + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	K054W	Embryo	0.06
		M28Y	Leaf base	0.34
		M162W	Leaf base	0.18
		D0940Y	Embryo	0.09
8	N6 + 0.25 mg L^{-1} 2,4-D + 2% (m/v) sucrose	M28Y	Leaf base	0.31
		M162W	Leaf base	0.07
		D0940Y	Embryo	0.07

9	MS + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	K054W	Embryo	0.11
		M28Y	Leaf base	0.26
		M162W	Leaf base	0.16
		D0940Y	Embryo	0.11
10	MS + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	M28Y	Leaf base	0.22
		M162W	Leaf base	0.09
		D0940Y	Embryo	0.07
11	N6 + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	M28Y	Embryo	0.23
		K054W	Embryo	0.07
		M28Y	Leaf base	0.31
		M162W	Leaf base	0.19
		D0940Y	Embryo	0.09
		K0315Y	Embryo	0.19
12	N6 + 3.00 mg L^{-1} 2,4-D + 6% (m/v) sucrose	M28Y	Leaf base	0.20
		M162W	Leaf base	0.07
		D0940Y	Embryo	0.10
13	MS + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin + 6% (m/v) sucrose	M28Y	Embryo	0.14
		K054W	Embryo	0.41
		D0940Y	Embryo	0.21
		F2834T	Embryo	0.39
		S0507W	Embryo	0.26
		K0315Y	Embryo	0.08
14	N6 + 0.30 mg L^{-1} 2,4-D + 0.50 mg L^{-1} kinetin + 6% (m/v) sucrose	M28Y	Embryo	0.20
		K054W	Embryo	0.23
		K054W	Leaf base	0.10
		M28Y	Leaf base	1.34
		D0940Y	Embryo	0.70
		F2834T	Embryo	0.41
		S0507W	Embryo	0.75
		K0315Y	Embryo	0.53
		K0315Y	Leaf base	0.30
		I137TN	Leaf base	0.38

[1]Source of callus refers to the tissue which was used to initiate callus (Leaf base = explants of *in vitro* germinated seedlings; Embryo = immature embryos).

Callus which was initially incubated for 10 d on auxin free regeneration medium supplemented with a high level of sucrose (6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8; Table 5.3; Experimental conditions 1 to 4), tended to develop a number of roots, and small, white root hairs. Some calli (e.g. those of M28Y derived from immature embryos) had developed many roots (Plate 46). Excessive root formation has previously been described as a common phenomenon in callus cultures incubated on reduced 2,4-D concentrations (Harms, *et al.*, 1976). It is widely

accepted that organogenic calli that produce roots before shoots will not differentiate further. Shoot differentiation must occur before root initiation. Embryogenic calli produce embryos which germinate, hence a root and shoot appear simultaneously.

Plate 46 Callus of M28Y (derived from an immature embryo) incubated on Regeneration medium 1 (N6 - 2,4-D + 6% (m/v) sucrose; Experimental condition 3). Note the abundance of root hairs (bar = 1.25 mm).

Nodular, white callus of D0940Y (derived from an immature embryo) on N6 auxin free medium (Experimental condition 3) developed a few small, green shoots on the surface of the callus (Plate 47). These shoots were pale green in colour and were curled. After 7 d on Regeneration medium 2, a shoot, which first appeared at the side of D0940Y callus (incubated on N6 based medium; Experimental condition 3), had developed into a small plantlet with three short roots (Plate 48). This plantlet appeared to have developed by embryogenesis as both roots and shoots developed simultaneously. The plantlet was carefully excised from the callus and transferred to Plantlet medium in a glass culture tube (70 cm^3; 15 mL medium). After 2 d in the glass culture tube, the plant was c.a. 6 cm in height and had two roots which penetrated the Plantlet medium. After a further 21 d, the plantlet had grown

to a height of 9 cm, had developed a number of roots and had two leaves. The first leaf had lost its green colour and become translucent, i.e. vitrified. Three days later the second leaf had begun to turn brown. Up to this point the development of the plantlet had been normal. However, after a further 3 d, three new leaves began to emerge simultaneously from the leaf whirl. At this stage the plantlet was transferred to Potting medium in a pot within a Coca-Cola® bottle. After transfer, however, the plantlet ceased to grow. It progressively turned brown and was discarded after one month. After 14 d on Regeneration medium 2, the remaining callus of D0940Y, and the calli of the other inbreds, had developed more roots. However, no further changes were noted in these regeneration cultures. After a further 2 wk on Regeneration medium 2, the calli had started to turn brown, had not increased very much in size, and had undergone no apparent changes. The calli were subcultured to fresh Regeneration medium 2 every 14 d. However, no further changes in the cultures were noted, and three months after the initiation of Experimental conditions 1 to 4, the regeneration cultures were discarded.

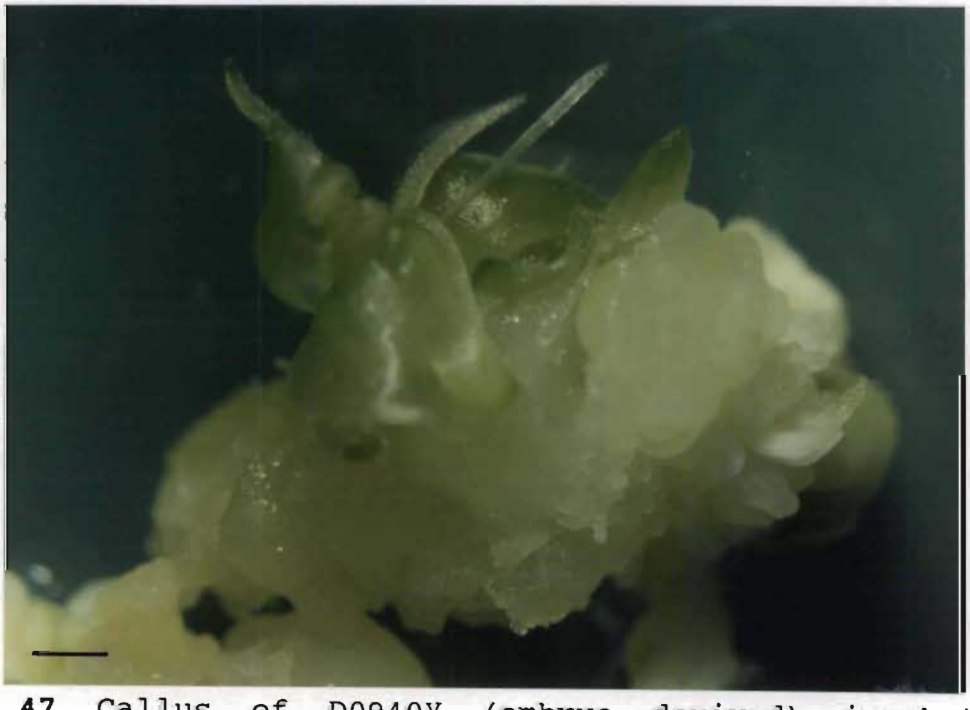


Plate 47 Callus of D0940Y (embryo derived) incubated on Regeneration medium 1 (N6 - 2,4-D + 6% (m/v) sucrose; Experimental condition 3). Small, curled green shoots visible after 10 d of incubation (bar = 1.25 mm).

Plate 48 Plantlet regenerated from nodular, white callus of D0940Y (embryo derived) incubated on Regeneration medium 1 (N6 - 2,4-D + 6% (m/v) sucrose; Experimental condition 3). Plantlet transferred to Plantlet medium in glass culture tube (70 cm^3; 15 mL medium) (bar = 5 mm).

Callus incubated on regeneration medium which contained 0.25 mg L^{-1} 2,4-D, 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8 (Table 5.3; Experimental conditions 5 to 8), had undergone no visible changes after 10 d. Two weeks after subculture to Regeneration medium 2, the calli had started to develop small green regions and had undergone a considerable increase in size. These green areas may have been either the formation of shoot primordia or cellular differentiation with cells producing chlorophyll. After a further 14 d, the calli had all started to turn brown, and most had developed a few roots. The calli had undergone no further apparent changes. The calli were continuously subcultured to fresh Regeneration medium 2 every 14 d. However, no further changes were noted in the regeneration

cultures, and three months after the initiation of Experimental conditions 5 to 8, the regeneration cultures were discarded. The non-regeneration of plantlets in these experiments indicates that the small green areas which developed were in fact differentiated cells, and not shoot primordia. Previously, researchers found that the use of regeneration medium containing 0.25 mg L^{-1} 2,4-D induced shoot-like structures and buds which did not develop fully into shoots (Harms, *et al.*, 1976).

Some callus cultures readily formed embryoids under normal callus maintenance conditions (e.g. M28Y; Plate 29; K054W; Plate 32; K0315Y; Plate 33). Therefore, callus was incubated for 10 d on Regeneration medium 1 which contained 3 mg L^{-1} 2,4-D, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8 (Table 5.3; Experimental conditions 9 to 12) to stimulate maturation of embryoids already present in the callus (Kamo, *et al.*, 1985; Armstrong, 1994). The calli developed a few small shoot-like structures at the surface. After 14 d on Regeneration medium 2, the calli had undergone no apparent changes, apart from a moderate increase in size and the development of a few nodular structures at the surface of the tissue. A further 2 wk later, the regeneration cultures had all started to turn brown. Most of the callus cultures had developed a few roots. Seven weeks after the initiation of the regeneration experiment, a shoot (c.a. 2 cm in length) developed from nodular, white callus of D0940Y (embryo derived; Experimental condition 12). The small plantlet was carefully excised from the callus and transferred to Plantlet medium in a glass culture tube. This plantlet, however, did not develop any further and eventually died. The remaining callus of D0940Y and the calli of the other inbreds on Regeneration medium 2 were continuously subcultured to fresh medium every 14 d. However, no further changes in the calli were noted, and three months after the initiation of Experimental conditions 9 to 12, the regeneration cultures were discarded.

The greatest regeneration response was observed in callus incubated on MS or N6 based medium supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8 (Table 5.3; Experimental conditions 13 and 14). After 10 d on this Regeneration medium 1, the calli had developed white, nodular embryoids at their surfaces (Plate 49). A number of short roots and small, green shoot primordia also became apparent (Plate 50). After 14 d on Regeneration medium 2, many shoot primordia were visible on the callus of D0940Y (embryo derived; Experimental condition 14) and S0507W (embryo derived; Experimental condition 13). Three plantlets had developed from callus of K0315Y (embryo derived; Experimental condition 13). These plantlets were carefully excised from the callus and transferred to 500 mL Schott bottles containing 100 mL Plantlet medium (Plate 51). After 3 d on this medium, the plantlets appeared to be thriving. One plantlet had developed an extensive root system and was c.a. 4 cm in height. However, after a further 10 d, when the plantlet had reached a height of 7 cm, the leaves began to turn brown. When this plantlet was c.a. 10 cm in height, with three leaves and c.a. 20 roots, it was transferred to Potting medium in a pot within a Coca-Cola® bottle. However, after transfer, the plantlet continued to turn brown and did not grow. After a month in Potting medium, the plantlet was discarded. The other two plantlets of K0315Y did not develop beyond c.a. 4 cm in height and were discarded after a further 3 wk of incubation on Plantlet medium.

One week after K0315Y plantlets were transferred to Plantlet medium, two plantlets of D0940Y and eleven plantlets of S0507W (Plate 52; Plate 53) were excised from Regeneration medium 2 and transferred to Plantlet medium in 500 mL Schott bottles. A further two plants of K0315Y were also transferred to Plantlet medium at this time. Unfortunately, due to microbial contamination, these plantlets were all lost. The remainder of the callus on Regeneration medium 2 did not produce any more plantlets. The calli turned brown progressively and three months after the start of the regeneration experiment, the cultures were

discarded. Large 500 mL Schott bottles were preferred to glass culture tubes for the growth of regenerated plantlets due to the larger amount of space and medium available to the plantlets. Plantlets grown in glass culture tubes developed at a slower rate than those grown in the larger Schott bottles.

Plate 49 White embryoids (a) developing at the surface of K0315Y callus (embryo derived) incubated on Regeneration medium 1 supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin and 6% (m/v) sucrose (Experimental condition 14) (bar = 1.25 mm).

Plate 50 Small, green shoot primordia developing at the surface of S0507W callus (embryo derived) incubated on Regeneration medium 1 supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin and 6% (m/v) sucrose (Experimental condition 14) (bar = 1.25 mm).

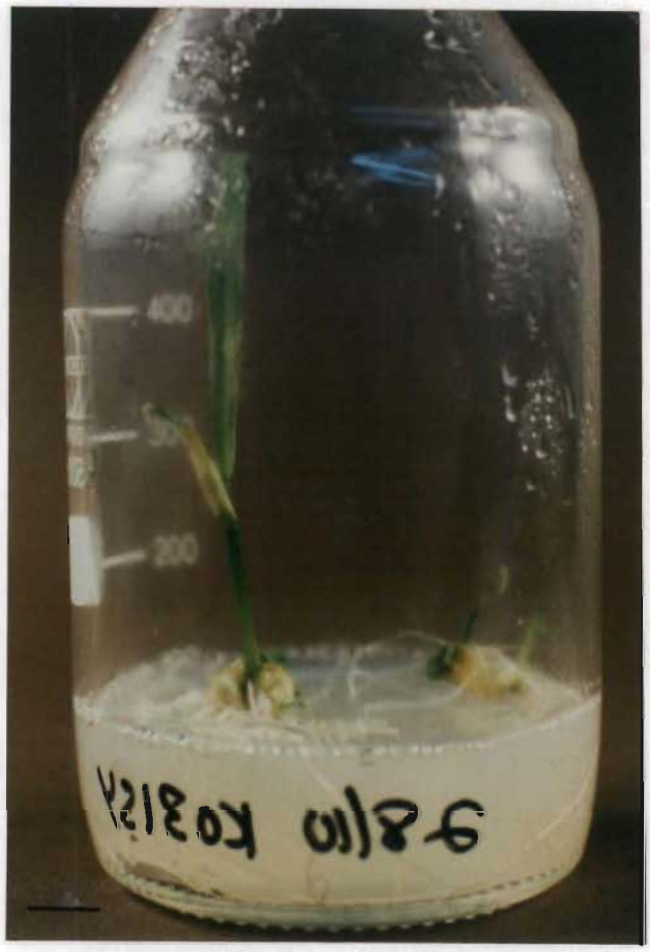


Plate 51 Regenerated plantlets of K0315Y (embryo derived) on Plantlet medium in a 500 mL Schott bottle (bar = 11.4 mm).

Plate 52 Plantlet of S0507W developing on Regeneration medium 2 (Experimental condition 13) (bar = 1.25 mm). Only shoot visible. Shoot removed from callus to reveal developing roots.

Plate 53 Plantlets of S0507W transferred to Plantlet medium in a 500 mL Schott bottle (bar = 11.4 mm).

At the start of the regeneration experiments, the fresh mass of the callus used in each experiment was determined (Table 5.3). Using this mass, the number of plantlets regenerated per mg fresh mass of callus was calculated (Table 5.4). The highest number of plantlets per mg fresh mass of callus were regenerated from callus of K0315Y and S0507W incubated on MS based medium supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8. This medium was subsequently chosen for further regeneration experiments (Section 5.2.2).

Table 5.4 Number of plantlets regenerated per mg fresh mass of callus subcultured to Regeneration medium 1.

Expt. condition	Inbred	Fresh mass of callus (mg)	No. plantlets regenerated per mg fresh mass of callus
3	D0940Y	380	0.0026
12	D0940Y	100	0.0100
13	K0315Y	80	0.0880
	S0507W	260	0.0420
14	D0940Y	700	0.0029

As ascertained by other researchers, MS based media were clearly superior to N6 for plantlet regeneration (Shillito, *et al.*, 1989). Kinetin was used to induce shoot formation in these regeneration experiments. Research has established that selected concentrations of both kinetin and BAP increased plantlet formation over controls without cytokinins, especially when added in combination with 0.25 mg L^{-1} 2,4-D (Shillito, *et al.*, 1989). In all of the regeneration experiments, no clear differences were observed between callus incubated in the dark or under a 12 h light/12 h dark photoperiod for the first 10 d. Plantlets were not regenerated when Regeneration medium 1 supplemented with 2% (m/v) sucrose was used (Experimental conditions 5 to 8). Therefore, it was concluded that the higher concentration of sucrose (6% (m/v)) used in the other regeneration media stimulated embryo enlargement and maturation, as determined by other researchers (Kamo, *et al.*, 1985; Armstrong, 1994). During the hardening off stage, temperature and light intensity should have been lower than that used for normal maize greenhouse cultivation (Armstrong, 1994). A lower temperature, and an alteration of the photoperiod from 12 h light/12 h dark to 16 h light/8 h dark may have improved the regeneration protocol still further. However, as only one growth chamber was available for the initiation and maintenance of maize callus, as well as regeneration experiments, the temperature and photoperiod were identical to that used for callus maintenance ($27\pm1^{o}C$; 12 h light/12 h dark). The photoperiod could therefore not be altered without the possibility of affecting the callus cultures maintained under the 12 h light/12 h dark regime.

In all of the regeneration experiments conducted on maize callus, plantlets were regenerated from nodular, white callus of the inbreds S0507W, D0940Y and K0315Y. Other researchers also found that regeneration occurred in hard, irregularly shaped, nodular, white or yellow callus (Duncan, *et al.*, 1985). Callus of all three regenerating inbreds had been initiated from immature embryos. Callus which had been initiated from young leaf bases of *in vitro* germinated seedlings, when placed on the various regeneration media, produced roots and shoot primordia. These shoots, however, did not develop into plantlets. It was therefore concluded that the regenerative ability of this callus was not as good as that of callus initiated from immature embryos. There are indications that, in grasses, only immature tissues proliferate readily in culture, and that plants may only be regenerated when the explants derive from a source close to an embryogenic state, e.g. the scutellum of immature embryos (Wernicke & Milkovits, 1984). Therefore, the immature embryos used for callus were competent for regeneration, and passed this competence on to the callus. However, the regenerative competence of maize leaf tissue is small (Chang, 1983) and as a consequence, no plantlets were regenerated from callus initiated from young leaf bases of *in vitro* germinated seedlings.

In conclusion, maize plantlets could be regenerated from callus of K0315Y, D0940Y and S0507W incubated on MS based regeneration medium supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8. Improvement of the hardening off process is, however, still required. During this phase, reduction in the amount of vitrification and microbial contamination (through inclusion of fungicides) is required. Additionally, a small mist bed would probably have enabled better control over humidity during the hardening off process.

5.3.2 Application of established regeneration protocol to maize callus which had been subjected to particle bombardment

Callus which had been subjected to particle bombardment was subcultured to regeneration medium. After 10 d of incubation on Regeneration medium 1 (MS medium supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8), the callus cultures were examined for signs of regeneration. Small, green shoot primordia were produced in all of the of the calli. No roots had been initiated. The calli were subcultured to Regeneration medium 2 (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8) and examined every second day. After 2 wk and 4 wk on the second regeneration medium, the shoot primordia had not developed any further, and the calli were starting to turn brown. The callus was subcultured twice more to fresh regeneration medium; however, no plantlets were regenerated. Callus which had originally been initiated from maize immature embryos produced more shoot primordia than callus which had been initiated from young leaf bases of *in vitro* germinated seedlings. Even though callus initiated from immature embryos was more competent for regeneration than callus initiated from young leaf bases of *in vitro* germinated seedlings, no plantlets were regenerated from callus which had been subjected to particle bombardment. The particle bombardment experiments were conducted with very small callus clumps (1.0 to 1.5 cm^3). It is therefore probable that these small pieces of callus were not competent for regeneration. Additionally, the particle bombardment process itself may have affected the callus in some way, thus affecting its competence for regeneration.

CHAPTER 6
OVERVIEW OF RESULTS AND FINAL CONCLUSIONS

The results obtained in this study on the *in vitro* culture and transformation of 10 South African elite maize inbreds, provides a basis for future research on the improvement of maize by tissue culture and genetic engineering techniques. Maize inbreds of diverse backgrounds were utilized to determine whether any South African inbreds used in commercial hybrid seed production would be amenable to *in vitro* manipulation. The initiation and maintenance of callus cultures, from both immature leaf base explants excised from *in vitro* germinated seedlings and immature embryos, was demonstrated on Murashige and Skoog (MS) medium supplemented with modified concentrations of the following supplements: glycine (7.7 mg L^{-1}), nicotinic acid (1.3 mg L^{-1}), thiamine-HCL (0.25 mg L^{-1}), pyridoxine-HCL (0.25 mg L^{-1}), calcium pantothenate (0.25 mg L^{-1}), casein hydrolysate (200 mg L^{-1}), L-asparagine (198 mg L^{-1}), sucrose (20 mg L^{-1}), 2,4-D (3 mg L^{-1}) and agar (10 g L^{-1}); pH 5.8. Callus cultures were incubated at $27\pm1^{o}C$ under a 12 h light/12 h dark photoperiod. In this study, two types of embryogenic callus were obtained. These corresponded in appearance to the compact, nodular Type I and soft, friable Type II embryogenic callus described previously (Vasil, *et al.*, 1985; Vasil & Vasil, 1986; Fransz & Schel, 1991). Immature embryos of maize are known to be a good source of totipotent cells with which to initiate embryogenic cultures (Hall, 1991; Vasil & Vasil, 1991; Gnanapragasam & Vasil, 1992), as was found in this study. The initiation of callus from immature embryos of South African maize inbreds resembled that observed by international researchers (Lu, *et al.*, 1982; Lu, *et al.*, 1983), with callus initially proliferating at the coleorhizal end of the embryo. Callus initiation from leaf, leaf roll and pith explants of maize tissue was unsuccessful probably because the ability of leaf cells to express morphogenic capability decreases as the leaf matures (Chang, 1983). The basal 1 to 4 cm leaf portion (above the ligule) of *in vitro* germinated seedlings produced

callus on callus initiation medium. These immature sections, taken from regions that are temporally and spatially closest to the meristem, are known to be a better source of leaf material with a high embryogenic response (Wernicke & Milkovits, 1984).

Friable callus was used to initiate cell suspension cultures of M28Y and M162W, which were maintained in liquid MS callus maintenance medium supplemented with sucrose (20 mg L^{-1}) and 2,4-D (3 mg L^{-1}); pH 5.8, and incubated in the dark at 27°C in an orbital shaker water bath operating at 150 rpm. The establishment of cell suspension cultures of maize is known to be extremely difficult, as was found with M28Y and M162W. Even though healthy, friable callus was used, the establishment of finely dispersed, rapidly growing suspension cultures was not accomplished. As the initiation of suspension cultures was not a complete study, all work on maize suspension culture conducted in this study is confined to an appendix. It is hoped that the work presented in Appendix E may help future researchers who may want to establish cell suspension cultures of maize.

Greenhouse grown leaf tissue, callus cultures and cell suspensions were used as sources for the isolation of viable maize protoplasts with commercial enzymes. An efficient protocol for the isolation of maize protoplasts from callus was established, with an enzyme combination of 2.5% (m/v) Cellulase Onozuka R-10, 0.5% (m/v) Macerase® Pectinase and 0.5% (m/v) Hemicellulase (Sigma®) being most effective. Subsequent to static digestion for 16 h, the digested callus was gently teased apart to release the protoplasts. An optimum concentration of mannitol (11% (m/v)) was determined for use as osmotic agent. Cells of South African maize plants may perhaps have thick cell walls as the plants have been bred to withstand harsh environmental conditions, in particular drought. This high concentration of mannitol is therefore necessary to protect the maize protoplasts, which without the cell wall, tend to imbibe excessive amounts of water through osmosis. The viability of the protoplasts was ascertained with Evan's Blue dye and Fluorescein diacetate.

Culture of maize protoplasts isolated from callus of elite South African inbreds was unsuccessful, with no cell wall regeneration being observed. The genotype of the original explant was found to critically affect the culture of protoplasts; therefore, it is probable that none of the 10 inbreds used in this study were competent for regeneration from protoplasts. Nurse cultures, critically important for the culture of maize protoplasts (Somers, *et al.*, 1987; Rhodes, *et al.*, 1988a; Rhodes, *et al.*, 1988b), could not be used for protoplast culture as a suspension culture was not correctly established. This may have contributed to the recalcitrance of the protoplast cultures. Time permitting, various other protoplast culture media and supplements may have resulted in some success with the protoplast cultures. For example, Nitsch and Nitsch medium (Imbrie-Milligan, *et al.*, 1987), or KM medium (Vasil & Vasil, 1987b; Prioli & Söndahl, 1989; Shillito, *et al.*, 1989) may have been used with supplements such as coconut water, NAA, BAP and cell wall precursors (Imbrie-Milligan, *et al.*, 1987). In addition, alteration of the growth conditions for protoplast culture may have been successful, e.g. an increase in humidity (Watt, 1996; Personal communication). Therefore, the regeneration of cells and callus from maize protoplasts may have been achieved through the use of finely dispersed embryogenic suspension cultures as the source material, through the use of nurse cultures or through the use of another culture medium. Additionally, at the beginning of the study a control maize genotype, highly amenable to tissue culture and regeneration, such as Black Mexican Sweet, was unfortunately not available for use as a standard.

Callus cultures, cell suspension cultures, and protoplasts isolated from maize callus cultures, were used in preliminary investigations involving gene transfer into maize by direct gene transfer methods. Protoplasts isolated from callus cultures, cell suspension culture cells and maize callus were subjected to electroporation with a Pro-Genetor™ or BIO-RAD Gene Pulser® electroporator. Protoplasts isolated from callus are ideal sources of material for transformation investigations on

transient gene expression. Transformation of maize protoplasts and cell suspension culture cells was unsuccessful in this study. It is possible that the reduction in protoplast viability, caused by the high voltages used during electroporation, decreased the viability to such an extent that any transformed protoplasts were not detected by the GUS assay. Additionally, the GUS assay used may not have been stringent enough to detect the transformed protoplasts and suspension culture cells as the blue colour was very sensitive to exposure to light. Successful transformation of maize callus was obtained with the Pro-Genetor™ electroporator set to a voltage of 350 V, with one pulse of 50 ms duration. The pBI221 (β-glucuronidase) plasmid construct was used. Maize callus was also successfully transformed by particle bombardment with tungsten particles coated with either the pAHC25 (β-glucuronidase) or the pDP687 (anthocyanin) plasmid construct.

As the essence of any transformation technique is the regeneration of transformed plantlets, callus was subcultured to regeneration medium. Plantlets were regenerated from nodular, white callus cultures of the maize inbreds S0507W, D0940Y and K0315Y. The plantlets were regenerated on N6 or MS medium supplemented with 2,4-D (0.3 mg L^{-1}), kinetin (0.5 mg L^{-1}), sucrose (6% (m/v)) and agar (10 g L^{-1}); pH 5.8, at 27±1°C under a 12 h light/12 h dark photoperiod. Callus of all three regenerating inbreds had been initiated from immature embryos. Callus which had been initiated from immature leaf bases of *in vitro* germinated seedlings produced roots and shoot primordia on regeneration medium. These shoots, however, did not develop into plantlets. It was therefore concluded that the regenerative ability of this callus was not as good as that of callus initiated from immature embryos. The immature embryos used for callus were competent for regeneration, and passed this competence on to the callus. However, the regenerative competence of maize leaf tissue is low (Chang, 1983) and as a consequence, no plantlets were regenerated from callus initiated from immature leaf bases of *in vitro* germinated seedlings. Manipulation of the environmental conditions in the custom-made Conviron (e.g. a

lower temperature, a different photoperiod (16 h light/8 h dark) and a lower light intensity) may have allowed for better regeneration from the callus. The fact that some inbreds produced embryogenic callus and plantlets, while others were incapable of regeneration illustrates the importance of the genotype in regeneration. As all three regenerating inbreds (S0507W, D0940Y and K0315Y) belong to the (M) heterotic group, it is likely that these inbreds all contain some common ancestor. This common ancestor may have a good tissue culture response, which was consequently passed on to the three inbreds successfully regenerated. The elite inbreds regenerated in this study are used as parents in commercial hybrid seed production in Natal. Therefore, the use of these three inbreds in transformation studies could enable supplementation of conventional breeding programmes by transformation with characteristics such as herbicide tolerance.

The production of callus from immature leaf bases of *in vitro* germinated seedlings requires less time than the production of callus from immature embryos (for which plants have to be grown to maturity). Therefore, should an efficient regeneration technique be established for callus initiated from young leaf bases of *in vitro* germinated seedlings, transformed maize plants for use in commercial breeding programmes could be produced in a matter of months. The limitations inherent in protoplast regeneration techniques represents a serious impediment to the advancement of *in vitro* methods for crop improvement (Imbrie-Milligan & Hodges, 1987). However, continued improvement of DNA-delivery techniques as well as continued development of transformable tissue targets should enhance and streamline genetic manipulation of maize in the future (Laursen, *et al.*, 1994). Most importantly, particle bombardment, or electroporation of embryos or regenerable callus, could considerably shorten the time required for the production of transgenic maize plants by the removal of difficult cell suspension and protoplast culture steps. In addition, as protoplast culture and regeneration is a source of somaclonal variation, the direct transformation of

callus or immature embryos by particle bombardment or electroporation may considerably decrease the amount of somaclonal variation produced in transformation experiments. The fact that microprojectile bombardment and electroinjection can be applied to intact totipotent tissues and organs of cereal crop species, in itself, is a sufficient cause for optimism to effect transformation in such recalcitrant but economically important crop species. Future research on the transformation of South African maize germplasm could be aimed at the isolation of an agriculturally important gene (e.g. resistance to grey leaf spot) for the genetic improvement of local cultivars. This gene would have to be cloned, sequenced and modified for expression in the maize genome. The agriculturally important gene could then be introduced into an inbred commonly used in commercial hybrid seed production through particle bombardment or electroporation of regenerable callus. Plantlets could then be regenerated and submitted for field trials prior to introduction into the commercial breeding programme.

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APPENDIX A
CALLUS INITIATION AND MAINTENANCE MEDIA

A.1 N6 Medium stock solutions (Rhodes & Gray, 1991)

100x Iron stock solution

Na_2EDTA	3.72 g
$FeSO_4.7H_2O$	2.78 g

The EDTA (Ethylene diamine tetra acetic acid, sodium salt) was boiled vigorously for 1-2 min in 200 mL distilled H_2O and added to the $FeSO_4$ dissolved in 200 mL distilled H_2O. The volume was brought to 1 L with distilled H_2O. The solution was sterilised by autoclaving at 121^{o}C (103.5 kPa) for 15 min.

10x N6 Major salts

KNO_3	28.30 g
$(NH_4)_2SO_4$	4.63 g
KH_2PO_4	4.00 g

The salts were added in the order specified to a beaker containing approximately 500 mL distilled H_2O. The solution was stirred until all salts were dissolved and the volume brought to 1 L with distilled H_2O. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 1210C (103.5 kPa) for 15 min.

100x N6 Minor salts

$MnSO4.1H_2O$	0.33 g
H_3BO_3	0.16 g
$ZnSO_4.7H_2O$	0.15 g
KI	0.08 g

The salts were added in the order specified to a beaker containing approximately 500 mL distilled H_2O. The solution was stirred until all salts were dissolved and the volume brought to 1 L with distilled H_2O. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

100x N6 Vitamin stock solution

Thiamine-HCl	0.02 g
Nicotinic acid	0.01 g
Pyridoxine-HCl	0.01 g
Glycine	0.04 g
Casein Hydrolysate	2.00 g

The vitamins were dissolved in 200 mL distilled H_2O and the pH adjusted to 5.8 with 1 M NaOH. The solution was filter sterilised through a 0.22 μm Millipore filter and stored in the door of a refrigerator.

$CaCl_2$ Stock solution

$CaCl_2.2H_2O$	0.40 g

The salt was dissolved in approximately 10 mL distilled H_2O and the final volume adjusted to 20 mL. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

$MgSO_4$ Stock solution

$MgSO_4.7H_2O$	3.70 g

The salt was dissolved in approximately 10 mL distilled H_2O and the final volume adjusted to 20 mL. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

2,4-Dichlorophenoxyacetic acid stock solution

2,4-D	0.05 g

The 2,4-D was dissolved in 2 mL ethanol. Distilled H_2O was slowly added to a volume of 100 mL and the pH adjusted to 5.8 with 1 M NaOH. The solution was filter sterilised through a 0.22 μm Millipore filter and stored in the door of a refrigerator.

Starter medium

Sucrose	20.00 g
L-asparagine	1.98 g

The sucrose and asparagine were dissolved in 870 mL distilled H_2O and the pH of the solution adjusted to 5.8 with 1 M NaOH. The solution was dispensed into 87 mL aliquots. Agar (Oxoid; 1 g) was added to each aliquot which was then sterilised by autoclaving at 121°C (103.5 kPa) for 15 min. For protoplast isolation, this medium was made up in a volume of 810 mL and dispensed into 81 mL aliquots, with agar (N6 Protoplast culture medium solidified with 6 g L^{-1} agar and supplemented with 11% (m/v) mannitol, 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D; pH 5.8 = N6(0.6)) or without agar (N6-PC) being added.

N6 medium composition (100 mL)

Starter medium	87 mL
10x N6 Major salts	10 mL
100x N6 Minor salts	1 mL
100x Iron stock solution	1 mL
$CaCl_2$ Stock solution	100 μL
$MgSO_4$ Stock solution	100 μL
100x N6 Vitamin stock solution	1 mL
2,4-D Stock solution	400 μL (2 mg L^{-1})
Final pH 5.8	

The stock solutions were aseptically added to Starter medium (N6) which had been melted. The medium was then dispensed into Petri dishes prior to setting.

Final composition of N6 medium

Salts	*Final concentration* (mg L^{-1})
$(NH_4)_2SO_4$	463.0
KNO_3	2830.0
$CaCl_2.2H_2O$	200.0
$MgSO_4.7H_2O$	185.0
KH_2PO_4	400.0
Na_2EDTA	37.2
$FeSO_4.7H_2O$	27.8
H_3BO_3	1.6
$MnSO_4.1H_2O$	3.3
$ZnSO_4.7H_2O$	1.5
KI	0.8
Organic constituents	
Thiamine-HCl	1.0
Glycine	2.0
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Casein Hydrolysate	100.0
L-asparagine	198.0
Sucrose	20000.0

A.2 Murashige-Skoog (MS) Modified Medium stock solutions (after Murashige & Skoog, 1962; after Green & Phillips, 1975; Hughes, 1984)

100x Iron stock solution

Na_2EDTA	3.72 g
$FeSO_4.7H_2O$	2.78 g

The EDTA was boiled vigorously for 1-2 min in 200 mL distilled H_2O and added to the $FeSO_4$ dissolved in 200 mL distilled H_2O. The volume was brought to 1 L with distilled H_2O. The solution was sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

MS Macronutrient stock solution

NH_4NO_3	16.5 g
KNO_3	19.0 g
KH_2PO_4	1.7 g

The salts were added in the order specified to a beaker containing approximately 500 mL distilled H_2O. The solution was stirred until all salts were dissolved and the volume brought to 1 L with distilled H_2O. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

MS Micronutrient stock solution 1

$MnSO_4.1H_2O$	2.23 g
$ZnSO_4.7H_2O$	0.86 g
H_3BO_3	0.62 g

The salts were added in the order specified to a beaker containing approximately 100 mL distilled H_2O. The solution was stirred until all salts were dissolved and the volume brought to 200 mL with distilled H_2O. The pH was adjusted to 5.8 with

1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

MS Micronutrient stock solution 2

KI	0.0830 g
$Na_2MoO_4.2H_2O$	0.0250 g
$CuSO_4.5H_2O$	0.0025 g
$CoCl_2.6H_2O$	0.0025 g

The salts were added in the order specified to a beaker containing approximately 100 mL distilled H_2O. The solution was stirred until all salts were dissolved and the volume brought to 200 mL with distilled H_2O. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

$CaCl_2$ Stock solution

$CaCl_2.2H_2O$	0.40 g

The salt was dissolved in approximately 10 mL distilled H_2O and the volume adjusted to 20 mL. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

$MgSO_4$ Stock solution

$MgSO_4.7H_2O$	3.70 g

The salt was dissolved in approximately 10 mL distilled H_2O and the volume brought to 20 mL. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

100x MS Vitamin stock solution

Glycine	0.0770 g
Nicotinic acid	0.0130 g
Thiamine-HCl	0.0025 g
Pyridoxine-HCl	0.0025 g
Ca_2 Pantothenate	0.0025 g
Casein Hydrolysate	2.0000 g

The vitamins were dissolved in 100 mL distilled H_2O and the pH adjusted to 5.8 with 1 M NaOH. The solution was filter sterilised through a 0.22 μm Miilipore filter and stored in the door of a refrigerator.

2,4-Dichlorophenoxyacetic acid stock solution

2,4-D	0.05 g

The 2,4-D was dissolved in 2 mL ethanol. Distilled H_2O was slowly added to a volume of 100 mL and the pH adjusted to 5.8 with 1 M NaOH. The solution was filter sterilised through a 0.22 μm Millipore filter and stored in the door of a refrigerator.

Starter medium

Sucrose	20.00 g
L-asparagine	1.98 g

The sucrose and asparagine were dissolved in 870 mL distilled H_2O and the pH of the solution adjusted to 5.8 with 1 M NaOH. The solution was dispensed into 87 mL aliquots. Agar (Oxoid; 1 g) was added to each aliquot which was then sterilised by autoclaving at 121^{o}C (103.5 kPa) for 15 min.

MS medium composition (100 mL)

Starter medium	87 mL
MS Macronutrient stock solution	10 mL
MS Micronutrient stock solution 1	200 µL
MS Micronutrient stock solution 2	200 µL
100x Iron stock solution	1 mL
$CaCl_2$ Stock solution	100 µL
$MgSO_4$ Stock solution	100 µL
100x MS Vitamin stock solution	1 mL
2,4-D Stock solution	400 µL (2 mg L^{-1})
Final pH 5.8	

The stock solutions were aseptically added to Starter medium (MS) which had been melted. The medium was then dispensed into Petri dishes prior to setting.

Final composition of MS medium

Salts	*Final concentration* mg L^{-1}
NH_4NO_3	1650.000
KNO_3	1900.000
KH_2PO_4	170.000
$CaCl_2.2H_2O$	200.000
$MgSO_4.7H_2O$	185.000
Na_2EDTA	37.200
$FeSO_4.7H_2O$	27.800
H_3BO_3	6.200
$MnSO_4.1H_2O$	22.300
$ZnSO_4.7H_2O$	8.600
KI	0.830
$Na_2MoO_4.2H_2O$	0.250
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic constituents	
Glycine	7.700
Nicotinic acid	1.300
Thiamine-HCl	0.250
Pyridoxine-HCl	0.250
Ca_2 Pantothenate	0.250
Casein Hydrolysate	200.000
L-asparagine	198.000
Sucrose	20000.000

A.3 MS Seedling medium (as modified by Wernicke & Brettell, 1982)

Seedling starter medium

Sucrose	10.00 g

The sucrose was dissolved in 880 mL distilled H_2O and the pH of the solution adjusted to 5.8 with 1 M NaOH. The solution was dispensed into 88 mL aliquots. Agar (Oxoid; 1 g) was added to each aliquot which was then sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

MS Seedling medium composition (100 mL)

Seedling starter medium	88 mL
MS Macronutrient stock solution (Appendix A.2)	10 mL
MS Micronutrient stock solution 1 (Appendix A.2)	200 µL
MS Micronutrient stock solution 2 (Appendix A.2)	200 µL
100x Iron stock solution (Appendix A.2)	1 mL
$CaCl_2$ Stock solution (Appendix A.2)	100 µL
$MgSO_4$ Stock solution (Appendix A.2)	100 µL
Final pH 5.8	

The stock solutions were aseptically added to Seedling starter medium which had been melted. The medium was then dispensed into Petri dishes prior to setting.

A.4 Controlled environment chamber

A controlled environment chamber was specifically designed for the incubation of tissue cultures (Plate 54). A Staycold "Coke" fridge (760 L; model no. 1140; Parys, Republic of South Africa) was fitted out with two Osram (L36W/77 Fluora; Germany) fluorescent tubes. The interior of the fridge was painted white to reflect the light to all parts of the chamber. The exterior of the fridge doors was painted black. Adjustable shelves, on which cultures could be placed, were positioned in the fridge. A Carel® universal control (cr7220; Brugine (Padova), Italy) was set to maintain the temperature at $27\pm1^{o}C$. The photoperiod was controlled by a Grässlin timer unit.

Plate 54 The controlled environment chamber designed for the incubation of tissue cultures.

APPENDIX B
PROTOPLAST ISOLATION AND CULTURE SOLUTIONS

B.1 Solutions for the isolation and culture of maize leaf protoplasts

CPW Salt solution (Power, Chapman & Wilson, 1984)

KH_2PO_4	0.027200 g
KNO_3	0.101000 g
$CaCl_2.2H_2O$	1.480000 g
$MgSO_4.7H_2O$	0.246000 g
KI	0.000160 g
$CuSO_4.5H_2O$	0.000025 g

The salts were dissolved in the order specified in approximately 700 mL distilled H_2O. Mannitol was added to the required percentage and the final volume adjusted to 1 L. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

Enzyme solution

2.0% (m/v) Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd)
0.5% (m/v) Macerozyme® R-10 (Yakult Honsha Co., Ltd)
0.5% (m/v) Hemicellulase (Sigma® Chemical Co.)

The enzymes were dissolved in CPW salt solution (containing the required percentage of mannitol). The pH was adjusted to 5.8 and the solution gently filter sterilised through a 0.45 μm Millipore filter. The enzyme solution was freshly prepared immediately preceding each protoplast isolation.

O-acetylsalicylic acid stock solution (Shillito, *et al.*, 1989)

0.2% (m/v) O-acetylsalicylic acid
2% (v/v) DMSO (Dimethyl sulphoxide)
0.1% (m/v) MES (4-Morpholine-ethanesulfonic acid)

DMSO and MES were dissolved in 80 mL distilled H_2O. The O-acetyl salicylic acid was dissolved in this solution, the final volume adjusted to 100 mL, and the pH adjusted to 5.8 with 1 M NaOH. The stock solution was filter sterilised through a 0.22 μm Millipore filter and stored in the freezer compartment of a refrigerator. The stock (50 mL L^{-1}) was aseptically added to the protoplast culture medium (final concentration of 100 mg L^{-1}).

Inositol stock solution (Rhodes & Gray, 1991)

Inositol	2 g

The inositol was dissolved in 20 mL distilled H_2O and the pH adjusted to 5.8 with 1 M NaOH. The stock solution was filter sterilised through a 0.22 μm Millipore filter and stored in a refrigerator.

N6 Protoplast culture (N6-PC) medium composition (100 mL)
(Shillito, *et al.*, 1989; after Rhodes & Gray, 1991)

Starter medium (N6) (without agar, Appendix A.1, required concentration of mannitol)	81 mL
10x N6 Major salts (Appendix A.1)	10 mL
100x N6 Minor salts (Appendix A.1)	1 mL
100x Iron stock (Appendix A.1)	1 mL
$CaCl_2$ Stock (Appendix A.1)	100 µL
$MgSO_4$ Stock (Appendix A.1)	100 µL
100x N6 Vitamin stock (Appendix A.1)	1 mL
2,4-D stock (Appendix A.1)	600 µL (3 mg L^{-1})
O-acetylsalicylic acid stock solution	5 mL
Inositol stock	200 µL
pH 5.6	

The stock solutions were aseptically added to Starter medium (N6). Solid protoplast culture medium (solidified with 6 g L^{-1} agar and supplemented with 11% (m/v) mannitol, 2% (m/v) sucrose and 3 mg L^{-1} 2,4-D; pH 5.8 = N6(0.6)) was made up by the addition of 0.6 g agar (Oxoid) to an 81 mL aliquot of Starter medium (N6).

Evan's Blue dye solution (Gaff & Okong'O-Ogola, 1971; Kanai & Edwards, 1973b; Fromm, Taylor & Walbot, 1985)

0.25 g Evan's Blue in 100 mL N6 liquid medium

The Evan's Blue was dissolved in the liquid medium (with the required concentration of mannitol) and filter sterilised through a 0.22 µm Millipore filter. The solution was stored in the freezer compartment of a refrigerator.

B.2 Solutions for the isolation and culture of maize callus protoplasts

Wash buffer (Rhodes & Gray, 1991)

80 mM $CaCl_2$
0.5% (m/v) MES
11% (m/v) Mannitol

The chemicals were dissolved in the order specified in distilled H_2O and the volume adjusted to 1 L. The pH was adjusted to 5.8 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

Fluorescein diacetate (FDA) solution (Widholm, 1972)

Stock solution

5 mg mL^{-1} Fluorescein diacetate dissolved in concentrated acetone

This solution was filter sterilised through a 0.22 µm Millipore filter and stored at -20°C.

Staining solution

20 µL stock solution into 1 mL 11% (m/v) mannitol solution (2.2 g mannitol dissolved in 20 mL distilled H_2O).

The staining solution was made up immediately before viability determinations. The solution was used fresh and then discarded.

APPENDIX C

ELECTROPORATION, PARTICLE BOMBARDMENT AND GUS ASSAY SOLUTIONS

C.1 Solutions for bacterial transformation and preparation of pBI221 plasmid DNA

Luria broth and agar (Sambrook, *et al.*, 1989)

	g L^{-1}
Bacto-tryptone	10.0
Bacto-yeast extract	5.0
NaCL	10.0
Agar (Oxoid)	15.0

The chemicals (without agar) were dissolved in distilled H_2O, and the volume made up to 1 L. The pH was adjusted to 7.0 with 1 M NaOH. The solution was poured into aliquots of 50 mL. For Luria agar, agar was added to each aliquot. The medium was sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

0.1 M $MgCl_2$ (Sambrook *et al.*, 1989)

$MgCl_2$	0.103 g

The $MgCl_2$ was dissolved in 5 mL of distilled H_2O and sterilised by autoclaving at 121°C (103.5 kPa) for 15 min. The solution was stored in a refrigerator.

0.1M $CaCl_2$ (Sambrook *et al.*, 1989)

$CaCl_2$	0.111 g

The $CaCl_2$ was dissolved in 10 mL distilled H_2O and filter sterilised through a 0.22 μm Millipore filter. The solution was stored in a refrigerator.

Ampicillin stock solution (Sambrook, *et al.*, 1989)

Ampicillin	0.05 g

The ampicillin was dissolved in 10 mL distilled H_2O. The solution was filter sterilised through a 0.22 μm Millipore filter, wrapped in aluminium foil and stored at -20^{o}C. For a concentration of 60 μg mL^{-1}, 600 μL was pipetted into a 50 mL aliquot of Luria agar.

Magic^{TM} Maxiprep DNA Purification Kit (Promega Corp., USA)

The following solutions were supplied with the Magic^{TM} Maxiprep DNA Purification Kit:

Cell resuspension solution

Tris-HCl (pH 7.5)	50 mM
EDTA	10 mM
RNase A	100 μg mL

Cell lysis solution

NaOH	0.2 M
SDS	1% (m/v)

Neutralization solution

Potassium acetate (ph 4.8)	2.55 M

TE Buffer

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM

Column wash solution

NaCL	200 mM
Tris-HCl (pH 7.5)	20 mM
EDTA	5 mM

Dilute 1:1 with 95% ethanol

Agarose Gel (Sambrook *et al.*, 1989)

Agarose	0.2 g

The agarose was dissolved in 25 mL TBE buffer (1X strength) by gentle heating over a bunsen burner.

TBE Running Buffer

Concentrated stock solution (pH 8.0) (Sambrook *et al.*, 1989)

5X:	Tris base	54 g
	Boric acid	27.5 g
	0.5 M EDTA (pH 8.0)	20 mL

The constituents were dissolved and the final volume adjusted to 1 L. The concentrated buffer was diluted to a working strength of 1X (i.e. a 1:5 dilution) for agarose gel electrophoresis. This running buffer could be used three times before it had to be discarded.

0.5 M EDTA (pH 8.0) (Sambrook *et al.*, 1989)

EDTA	146.125 g

The EDTA was added to 800 mL distilled H_2O and stirred vigorously on a magnetic stirrer. The volume was adjusted to 1 L and the pH adjusted to 8.0 with 1 M NaOH.

Loading Buffer (Sambrook *et al.*, 1989)

Sucrose	2 g
Bromophenol blue	0.2 g

The above constituents were dissolved in 10 mL 1X TBE Running buffer and stored at $4^{o}C$.

Ethidium Bromide (Sambrook *et al.*, 1989)

Ethidium bromide was prepared as a stock solution by dissolving 0.1 g ethidium bromide in 10 mL distilled H_2O i.e a 10 mg mL^{-1} stock solution. This solution was stirred for several hours on a magnetic stirrer to ensure the dye had dissolved. The stock solution was stored at room temperature in a bottle wrapped in aluminum foil. The dye was incorporated into the gel and electrophoresis buffer at a concentration of 0.5 μg mL^{-1}.

C.2 Electroporation solutions

2 M KCL (Rhodes & Gray, 1991)

KCl	2.98 g

The KCl was dissolved in 15 mL distilled H_2O and the final volume adjusted to 20 mL. The pH was adjusted to 8.0 with 1 M NaOH and the solution sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

EPM buffer (D'Halluin, *et al.*, 1992)

5 mM $CaCl_2$
10 mM Hepes
0.425 M mannitol
80 mM KCl

The EPM buffer was made up in distilled H_2O. The pH was adjusted to 7.2 with 1 M NaOH, and the solution sterilized by autoclaving at 121°C (103.5 kPa) for 15 min.

N6aph Solution (D'Halluin, *et al.*, 1992)

N6aph Starter medium

Sucrose	3.00 g
L-asparagine	0.09 g
Mannitol	5.40 g

The sucrose, L-asparagine and mannitol were dissolved in 88 mL distilled H_2O and the pH of the solution adjusted to 5.8 with 1 M NaOH. The solution was dispensed into 8.8 mL aliquots, which were sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

N6aph Vitamin stock solution

Thiamine-HCl	0.002 g
Nicotinic acid	0.001 g
Casein Hydrolysate	0.200 g
Inositol	0.200 g

The vitamins were dissolved in 20 mL distilled H_2O. The pH was adjusted to 5.8 with 1 M NaOH and the solution filter sterilised through a 0.22 μm Millipore filter. The N6aph vitamin stock was stored in a refrigerator.

N6aph Solution composition (10 mL)

N6aph Starter medium	8.8 mL
10x N6 Major salts (Appendix A.1)	1 mL
100x N6 Minor salts (Appendix A.1)	100 μL
N6aph Vitamin stock solution	100 μL

The stock solutions were aseptically added to N6aph Starter medium. The N6aph Solution was used fresh and then discarded.

Enzyme solution (D'Halluin, *et al.*, 1992)

0.3% (m/v) Macerozyme® R-10
CPW Salt solution (Appendix B.1)
10% (m/v) Mannitol
5 mM MES

The chemicals were dissolved in CPW Salt solution, and the pH adjusted to 5.6 with 1 M NaOH. The enzyme solution was filter sterilised through a 0.45 μm Millipore filter and used fresh.

C.3 GUS assay solutions

Fixer buffer (after Jefferson, *et al.*, 1987)

Formaldehyde	3.0 mL
Mannitol	9.1 g
MES	0.195 g

The chemicals were dissolved in the order specified in 100 mL distilled H_2O, and the pH adjusted to 5.8 with 1 N NaOH. The solution was filter sterilised through a 0.22 μm Millipore filter and stored in a refrigerator.

Sodium phosphate buffer (after Jefferson, *et al.*, 1987)

Na_2HPO_4	0.2 M
NaH_2PO_4	0.2 M

The salts were dissolved in distilled H_2O, the pH was adjusted to 7.0 and the solution was sterilised by autoclaving at 121^{o}C (103.5 kPa) for 15 min. The buffer was stored in a refrigerator.

Incubation buffer (after Jefferson, *et al.*, 1987)

0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 (pH 7.0)	25.0 mL
0.1 M $K_3[Fe(CN)_6]$	0.25 mL
0.1 M $K_4Fe(CN_6).3H_2O$	0.25 mL
0.1 M Na_2EDTA	0.5 ml
Mannitol	4.6 g

The solutions and mannitol were added to 24 mL distilled H_2O. The buffer was sterilised by autoclaving at 121^{o}C (103.5 kPa) for 15 min and stored at -20^{o}C.

5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid stock (X-Glu) (after Jefferson, *et al.*, 1987)

X-Glu (10 mM)	0.0105 g
DMSO	0.5 mL
Incubation buffer	1.5 mL

X-Glu (Sigma® Chemical Co.) was dissolved in DMSO. The incubation buffer was added and the solution was vortexed. The solution was wrapped in aluminium foil and stored at -20°C.

X-Glu Substrate buffer (50 mL) (after Jefferson, 1987)

0.2 M NaH_2PO_4	9.7 mL
0.2 M Na_2HPO_4	15.2 mL
ferricyanide	0.0825 g
ferrocyanide	0.1050 g
X-Glu	0.025 g

The ferricyanide, ferrocyanide and X-Glu were dissolved in the order specified in 25 mL distilled H_2O. The NaH_2PO_4 and Na_2HPO_4 solutions were added and the pH adjusted to 7.0. The buffer was filter sterilised through a 0.22 μm Millipore filter, wrapped in aluminium foil and stored at -20°C. Triton X-100 (3 μL mL^{-1}) was added to the X-Glu Substrate buffer after vacuum infiltration.

APPENDIX D
REGENERATION MEDIA AND SOLUTIONS

Kinetin stock solution (Gamborg, 1991)

Kinetin	0.05 g

The kinetin was dissolved in 2 mL 0.5 N HCl and heated slightly. Distilled H_2O was slowly added to a volume of 100 mL and the pH adjusted to 5.8 with 1 M NaOH. The solution was filter sterilised through a 0.22 µm Millipore filter and stored in the door of a refrigerator.

Plantlet medium (100 mL) (Wernicke & Brettell, 1982)

Seedling starter medium (Appendix A.3)	88 mL
MS Macronutrient stock solution (Appendix A.2)	10 mL
MS Micronutrient stock solution 1 (Appendix A.2)	200 µL
MS Micronutrient stock solution 2 (Appendix A.2)	200 µL
100x Iron stock solution (Appendix A.2)	1 mL
$CaCl_2$ Stock solution (Appendix A.2)	100 µL
$MgSO_4$ Stock solution (Appendix A.2)	100 µL
100x MS Vitamin stock solution (Appendix A.2)	500 µL

The stock solutions were aseptically added to Seedling starter medium which had been melted. The medium was then dispensed into glass culture tubes (70 cm^3; 15 mL medium) or 500 mL Schott glass bottles (100 mL medium) prior to setting.

Potting medium

Perlite	1 part
Sawdust (fine)	1 part
Vermiculite	1 part
Sand (Umgeni)	1 part
Koya	4 parts

The koya was soaked in warm water and broken up into fine crumbs. The perlite, sawdust, vermiculite and sand were mixed with the koya. The Potting medium was placed into a 1 L beaker and sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

Hoogland nutrient solution (Gamborg, 1991)

	Concentration g L^{-1}	Final molar concentration
Macronutrients		
$Ca(NO_3)_2.4H_2O$	0.94	4.0 mM
$MgSO_4.7H_2O$	0.52	2.0 mM
KNO_3	0.66	6.0 mM
$NH_4H_2PO_4$	0.12	1.0 mM
Sequestrene 330 Fe[1]	0.07	
Micronutrients		
H_3BO_3	28.0	45 μM
$MnSO_4.H_2O$	34.0	20 μM
$CuSO_4.5H_2O$	1.0	0.4 μM
$ZnSO_4.7H_2O$	2.2	0.7 μM
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.0	0.2 μM
H_2SO_4 (concentrated)	5 mL	

[1]Sequestrene 330 Fe = Ethylenediamine tetraacetic acid (Ferric sodium salt)

A 0.1 mL volume of the micronutrient solution was mixed with 1 L of macronutrients, and the pH adjusted to 6.7. The solution was sterilised by autoclaving at 121°C (103.5 kPa) for 15 min.

APPENDIX E
CELL SUSPENSION CULTURES OF MAIZE

E.1 Introduction

Cell suspension cultures are populations of single plant cells or small cell clumps cultured in an agitated liquid medium (Imbrie-Milligan & Hodges, 1986; Vasil, 1988; Hall, 1991). Maize callus cultures are commonly used as an intermediate step towards the initiation of cell suspension cultures for the production of plants from protoplasts (Shillito, *et al.*, 1989; Hall, 1991). Suspension cell cultures of maize are frequently initiated and maintained in liquid callus maintenance medium (Emons & Kieft, 1991) in the dark (Kamo, *et al.*, 1987; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992), but may also be maintained under a 12 h light/ 12 h dark (Fromm, *et al.*, 1987) or 16 h light/8 h dark photoperiod (Somers, *et al.*, 1987; Emons & Kieft, 1991). The temperatures at which maize cell suspensions are maintained range from 24^{o}C (Huang & Dennis, 1989) to 30^{o}C (Maas & Werr, 1989), with an average of 27 to 28^{o}C (Chourey & Zurawski, 1981; Shillito, *et al.*, 1989; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992). Suspensions are shaken at speeds from 100 rpm (Howard, *et al.*, 1987; Emons and Kieft, 1991) to 150 rpm (Chourey & Zurawski, 1981; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992). Subculturing usually takes place every 7 d (Chourey & Zurawski, 1981; Carswell, *et al.*, 1989; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992).

Variation in the conditions required for the isolation of protoplasts from maize leaves, callus and cell suspension cultures (Table 3.1), emphasises the need to optimize isolation conditions for the specific experimental system utilized. For example, a substantially higher yield and viability of protoplasts is obtained from maize cell suspension cultures maintained on MS medium rather than N6 medium (Imbrie-Milligan

& Hodges, 1986; Lyznik, *et al.*, 1989a). Maize protoplasts obtained from Type I and Type II calli from several genotypes were shown to be capable of synthesizing cell walls and forming small clusters of cells (Imbrie-Milligan & Hodges, 1986; Imbrie-Milligan, *et al.*, 1987), while protoplasts obtained from suspension cultures are capable of forming callus (Kamo, *et al.*, 1987). It is generally known that during prolonged culture periods, suspension cells often lose their morphogenic potential due to the occurrence of epi-genetic instability. Consequently, new cell suspensions have to be initiated at regular intervals to maintain regeneration capacity (Roest & Gilissen, 1989).

Transgenic maize has been successfully produced thorough the use of direct gene transfer techniques such as particle bombardment (Gordon-Kamm, *et al.*, 1990; Aves, *et al.*, 1992; Walters, *et al.*, 1992; Murry, *et al.*, 1993), electroporation of protoplasts (Rhodes, *et al.*, 1988b; Murry, *et al.*, 1993), and electroporation of intact maize tissues including cell suspension culture cells and callus (D'Halluin, *et al.*, 1992; Laursen, *et al.*, 1994).

The aim of work presented in this appendix, was to initiate cell suspension cultures of South African elite maize inbreds. Although a complete study was not accomplished, the work is presented here in the hope that it will help future researchers in South Africa who may wish to establish maize cell suspension cultures. Friable callus of established cultures was used in an attempt to develop the cell suspension cultures. Although not correctly established, the suspensions were digested with enzyme preparations to determine if any protoplasts could be isolated. In preliminary transformation studies, maize suspension culture cells were electroporated in the presence of a plasmid encoding the GUS gene, and assayed for transient GUS activity using a histochemical GUS assay. The regeneration protocol established in Chapter 5 was used in an attempt to regenerate plantlets from the maize cell suspension cultures.

E.2 Materials and methods

E.2.1 Cell suspension culture initiation

Rapidly-growing, friable maize callus of M28Y and M162W was used (1 wk after subculture) in an attempt to establish cell suspension cultures. The callus was initiated from the leaf bases of young seedlings germinated *in vitro* (Section 2.2.4). M28Y and M162W callus cultures were used as they produced the most callus under the incubation conditions used. Any callus showing brown, damaged or diseased areas was not used. All initiation and subculture procedures were performed on a laminar flow bench under aseptic conditions.

Autoclaved Erlenmeyer flasks (100 or 250 mL), capped with non-absorbent cotton wool and a double layer of aluminium foil, were used as culture vessels for establishment of the cell suspension cultures. The foil caps of the Erlenmeyer culture flasks were quickly flamed and loosened. As suspension cell cultures are frequently initiated and maintained in liquid callus maintenance medium (Emons & Kieft, 1991), liquid MS-CM medium (Section 2.2.5) (MS modified medium (according to Murashige & Skoog, 1962; Green & Phillips, 1975; Hughes, 1984; Appendix A.2) + 2% (m/v) sucrose + 3 mg L^{-1} 2,4-D; pH 5.8) was used. The suspension culture medium was added to the flasks (20 mL medium per 100 mL flask; 50 mL medium per 250 mL flask) after the foil caps had been removed and the neck of the open flasks had been flamed. The foil caps were replaced on the flasks. In a sterile glass Petri dish, the callus to be used for suspension initiation was weighed (2 g fresh weight) and then sliced into smaller pieces with a sterile scalpel. This callus was then transferred to the suspension culture medium in the flasks. All flasks were secured firmly in a rotary shaker water bath. The cultures were incubated at 27°C in the dark at a speed of 150 rpm.

The cultures were checked after 7 d and any showing signs of microbial contamination were discarded. After 10 d, the suspensions were subcultured (1 part cell suspension : 4 parts fresh medium) into fresh suspension culture medium using sterile 10 mL pipettes. This subculture method therefore restricted the size of the cell clumps transferred to fresh medium to those that could fit through the mouth of the pipette. After a further 10 d the extent of cell aggregation was checked with an Olympus (CK2) inverted microscope. Microphotography of suspension cultures was carried out with a Zeiss Axiophot® microscope. Subsequently, the suspension cultures were subcultured once a week (Chourey & Zurawski, 1981; Carswell, *et al.*, 1989; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992), at which time routine checks for cell aggregation were performed.

E.2.2 Protoplast isolation from suspension cultures

A suspension of M28Y was used for the isolation of protoplasts. This suspension was, however, not correctly established and consisted of large non-embryogenic cells interspersed amongst smaller cytoplasmically dense embryogenic cells. All procedures were performed under aseptic conditions on a laminar flow bench. Ten millilitres of the M28Y suspension culture were pipetted into a 15 mL gradated conical centrifuge tube. The suspension had been agitated to obtain a fully suspended solution prior to sampling of the 10 mL. The suspension was centrifuged at 50 g for 10 min in a Hettich Universal swinging bucket benchtop centrifuge to pellet the cells. The pellet was resuspended in 10 mL digestion buffer (containing 2.5% (m/v) Onozuka R-10; 0.5% (m/v) Macerase® pectinase and 0.5% (m/v) Hemicellulase). The resulting suspension was gently poured into a sterile glass Petri dish (50 mm diameter), which was covered and wrapped in aluminium foil. The suspended cells, in digestion buffer, were incubated for 16 h in the dark at 25°C. After 16 h digestion, the Petri dish was gently swirled to release the protoplasts. The procedure for the isolation of callus protoplasts (Section 3.2.2.1) was then used, starting with the first centrifugation step.

E.2.3 Electroporation of maize suspension culture cells

A suspension of M28Y was used for an investigation into the electroporation of maize suspension culture cells. Gene transfer was attempted with the GUS plasmid pBI221 (Clontech Laboratories, Inc., CA). The cell suspension used for electroporation was, however, not well-established and consisted of large non-embryogenic cells interspersed amongst smaller cytoplasmically dense embryogenic cells. Ten millilitres of M28Y suspension were gently pipetted into a 15 mL gradated conical centrifuge tube and centrifuged at 50 g for 10 min in a Hettich Universal swinging bucket benchtop centrifuge to pellet the cells. The supernatant was removed and replaced with 10 mL EPM buffer (without KCl; Appendix C.2). The suspension cells were gently resuspended and preplasmolysed at room temperature (25°C) for 3 h in this solution.

After 3 h preplasmolysis, the suspension was centrifuged at 50 g for 10 min in a Hettich Universal swinging bucket benchtop centrifuge to pellet the cells. The supernatant was removed, and the cells gently resuspended in 5 mL EPM buffer (with KCl; Appendix C.2). Aliquots (600 µL) of suspended cells were transferred to wells of a Costar® dish. pBI221 Plasmid DNA (25 µg) was added to each well and co-incubated with the preplasmolysed suspension cells for 1 h.

After 1 h, the Costar® dish was transferred to an ice bath for 10 min. After 10 min, the cells were electroporated on ice with the Pro-Genetor™ electroporator, using a range of voltages (300 V; 350 V and 400 V). Two controls were used: incubation of suspension cells with 25 µg pBI221 plasmid DNA, but no electroporation (Control 1); and electroporation at 350 V in the absence of pBI221 plasmid (Control 2). Immediately after electroporation, 200 µL of fresh liquid N6aph solution (Appendix C.2) was added to each well of the Costar® dish. The Costar® dish was incubated on ice for 10 min and then transferred to an incubator at 25°C. The suspension cells were incubated in

the dark for 24 h to enable the expression of the GUS gene. After 24 h, the suspension cells were assayed for GUS expression. The histochemical assay for electroporated protoplasts (Section 4.2.3) was used.

E.2.4 Regeneration of callus from maize cell suspension cultures

Cell suspension cultures of M28Y and M162W were removed from the incubator and allowed to settle for 2 min on a laminar flow bench. A sample (5 mL) from each culture was pipetted onto callus regeneration medium (MS modified supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8) in Petri dishes. The Petri dishes were sealed with Parafilm "M" and incubated at $27\pm1^{o}C$ under a photoperiod of 12 h light/12 h dark. The cultures were observed every second day. After 21 d, the developing callus was scraped off of the regeneration medium with the aid of a sterile spatula, and placed onto the second regeneration medium (MS modified supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8). The regeneration cultures were examined every second day for signs of plantlet regeneration. Every 2 wk the callus was subcultured to fresh medium.

E.3 Results and discussion

E.3.1 Cell suspension culture initiation

Correctly established embryogenic cell suspension cultures are rapidly dividing, and are therefore an excellent source of protoplasts for transformation experiments, where protoplast numbers need to be high to ensure a transformation event. Cell suspension cultures of cereals are very difficult to establish. As embryogenic cell suspension cultures of maize are an excellent source of dividing and totipotent protoplasts (Imbrie-Milligan

& Hodges, 1986; Kamo & Hodges, 1986; Horn, 1991a; Vasil & Vasil, 1991; Taylor, *et al.*, 1994), the initiation of cell suspensions of two South African maize inbreds was attempted.

The inbreds M162W and M28Y were used for the initiation of maize cell suspensions for two reasons. Firstly, these inbreds are important parents in the production of hybrids in maize breeding programmes in South Africa. Secondly, when immature leaf bases of young *in vitro* germinated seedlings were placed on callus initiation medium, these inbreds produced callus that was friable and fast growing under the culture conditions used. They therefore produced the most callus, which could consequently be used for a number of different applications, including the establishment of conditions required for protoplast isolation (Chapter 3). Additionally, as only small amounts of callus were initiated from immature embryos, the majority of this totipotent callus was utilized for regeneration studies, rather than in an attempt to initiate cell suspension cultures.

Initially, 50 mL of suspension culture medium was used in 250 mL Erlenmeyer flasks. After 10 d in suspension culture medium, the cell cultures of M28Y and M162W were cream in colour and had a cloudy appearance due to the presence of many cells in suspension. The large callus clumps used for suspension initiation had increased in size and were beginning to turn brown. The cultures were subcultured by transferring 1 part of a well mixed cell suspension into 4 parts fresh suspension culture medium. As a sterile 10 mL pipette was used for transfer of the cells to fresh medium, the size of cell aggregates transferred was restricted to the smaller aggregates which could pass through the mouth of the pipette (1.0 to 1.5 mm diameter).

Seven days after the first subculture, the suspensions were examined macroscopically and microscopically and hence subcultured. At the macroscopic level, M28Y suspensions consisted of small cream coloured clumps. Not much growth seemed to have occurred since the first subculture. Suspensions of M162W had a

cloudy, cream-coloured appearance with small cell clusters. These suspensions also did not appear to have grown since the first subculture. When examined with the inverted microscope, cell suspensions of M28Y consisted of some large cells (non-embryogenic) which were single or in clusters. There were also a number of cell clusters (of various sizes) consisting of smaller cells which varied in shape from elongated to round (Plate 55). Suspensions of M162W also contained small clumps of cells (Plate 56). However, more single cells were apparent for the suspensions of M162W than those of M28Y. Most of these single cells of M162W (and a few in clusters) were elongated and sausage-shaped. The majority of the small cell aggregates consisted of small round cells.

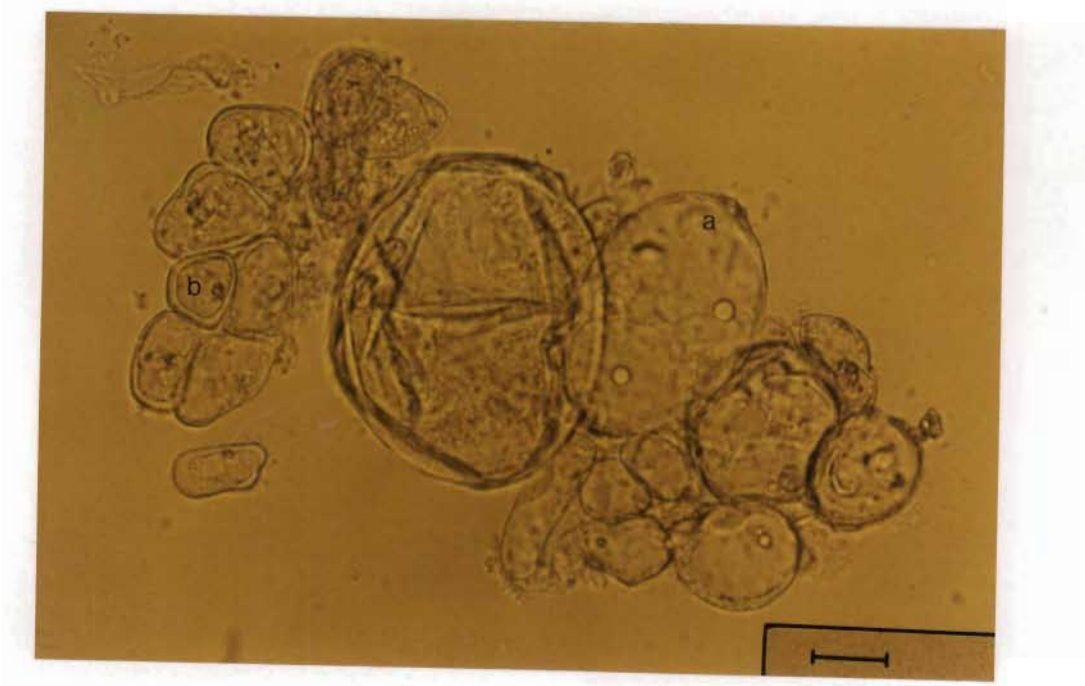

Plate 55 Microscopic examination of suspension culture cells of M28Y, showing large non-embryogenic cells (a) interspersed amongst smaller cytoplasmically dense embryogenic cells (b) (bar = 24.6 μM).

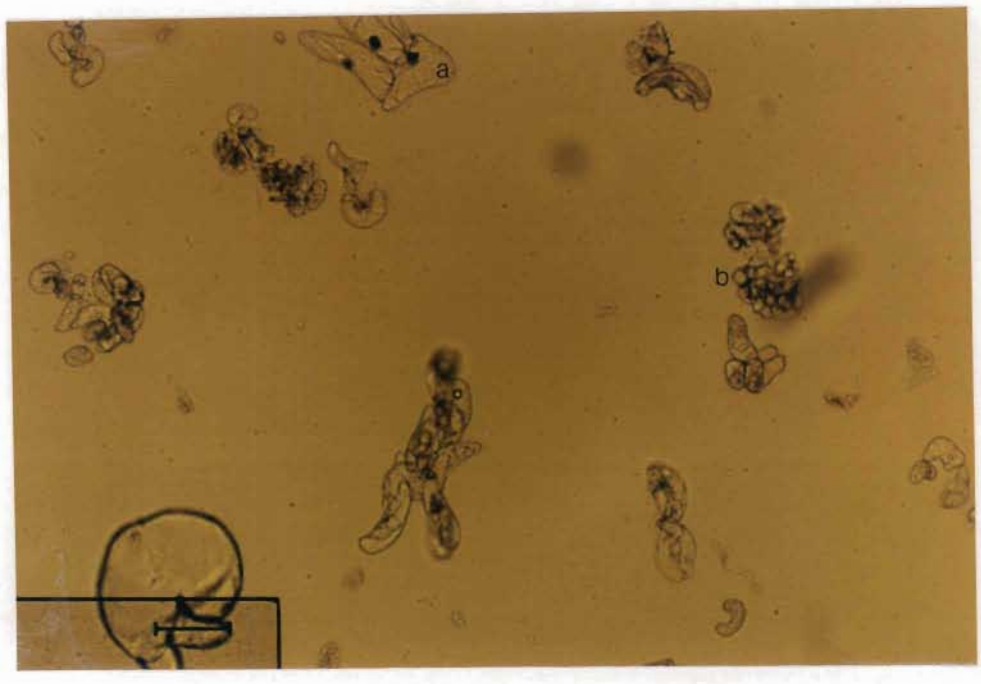

Plate 56 Microscopic examination of suspension culture cells of M162W, showing large non-embryogenic cells (a) interspersed amongst smaller cytoplasmically dense embryogenic cells (b) (bar = 100 μM).

At the next subculture (1 wk later), the suspensions were again examined. M162W suspensions had a bacterial contamination and were discarded. Macroscopically, M28Y cell cultures did not appear to have grown. The cultures had a slightly brown tinge, and when examined with the inverted microscope, there was no apparent change from the last subculture. Due to the lack of growth of any of the suspensions, the cultures were discarded.

Suspension cultures of M28Y and M162W were also initiated in 20 mL suspension culture medium in 100 mL Erlenmeyer flasks. This small volume increased the relative concentration of cells to solution, thereby increasing the initial cell inoculum density. The subculture routine consisted of pipetting 1 part cell suspension into 4 parts fresh suspension culture medium. At this time, the suspensions were examined. Both suspensions were healthy looking with lots of small clumps of cells in suspension. At the macroscopic level, M28Y suspension cultures were browning

slightly. The small clumps of cells in suspension gave the cell cultures a cloudy appearance. The original callus material used to initiate the suspensions of M28Y and M162W had increased in size. M162W suspension cultures were cream in colour with a very cloudy appearance (many small cell aggregates in suspension). When inspected with the inverted microscope, the M28Y suspensions consisted of small clumps of small cells (which varied in shape from regular to irregular). A number of large non-embryogenic cells were present amongst many sausage-shaped cells. M162W cultures were composed of many single cells in culture. These cells ranged in shape from sausage-shaped to regular (isodiametric). Small aggregates of regularly shaped cells (5 to 10 cells aggregate^{-1}) were also present, as well as a great number of sausage-shaped cells amongst large non-embryogenic cells. The cell integrity of all suspension cultures was very good, with hardly any evidence of damaged cells. There would therefore appear to be no shearing effect from the rotary shaker water bath used for incubation.

At the time of the second subculture, M162W suspensions were still cloudy and cream in colour. Some large clumps of cells were visible to the naked eye. Not much growth appeared to have taken place in the M28Y suspension cultures. These suspensions were also cream in colour with a slight brown tinge. The M28Y cultures did not have a cloudy appearance as there were not may cells in suspension. When examined with the inverted microscope, the M162W cultures contained many single sausage-shaped cells and aggregates of small cells (more regularly shaped cells; 5 to 15 cells aggregate^{-1}). The cell integrity was still very good. Some large cells were still evident. The M28Y cultures appeared to be very similar to the M162W cultures, except that there were very few cells in suspension. Very little growth, if any, had occurred. These cultures showed some evidence of shearing as broken cells were present in the suspension.

Shortly before the next subculture, the cell suspensions were examined. M162W suspensions had grown slightly and contained

small cell clusters which were visible to the naked eye. The cultures were cream in colour, but were not as cloudy as previously, suggesting that not as many cells were present in suspension. Microscopically, the M162W suspensions appeared similar to those previously obtained, with single cells, sausage-shaped cells and non-embryogenic cells. However, more aggregates of regularly shaped, small cells than previously observed, were apparent. M28Y cultures had undergone no change since the previous subculture.

After this subculture, no cell growth was observed for either of the two inbreds. All suspensions were examined microscopically. M28Y suspensions had undergone no change since the previous subculture. There was no apparent change to the M162W suspensions, except that cell integrity seemed to have been compromised in some way as far more debris was evident in suspension. The cell suspensions were subcultured a further three times into fresh suspension culture medium. However, no changes were observed and the suspensions were finally discarded.

Maize cell suspension cultures of M28Y and M162W, grown in 20 mL suspension culture medium in 100 mL Erlenmeyer flasks, were also subjected to a different subculture regime. This consisted of a 1:1 dilution of the suspension with fresh suspension culture medium. At each subculture therefore, a much higher inoculation density was used. This high density may have been necessary for cell division to occur.

Seven days after initiation of the experiment, the M162W suspension was cream-coloured and had a very cloudy appearance. It appeared to be a very thick suspension. The original callus clumps in the M28Y and M162W suspensions had increased in size. The cell suspension of M28Y appeared similar to that of M162W, except for the medium, which had a slightly brown tinge. In this suspension, the callus pieces were still green in colour. Microscopically, single cells of a sausage-shape were apparent in the M162W suspension, interspersed amongst small

isodiametrically shaped cells. Large non-embryogenic cells were also apparent, although fewer than in previous experiments. The cell integrity of the M162W cell suspension was good. The M28Y suspension appeared similar to that of M162W, except that some cells were far larger than the largest seen in the M162W culture. More large aggregates consisting of many cells were present than single cells or small cell aggregates. Cell damage was apparent for this suspension. The suspension cultures developed as in the previous experiment conducted with a higher initial inoculum density. The suspensions were cream-coloured, with M162W forming a finer suspension than that of M28Y, which tended to form a more coarse suspension.

The fact that no growth occurred in the suspension cultures of M162W and M28Y (in 50 mL suspension culture medium) after subculture, may be due to the small inoculum size that was used at subculture (1 part suspension: 4 parts fresh medium). This small inoculum size may have either caused an extended lag phase after subculture, or may have possibly resulted in no growth at all as the cell density may have become too low for cell division to take place. In subsequent experiments, therefore, the initial cell inoculum density was increased through the use of smaller cell suspension culture media volumes, and the subculture regime used to establish the cell suspensions was altered to a 1:1 dilution of the suspension cultures with fresh medium (Kamo & Hodges, 1986). These alterations of the protocol enabled denser cell suspensions to be established. Although the suspensions were subcultured further, they gradually lost their ability for cell division (competence) and were subsequently discarded. A further reason for the non-division of the suspensions is provided by other workers (Horn, 1991a). In cell suspensions that are initially composed largely of non-embryogenic cells, these cells are most probably derived from the suspensors or root apices of the mature embryos in the callus culture. Therefore, use of younger callus to initiate the suspension cultures, so that they contain no mature embryos, would reduce the production of non-embryogenic cells (Horn, 1991a). The callus cultures therefore

used for suspension initiation in this study, may have been maintained in culture for too long and therefore contained too many non-embryogenic cells.

As this study was by no means a complete study into the establishment of maize suspension cultures, no growth kinetic were investigated. The subculture regime was based on the 7 d regime used by other workers (Chourey & Zurawski, 1981; Carswell, *et al.*, 1989; Antonelli & Stadler, 1990; Petersen, *et al.*, 1992). As no growth was observed in the suspension cultures, it is possible that this subculture regime was too short. The cells, therefore, were probably unable to recover and divide properly before the next subculture. Consequently, growth kinetic studies are important for the establishment of an effective subculture regime, and as such should be incorporated into future research on maize cell suspension cultures.

E.3.2 Protoplast isolation from suspension cultures

Even though an optimized, vigorous maize cell suspension culture was not established, an attempt at protoplast isolation was made, as correctly established embryogenic suspension cultures are an ideal source of totipotent protoplasts (Imbrie-Milligan & Hodges, 1986; Kamo & Hodges, 1986; Horn, 1991a; Vasil & Vasil, 1991; Taylor, *et al.*, 1994). After 16 h of digestion, however, no protoplasts were released from the M28Y suspension culture used. Single cells only were present in suspension. Therefore, yield and viability determinations were not undertaken, and the suspension was discarded.

Other researchers have found that primary suspension cultures which contained aggregates of densely cytoplasmic cells, along with large, expanded cells, gave poor yields of protoplasts that rarely divided. Generally, freshly initiated suspensions yielded protoplasts that divided poorly or not at all. After further subculturing, the suspensions yielded large numbers of protoplasts that divided at a reasonable frequency (Shillito, *et*

al., 1989). Protoplasts were possibly not isolated from the suspension cultures, as the cultures were not actively growing or metabolically active. Additionally, a different range of enzymes and concentrations may have yielded protoplasts, as the thickness of the cell wall of a suspension culture cell may differ to that of callus and leaf. Alternatively, prior incubation in preplasmolysing solution, or the inclusion of enzymes in the suspension culture medium a few days before protoplast isolation, may have softened the suspension cells and enabled protoplast isolation. However, due to time constraints, and shortage of material, these approaches could not be taken.

E.3.3 Electroporation of maize suspension culture cells

Suspension culture cells of M28Y, electroporated with three different voltages, did not express the GUS gene. No positive results were obtained with the histochemical GUS assay, i.e. electroporation had been unsuccessful. It was therefore concluded that the electroporation conditions were not optimal, and that the suspension cells may not have been competent for transformation. Suspension culture cells of one of the other nine inbreds used in this study may possibly have been competent for transformation. A suspension culture of M162W had been established; however, due to time constraints and problems with microbial contamination, electroporation of this culture was not attempted.

E.3.4 Regeneration of callus from maize cell suspension cultures

After 21 d on Regeneration medium 1 (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8), callus of M28Y and M162W had formed on the surface of the medium. Some larger clumps of callus (c.a. 5 mm^3) were produced from small callus aggregates which were pipetted onto the medium. Smaller callus clumps (c.a. 2 mm^3) appeared to be produced from small aggregates of cells (invisible to the naked

eye) which had been present in the cell suspension cultures. These callus aggregates were subcultured to Regeneration medium 2 (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 2% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8). On this medium, the M28Y and M162W callus grew very fast. M162W callus was cream and had a soft, friable appearance (Plate 57). This callus did not produce any roots or shoot primordia on regeneration medium. Callus of M28Y was also cream with a soft, friable morphology. However, some small green areas of cells with differentiated chloroplasts developed on M28Y callus (Plate 58). No shoot primordia, however, developed and therefore no plantlets were obtained.

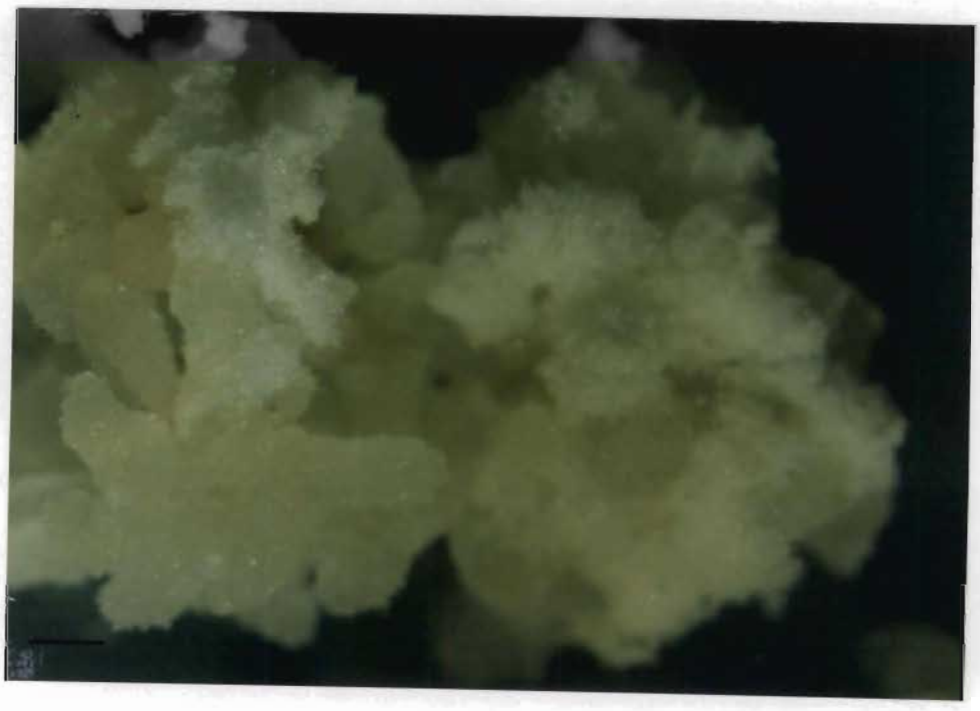

Plate 57 Soft, friable cream callus of M162W formed from suspension culture cells plated onto regeneration medium (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8) (bar = 1.25 mm).

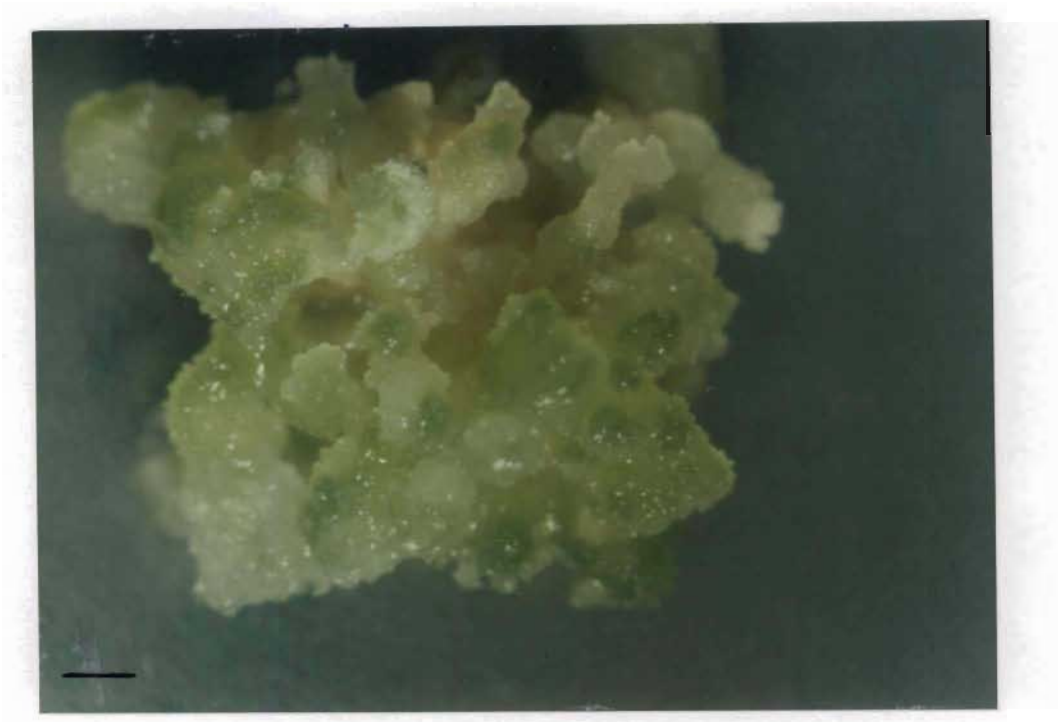

Plate 58 Soft, friable callus of M28Y formed from suspension culture cells plated onto regeneration medium (MS supplemented with 0.3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} kinetin, 6% (m/v) sucrose and 10 g L^{-1} agar; pH 5.8). Small, green areas of cells with differentiated chloroplasts visible (bar = 1.25 mm).

As both M162W and M28Y developed no shoot primordia, it was assumed that this was due to the source of the explants (young leaf bases of *in vitro* germinated seedlings) used to initiate the callus originally employed to establish the suspensions. The callus initiated from young leaf bases of *in vitro* germinated seedlings was not competent for regeneration. Therefore, the callus regenerated from the suspension cultures was also not competent for regeneration. Consequently, no plantlets were regenerated from callus which was regenerated from the M28Y and M162W suspension cultures.

In conclusion, work on cell suspension cultures of maize was unsuccessful. As no actively growing and metabolically active suspensions were established, the subsequent protoplast isolation and transformation studies were unsuccessful. The inclusion of growth kinetic studies may have greatly improved matters. The suspensions would therefore have been subcultured at the right time, and would thus have been more successfully established. As

a consequence of this, the protoplast isolation and transformation studies may have yielded better results. However, as this study on the suspension cultures of maize was in no way complete, the results were presented in this appendix in the hope that they may benefit future researchers who may wish to establish cell suspension cultures of maize.