REGENERATION AND BIOTRANSFORMATION OF SOME MEMBERS OF THE CUCURBITACEAE

by

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PREFACE

The experimental work described in this thesis was carried out at the ARC - Roodeplaat, Vegetable and Ornamental Plant Institute, Pretoria under supervision of Professor J. van Staden.

These studies represent original work and have not otherwise been submitted in any form for any degree or diploma to any University.

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ABSTRACT

Five cultivars, all belonging to the family Cucurbitaceae, have been tested for the ability to regenerate shoots or somatic embryos from cotyledonary explants. The influence of several combinations of growth regulators on regeneration from cotyledonary and other explants was tested.

No regeneration was obtained from the two cultivars *Cucurbita maxima* Duch. cv A-Line and *Cucurbita pepo* L. cv Rolet. Somatic embryos developed on *Cucurbita maxima* Duch. cv Chicago Warted, a Hubbard squash. A shoot regeneration response was observed for the cultivar *Cucumis sativus* L. cv Ashley, but the frequency was low and results could not be repeated in subsequent experiments. A reliable shoot regeneration protocol was developed for *Cucumis melo* L. cv Hales Best 36.

The influence of the antibiotics kanamycin sulphate and cefotaxime on shoot regeneration from cotyledonary explants of *Cucumis melo* L. cv Hales Best 36 was tested. The plasmid pBI121 was transferred from *Escherichia coli* strain HB101 into *Agrobacterium tumefaciens* strain LBA4404 via a triparental mating. The plasmid pBI121, contains the screenable marker gene ß-glucuronidase (GUS) and the selectable neomycin phosphotransferase-II gene (NPT-II) that confers kanamycin resistance. Cotyledonary tissue was transformed using this *Agrobacterium tumefaciens* transformation system. The influence of co-cultivation time, inoculation time and the wound factor acetosyringone on transformation was established. Rooted plantlets were regenerated from transformed cotyledonary tissue placed on kanamycin supplemented regeneration media. Plantlets tested positive for the presence of the GUS gene, using fluorometric and histochemical assays.

The developed protocol was used to transform *Cucumis melo* cv Hales best 36 with the *pat* gene that provides resistance to the herbicide Ignite®. A selection medium was developed containing phosphinothricin, the active ingredient of the herbicide.

Transformants were selected on this medium and five lines were recovered. These plants were acclimatized and the herbicide resistance was confirmed in greenhouse spray tests. The ploidy level of these plants was deduced from indirect evidence of micro- and macroscopic characteristics that have been shown to have a correlation with the chromosome number of melon plants.

The five lines were subjected to molecular analysis. The polymerase chain reaction was used to give an indication of the transformed nature of the selected plants. Agarose gel electrophoresis confirmed that the correct size band could be obtained from the putative transformants and the presence of *pat* in the product was verified using a non-radioactive system for nucleic acid analysis. Stable gene insertion into the genome of the plant was verified with a Southern blot of the total genomic DNA. This was achieved by hybridising a radioactively labelled ^{32}P probe specific for the *pat* gene to a blot of restriction digested plant DNA.

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ABA	abscisic acid
ANOVA	analysis of variance
BA	6-benzylaminopurine
bar	the bialaphos resistance gene
BSA	bovine serum albumine
°C	degrees celsius
CCC	(2-chloroethyl)-trimethylammoniumchloride
CMV-WL	cucumber mosaic virus white leaf strain
CMV-C	cucumber mosaic virus strain C
cv	cultivar
Cx	cefotaxime
2,4-D	2,4-dichlorophenoxyacetic acid
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxy-nucloetide triphosphate
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
GUS	ß- glucuronidase
g	grams
kinetin	6-furfurylaminopurine
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IBA	3-indolebutyric acid
iP	isopentenylaminopurine
Km	kanamycin sulphate
l	litre
MU	methyl umbelliferone
MUG	4-methyl umbelliferryl ß-D-glucuronic acid
µg	microgram
µl	microlitre

mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
min	minute
M	molarity
MS medium	MURASHIGE and SKOOG (1962) medium
MXT	methotrexate
NAA	1-naphthaleneacetic acid
ng	nanogram
NOA	ß-naphthoxyacetic acid
NPT II	neomycin phosphotransferase II
NaOCl	sodium hypochlorite
PAT	phosphinothricin acetyl transferase
pat	the phosphinothricin acetyl transferase gene
PCR	polymerase chain reaction
pH	log hydrogen ion concentration
PPT	phosphinothricin
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sp	species
T_m	melting temperature
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
TDZ	thidiazuron
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
v/v	volume per volume
X-gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronide

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

The family Cucurbitaceae includes a number of cultivated species that are of economic importance. Pumpkins (*Cucurbita maxima* Duch.) and squashes (*Cucurbita pepo* L.) originate from North and South America from where they spread to the rest of the world. They are related to cucumbers (*Cucumis sativus* L.), sweet melons (*Cucumis melo* L.) and watermelons (*Citrullus lanatus* [Thunb.] Matsum & Nakai) that were developed from plants indigenous to Africa and Asia. (BOSCH and OLIVIER, 1978).

Cucurbits are grown on a small scale throughout South Africa, but the main production areas are situated in areas of Mpumalanga, Gauteng, the eastern and western Cape and the Free State. In most areas cucurbits are only grown in the summer months, but in Mpumalanga crops are produced throughout the year (VAN DER MEER, 1985). In the 1994/5 season, 5 200 tonnes of cucumbers, 20 485 tonnes of muskmelons, 47 800 tonnes of pumpkins, 51 900 tonnes of hubbard squashes and 26 600 tonnes of gem squashes were sold at the fifteen major Fresh Produce markets in the Republic of South Africa (ANONYMOUS, 1996).

Many species belonging to the Cucurbitaceae are susceptible to infection by *Agrobacterium tumefaciens* pathogens (SMARRELLI *et al.*, 1986) and a number

of these species can be regenerated *in vitro*. Furthermore, transformation of cucumber (CHEE, 1990b; CHEE and SLIGHTOM 1991; SARMENTO *et al.*, 1992), watermelon (CHOI *et al.*, 1994) and melon (FANG and GRUMET, 1990; DONG *et al.*,1991; GONSALVES *et al.*, 1994) by *Agrobacterium tumefaciens* has been accomplished. This system has potential applications in the transfer of genetic traits that are difficult or impossible to transfer via conventional plant breeding techniques. Practical uses of this technology include the transfer of resistance to herbicides and insects (TRULSON *et al.*, 1986). The incorporation and expression of virus capsid protein genes has proven to be one of the most effective ways to obtain resistance to virus infection (BEACHY *et al.*, 1990).

1.2. **GENERAL PLANT TISSUE CULTURE**

The aim of the majority of experiments using genetic transformation technology is the production of whole, fertile, transformed plants. This aim would be impossible to achieve without a reliable plant regeneration methodology, to develop plants from transformed tissues (DRAPER *et al.*, 1988).

The term plant tissue culture is a convenient expression to describe all types of aseptic plant culture procedures pertaining to the growth of plant protoplasts, cells, tissues, organs, embryos and plantlets. Here it will be used to describe the production of plantlets in *in vitro* culture. Procedures for plant tissue culture have developed rapidly and numerous species can be regenerated *in vitro* through appropriate protocols (TISSERAT, 1985; GEORGE, 1996). It must however, be remembered that regeneration success varies greatly from one species to another and even within a species. For each species there may be several processes of plant

regeneration, but usually one will be more efficient than the others (MURASHIGE, 1977; THORPE, 1994). The establishment of a procedure for use on a specific species will benefit from an extensive literature review. Aspects to bear in mind are that; (a) prior reports can be conflicting or fragmentary; (b) lack of standardisation of techniques among researchers contributes to non-reproducible results; (c) biological factors such as genotype, condition of the source material and culture conditions might prevent successful repetition; and (d) that knowledge and techniques of plant tissue culture and plant regeneration are changing and improving fast, often relegating earlier work to insignificance (TISSERAT, 1985; CONSTABEL and SHYLUK, 1994).

1.3. **PLANT REGENERATION IN CUCURBITS**

1.3.1 **Regeneration research**

The *in vitro* regeneration of some of the economically important members of the Cucurbitaceae has been studied. These are mainly the cucumber, *Cucumis sativus* L., the muskmelon, *Cucumis melo* L., the watermelon, *Citrullus lanatus* ([Thunb.] Matsum & Nakai) and squash and pumpkin, *Cucurbita pepo* L.. The explant types, media formulations and growth regulators that were used in, as well as the results obtained from these studies, are summarised in Tables 1.1 to 1.4. In these tables, the abbreviation MS is used to indicate the medium formulated by MURASHIGE and SKOOG (1962).

Table 1.1.

The use of different explant types, media formulations, growth regulators and the results obtained from experiments on the culture of *Cucumis sativus* L. *in vitro*.

EXPLANT TYPE	MEDIUM	GROWTH REGULATORS	RESULTS	REFERENCE
Axillary buds.	MS salts. Organic additives: 0.4 mg l^{-1} thiamin, 1.0 mg l^{-1} pyridoxine, 5.0 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 3 %(w/v) sucrose, 0.7 % (w/v) agar.	Combinations of 0 - 0.5 mg l^{-1} NAA and 0 - 20 mg l^{-1} kinetin.	Callus culture was unsuccessful. Best plantlet formation was observed on 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} kinetin. Plantlets were transferred to soil.	HANDLEY and CHAMBLIS, 1979.
Hypocotyl, cotyledon explants of 85 genotypes.	MS salts and vitamins, 3 % (w/v) sucrose.	1 mg l^{-1} BA + 1 mg l^{-1} NAA.	Callus formed on both types of explant. Shoots formed on 28 genotypes and roots on 32 genotypes using cotyledon explants. Only roots formed on hypocotyl explants.	WEHNER and LOCY, 1981.
Leaf.	MS medium with 250 g l^{-1} edamin, 3 %(w/v) sucrose, 0.45 % (w/v) agar.	Use primary medium for 6 - 8 weeks, before switching to a secondary medium. All media contained 2,4,5 -T, 2,4-D, BA or iP in various concentrations.	Numerous plantlets were regenerated through somatic embryogenesis. This was observed on primary medium containing 0.8 mg l^{-1} BA, with the secondary medium 0.4 mg l^{-1} BA or no regulators. Alternatively, 1.2 mg l^{-1} 2,4,5-T in the primary medium with 0.4 mg l^{-1} BA or 0.06 mg l^{-1} 2,4,5-T in the secondary medium.	MALEPSZY and NADOLSKA-ORCZYK, 1983.
Cotyledon, root explants of 14 genotypes.	Modified MS medium with 100 mg l^{-1} cefotaxime, 0.7 % (w/v) agar.	1.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} BA.	Somatic embryos were induced on this medium, matured on medium without 2,4-D and plantlets developed on MS medium.	TRULSON and SHAHIN, 1986.

Hypocotyl.	Liquid MS medium with 0.1 g l^{-1} casein hydrolysate. For tertiary medium: 50 g l^{-1} or 10 % (w/v)sucrose, or 18 g l^{-1} mannitol + 0.5 % (w/v) activated charcoal + agar. OR: Double layer medium with 0.5 % (w/v)activated charcoal in solid bottom layer, half strength liquid medium with full strength $CaCl_2$ on top.	Primary medium: 1 mg l^{-1} 2,4-D + 0.2 mg l^{-1} BA. Secondary medium: No additives. Tertiary medium: 0.2 mg.l^{-1} zeatin + 0.1 mg.l^{-1} ABA. Double layer medium: with or without 0.1 mg.l^{-1} ABA.	Callus was initiated on primary medium and embryos on secondary medium. Vitreous plantlets developed on the double layer cultures, flowered and were transferred to the greenhouse.	ZIV and GADASI, 1986.
Cotyledon explants of 14 different genotypes.	MS salts. Organic additives: 100 mg l^{-1} myo-inositol, 0.8 mg l^{-1} thiamine HCl, 2.0 mg l^{-1} nicotinic acid, 0.8 mg l^{-1} pyridoxine HCl, 3 % (w/v) sucrose and 0.8 % (w/v) agar.	Combinations of 2,4-D or NAA and BA or kinetin.	Callus formed on 0.1 mg l^{-1} 2,4-D + 1.1 mg l^{-1} BA, 3 genotypes developed shoots on 0.1 mg l^{-1} NAA + 1.1 mg l^{-1} BA and all genotypes developed roots on 0.1 mg l^{-1} NAA + 0.002 mg l^{-1} BA. Only a few plants were soil acclimated.	KIM *et al.*, 1988.
Cotyledons of 4 genotypes.	MS medium with 0.8 % (w/v) agar and 3 % (w/v) sucrose. Or MS medium with 3 % (w/v) sucrose, 1 % (w/v) agar and 0.5 % (w/v) agar.	Combinations of 0.0, 0.25,0.5, 1.0, or 2.0 mg l^{-1} 2,4-D and kinetin. Maturation: 0.5 mg l^{-1} kinetin. 1 mg l^{-1} NAA + 1 mg $^{-1}$ 2,4-D + 0.5 mg l^{-1} kinetin.	Embryo initiation was best on 1 or 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} kinetin. Embryos matured and germinated. Charcoal inhibited number of normal embryos and conversion to plants.	CADE *et al.*, 1990.
Cotyledons.	MS medium with 0.7 % (w/v) agar.	Combinations of 1.2 mg l^{-1} 2,4,5-T + 0.8 mg l^{-1} BA, and 1 or 2 mg l^{-1} 2,4-D + 0.5 - 2 mg l^{-1} kinetin.	Somatic embryos were induced on 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} kinetin and embryos matured on 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} kinetin. Plantlets developed on MS medium and were transferred to a greenhouse.	CHEE, 1990a.
Cotyledons.	MS medium with 20 g l^{-1} sucrose and 0.8 % (w/v) agar.	BA, kinetin, iP, NAA or IAA, or combinations thereof.	High shoot formation from adventitious buds, with little callus development, occured on 2-5 mg l^{-1} kinetin, as well as on 1- 5 mg l^{-1} iP, and 0.5 mg l^{-1} BA. Plantlets were formed.	GAMBLEY and DODD, 1990.

Cotyledons, embryo axis.	MS medium with 0.7 % (w/v) agar.	Combinations of 2 - 5 mg l^{-1} BA and 1 - 1.3 mg l^{-1} NAA.	Shoots formed on all media but best on 2 or 4 mg l^{-1} BA + 0.3 mg l^{-1} NAA. Male and female flowers developed.	MSIKITA *et al.*, 1990.
Cotyledons of 6 cultivars. Seeds were stored for 10 years.	MS medium with 0.8 % (w/v) agar.	0, 1, 2 or 3 mg l^{-1} BA + 0.0, 0.1, 0.2, or 0.3 mg l^{-1} NAA, or 4 mg l^{-1} BA + 0.2 mg l^{-1} NAA.	All the cultivars placed on 1 mg l^{-1} BA in combination with NAA formed shoots. Some shoot development occurred on 2 and 3 mg l^{-1} BA combinations. Rooted plantlets developed in the presence of 1.5 mg l^{-1} NAA.	ALI *et al.*, 1991.
Cotyledons.	Modified MS medium with 3 % (w/v) sucrose (or glucose or fructose or maltose) and 0.35 %, 0.7 % or 1.4 % (w/v) agar or 0.15 % 0.3 % or 0.6 % (w/v) gelrite.	1.1 mg l^{-1} 2,4-D + 0.2796 mg l^{-1} NAA + 0.226 ng l^{-1} BA.	Induction of embryos was best on 0.7 % agar. Multiplication was similar on 0.15 % gelrite and 0.7 % agar. The embryos germinated best on gelrite and sucrose supplemented media.	LADYMAN and GIRARD, 1992.
Four week old shoot tips.	Modified MS medium with 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} thiamine HCl, 0 %, 0.3 %, 1 % or 3 % (w/v) sucrose and 0 % or 0.8 % (w/v) agar.	0.0, 0.01, 0.1, and 1.0 mg l^{-1} NAA or 0.01 mg l^{-1} NAA + 0.1 mg l^{-1} BA.	*In vitro* flower production decreased with increasing NAA concentration. Parthenocarpic fruit developed *in vitro*.	TISSERAT and GALLETTA, 1993.
Cotyledon, young first leaf, internode explants from 7 cultivars.	MS medium with Gamborg B5 vitamins, 3%, 6%, 9% or 12% (w/v) sucrose and 0.25% (w/v) gelrite.	2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BA , then 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} kinetin or no growth regulators.	Six of the cultivars formed embryos from cotyledonary explants, five from first leaf explants, one from internodes. Sucrose concentrations of 9% and 12% (w/v) in the initiation medium was optimal for embryo production. Plantlets were hardened off.	LOU and KAKO, 1994.
Exised radicles.	MS medium with 3 % (w/v) sucrose. Complex medium for maintainence.	2 mg l^{-1} 2,4-D + 1 mg l^{-1} kinetin, diluted during culture. 1.5 mg l^{-1} ABA.	Pro-embryogenic cell masses formed and were maintained for 3 years without loss of embryogenic potential. Embryos matured on MS medium with ABA and developed into plantlets on Sorbarod containers.	KREUGER *et al.*, 1996.

Table 1.2.

The use of different explant types, media formulations, growth regulators and the results obtained from experiments on the culture of *Cucumis melo* L. *in vitro*.

EXPLANT TYPE	MEDIUM	GROWTH REGULATORS	RESULTS	REFERENCE
Cotyledon, half-leaf explants of 5 genotypes.	a) NITSCH and NITSCH, (1969) medium H (NN) with 0.8 % (w/v) agar and 4 %(w/v) sucrose. b) As above, but with 250 mg l^{-1} $NaH_2PO_4.H_2O$ + 28 mg l^{-1} $FeC_6H_5O_7.nH_2O$ +100 mg l^{-1} inositol.	a) 0.05 mg l^{-1} NOA + 10 mg l^{-1} iP + 0.1 mg l^{-1} CCC. b) 0.1 mg l^{-1} NOA + 20 mg l^{-1} iP + 0.1 mg l^{-1} CCC.	For three genotypes shoot formation was observed on leaf explants, but only using the first medium (a). For another genotype, shoot development occured on both media using cotyledon explants, but only on the second (b) medium for leaf explants. Roots formed on NN medium.	BLACKMON and REYNOLDS, 1982.
Cotyledon, hypocotyl.	MS salts, with 3 % (w/v) sucrose, 100 mg l^{-1} myoinositol, 1 mg l^{-1} thiamine HCl, and 0.8 % (w/v) agar.	Combinations of 0.0, 1.5, 3.0, 4.5 or 6.0 mg l^{-1} IAA and 0.0, 1.5, 3.0, 4.5 or 6.0 mg l^{-1} kinetin.	Maximum shoot formation was obtained on 1.5 mg l^{-1} IAA + 6 mg l^{-1} kinetin using cotyledon explants, and best root formation on 1.5 mg l^{-1} IAA + low kinetin. Finally, plantlets developed. On hypocotyl explants, no shoot formation was observed. Roots formed only in absence of kinetin. Embryogenesis was found on high IAA levels, but these did not develop further.	MORENO *et al.*, 1985.
Hypocotyl.	MS medium, with 2 % (w/v) sucrose and 0.8 % (w/v) agar.	Combinations of IAA, kinetin, BA, NAA, iP, ABA, zeatin and GA_3.	Callus was initiated on 1 mg l^{-1} IAA + 0.5 mg l^{-1} kinetin. Nodules developed on 3 or 4 mg l^{-1} BA + 2 mg l^{-1} NAA, or on 0.5 mg l^{-1} BA. Normal shoots were formed on 0.5 mg l^{-1} BA + 0.5 mg l^{-1} iP + 0.13 mg l^{-1} ABA / 0.04 mg l^{-1} GA_3.	KATHAL *et al.*, 1986.

Cotyledons of 15 genotypes.	MS salts with 3 % (w/v) sucrose, 100 mg l^{-1} myoinositol, 1 mg l^{-1} thiamine HCl and 0.8 % (w/v) agar.	6 mg l^{-1} kinetin + 1.5 mg l^{-1} IAA.	The percentage of calli with shoot buds varied between 91.8 % and 1.8 %. The percentage of calli with normally developed shoots was far lower. Shoots could be rooted on media containing no growth regulators. A significant variation in the response from different genotypes was found.	ORTS *et al.*, 1987.
Leaf.	MS medium, with 3 % (w/v) sucrose and 0.8 % (w/v) agar.	Combinations of 0.1 - 2 mg l^{-1} BA, 0.2 - 2 mg l^{-1} iP and 0.1 mg l^{-1} IAA.	Best shoot regeneration was seen on 0.2 mg l^{-1} BA + 0.2 mg l^{-1} iP, shoot elongation on 0.2 mg l^{-1} BA and root formation on 0.01 mg l^{-1} IAA. Plants were successfully transferred to soil.	KATHAL *et al.*, 1988.
Leaf and cotyledon.	MS medium with 3 % (w/v) sucrose.	Combinations of 1, 2.5 or 5.0 mg l^{-1} BA and 0, 0.1, 0.25 or 1.0 mg l^{-1} IAA.	Best shoot regeneration was obtained on medium containing 1 mg l^{-1} BA. Roots formed on MS medium with 2 % (w/v) sucrose. Rooted plantlets were transferred to the greenhouse.	DIRKS and VAN BUGGENUM, 1989.
Cotyledon.	MS medium with 3%(w/v) sucrose and 0.7% (w/v) agar.	a) 2 mg l^{-1} IAA (root induction). b) 0.2 mg l^{-1} BA (shoot induction). c) triiodobenzioc acid .	Direct shoot regeneration occured only on the basal side of explants after 5 days on shoot induction medium. Root development occured on root induction medium. Triiodobenzioc acid inhibited organogenesis.	LESHEM, 1989
Cotyledons of 4 genotypes.	MS medium with 165 mg l^{-1} NH_4NO_3.	a) Combinations of 1 mg l^{-1} IAA and various concentrations of kinetin, BA, iP and zeatin. b) 0.1 or 1.75 mg l^{-1} IAA + 1 or 2.2 mg l^{-1} BA. c) Combinations of 1 mg l^{-1} IAA + 1 mg l^{-1} BA + ABA or GA_3 or thidiazuron.	Significant differences were observed in the bud initiation response of the different genotypes. Best shoot regeneration response was found on media containing BA, but no buds were formed on media containing iP. Young explant age (7 days), low light intensity (5 - 30 µ mol m^{-2} s^{-1}) and addition of ABA increased bud formation.	NIEDZ *et al.*, 1989.

Stem, leaf and shoot apex (several varieties).	MS medium with 3 % or 7 % (w/v) sucrose and 0.2 % (w/v) gelrite.	Combinations of 1, 2, or 4 mg l^{-1} 2,4-D, 0, 1, 2 or 4 mg l^{-1} NAA and 0.1 mg l^{-1} BA. Maturation: No growth regulators.	Somatic embryos developed from stem, leaf and shoot apex cultures in a number of the varieties tested. Plantlets were regenerated.	KAGEYAMA *et al.*, 1990.
Cotyledon.	MS medium with 3% (w/v) sucrose and 0.7% (w/v) agar.	a) 1 mg l^{-1} BA (shoot induction) b) 2 mg l^{-1} IAA (root induction)	The presence or disappearance of a group of polypeptides were identified as markers for regeneration of roots and shoots respectively.	LESHEM and SUSSEX, 1990.
Cotyledonary, leaf and hypocotyl of three genotypes.	MS salts, with 3 % (w/v) sucrose, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine HCl and 0.7 % (w/v) agar.	a) 1.5 mg l^{-1} IAA + 6.0 mg l^{-1} kinetin b) 2.5 mg l^{-1} NAA + 2.5 mg l^{-1} iP.	Shoot regeneration and embryogenesis were obtained with all genotypes, from both explant types. Embryoids showed abnormal development.	BORDAS *et al.*, 1991.
Cotyledon.	MS medium with 3 % (w/v) sucrose 0.6 % (w/v) agar.	a) 0.0, 0.5, 0.75 or 1.0 mg l^{-1} BA. b) 1.5 mg l^{-1} IAA + 6.0 mg l^{-1} kinetin. c) 0.0,0.001, 0.01 or 0.1 mg l^{-1} NAA.	Significantly more organogenic callus developed on 1 mg l^{-1} BA than any of the other media. Rooting was obtained on 0.01 mg l^{-1} NAA. Rooted plantlets flowered and produced fruit with seed.	CHEE, 1991a.
Cotyledonary and leaf protoplasts.	MS medium with 0.3 % (w/v) gelrite and 3 % (w/v) sucrose or maltose.	a) 1 mg l^{-1} BA. b) 1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} BA.	Shoots developed on the first medium (a) and somatic embryos on the second medium (b).	DEBEAUJON and BRANCHARD, 1991.
Cotyledonary suspension culture.	Liquid MS medium with 3 % (w/v) sucrose. Wash medium: liquid MS medium with 0.5 % (w/v) charcoal.	1 mg l^{-1} 2,4-D + 1 mg l^{-1} NAA + 0.1 mg l^{-1} BA. Maturation: no growth regulators.	The culture period on initiation medium affected embryo development, with the best development observed in 7 day cultures. Washed embryos developed normally. Plantlets were vitrified.	KAGEYAMA *et al.*, 1991.
Seed derived and mature cotyledons, hypocotyls, leaves and petioles.	MS medium with 3 % (w/v) sucrose and 0.7 % (w/v) agar.	0.01 to 2.0 mg l^{-1} 2,4-D or 0.01 to 25 mg l^{-1} NAA or 0.01 to 300 mg l^{-1} IAA, with 0.1 mg l^{-1} BA.	Adventitious shoots formed at low auxin concentration; somatic embryos at high auxin concentrations. No regeneration was seen on mature cotyledons. The best results were obtained using cotyledons of mature seeds on IAA containing media.	TABEI *et al.*, 1991.

Cotyledons of two genotypes.	MS medium with B5 vitamins (GAMBORG *et al.*, 1968), 100 mg l^{-1} myo-inositol and 3 % (w/v) sucrose.	a) 1 mg l^{-1} BA (first genotype). b) 1 mg l^{-1} BA + 2.8 mg l^{-1} IAA (second genotype).	Rooted plantlets were produced and transferred to greenhouse. Variation in morphology and chromosome number (tetraploids) were observed.	FASSULIOTIS and NELSON, 1992.
Cotyledons and hypocotyls of 18 genotypes.	N6 liquid medium (CHU *et al.*, 1975).	3.0 mg l^{-1} 2,4-D + 0.1 mg l^{-1} BA.	Variation in somatic embryogenesis capacity exist between the cultivars used. The inheritance of embryogenic capacity was studied.	ORIDATE *et al.*, 1992.
Cotyledons.	MS medium, with 3% (or 1 %) (w/v) sucrose and 0.2% (w/v) gelrite.	1 mg l^{-1} BA, combined with various concentrations of proline (0 - 100 mM), salisylic acid (0 - 1000 µM), aspirin (0 - 1000 µM) and thioproline (0.5 mM).	Shoot regeneration was enhanced by the addition of 5 - 20 mM proline and 50 - 200 µM aspirin or salisylic acid. These effects were reduced with the addition of thioproline.	SHETTY *et al* ., 1992.
Cotyledons and shoot apices.	MS medium with 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 0.2 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} nicotinic acid, 3 % (w/v) sucrose, 0.7 % (w/v) agar	2.5 mg l^{-1} BA (shoot regeneration) 0.25 mg l^{-1} BA (shoot development)	Plantlets developed and were hardened off. Ploidy was determined after 10 weeks and 9 months. Polyploids developed during the first few cell divisions in culture, and was not produced during long term tissue culture.	ADELBERG *et al.*, 1993.
Cotyledons of 52 cultivars.	MS medium with 1000 mg l^{-1} myo-inositol, 1 mg l^{-1} nicotinic acid, 10 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxine HCl, 1.5 % or 3 % (w/v) sucrose and 0.7 % agar or 0.2% (w/v) gelrite.	0, 0.1, 1, or 5 mg l^{-1} 2,4-D + 0, 0.1, or 1 mg l^{-1} BA. 5 mg l^{-1} 2,4-D + 0.1, 1 or 5 mg l^{-1} BA or kinetin or TDZ or iP. 12 TDZ concentrations + 5 mg l^{-1} 2,4-D	Explants placed on induction medium with 5 mg l^{-1} 2,4-D and 0.075 mg l^{-1} TDZ in the dark for 2 weeks, followed by a subculture to development medium (MS with 3 % (w/v) sucrose) formed embryos within 2 weeks. Highest number of embryos were seen after 7 weeks.	GRAY *et al.*, 1993.

Immature and mature cotyledons, whole or sectioned, shoot apices.	MS medium with 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 0.2 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} nicotinic acid, 3 % (w/v) sucrose and 0.7 % (w/v) agar.	2.5 mg l^{-1} BA (shoot regeneration) 0.25 mg l^{-1} BA (shoot development) 1 mg l^{-1} IBA (rooting)	More tetraploid regenerants were recovered from the proximal region of cotyledons than from whole cotyledons or from the distal region. Immature cotyledons gave rise to more tetraploids than mature explants.	ADELBERG *et al.*, 1994.
Root explants.	MS medium with 3 % (w/v) sucrose and 0.8% (w/v) agar.	Various concentrations of BA, iP, kinetin, zeatin, IBA, IAA, 2,4-D and NAA.	Optimal nodule formation was seen on 0.7 mg l^{-1} BA + 0.6 mg l^{-1} iP, obtained from basal root sections of 21 day old seedlings. Shoot differentiation occured on 0.25 mg l^{-1} BA. Plantlets formed roots on 0.2 mg l^{-1} IBA.	KATHAL *et al.*, 1994.
Cotyledons.	MS medium with 3 % (w/v) sucrose and 0.7 % (w/v) agar.	Various concentrations of BA, IAA, or both.	The influence of BA and/or IAA on regeneration and the disappearance of a group of polypeptides was studied over a culture period of 15 days. Root and shoot regeneration and the pattern of polypeptide disappearance was dose dependent.	LESHEM *et al.*, 1994.

Table 1.3.

The use of different explant types, media formulations, growth regulators and the results obtained from experiments on the culture of *Citrullus lanatus* ([Thunb.] Matsum & Nakai) *in vitro*.

EXPLANT TYPE	MEDIUM	GROWTH REGULATORS	RESULTS	REFERENCE
Cotyledon.	a) NITSCH and NITSCH, (1969) medium H (NN) with 8 $g.l^{-1}$ agar and 40 $g.l^{-1}$ sucrose. b) As above, but with 250 $mg.l^{-1}$ $NaH_2PO_4.H_2O$ + 28 $mg.l^{-1}$ $FeC_6H_5O_7.nH_2O$ +100 $mg.l^{-1}$ inositol.	a) 0.05 mg l^{-1} NOA + 10 mg l^{-1} iP + 0.1 mg l^{-1} CCC. b) 0.1 mg l^{-1} NOA + 20 mg l^{-1} iP + 0.1 mg l^{-1} CCC.	Shoot development occurred on both media, but to a lesser extent on medium b. A high rooting percentage was obtained on NN medium.	BLACKMON and REYNOLDS, 1982.
Cotyledon and hypocotyl.	MS medium with 30 g l^{-1} sucrose, 100 mg l^{-1} inositol and 0.8 % (w/v) agar.	0.05 - 0.2 mg l^{-1} NAA, 0.04 - 0.2 mg l^{-1} IAA, 0.6 - 1.0 mg l^{-1} BA or 0.6 - 1.0 mg l^{-1} kinetin.	For cotyledonary explants, best shoot formation was observed on 1 mg l^{-1} BA, and best root formation on 0.2 mg l^{-1} NAA. Callus formed on all auxin containing media. Using hypocotyl explants, the same medium could be used for shoot development, but for rooting, 0.1 $mg.l^{-1}$ NAA was used. Callus formed as above.	SRIVASTAVA *et al.*, 1989.

Cotyledons of 8 genotypes.	MS medium with 3 % (w/v) sucrose and 0.85 % (w/v) agar.	a) 5.0 mg l^{-1} BA (or zeatin, kinetin or iP) + 0.5 mg l^{-1} IAA. b) 1.0 - 12.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA. c) BA + 0.0 - 3.0 mg l^{-1} IAA. d) 0.2 mg l^{-1} kinetin. e) 0.1 mg l^{-1} NAA.	The genotypes used differed in their shoot regeneration response. Of the four cytokinins that were tested, best results were obtained on media containing BA and zeatin. Concentrations of 5.0 or 7.0 mg l^{-1} BA + 0.5 - 1.0 mg l^{-1} IAA were most effective for shoot regeneration. Five day old seedlings were shown to be the optimal explant source. Shoot regeneration decreased in seedlings older than 7 days. Shoot buds elongated when placed on medium d. Roots formed when explants were cultured on medium e.	DONG and JIA 1991.
Cotyledons; diploid (11 cultivars), triploid and tetraploid (3 genotypes each)	MS medium with 100 mg l^{-1} *myo*-inositol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} pyridoxine HCl, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamine HCl, 3%(or 2 % for germination) (w/v) sucrose, 0.7 % (w/v) agar.	a) 0, 1.15, 2.3, or 4.6 mg l^{-1} BA + 0, 0.1, or 0.85 mg l^{-1} IAA. b) BA, or kinetin or TDZ at various concentrations. c) 0, 4.15, 2.3, or 4.6 mg l^{-1} BA. d) 0.02 mg l^{-1} NAA (rooting).	Addition of IAA supressed regeneration, that occurred on basal sides of the explant. BA was the best cytokinin tested. Diploid lines regenerated more shoots than triploid or tetraploid lines. Plants were acclimised and placed in the glasshouse.	COMPTON and GRAY 1993.

Table 1.4.

The use of different explant types, media formulations, growth regulators and the results obtained from experiments on the culture of *Cucurbita pepo* L. *in vitro*.

EXPLANT TYPE	MEDIUM	GROWTH REGULATORS	RESULTS	REFERENCE
Hypocotyls.	MS medium with 3 (w/v) sucrose and 0.9 % (w/v) agar.	IBA, 2,4-D, IAA, NAA, adenine, kinetin, watermelon sap, yeast extract and combinations thereof.	Embryogenic callus developed on a small percentage of explants; the majority forming roots. Adventitious buds and plantlets formed on IAA, IBA, NAA containing media.	JELASKA, 1974.
Meristem tips.	MS salts with 3 (w/v) sucrose, 0.1 mg l^{-1} thiamine HCl and 100 mg l^{-1} inositol as a liquid medium on filter paper bridges. Only rooting medium: 0.1 (w/v) agar.	a) 0.0, 2.56, 12.8 and 25.6 mg l^{-1} kinetin + 0.0, 8.0, 16.0 and 32.0 mg l^{-1} IAA. b) 1.0, 2.5 and 5.0 mg l^{-1} BA + 1 mg l^{-1} IAA, IBA or NAA. c) 0.0, 0.5, 1.0, 1.5 or 2.5 mg l^{-1} BA + 0.0, 1.0, 2.0, 4.0 or 8.0 mg l^{-1} IAA.	Maximum shoot proliferation occurred on medium with 1 mg l^{-1} BA. No shoot proliferation was seen on medium a. Shoots were rooted on medium containing 8 mg l^{-1} IAA. Plantlets were established in soil.	PINK and WALKEY, 1984.
Shoot apices.	MS medium.	a) 1.2 mg l^{-1} 2,4,5-T + 0.8 mg l^{-1} BA + 0.0, 0.1, 0.2 or 0.5 mg l^{-1} kinetin. b) 0.05 mg l^{-1} NAA + 0.05 mg l^{-1} kinetin.	Somatic embryogenesis was induced on 1.2 mg l^{-1} 2,4,5-T + 0.8 mg l^{-1} BA + 0.1 mg l^{-1} kinetin. Plantlets developed on 0.05 mg^{-1} NAA + 0.05 mg^{-1} kinetin. Regenerated plants produced flowers.	CHEE, 1991b.
Hypocotyl.	MS medium with 3 or 1.5 % (w/v) sucrose, or MS with thiamin HCl, pyridoxine HCl and myo-inositol. 2 % (w/v) activated charcoal.	Induction: 1.0 mg l^{-1} 2,4-D, or 10 mg l^{-1} IBA, or 10 mg l^{-1}IAA. Coversion: 0, 0.5, 1.0, or 2.0 mg l^{-1} IAA, or 12 µM indole-3-ethanol, or 1.0 mg l^{-1}ABA.	Optimal conversion of embryos from newly established lines and 15 year old lines was seen on 2.0 mg l^{-1} IAA with 1.5 (w/v) sucrose, or on 2 % (w/v) activated charcoal.	JURETIĆ and JELASKA, 1991.

Cotyledon explants of 6 cultivars.	MS medium with 500 mg l^{-1} carbenicillin, 3 (w/v) sucrose and 0.8 % (w/v) agar.	a) 1.2 mg l^{-1} 2,4,5-T + 0.8 mg l^{-1} BA + 0.0, 0.1, 0.2 or 0.5 mg l^{-1} kinetin. b) 0.05 mg l^{-1} NAA + 0.05 mg l^{-1} kinetin.	Somatic embryogenesis occurred in all the cultivars. The embryos developed into plants that produced fruit and seed.	GONSALVES *et al.*, 1995.

1.4. THE *AGROBACTERIUM TUMEFACIENS* TRANSFORMATION SYSTEM

Members of the genus *Agrobacterium* which forms part of the family Rhizobiaceae are distinguished by their phytopathogenicity. *Agrobacterium tumefaciens* is a soil bacterium which is widely studied because of it's ability to induce crown gall in agriculturally important plants (SMITH and TOWNSEND, 1907). The host range of *Agrobacterium tumefaciens* is very wide amongst dicotyledonous plants (DE CLEENE and DE LEY, 1976). Because the cell response of monocotyledonous plants is much less pronounced or absent, it was long thought that these plants were not susceptible to infection by *Agrobacterium*. Evidence to the contrary has now been presented and members of the families Amaryllidaceae, Gramineae and Liliaceae have been shown to be susceptible to infection (HERNALSTEENS *et al.*, 1984; GRAVES and GOLDMAN, 1986, 1987; GRIMSLEY *et al.*, 1987). These findings increased the importance of the research on *Agrobacterium*, both as a cause of an agricultural disease of major economic importance and because of the extended potential for use of the *Agrobacterium* transformation system in agricultural applications.

1.4.1. Tumor induction by *Agrobacterium tumefaciens*

Infection of plants with *Agrobacterium* occurs at fresh wound sites (LIPETZ, 1965). Neoplastic growth, commonly called the crown gall, proliferates at the point of infection. The crown gall does not show organised growth and no shoots and roots are formed (CHILTON, 1983). This growth is characterized by two peculiarities. The first is the production of novel amino acid and sugar derivates called opines.

The type of opine which is synthesized by the gall (eg. nopaline, octopine, agrocinopine, mannopine and agropine) is dependant on the *Agrobacterium tumefaciens* strain which initiated the crown gall in the plant (PETIT *et al.*, 1970) and not on the host plant. The type of opine synthesized by *Agrobacterium tumefaciens* is used as the basis for the classification of strains.

The second characteristic of a crown gall is the ability to proliferate in the absence of exogenous growth regulators after being established. Whereas normal plants generally require the addition of exogenous auxins and cytokinins for *in vitro* propagation, crown gall tissue can grow indefinitely without them. These findings indicate the ability of crown galls to produce all the factors necessary for maintaining growth (THOMASHOW *et al.*, 1986).

1.4.1.1 **Ti-Plasmids of *Agrobacterium tumefaciens***

In 1975 WATSON *et al.* demonstrated that both tumour induction and opine production are dependant on the presence of a megaplasmid, called the Ti or tumour inducing plasmid. The plasmid is around 200 - 250 kilobases (kb) in size. It is maintained and stable in *Agrobacterium* at temperatures below 30°C. At higher temperatures, the plasmid can be lost, giving rise to avirulent strains of *Agrobacterium* (HAMILTON and FALL, 1971).

Comparisons of different Ti plasmids have revealed four regions of extensive homology (DRUMMOND and CHILTON, 1978). Various methods such as transposon insertion, deletion mutagenesis and sequence comparisons have been used to elucidate the functions of the gene products encoded by these regions. Two

of the homologous regions do not take part in the oncogenic process. The first of these is the *ori* region that codes for the origin of transfer that plays a role in conjugative transfer and replicative maintenance of the plasmid within *Agrobacterium*. This region also includes an incompatibility function (GALLIE *et al.*, 1985). The second non-oncogenic region is known as the *con* region and it encodes bacterial transfer functions (HOOYKAAS *et al.*, 1977). The other two regions are respectively known as the T-region, the mobile DNA that is transferred to the plant cell, and the *vir* region that codes for proteins involved in the T-DNA transfer process. Both of these are essential for the transformation of a plant cell.

1.4.1.1.1 **The T-DNA region**

It is generally accepted that the T-DNA is the only section of DNA that is transferred from *Agrobacterium* and stably integrated and maintained in the plant nuclear genome (CHILTON *et al.*, 1980; ZUPAN and ZAMBRYSKI, 1995). It has, however, been shown that DNA from outside the classically defined T-DNA region occurs in 20 % to 30 % of transformed plants (MARTINEAU *et al.*, 1994). The T-DNA functions solely as a structural element during transfer and does not encode any of the products essential for this process (GARFINKEL *et al.*, 1981). The length of the T-DNA does not seem to influence the transfer process. In nopaline strains, a single T-DNA segment of approximately 24 kb is transferred to the plant (LEMMERS *et al.*, 1980). In octopine strains, two contiguous segments are found, the T_L of 14 kb, and the T_R of 7 kb (DE BEUCKELEER *et al.*, 1981). These can be transferred to the plant cell either independent of each other, or as a continuous stretch of DNA. Although the structural organisation of these two types of Ti-plasmid differ, the T-DNA regions of both types are flanked by 25 base pair (bp)

direct repeat sequences. These provide recognition sites for T-DNA transfer (WANG *et al.*, 1984).

The T-DNA contains all the plasmid DNA sequences found in established tumour lines. Hybridisation of a Ti-plasmid specific probe to tumour DNA has shown that the T-DNA found in the plant cell is co-linear with the T-DNA found in the Ti-plasmid of *Agrobacterium* (ARMITAGE *et al.*, 1988). This is an indication that no major rearrangements of the DNA sequence takes place during tumour establishment. The site of integration into the plant genome is random and multiple copies can be present, either in direct repeats, or in different regions of the plant DNA (ZAMBRYSKI, 1988).

Synthesis of opines are encoded by T-DNA genes. These compounds are used by *Agrobacterium* as a sole source of carbon and nitrogen (BOMHOFF *et al.*, 1976). Tumor formation by *Agrobacterium tumefaciens* seems to be primarily due to the activity of three oncogenes present on the T-DNA segment. Mutations in the *tmr* (tumor morphology root) locus resulted in root proliferation in the tumor. The *tmr* locus (also called *cyt*, *ipt* or *roi* [HOOYKAAS and SCHILPEROORT, 1992]) encodes an isopentenyl transferase enzyme, which converts 5' AMP and isopentenylpyrophosphate into the active cytokinin isopentenyladenosine-5-monophosphate (AKIYOSHI *et al.*, 1984). Two loci, which influence the balance of auxins in the plant, were defined when deletion mutations resulted in shoot proliferation. The *tms1* (also known as *aux1* or *iaaM*) and *tms2* (*aux2* or *iaaH*) loci code for enzymes which establish a new auxin biosynthetic pathway for the transformed plant cell. The *tms1* product is tryptophan-2-mono-oxygenase. It catalyses the biosynthesis of indole-3-acetamide which is converted to the natural auxin, indole-3-acetic acid, by the product of *tms2* (INZE *et al.*, 1984). The

expression of these genes will therefore lead to a deregulated phytohormone production that overrides the normal metabolic pathways. Neoplastic proliferation of these cells as well as their neighbouring cells results from the expression of these genes which can be considered to be oncogenes (ZAMBRYSKI *et al.*, 1989). The simultaneous transfer of the growth controlling genes and the genes encoding for opine synthesis on a single T-DNA segment ensures that the opine producing cells will actively proliferate and ensure a continuous supply of carbon and nitrogen compounds to the bacterium.

1.4.1.1.2 **The virulence (*vir*) region**

None of the elements necessary for T-DNA transfer are part of the T-DNA itself. Most of these are provided by the Ti-plasmid's *trans* acting virulence (*vir*) region (KLEE *et al.*, 1982). This 40 kb region consists of twenty four genes, present in 8 operons, that are either essential for (*vir*A, *vir*B, *vir*D and *vir*G), or that enhance the efficiency of (*vir*C, *vir*E, *vir*F and *vir*H), plant cell transformation (STACHEL and NESTER, 1986). OKKER *et al.* (1984) have shown that the *vir*A and *vir*G loci are constitutively expressed. The other *vir*-promotors are tightly regulated and activation of gene expression is mediated by low molecular weight phenolic compounds secreted by wounded plant cells. The most important of these are acetosyringone and α-hydroxy-acetosyringone (STACHEL *et al.*, 1985). An important characteristic of the *vir* region is that all of these loci can operate *in trans* for the transfer of T-DNA to the host cells. This proved to be very useful in the construction of transformation vectors.

1.4.1.2 **Mechanism of T-DNA transfer**

Three elements are necessary for T-DNA transfer, namely four chromosomal virulence genes (DOUGLAS *et al.*, 1985), the T-DNA border sequences (WANG *et al.*, 1984) and the *vir* region (STACHEL and NESTER, 1986). The transfer of T-DNA to the plant cell consists of a number of identifiable steps. The first step in the transfer process is the recognition of a susceptible plant cell by *Agrobacterium* and subsequent attachment of the bacterium to the cell. Wounding of the cell is a requirement for attachment and subsequent transformation to take place (KAHL, 1982). The bacterium posseses a chemotaxis system which responds to various compounds present in wounded cells (ASHBY *et al.*, 1987). Four chromosomal genes are responsible for the attachment of the bacterium to the cell. These genes are known as the *chv*A, *chv*B, (chromosomal virulence) *exo*C and *att* and are constitutively expressed (DOUGLAS *et al.*, 1985).

The second step in the plant cell transformation is the induction of *vir* gene expression. As mentioned previously, this involves the recognition of phenolic compounds such as acetosyringone and α-hydroxy-acetosyringone (STACHEL *et al.*, 1985). These are both released by wounded cells and are the main signal compounds for the activation of *vir* gene expression. Even in the presence of these inducers, certain conditions have to be met to obtain optimal *vir* induction. These are (a) the pH of the medium must be between 5 and 6, (b) the temperature must be between 20 and 30 °C, (c) the presence of yeast extract must be avoided and (d) a high sugar content must be present in the medium (STACHEL and ZAMBRYSKI, 1986; ALT-MOERBE *et al.*, 1988; MELCHERS *et al.*, 1989; TURK *et al.*, 1991). NIXON *et al.* (1986) proposed that the activation of the *vir* gene functions depend on a two component sensor/regulator system. The *vir*A gene functions as the

sensor and the *vir*G as the regulatory component in this system. Both of these loci are constitutively expressed at low levels. The level of expression increases substantially in response to the presence of the phenolic *vir* inducers.

The *vir*A locus specifies an inner membrane protein that most likely functions as a chemoreceptor to sense the presence of plant wound factors (LEROUX et al., 1987). WINANS *et al.*, (1989) suggested a possible structure for the *vir*A gene product. It was suggested that the product is a two domain protein with an amino terminus situated to the outside of the cytoplasmic membrane which binds to the phenolic inducers and a carboxy terminus localised in the cytoplasm. It is thought that this protein can convert the *vir*G product to an active form that can bind to *vir* promotors to activate the transcription of these genes (POWELL *et al.*, 1986). The conversion of the *vir*G product is likely to be a phosphorylation process.

The *vir*B, *vir*C, *vir*D and *vir*E loci are multigenic operons. Their products mediate the generation and transfer of single stranded T-DNA molecules, called the T-strands to the plant cell (STACHEL *et al.*, 1986b). The production of this transferable T-DNA copy represents the third step in the transformation process. A model of the generation of the T-strands is shown in Figure 1.1. Two of the most important elements influencing this step of the transfer process are the T-DNA border regions. The T-DNA of all naturally occurring Ti-plasmids are flanked by two near perfect 25 bp direct repeat sequences (WANG *et al.*, 1984). These regions enable the *vir* genes involved in the production of the T-strands to recognise the limits of the T-DNA and are the only *cis* elements needed for T-DNA processing.

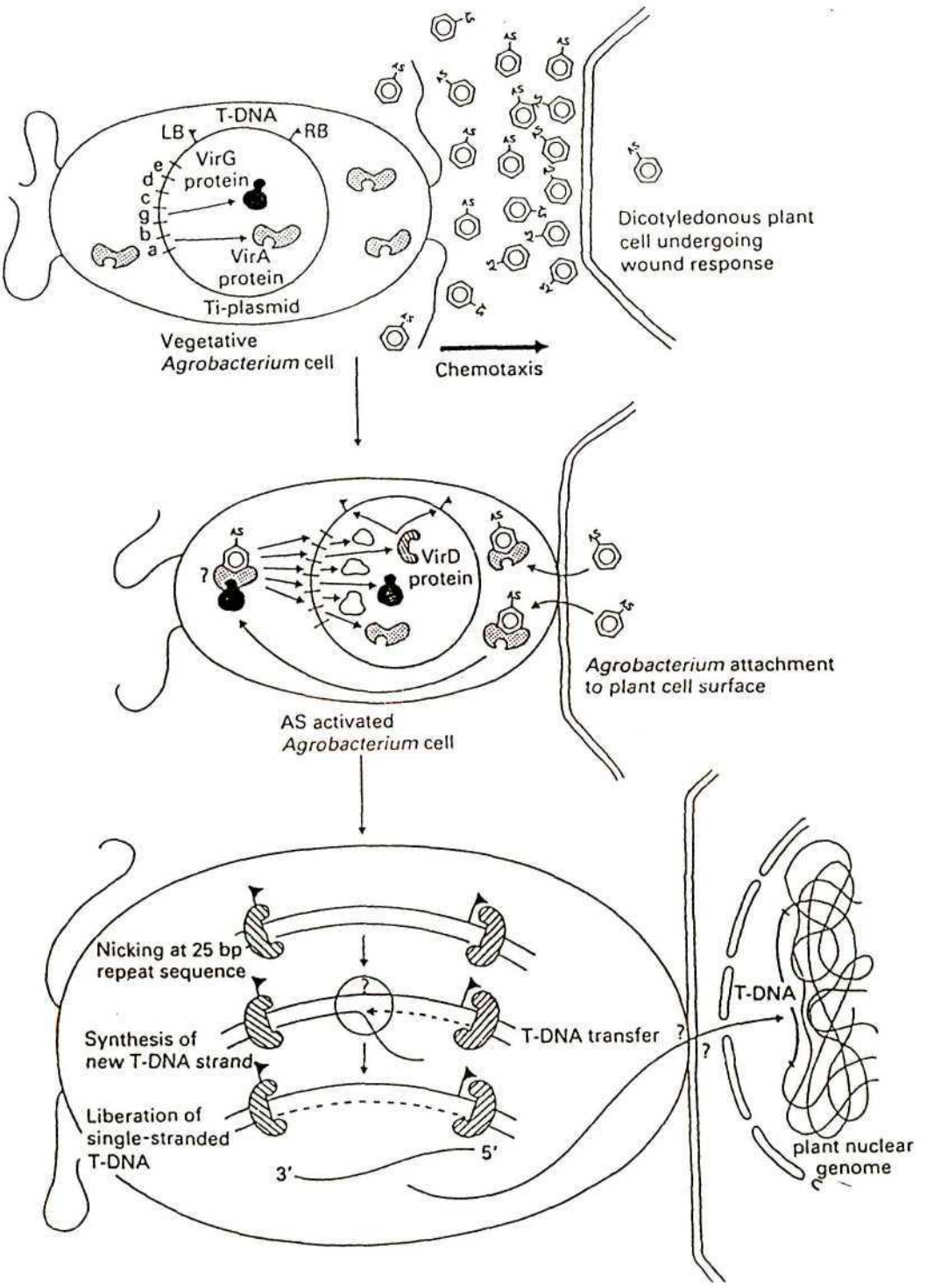


Figure 1.1. *Agrobacterium* / plant interaction and mechanism of T-DNA transfer (AS - acetosyringone) (ARMITAGE *et al.*, 1988).

A clear difference exists between the right and left border sequences. The right border sequence is essential for T-DNA transfer to the plant cell (WANG *et al.*, 1984). Deleting or reversing the orientation of this border abolishes T-DNA transfer, while manipulation of the left border sequence has little or no effect (HILLE *et al.*, 1983). The homology between the left and right 25 bp repeats at sequence level is such that it is unlikely that these differences can be the cause of the observed functional dissimilarity (VAN HAAREN *et al.*, 1987). A sequence of 24 bp has been found in octopine Ti-plasmids that might explain this discrepancy. This sequence is present on the right side of the right border repeat but not at the left border. It cannot mediate T-DNA transfer by itself, but enhances the process and is called "overdrive" (PERALTA *et al.*, 1986). The position of this enhancer can be varied considerably and it will still have an effect when placed up to 6 kb from the border repeat (VAN HAAREN *et al.*, 1987).

The appearance of nicks in the 25 bp border region of the T-DNA is the first indication of T-strand production. The nicks are involved in the initiation and termination of T-strand formation (WANG *et al.*, 1987). By using radioactive probes of the positive and negative strands of the T-DNA region, it could be shown that T-strands correspond to the negative strand of the T-DNA region, such that it's 5' and 3' ends map to the right and left T-DNA borders respectively (STACHEL *et al.*, 1986b). These T-strands are produced at approximately one copy per *vir* induced bacterium.

The production of both border nicks and eventually of T-strands seem to be under the control of the polypeptides specified by the *vir*D locus (STACHEL *et al.*, 1987). It has been shown that two of the genes encoded by this locus, *vir*D1 and *vir*D2 are involved in the recognition of the T-region boundaries and the production of T-DNA

intermediates (STACHEL *et al.*, 1986b; FILICHKIN and GELVIN, 1993). Together they produce a single stranded endonucleolytic cleavage in the bottom strand of each border. The *vir*D2 product stays bound to the 5' end of the nick, as a T-strand-protein complex, even during extremely harsh treatment. This complex remains present during transfer of the T-strand from the Ti-plasmid (HOWARD *et al.*, 1989; FILICHIN and GELVIN, 1993), giving it a polar character. Transfer of the T-DNA complex to the bacterial membrane represents the fourth step in the transfer of T-DNA to the plant cell. The function of the complex is unknown, but possible explanations that have been offered are that; (a) it may act as unwinding protein during T-strand synthesis; (b) it may protect the 5' end from endonucleases; or (c) it may facilitate the transfer to the bacterial membrane (WEBSTER, 1990).

Mutations of the *virB* gene causes transformation to be abolished. The gene is very large and codes for eleven open reading frames (WARD *et al.*, 1988). These products have been found in large quantities in the membranes of induced bacteria (ENGSTROM *et al.*, 1987). These products seem to form a transmembrane apparatus (a conjugal pore or pilus) through which the T-DNA-protein complex might be exported, as they exhibit the predicted features associated with such an structure. The membrane association of seven of the *vir*B proteins was confirmed using subcellular localisation studies (ZUPAN and ZAMBRYSKI, 1995). Two of the *vir*B proteins, *vir*B4 and *vir*B11 provide the energy for the translocation of the T-complex (BERGER and CHRISTIE, 1994).

The *virC* locus seems to consist of two open reading frames (CLOSE *et al.*, 1987). This gene has been shown not to be essential for plant cell transformation, but the process is considerably diminished in it's absence. From experiments done by ALT-MOERBE *et al.*, (1986) it was suggested that the *virC* proteins might play a role in

stimulating the formation of T-DNA intermediates, by facilitating the interaction between the *virD* proteins and the 25 bp border repeats. Without this stimulating effect, it is possible that the T-DNA transfer becomes too inefficient to produce a tumerous response in some plants.

Mutations in the *vir*E region influence the host range for the *Agrobacterium* (HOOYKAAS *et al.*, 1984; HIROOKA and KADO, 1986). Further investigation revealed that some of the products encoded by these genes are excreted by the *Agrobacterium* (OTTEN *et al.*, 1984). This could indicate that they play a role in the conditioning of the plant cells at the wound site. *Vir*E2 is a nucleic acid binding protein that binds tightly to the T-strand during transfer, coating it completely. It prevents nucleolytic degradation, and unfolds and extends the single stranded DNA to a narrow diameter of 2 nm to facilitate transport through membrane channels (ZUPAN and ZAMBRYSKI, 1995). This transport of the T-DNA complex through the bacterial and plant cell membranes, influenced by the products of the *vir*B and *vir*E loci, represents the fifth step in the transfer process.

Finally, the T-DNA complex is transferred through the cytoplasm of the plant cell, through the nuclear membrane and then integrated into the plant nuclear genome. The *vir*E2 protein appears to be functionally important for nuclear import of the T-complex through the nuclear pore (reviewed by ZUPAN and ZAMBRYSKI, 1995). GHEYSEN *et al.* (1991) proposed a general model for T-DNA integration. According to this model, the right border of the T-strand as well as its accompanying *vir*D2 protein interacts with a nick in the plant DNA. A second nick is formed on the opposite strand of the genomic DNA due to the stress caused by this interaction. This second nick can appear at varing distances from the first nick. The T-strand is ligated to the plant DNA and the homologous strand replicates.

Replication and repair of the nick leads to both repeated sequences and sequence rearrangements at the ends of the T-DNA insertions. The insertion of the T-DNA is random with respect to the plant DNA sequences.

After integration into the plant genome, the T-DNA is transcribed and translated, and proteins involved in tumor formation and opine production are formed. These genes are only expressed after transfer to the plant cell. The reason for this only became clear when plant promotor sequences such as the TATA box sequence and the CAAT box region were found upstream from these genes (BARKER *et al.*, 1983; SLIGHTOM *et al.*, 1986). Most plant genes are highly regulated and function only at certain stages of plant development, but the T-DNA genes are constitutively expressed by plants (DE GREVE *et al.*, 1982). This discovery has made it possible to exploit these promotors for the expression of foreign genes in plants.

1.4.2 **The *Agrobacterium tumefaciens*-system used as transformation vector**

Three fundamental observations form the basis of the development of plant transformation vectors based on the *Agrobacterium tumefaciens* Ti-plasmids (SCHELL, 1987):

(a) Foreign DNA sequences inserted within the T-region of Ti-plasmids are transferred to the plant genome (HERNALSTEENS *et al*, 1980) and are stably maintained in the plant through meioses (BARTON *et al.*, 1983);

(b) None of the genes in the T-DNA region are responsible for the transfer of the DNA or the integration into the plant genome (ZAMBRYSKI *et al.*, 1983); and

(c) The T-region does not have to be physically linked to the *vir* genes of the Ti-plasmids to be transferred (HOEKEMA *et al.*, 1983). Any DNA segment flanked by the 25 bp border sequences will be transferred from the *Agrobacterium* host to the plant genome provided functional *vir* and chromosomal genes are present in the *Agrobacterium* strain.

From this information, a system of binary or *trans* acting Ti gene vectors was developed. Binary vectors are composed of two elements. Both of these elements must be located in a *Agrobacterium* strain harbouring functional chromosomal virulence genes. The first element is a helper Ti-plasmid from which the whole T-region, including the border 25 bp sequences, has been removed by deletion. This plasmid still contains a fully functional *vir* region that can act *in trans* (HOEKEMA *et al.*, 1983).

The second element is a plasmid containing the right and left T-DNA border sequences in the correct polarity. The phytohormone genes must be deleted from this plasmid, to ensure the normal development of transformed plants. This plasmid must be capable of transforming a broad host range and contain cloning sites and marker genes for the identification or selection of transformed plants (SCHELL, 1987).

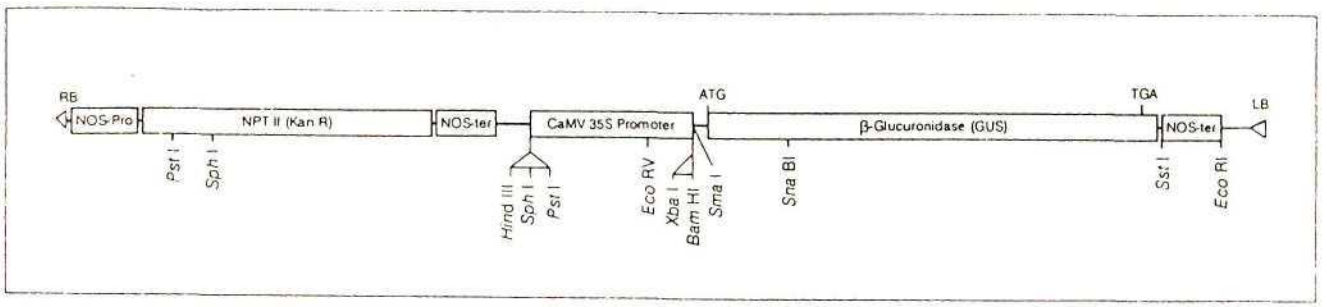
An example of one such binary vector is the 10 kilo base plasmid pBin19 (BEVAN, 1984). This vector was based on the wide host range plasmid pRK252 and can replicate in both *Escherichia coli* and *Agrobacterium tumefaciens*. The vector contains a kanamycin resistance gene that allows for the selection in bacteria, as well as a dominant selectable marker, a chimeric nopaline synthase-neomycin phosphotransferase II (NPT II) gene which confers kanamycin resistance to

transformed plant tissue (BEVAN, 1984). A multiple cloning site, the *lacZα* region from the phage vector M13mp19 is present. The presence of this region facilitates the detection of inserted DNA.

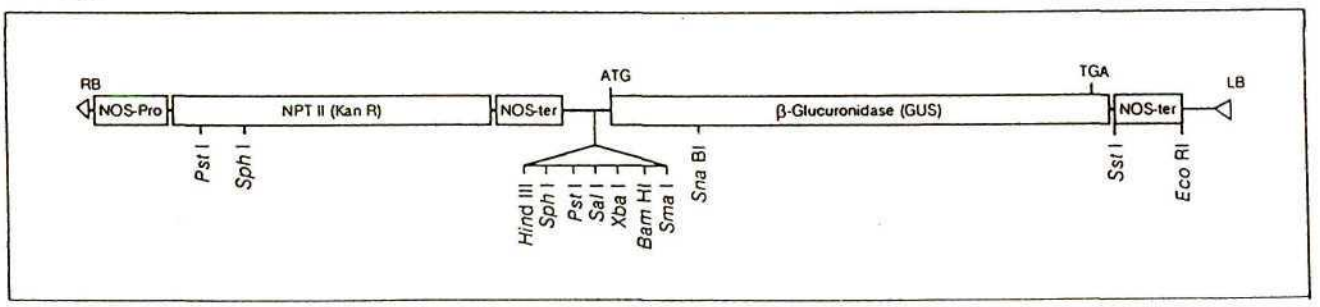
The plasmid can be transferred efficiently from *Escherichia coli* into the *Agrobacterium* strain LBA4404 by triparental mating using the helper plasmid pRK2013. This *Agrobacterium* strain contains the plasmid pAL4404 from which the T-DNA has been deleted, but which contains an intact *vir* region (HOEKEMA *et al.*, 1983). Selection for transconjugants can be done on the basis of their resistance to kanamycin and rifampicin. Transconjugants can be used for transformation of plant material.

Although reporter genes such as those conferring kanamycin resistance has proved to be very useful selectable transformation markers, screenable marker genes are often included on transformation vectors to allow independent verification of the transformed status of tissues (ARMITAGE *et al.*, 1988). Where these markers are used, the ease, expense and specificity of the detection methods are important considerations. Frequently utilised screenable markers include chloramphenicol acetyl transferase (CAT), octopine and nopaline synthase, ß-glucuronidase (GUS), ß-galactosidase, firefly luciferase and phosphinothricin (PPT) .

Examples of plasmids containing screenable marker genes are the plasmids pBI 101/*pat* and pBI121 that will be used during this study (Figure 1.2). Both plasmids are derivatives of pBIN19, into which a GUS casette has been cloned (JEFFERSON *et al.*, 1987). In the plasmid pBI101/*pat*, the GUS cassette is promotorless , but in pBI121, an 800 bp fragment containing the promotor from the cauliflower mosaic



A



B

Figure 1.2 A) The GUS cassette with the CaMV 35S promotor in pBI121.
B) The 'promotorless' GUS cassette in pBI 101. The *pat* gene was ligated into the plasmid between the *HindIII* and *XbaI* sites.

virus responsible for the transcription of the 35S RNA fragment, was cloned upstream of the GUS gene.

1.4.2.1 **The ß-glucuronidase system for screening transformants**

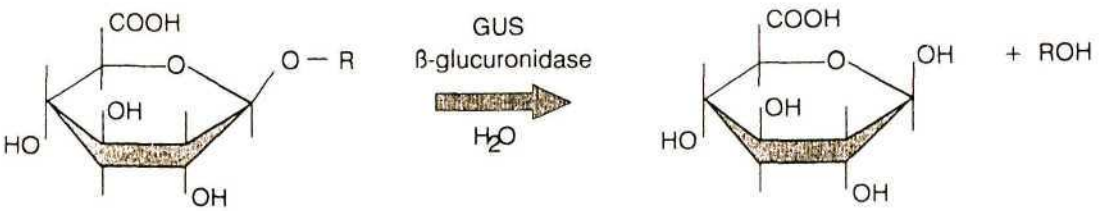
The ß-glucuronidase gene fusion system is based on the ß-glucuronidase enzyme (EC.3.2.1.31), that was isolated from *Escherichia coli*. The enzyme is a hydrolase which catalyses the cleavage of a wide variety of ß-glucuronides (Figure 1.3), many of which are available as spectrophotometric, fluorometric and histochemical substrates. Plants lack detectable glucuronidase activity, making sensitive assays of enzyme activity possible. Accurate measurements of glucuronidase activity in plants expressing the gene can be made from very small amounts of transformed plant tissue (JEFFERSON *et al.*, 1987).

1.4.2.2 **Phosphinothricin as selectable marker for transformation**

The bioactive ingredient of Ignite®, phosphinothricin (PPT) is a compound produced by *Streptomyces* species. These species have evolved mechanisms to inactivate this compound and show no signs of self toxification. Two research groups independently discovered and isolated phosphinothricin resistance genes in separate *Streptomyces* genomes (DONN, 1991). The first is the *bar* gene that confers bialaphos resistance that was isolated from *Streptomyces hygroscopicus*. Bialaphos is produced by fermentation of *Streptomyces hygroscopicus* and consist of phosphinothricin, an analogue of L-glutaminic acid and two L-alanine residues (DE BLOCK *et al.*, 1987). The second gene, *pat*, was isolated from *Streptomyces*

Reaction Catalyzed by ß-Glucuronidase

(E.C. 3.2.1.31)



Analytical Substrates for GUS

Type of Substrate	ROH =
Fluorogenic	4-methylumbelliferone
	3-cyano-4-methylumbelliferone
	4-trifluoromethylumbelliferone
	fluorescein
	3-O-methylfluorescein
	resorufin
Chromogenic	5-bromo-4-chloro-3-indoxyl (X-gluc)
	Naphthol ASBI
	phenolphthalein
	p-nitrophenol

Figure 1.3. The reaction catalysed by ß-glucuronidase.

viridochromogenes and confers resistance to chemically synthesised PPT sold in South Africa as the herbicide Ignite® (Hoechst AG) (STRAUCH *et al.*, 1988). The genes are conserved in these two species and encodes phosphinothricin acetyl transferases (PAT), that convert phosphinothricin into the inactive form N-acetylphosphinothricin (HARA *et al*,. 1991). A third and distinct gene was developed by Hoechst. Both the *pat* and *bar* genes show a high content of guanine and cytosine bases of 70 %, while typical plant genes have only 50 % GC pairs. Cytosine in the DNA are targets for gene inactivation through methylation. A synthetic form of *pat* was therefore developed with a lower GC content of 50 % (DONN, 1991). This latter form of the gene was used in this study.

Phosphinothricin acts by inhibiting a specific amino acid biosynthesis pathway and acts as a potent inhibitor of the enzyme glutamine synthetase (DE BLOCK *et al.*, 1987). Glutamine synthetase is the only enzyme in plants that catalyses the conversion of ammonia to glutamine, preventing the accumulation of toxic levels of ammonia in plant tissues. Ammonia is naturally formed through a number of processes such as nitrate reduction, amino acid degradation and photorespiration (TACHIBANA *et al.*, 1986). Inhibition of glutamine synthetase leads to a rapid accumulation of ammonia and death of the cell. The action of the *pat* gene is summarised in Figure 1.4.

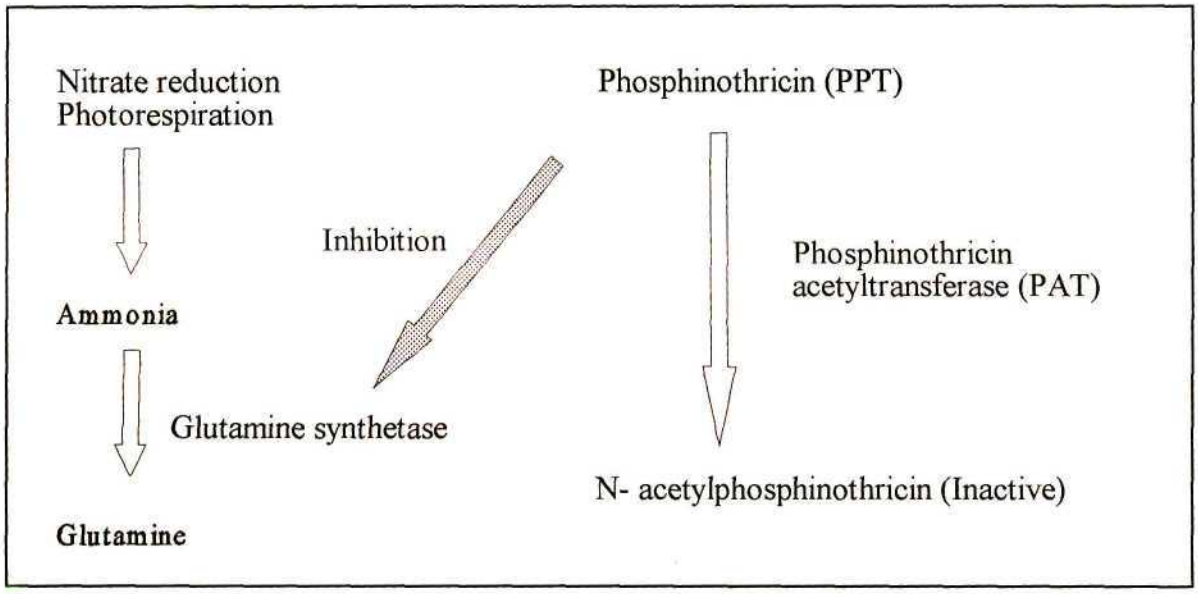


Figure 1.4 The action of phosphinothricin and phosphinothricin acetyltransferase in plants.

1.5. **TRANSFORMATION OF CUCURBITS**

A number of reports have appeared describing the successful transformation and regeneration of cucurbits. The first of these appeared in 1986 when TRULSON *et al.* described the transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes*. *Agrobacterium rhizogenes* differs from *Agrobacterium tumefaciens* by inducing roots rather than tumors in the host (CHILTON *et al.*, 1980). Transformed plants were regenerated from roots induced by inoculation of inverted hypocotyl sections with *Agrobacterium rhizogenes* containing the vector pARC8 in addition to the resident Ri-plasmid (root inducing-plasmid).

The T-DNA transformation cassette included the neomycin phosphotransferase II (NPT II) gene that confers kanamycin resistance to the plant. Selection was done on kanamycin supplemented media. Bacterial carry-over was eliminated on media containing cefotaxime. Transgenic plants appeared normal and tested positive in assays for the neomycin phosphotransferase II. Southern blot analyses revealed that all transgenic plants contained the T-DNA from the vector, but only some of them contained DNA from the resident Ri-plasmid.

CHEE (1990b) reported transformation of cucumber using a *Agrobacterium tumefaciens* transformation system. A binary vector was used. It consisted of the *Agrobacterium tumefaciens* strain C58Z707 containing the vector plasmid pGA482. The T-DNA region also contained a plant expressable neomycin phosphotransferase II (NPT II) gene. Cotyledon sections were inoculated and grown on selective medium containing kanamycin. Plants were regenerated via somatic embryogenesis using the method described by CHEE (1990a).

NPT II enzyme activity in regenerated plants confirmed the transformation of the plants. Total plant DNA was isolated and digested with *Bam*HI and *Hind*III. The fragments were hybridised against ^{32}P-labeled NPT II gene fragments and a predicted hybridising fragment was observed. Copy number constructions suggested that each of the transformed plants contained one gene copy of the NPT II gene.

Transgenic muskmelon plants were obtained from cotyledonary explants inoculated with *Agrobacterium tumefaciens* strain LBA4404 (FANG and GRUMET, 1990). The *Agrobacterium* strain contained the binary vector pCIB10 (ROTHSTEIN *et al.*, 1987), with the selectable marker for kanamycin resistance, the neomycin phosphotransferase gene (NPT II). Several factors that can influence transformation were investigated. An inoculation time of 10 minutes and co-cultivation time of three days with freshly prepared bacteria proved to be the most efficient protocol. Rooted plantlets, developed on kanamycin supplemented media, were transferred to the greenhouse. Southern blot analysis of the rooted plants was performed and hybridisation of the NPT II probe was reported only in regenerated plants, not in any control plants. The progeny of these plants were tested and showed the expected 3:1 (NPT positive: NPT negative) ratio for the incorporation of a single gene. The time from the initiation of the experiment to establishment of plants in the greenhouse was approximately three months.

In 1991, CHEE and SLIGHTOM reported successful transfer and expression of the cucumber mosaic virus strain C (CMV-C) coat protein gene in the genome of cucumber. The system that was used by CHEE (1990b), was adapted for use in this transformation procedure. The plasmid referred to as pGA482GG/cpCMV19, containing the virus coat protein gene, was used instead of the plasmid pGA482.

Transformed cotyledonary tissue was regenerated via somatic embryogenesis. All regenerated plants appeared morphologically normal and tested positive for NPT II. Southern blot analysis of the DNA of selected cucumber plants indicated that NPT II, GUS and CMV-C coat protein genes were integrated into the plant genome. ELISA and Western blot analysis confirmed that the CMV-C coat protein was present in the protein extracts of the progeny plants.

DONG *et al.*, (1991) recovered transgenic melon plants at frequencies of 4 - 6 %. Cotyledon explants were co-cultivated with *Agrobacterium tumefaciens* strain GV3111SE, containing the plasmids pFM201, ROA93 or pMON530-LUC-E9 (OTT *et al.*, 1990; JIA *et al.*, 1989). The pFM201 plasmid contains ß-glucuronidase (GUS) coding sequences and confers methotrexate (MTX) resistance through the dihydrofolate reductase gene. The plasmid pROA93 contains a NPTII gene for kanamycin resistance, as well as GUS. pMON530-LUC-E9 contained both NPTII and the firefly luciferase gene as a reporter gene. Various aspects of the protocol were optimised using these plasmids alone or in combination. Despite several attempts at improving the selection media, 'escapes' remained a problem. The expression pattern of the CaMV 35S promoter in transgenic melon was studied histochemically using the GUS gene. It was shown that the expression pattern changes during plant development and that it is preferentially expressed in certain tissues. Transgenic plants were recovered and the progeny tested for GUS expression and methotrexate resistance. The transgenes were transmitted as dominant Mendelian traits.

SARMENTO *et al.*, (1992) investigated some of the factors infuencing *Agrobacterium* mediated transformation of pickling cucumber. Leaf and petiole explants were transformed using the *Agrobacterium* strain LBA4404 containing the

plasmids pBIN19 (BEVAN 1984) or pCGN783 (HOUCK *et al.*, 1991). The NPT II expression was used as selectable marker. Transformation of plants recovered from both explant types, using both plasmids was confirmed using Southern analyses and dot blot assays (RADKE *et al.*, 1988). The optimal petiole length was found to be 4 to 6 mm, using an inoculation time of five minutes and co-cultivating the explants on a tobacco feeder layer. Acetosyringone treatment did not improve the transformation efficiency.

The first report of watermelon transformation was that of CHOI *et al.*, (1994). *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid pBI121 (see Figure 1.2 a above) was used to transform cotyledonary explants. Transgenic plants were selected on kanamycin and carbenicillin containing media. A 5 day preculture period enhanced the transformation competence. The GUS histochemical assay and Southern blot analysis were used to confirm transformation. Transformants were grown to maturity.

The coat protein of the cucumber mosaic virus- white leaf strain(CMV-WL) was transferred to 5 *Cucumis melo* L. cultivars (GONSALVES *et al.*, 1994). The vector pGA482GG/cpCMV-WL, containing the coat protein, NPTII and GUS genes was used for the transformation. *Agrobacterium* mediated transformation and microprojectile bombardment were tested. GUS and NPTII expression of putative transgenics were analysed, followed by PCR analysis, coat protein ELISA and Western blot analysis. Although the results of all the tests were not always consistent for a specific line, transgenic plants were obtained for all the cultivars. A number of morphological abnormalities were observed and several plants were tetraploid or mixoploid. Generally, the resistance to the virus was low.

1.6. **PURPOSE OF THIS INVESTIGATION**

Since the first transgenic plants were regenerated, much progress has been made in the development of improved crops. A variety of techniques exist that can be utilised for the development of plants with novel characteristics.

The fact that many species of the Cucurbitaceae are susceptible to infection by *Agrobacterium* pathogens (SMARRELLI *et al.*, 1986) and that procedures for regenerating several of these species have been established, suggest that the transfer of genetic information into the genome of a number of these species should be possible. Such a system would be useful for the transfer of genes that confer resistance against viral infection (POWELL-ABEL *et al.*, 1986), or herbicides (COMAI *et al.*, 1985). These traits are often difficult or impossible to aquire via conventional plant breeding. The reports that exist regarding transformation of the Cucurbitaceae indicates that such a project could be feasible.

The aim of this study was to develop a reliable regeneration and transformation protocol for selected members of the Cucurbitaceae. Several factors that can influence shoot regeneration were investigated. The usefulness of *Agrobacterium tumefaciens* strain LBA4404 for transformation of the Cucurbitaceae had to be investigated. The binary vector pBI121 was mobilised into this *Agrobacterium* strain using the helper functions of pRK2013. This vector contains the screenable marker gene ß-glucuronidase (GUS) (JEFFERSON *et al.*, 1987) and the selectable neomycin phosphotransferase-II gene (NPT-II). Shoots that developed on kanamycin containing media and formed roots had to be tested for the presence of the GUS gene using fluorometric and histochemical assays.

Subsequently, this protocol was used to transfer the herbicide resistance gene phosphinothricin acetyltransferase (*pat*) into *Cucumis melo* L. Hales Best 36. The same *Agrobacterium* strain was used, with the plasmid pBI101/*pat*. The selection was done on both kanamycin and phosphinothricin. Putitive transformants were examined with PCR (polymerase chain reaction) and Southern blot analyses. The recovered lines were examined for morphological and ploidy changes.

CHAPTER 2

REGENERATION OF FIVE CUCURBIT CULTIVARS *IN VITRO*

2.1 INTRODUCTION

A large number of reports have appeared describing *in vitro* regeneration of some of the economically important members of the family Cucurbitaceae (Chapter 1 Tables 1.1 - 1.4). These included mainly cucumber, *Cucumis sativus* L., melon, *Cucumis melo* L., watermelon, *Citrullus* spp. and squash and pumpkin cultivars belonging to *Cucurbita pepo* L. A few cultivars which have been used in regeneration studies overseas are available and cultivated in South Africa. These include the cucumber cultivar Marketer (ALI *et al.*, 1991) and the melon cultivar Hales Best Jumbo (NIEDZ *et al.*, 1989).

No reports have appeared on the regeneration of cultivars developed specifically for the South African market. Examples of locally developed cultivars include the cultivar A-line (the common Flat White Boer pumpkin), which belongs to the species *Cucurbita maxima* L. and *Cucurbita pepo* cv Rolet (gem squash). Both these cultivars were developed and released by the ARC-Roodeplaat (BOSCH and OLIVIER, 1978).

Techniques used by different researchers for regeneration of cucurbits vary greatly, even where the goals of investigations were similar. Variations in the sterilisation methods, explant types, media formulations and growth regulators used necessitated

the investigation of the influence of some of these parameters on regeneration, in order to try and develop more uniform protocols.

In this investigation methods to establish an effective sterilisation procedure for seed derived explants were evaluated. The influence of two growth regulators, the cytokinin, 6-benzyladenine, and the auxin, indole 3-acetic acid, on regeneration of shoot buds was assessed. The influence of the cytokinins 6-benzyladenine, kinetin, thidiazuron and iso-pentenyladenine in combination with the auxin indole 3-acetic acid on regeneration was also tested.

2.2 **MATERIALS AND METHODS**

2.2.1 **Surface sterilisation of donor material**

Seeds of *Cucurbita maxima* L cv. A-line were obtained from two different sources and used to establish a reliable seed sterilisation protocol. Initially seeds were obtained from the Genebank of the ARC-Roodeplaat. A second batch of seed was bought from Hygrotech Seed, a commercial seed supplier in Silverton, Pretoria.

A number of different procedures had to be tested due to initial difficulties in obtaining uncontaminated explants. Sodium hypochlorite (commercial Jik, containing 3.5 % (w/v) NaOCl, diluted with distilled water to provide the appropriate concentration of NaOCl) was used as sterilising agent. Combinations of three concentrations of NaOCl (0.5 %, 1.0 % and 2.0 % w/v) and three

sterilisation times (10 minutes, 20 minutes and 30 minutes) were tested, using the seed obtained from Hygrotech. The test treatments are outlined in Table 2.1.

The influence of the seed coat on effective sterilisation was determined by exposing equal numbers of intact seeds and exised embryos to each of the treatments. Embryos were exised after the seeds had been soaked in tap water for 10 -15 minutes to soften the coats. The influence of an ethanol pre-treatment on sterilisation and germination was also tested. Exised embryos were rinsed in 70 % (v/v) ethanol for 2 minutes. The ethanol was removed under aseptic conditions and the seeds then exposed to a 1 % (w/v) solution of NaOCl for 20 minutes. The solution was removed and the seeds rinsed three times with sterile distilled water.

A preliminary sterilisation study was done with the seed obtained from the Genebank, ARC-Roodeplaat. Only a few treatments were tested. These corresponded to treatments 2, 4, 5, 6 and 8 described in Table 2.1. Only exised embryos were used. The influence of a 2 minute ethanol pre-treatment was determined using both intact seeds and exised embryos. This treatment was conducted as described previously for seed obtained from Hygrotech. In this preliminary study, intact seeds were decoated aseptically with a sterile scalpel blade three days after commencing with the experiment.

In all the treatments (including the preliminary test), seeds were placed in 100 ml Schott bottles. The appropriate solution of NaOCl was added. The seeds were soaked in this solution for 10, 20 or 30 minutes, depending on the treatment. The bottles were shaken regularly to remove air bubbles and to ensure that the sterilising agent coated the whole surface. After the treatment period, the NaOCl was poured off and the seeds were rinsed three times with sterile distilled water.

Table 2.1. The sodium hypochlorite treatments which were used to determine an effective seed sterilisation protocol for seeds of *Cucurbita maxima* cv A-line obtained from Hygrotech. All treatments were repeated with intact seeds and exised embryos. Treatments marked with an asterisk were used in a preliminary study where seeds were obtained from the Genebank at ARC-Roodeplaat.

PERCENTAGE NaOCl USED	SOAKING TIME (minutes)		
	10	20	30
0.5	1	2 *	3
1.0	4 *	5 *	6 *
2.0	7	8 *	9

All treated seeds were placed in sterile disposable petri-dishes containing approximately 20 ml MURASHIGE and SKOOG (1962) (MS medium), supplemented with 3 % (w/v) sucrose and solidified with 0.75 % (w/v) agar. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes. Each petri-dish contained five seeds and served as one replicate. Each treatment consisted of five replications. Petri-dishes were sealed with Parafilm® and were placed in a growth room at 26 °C ± 2 °C with a 16 hour/8 hour light/dark cycle. Contamination and germination were noted each day for four consecutive days and final results were noted after one week. The appearance and elongation of the radicle was used as an indication of germination having occurred.

2.2.2 **The influence of different plant growth regulators on shoot regeneration from cotyledonary explants of members of the Cucurbitaceae**

The following cultivars were tested for their ability to regenerate shoots from cotyledonary explants:

Cucurbita maxima Duch. cv. A-line (Flat White Boer pumpkin)

Cucurbita maxima Duch. cv. Chicago Warted (Hubbard squash)

Cucurbita pepo L. cv. Rolet (Gem squash)

Cucumis melo L. cv. Hales Best 36 (Melon)

Cucumis sativus L. cv. Ashley (Cucumber).

Seeds of the A-line, Chicago Warted and Hales Best 36 were obtained from Hygrotech Seed in Silverton Pretoria; Rolet seeds were obtained from the Genebank of the ARC-Roodeplaat and the Ashley seeds were produced by Kirchhoff Seeds and bought from Lion Bridge, Pretoria. Seeds of all five cultivars were soaked in tap water for 10 to 15 minutes to soften the seed coat and embryos then exised. Seeds were surface sterilised by soaking them in a 1 % (w/v) NaOCl solution for 20 minutes, stirring regularly. The NaOCl solution was poured off and the seeds were rinsed three times with sterile distilled water.

In the case of *Cucumis melo* cv Hales Best 36 and *Cucumis sativus* cv Ashley. two explants were obtained from each seed. The proximal and distal ends of the seeds were cut away with a scalpel blade, making sure the whole embryo axis was removed. The remaining edges were removed as well. The two cotyledons were separated and were used as explants. The bigger seeds of the other three cultivars were treated in the same way, but were cut in half longitudinally to provide four explants per seed.

Explants were placed on MS medium supplemented with 3 % (w/v) sucrose, 0.75 % (w/v) agar and a combination of growth regulators. The combinations of growth regulators which were used are given in Table 2.2. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes.

Table 2.2. The combinations of the growth regulators BA and IAA which were used to supplement MS media in a study to test the shoot regeneration ability of five cultivars belonging to the family Cucurbitaceae.

		CYTOKININ CONCENTRATION (mg l^{-1})				
		0.0	1.0	2.0	5.0	10.0
AUXIN	0.0	1	2	3	4	5
CONCENTRATION	0.1	6	7	8	9	10
(mg l^{-1})	0.5	11	12	13	14	15
	1.0	16	17	18	19	20
	2.0	21	22	23	24	25

Each treatment consisted of twelve petri-dishes containing two explants each. Explants were subcultured after four weeks. Very little contamination occurred and only in a few treatments results were recorded for fewer than 24 explants. Results were recorded after 8 to 9 weeks. The percentage of explants which developed roots and shoot buds and the number of distinguishable shoot buds on each explant were recorded. Results were plotted on graphs primarily to visualise trends and where necessary, the data were further analysed statistically and presented in tables.

A further study was done to investigate the effects of four different cytokinins on the shoot regeneration response of the five cultivars. Explants were obtained as described above. The effects of kinetin (Kin), iso-pentenyladenine (iP), thidiazuron (TDZ) and 6-benzyladenine (BA) were investigated. Three concentrations of each cytokinin were used: 1 mg l^{-1}, 2 mg l^{-1} and 5 mg l^{1}. All cytokinin treatments were supplements to basic MS medium with IAA added at a concentration of 0.75 mg l^{-1}. The MS medium contained 3 % (w/v) sucrose and 0.75 % (w/v) agar. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes.

Each treatment consisted of fifteen petri-dishes containing two explants each, giving a total of 30 explants per treatment. Petri-dishes were sealed with Parafilm® and cultivated in the same growth room used for the previous study. Explants were transferred to fresh medium after 4 weeks. The percentage of explants with roots and shoot buds, as well as the number of shoot buds per explant were recorded after 8 to 9 weeks.

2.3 **RESULTS AND DISCUSSION**

2.3.1 **Surface sterilisation of donor material**

The results which were obtained in the study utilising seeds purchased from Hygrotech differed from the results from the preliminary study. No contamination occurred on any of the treatments in the main experiment. This was true for both intact seeds and exised embryos. In the preliminary experiment some contamination was observed. The percentage of explants which were contaminated in each of the treatments is listed in Table 2.3.

The highest percentage of contaminated explants occurred in the treatment where exised embryos were soaked in NaOCl for 10 minutes. The three treatments where seeds were soaked for 20 minutes had very little contamination, regardless of the NaOCl concentration (0.5 %, 1.0 % or 2.0 % w/v) used. Two embryos were contaminated in the 1 % (w/v) NaOCl, 30 minute treatment. It therefore appears that a sterilisation time of 20 minutes is sufficient to provide an acceptable measure of

Table 2.3. The percentage of contaminated and germinated seeds of *Cucurbita maxima* cv A-line observed for each of the sterilisation treatments used in a preliminary study. Results were recorded after 7 days. Seeds were obtained from the ARC-Roodeplaat.

TREATMENT (See text for details)	**CONTAMINATION (%)**	**GERMINATION (%)**
Exised embryo, 1 % (w/v) NaOCl, 10 minutes	24	96
Exised embryo, 1 % NaOCl, 20 minutes	4	100
Exised embryo, 1 % NaOCl, 30 minutes	8	100
Exised embryo, 0.5 % NaOCl, 20 minutes	0	100
Exised embryo, 2 % NaOCl, 20 minutes	4	100
Exised embryo, EtOH pre-treatment, 1 % NaOCl, 20 minutes	8	64
Intact seed, EtOH pre-treatment, 1 % NaOCl, 20 minutes, embryos exised on third day	76	100

disinfection. The concentration of NaOCl did not seem to influence the sterilisation efficiency, as long as seeds were soaked for a sufficient time. Soaking explants in 0.5 % NaOCl for 20 minutes resulted in complete sterilisation of explants.

In the main study, the germination of exised embryos was 100 % except in the case of treatment 9, (2 % NaOCl, 30 minutes) where one seed failed to develop. On closer examination, this seed was found to be damaged. These results differed from the results obtained in the preliminary experiment, where higher NaOCl concentrations had a slightly stimulatory effect on the germination. Although 100 % germination was recorded for the 0.5 %, 20 minute NaOCl treatment after 7 days, there was an initial delay in the germination compared to that for the 1.0 % and 2.0 % NaOCl treatments (Figure 2.1). In both the 1.0 % and the 2.0 % treatments 96 % germination was achieved on the second day of the experiment. By day three

germination was 100 %. The 0.5 % NaOCl treatment only gave 100 % germination on day four (Figure 2.1).

These preliminary results indicated that sterilisation with a 1 % NaOCl concentration for 20 minutes gave the best results. It provided effective sterilisation without causing discernable harm to seeds and seemed to have a slight stimulatory effect on embryo germination. Within the literature studied for this investigation, a total of more than 25 papers, no mention was made about the influence of the sterilisation treatment on the germination of treated seeds. Due to this lack of information from the literature, as well as the ambiguous experimental evidence that was obtained in this study, it was decided to use a concentration of 1 % NaOCl, a concentration recommended by PIERIK (1987), for all subsequent experiments.

The germination of intact seeds varied between 48 % and 92 % in the main study. The final germination obtained from each of the treatments are given in Table 2.4. No pattern in seedling emergence was observed for the different treatments, but it was clear that the seed coat provided a mechanical barrier which had a negative effect on germination.

In the preliminary study, the only treatment which included intact seeds was subjected to the ethanol pre-treatment. Germination was 100 %, but 76 % of the explants were contaminated. In this case the seed coat seemed to provide protection against the harmful effects of the ethanol, but at the same time it provided a barrier to effective sterilisation. This must be taken into account when seeds that could be heavily contaminated are used for experimental purposes.

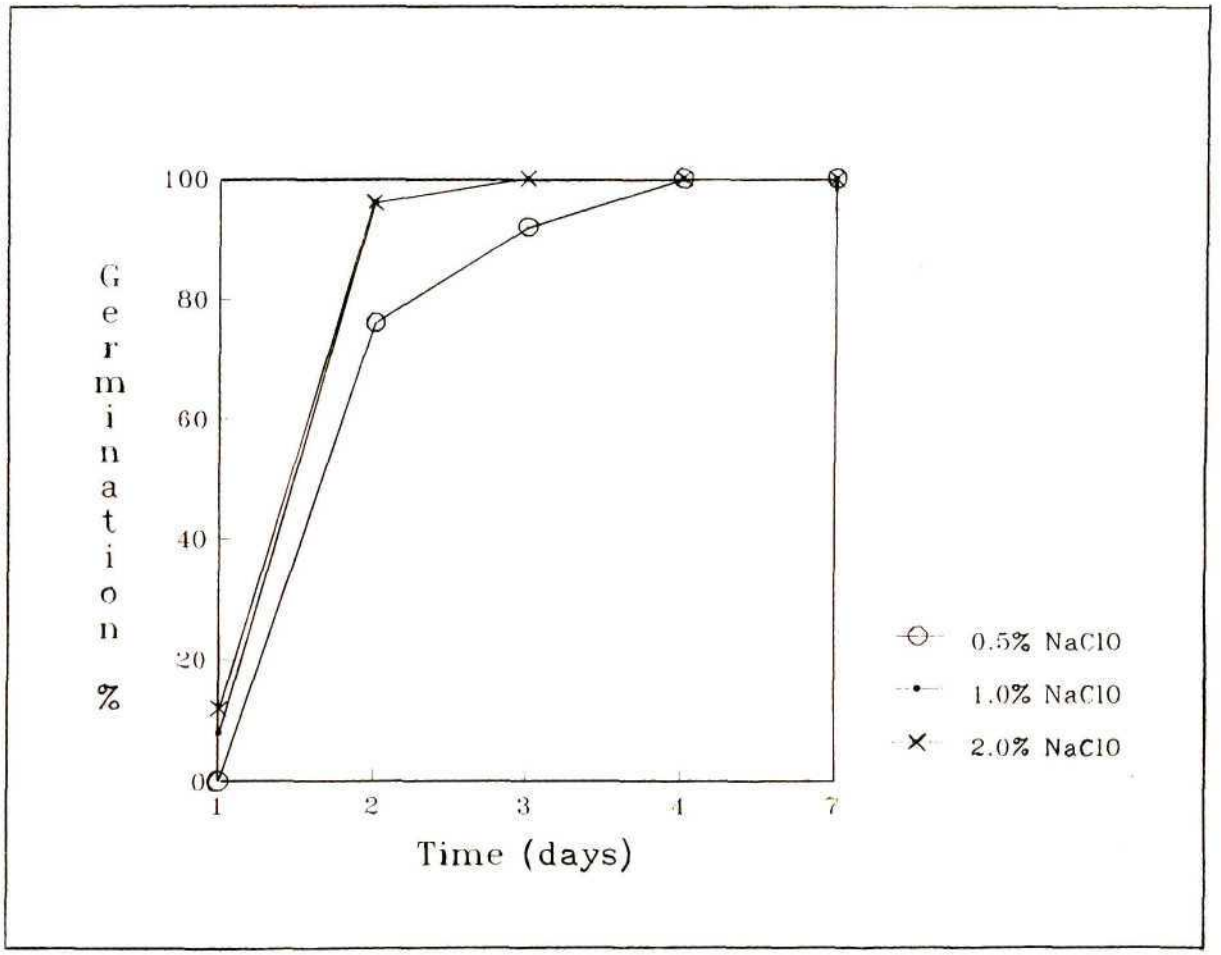


Figure 2.1. The germination rate of *Cucurbita maxima* cv A-line embryos sterilised in 0.5%, 1.0% or 2.0% NaOCl for 20 minutes respectively.

Table 2.4. Germination (%) of intact seeds of *Cucurbita maxima* cv A-line, for each of the sterilisation treatments after 7 days. Seeds were obtained from Hygrotech Seed.

		TIME (minutes)		
		10	20	30
NaOCl	0.5	92	64	60
CONCENTRATION (%	1.0	72	48	84
w/v)	2.0	76	68	52

The difference in final germination of intact seeds in the two studies can be ascribed to the fact that the seed coats were left intact during the main study, but in the preliminary study, they were removed after three days, after it was observed that uniform germination was inhibited. GEORGE and SHERRINGTON (1984a) recommended that all dead and superfluous tissue (such as the seed coat) should be removed before disinfection. In the present study, the presence of the seed coat provided no benefits and was linked to increased contamination of explants and sporadic germination. These factors indicated the advisability of seed coat removal prior to sterilisation.

The germination of the ethanol pre-treated seeds and seeds which were treated with 1 % NaOCl for 20 minutes are plotted in Figure 2.2. Results of both the preliminary experiment and the main experiment are depicted. In the main study, seeds which received the ethanol pre-treatment had no contamination and gave 88 % germination. In the preliminary study, effective sterilisation was achieved when an ethanol pre-treatment was used on exised embryos and germination of 64 % was achieved. The germination for all other treatments was 100 %, with one exception, the 1 % NaOCl, 10 minute treatment where 96 % of the seeds germinated. The difference in germination between the ethanol-treated embryos and the embryos

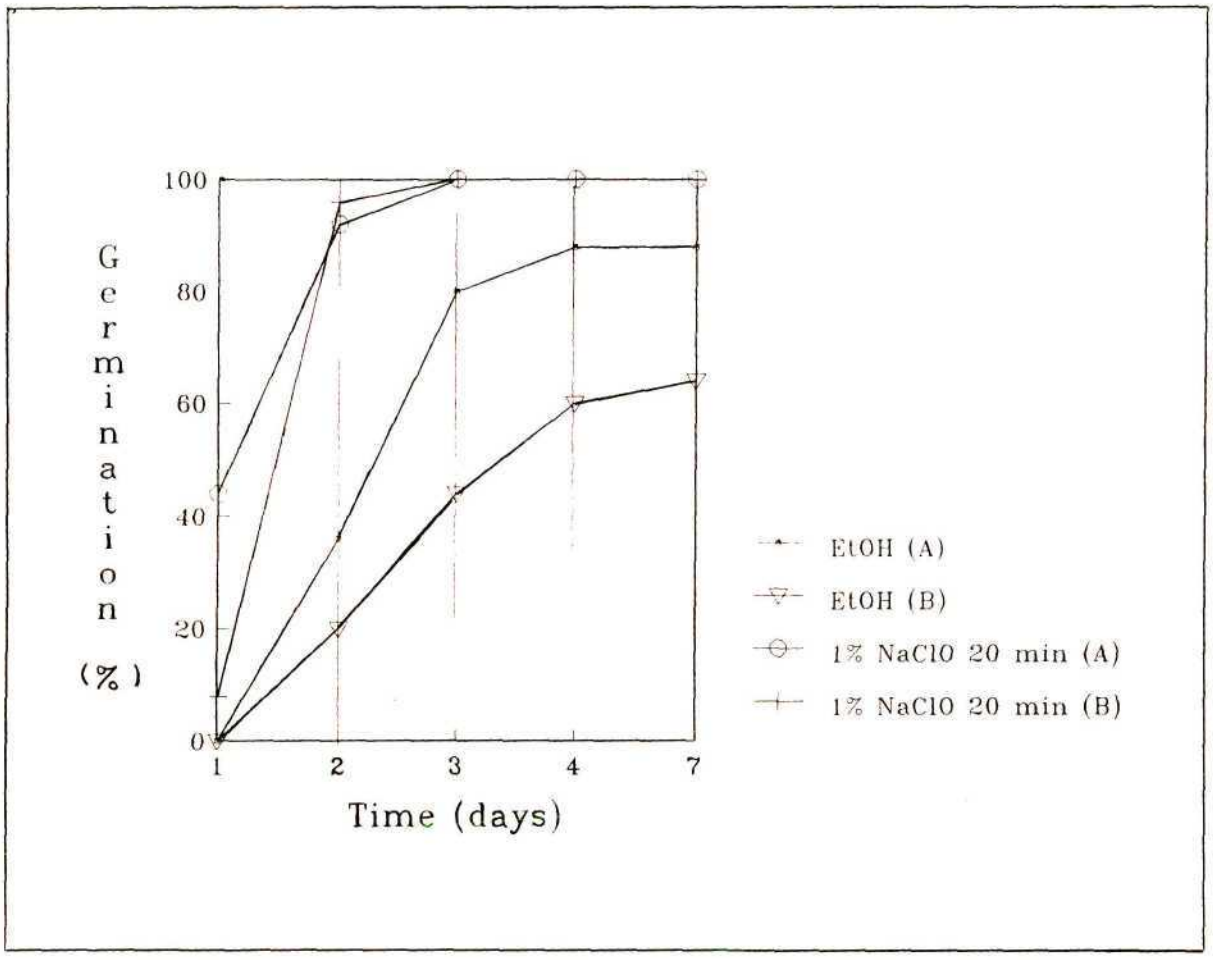


Figure 2.2. Rate of germination of seeds of *Cucurbita maxima* cv A-line, sterilised in 1 % (w/v) NaOCl for 20 minutes with or without an ethanol pretreatment. Germination was recorded each day for four consecutive days and again after one week. Results from the preliminary (B) and the main studies (A) are plotted.

not treated with ethanol was significant (5 %) (Figure 2.2). This lower germination can possibly be ascribed to dehydration of ethanol-treated seed (PIERIK, 1987). The ethanol pre-treatment proved to be unnecessary as effective sterilisation was achieved without it. Due to it's detrimental effects on germination, ethanol was eliminated from the sterilisation protocol.

Investigation into the way fruits from the two sources were treated to obtain the seeds, revealed that the seeds from ARC-Roodeplaat were obtained using a fermentation process (STORK, P. personal communication, 1992). Fruits were first broken and then placed in a wooden barrel and covered with water. After 24 hours, seeds were fairly loose due to fermentation and could be washed to separate the seeds from the flesh. Seeds were then spread to dry. Dried seeds were rubbed by hand to clean the surface of the seed coat. The seeds which were bought from Hygrotech were scooped out of the fruit and left to dry (GROBLER, P. personal communication, 1992). Dried seeds were placed in a mechanical cleaner to remove the dried flesh and finally treated with the fungicide Captan.

It is clear that seeds which had been allowed to ferment had a greater potential for contamination than dry harvested seed. This was reflected in the results which were obtained with these seeds. Treatment with a fungicide also had a beneficial effect on seed sterilisation. It is not always possible to establish how seeds have been treated when they are bought from a commercial supplier. Under such circumstances it would be advisable to choose a sterilisation method which would provide adequate disinfection of contaminated seed such as fermented seed, without causing chemical harm to the seedlings obtained.

From the two above experiments, a simple, effective sterilisation procedure was developed and this could be used for all subsequent seed sterilisation. According to this procedure seeds were placed in tap water for 10 to 15 minutes to soften the seed coat. Seeds were manually decoated and the exised embryos soaked in a 1 % solution of NaOCl for 20 minutes. The NaOCl solution was removed under aseptic conditions and the embryos rinsed three times in sterile distilled water to remove traces of the sterilising agent. Embryos were then ready for use in *in vitro* studies.

No attempt was made to adapt this procedure for the other cultivars which were used. The assumption was made that seeds of other members of the family Cucurbitaceae would respond in a similar way and could thus be sterilised adequately as outlined above.

2.3.2 **The influence of different plant growth regulators on shoot regeneration from cotyledonary explants in members of the Cucurbitaceae**

2.3.2.1 ***Cucurbita maxima* A-line**

No shoot bud regeneration occurred on any of the 6-benzyladenine (BA) and indole 3-acetic acid (IAA) treatments applied to the cultivar *Cucurbita maxima* A-line. Callus formation was however, noted with most of the media used.

Root formation occurred on all the media containing no cytokinin (Figure 2.3). The percentage of explants forming roots increased with increasing auxin concentration. Where the A-line was cultivated in the presence of different cytokinins, no shoot regeneration was seen on any of the treatments, but root formation occurred on

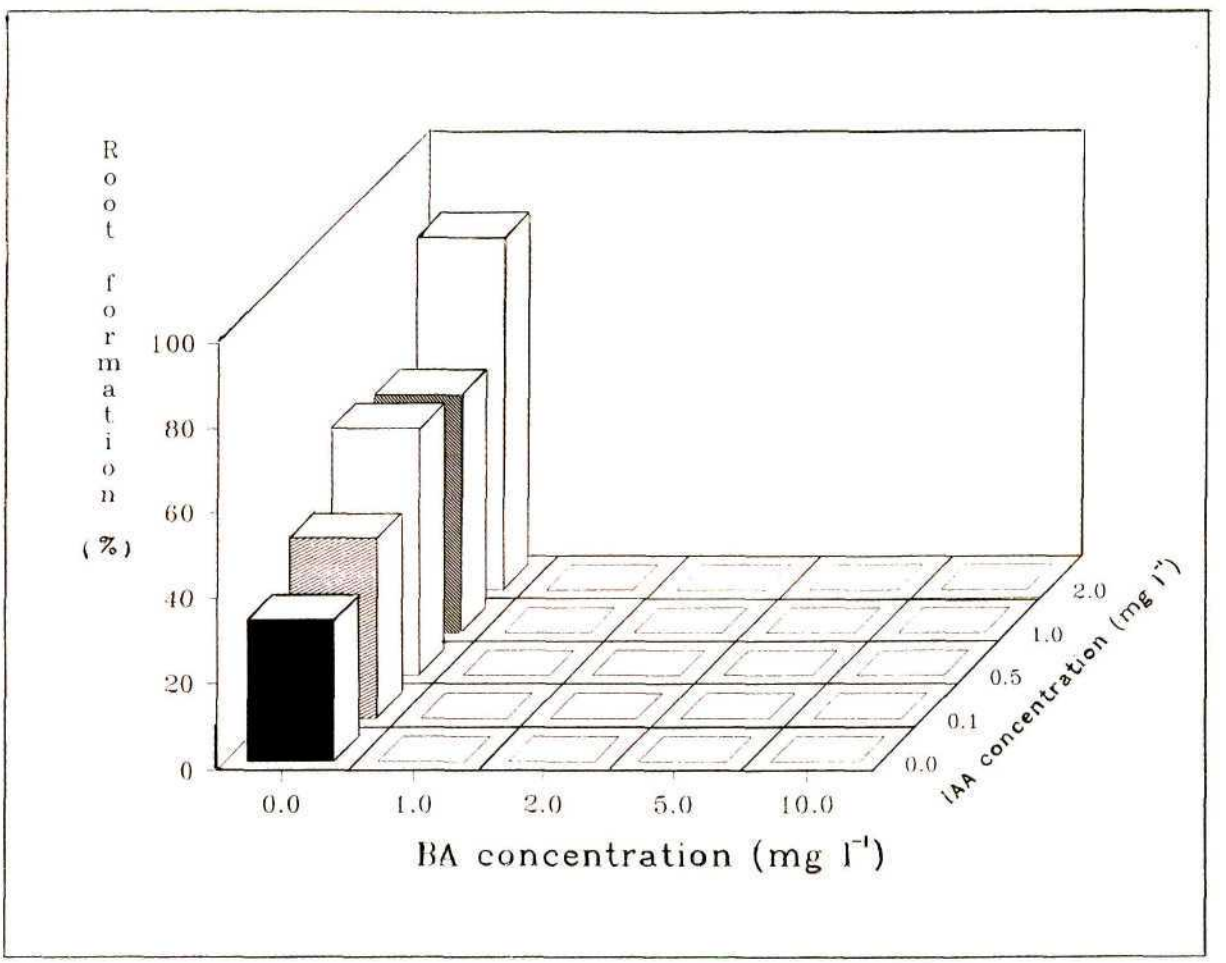


Figure 2.3. The percentage of cotyledonary explants of *Cucurbita maxima* cv A-line that formed roots using combinations of the growth regulators BA and IAA.

media containing iso-pentenyladenine (iP) and kinetin (Figure 2.4). The rooting response decreased with increasing iso-pentenyladenine concentration, but this was not significant. No roots were observed on media containing thidiazuron or BA. This corresponds with the results obtained in the previous study where roots only developed in the absence of BA.

2.3.2.2 *Cucurbita maxima* cv Chicago Warted

Cucurbita maxima cv Chicago Warted, also known as Green Hubbard squash, did not show shoot regeneration on any of the treatments and very little root development was observed. Roots formed on media 1 (MS medium), 11 (MS medium with 0.5 mg l^{-1} IAA) and 16 (MS medium with 1.0 mg l^{-1} IAA). The percentage of explants which developed roots were 5 % for medium 1 and 22 % and 17 % for medium 11 and 16 respectively. No roots were obtained on any of the media where combinations of 0.75 mg l^{-1} IAA and the four different cytokinins listed previously were used, except where IAA was used in combination with 1 mg l^{-1} kinetin. In this treatment, 8 % of the explants developed roots.

Somatic embryos developed on 5 of the media; the four auxin supplemented media and the medium with the lowest levels of both IAA and BA (Figure 2.5). The medium with the lowest level of IAA (0.1 mg l^{-1}) showed the highest response with 75 % of the explants producing somatic embryos. The embryos arose on the surface of the explants. No intervening callus stage was observed (Figure 2.6). Very little callus formed on the cut edges of the explants and callus was not associated with embryo formation. Embryos were not attached to the explant and often fell off if the

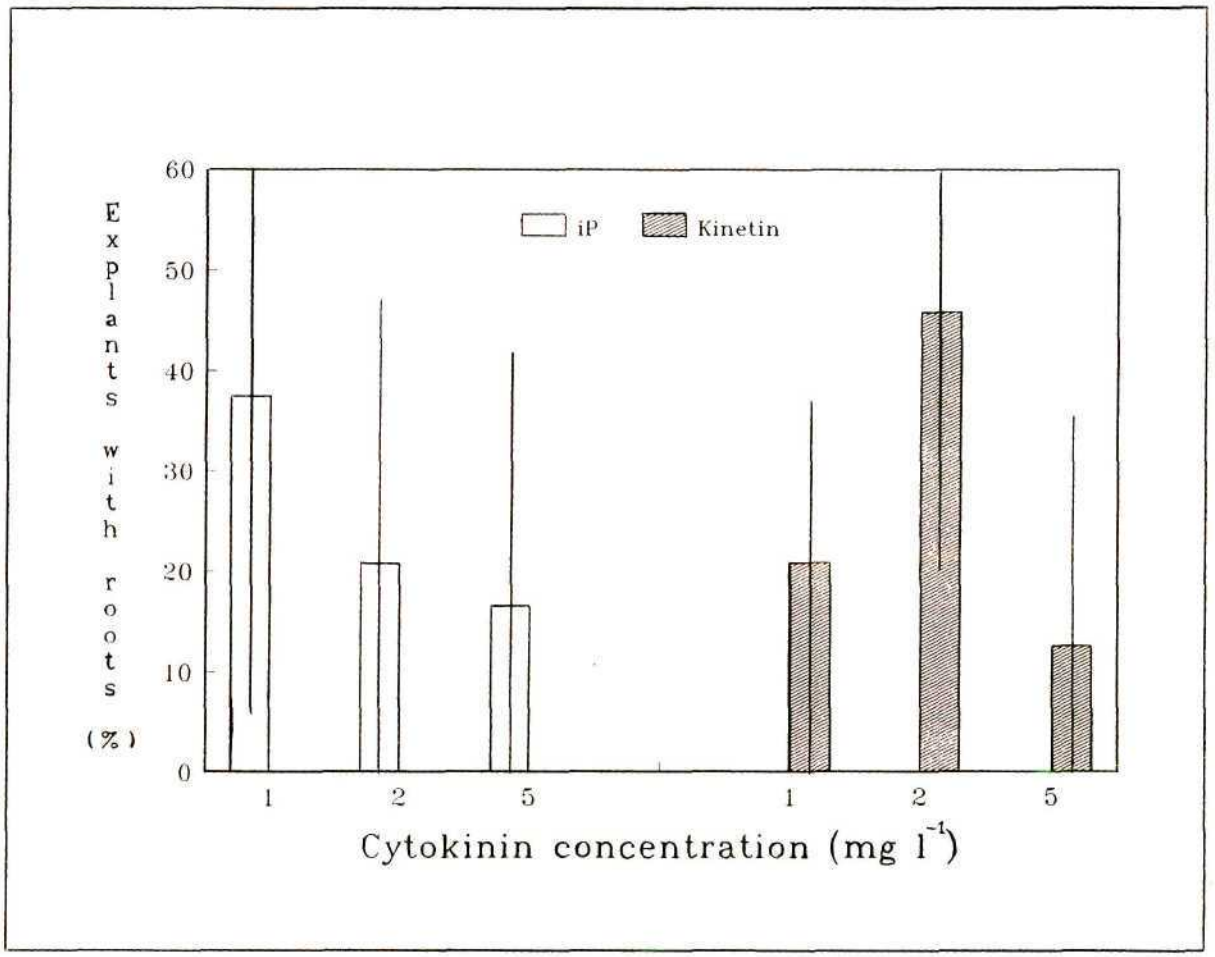


Figure 2.4. The percentage of cotyledonary explants of *Cucurbita maxima* cv A-line which formed roots on media containing different concentrations of the cytokinins iP and kinetin in combination with 0.75 mg l^{-1} IAA. The bars represent the standard errors.

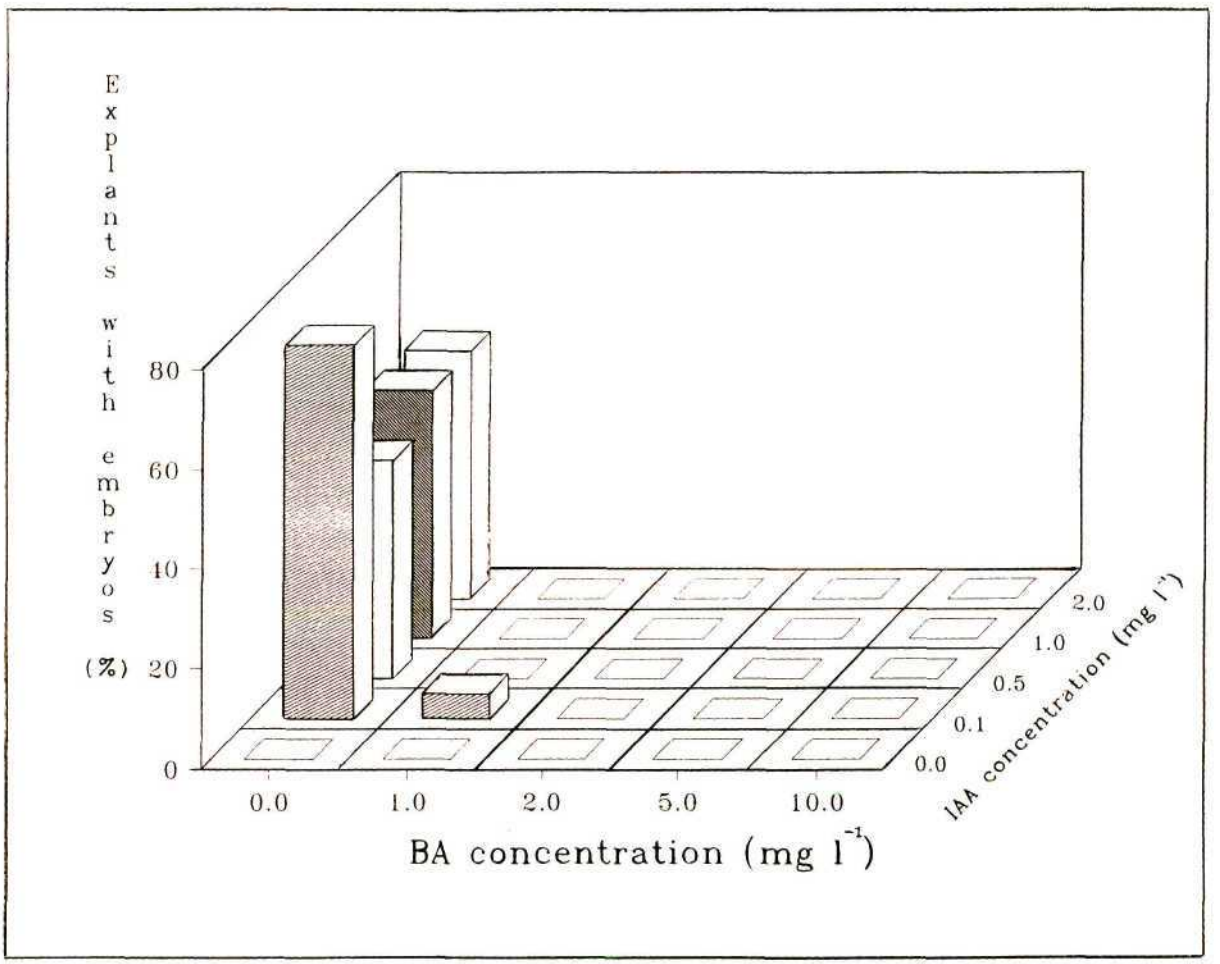


Figure 2.5. The percentage cotyledonary explants of *Cucurbita maxima* cv Chicago Warted that formed embryos.

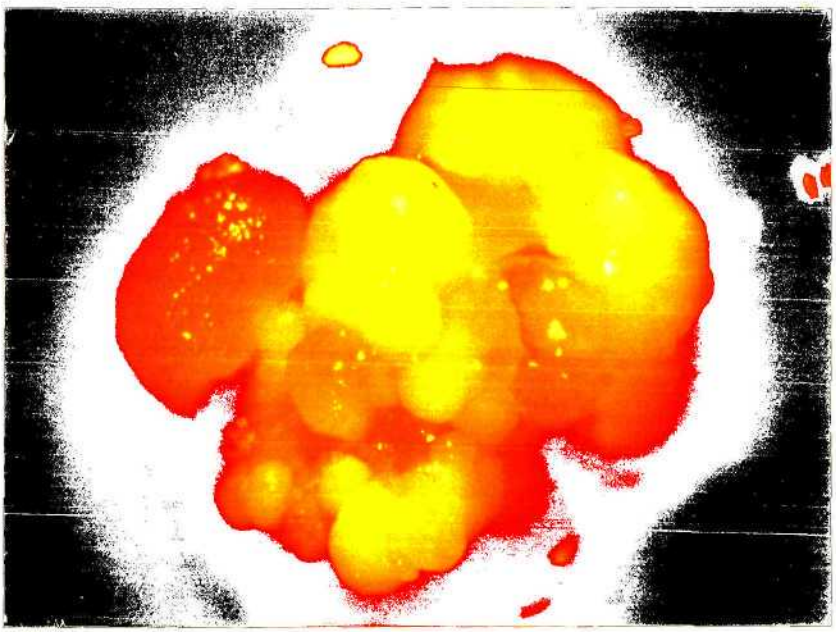


Figure 2.6. Direct somatic embryogenesis from cotyledonary explants of *Cucurbita maxima* cv Chicago Warted cultured on MS medium supplemented with 0.1 mg l^{-1} IAA.

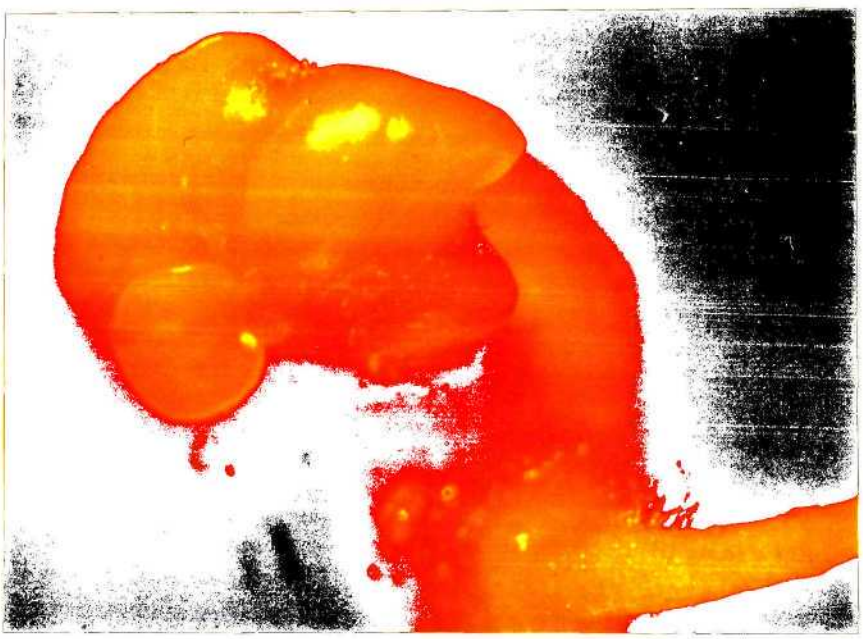


Figure 2.7. A malformed plantlet that developed from a somatic embryo of *Cucurbita maxima* cv Chicago Warted.

explant was disturbed in any way. Some of the embryos developed into malformed plantlets, which turned green for a while (Figure 2.7). These plantlets died within a month. No further attempts were made to develop a medium for development of these embryos.

Somatic embryogenesis usually occurs after callus formation, but it can arise directly on explants (THORPE, 1988). This phenomenon is known as direct somatic embryogenesis and has been described for a number of species, most notably some of the *Citrus* species, where it may be the only embryos formed (PIERIK, 1987). According to THORPE (1988), embryogenesis can be induced from a range of tissues, but immature or young explants are usually more responsive. In this study explants were isolated from cotyledons, which would conform with this general observation. Genotype is also a determining factor in the formation of somatic embryos (THORPE, 1988). Although two cultivars belonging to *Cucurbita maxima* were subjected to the same treatments, only one gave rise to somatic embryos. It is obvious that the most likely explanation for the dissimilarity in the response of the two cultivars would be the existence of genotypic differences.

According to GEORGE and SHERRINGTON (1984b) the auxin concentration is critical for the formation of somatic embryos on responsive explants. Often, a high concentration of an auxin such as 2,4-D is required in the initial culture medium, with a reduction or an absence of auxin in the secondary medium (PIERIK, 1987). The auxin 2,4-D was used in 57 % and NAA in 25 % of the successful attempts to induce embryogenesis which were studied by EVANS *et al.*, (1981). SHARP *et al.*, (1980) proposed that auxin induces an embryogenic determination in a proportion of the cells, but at the same time causes these cells to cease cell division. When explants are subcultured to auxin free media, or media with reduced auxin, cell

division is resumed and active proliferation of cells take place (THORPE, 1982). The formation of somatic embryos in this study was surprising as it occurred on media containing relatively low concentrations of IAA. The requirement of reduced auxin levels for somatic embryos to develop, was probably met through the breakdown of auxin in the medium or through metabolism by the explants.

A wide range of basal media can be used for somatic embryogenesis, but over 70 % of successful cases made use of MURASHIGE and SKOOG (1962) (MS) medium (THORPE, 1988). This medium contains high levels of salts with both NH_4^+ and NO_3^- included. The presence of NH_4^+ in the medium is important for embryo induction (THORPE, 1988). MS medium was used throughout this study and could have contributed to the absence of limiting factors for embryogenesis. The cultural environment influences the induction of somatic embryos and in general explants should be cultured in darkness. This was not the case in this study, as somatic embryogenesis was not the primary objective.

It did not fall within the scope of this study to fully develop a protocol for somatic embryogenesis in this cultivar. It is clear however, that further study of the potential of the *Cucurbita maxima* cultivar Chicago Warted to produce somatic embryos could be fruitful.

2.3.2.3 *Cucurbita pepo* cv Rolet

The cultivar, *Cucurbita pepo* cv Rolet, also failed to show any shoot regeneration response on any of the treatments where 6-benzyladenine and indole 3-acetic acid combinations were used. Similar to the results obtained for the cultivar A-line, roots

developed on all the treatments not supplemented with additional cytokinin (Figure 2.8). The percentage of explants which developed roots increased with increasing indole 3-acetic acid (IAA) concentration up to 0.5 mg l^{-1} IAA, but decreased again at 1 mg l^{-1} and 2 mg l^{-1} IAA. A number of BA supplemented media also showed a rooting response, but only in the presence of an additional auxin supplement. Again this response increased with increasing auxin concentration. At an IAA concentration of 2 mg l^{-1} roots were observed on all the BA containing media.

Rooting was observed on all the media supplemented with the cytokinins iP, kinetin and BA in combination with 0.75 mg l^{-1} IAA (Figure 2.9). No rooting response was observed where thidiazuron was used in combination with IAA (results not shown).

2.3.2.4 *Cucumis melo* cv Hales Best 36

Shoot bud regeneration occurred on all the media containing BA. No shoot bud regeneration was observed on media containing only IAA. The results are illustrated in Figure 2.10.

The percentage of explants that showed shoot bud regeneration increased with increasing IAA concentration up to 0.5 to 1.0 mg l^{-1}, but decreased with higher levels of this auxin. The response decreased with increasing BA concentration, although at levels of 1 or 2 mg l^{-1} IAA, there was an initial increase in the percentage of responsive explants when BA was increased from 1 to 2 mg l^{-1}.

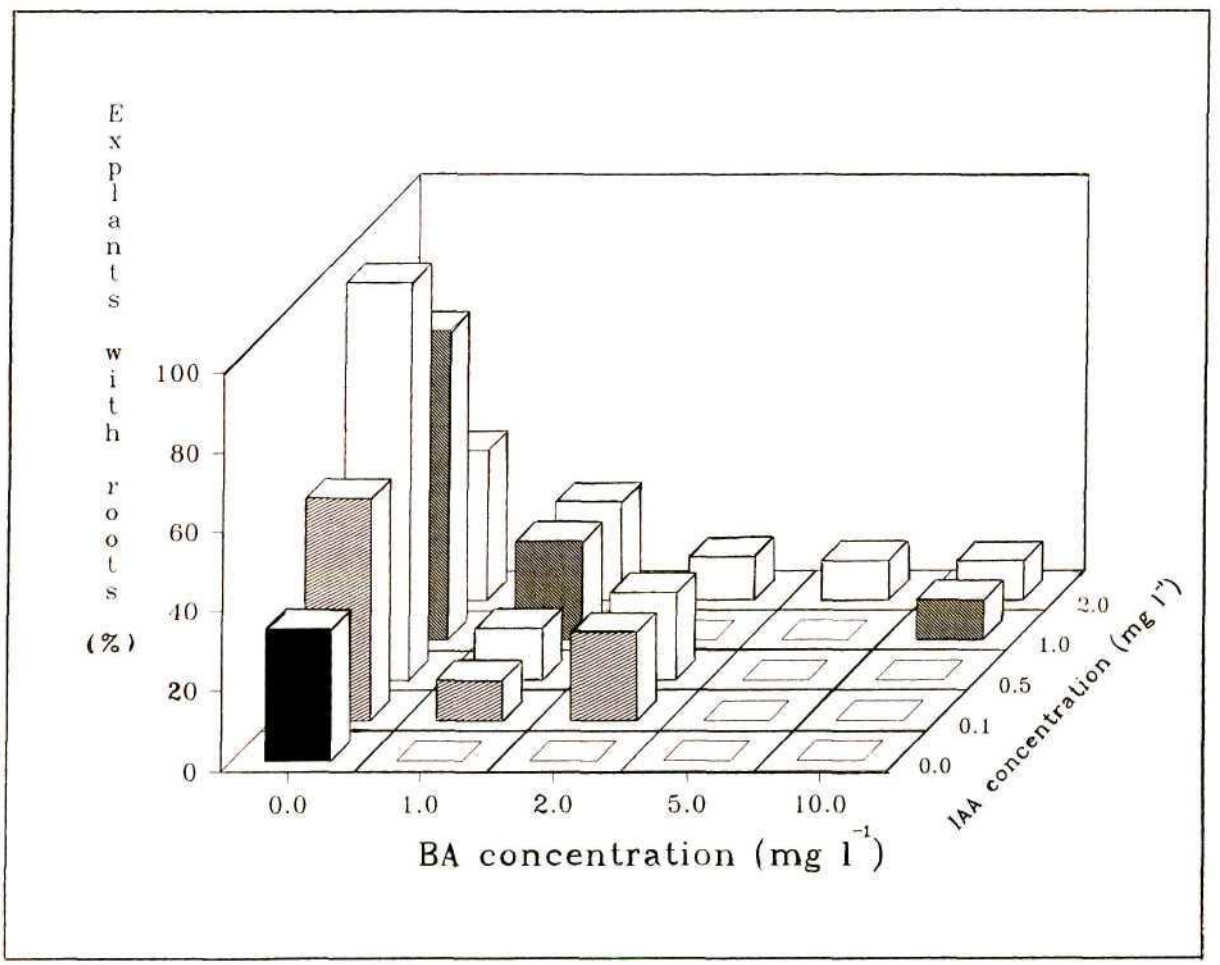


Figure 2.8. The percentage of cotyledonary explants of *Cucurbita pepo* cv Rolet that formed roots on various combinations of the growth regulators BA and IAA.

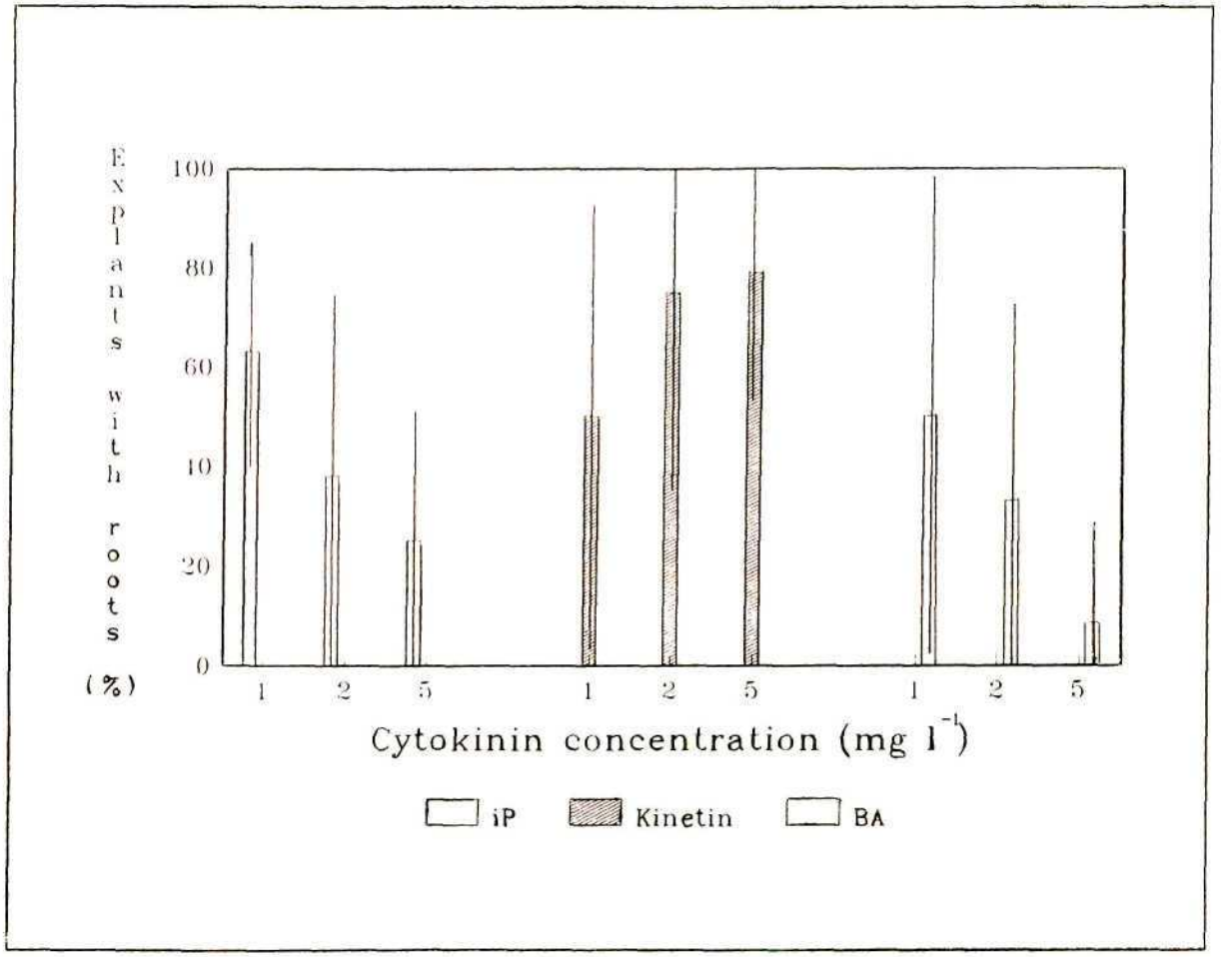


Figure 2.9. The percentage of cotyledonary explants of *Cucurbita pepo* cv Rolet that formed roots on media containing three different concentrations of the growth regulators iP, kinetin and BA in combination with 0.75 mg l^{-1} IAA. The bars represent the standard error.

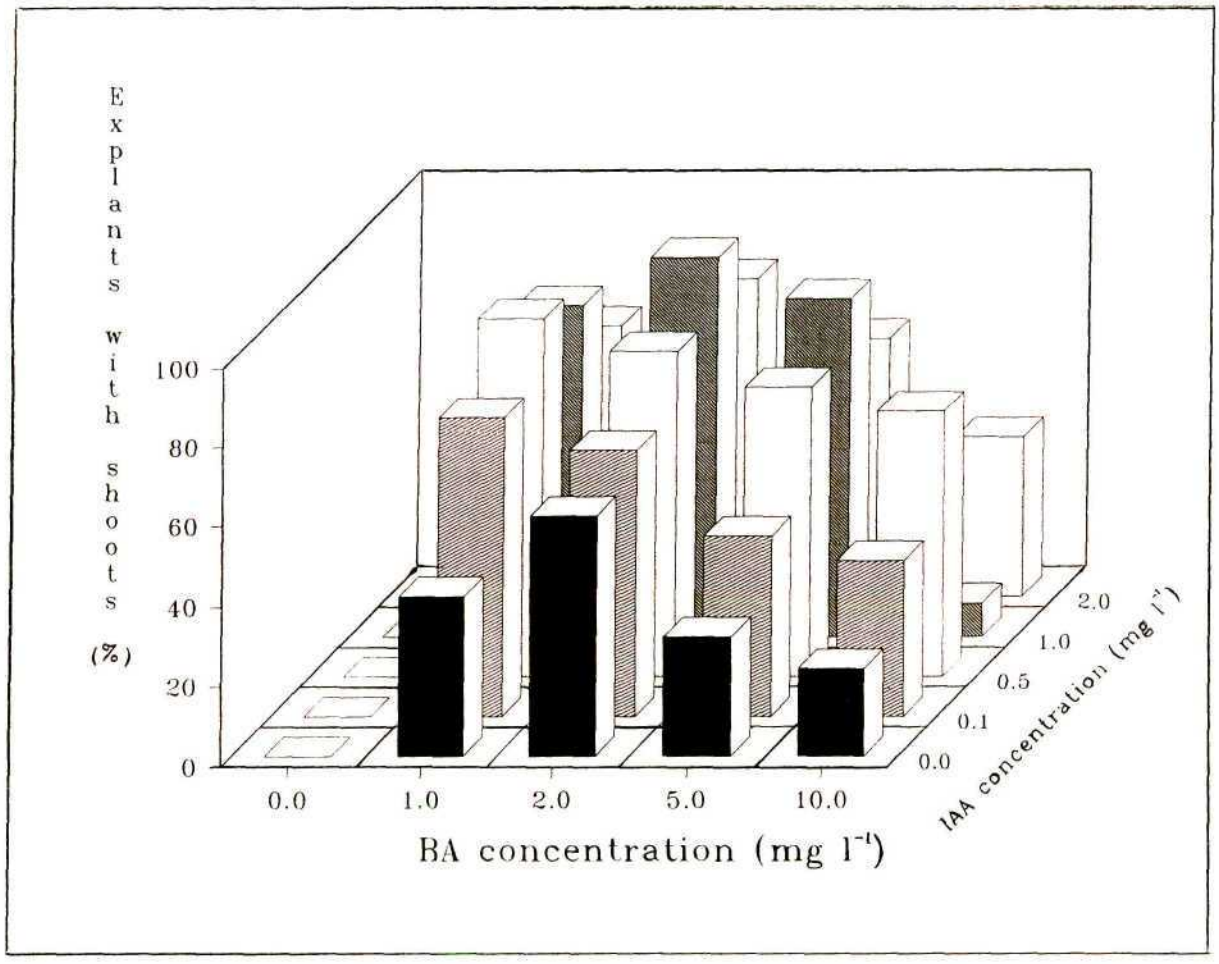


Figure 2.10. The percentage of *Cucumis melo* cv Hales Best 36 cotyledonary explants that showed shoot bud regeneration on media containing various combinations of the growth regulators BA and IAA.

The average number of shoot buds which developed per explant on each of the 25 treatments was plotted and presented in a histogram (not shown). A regression analysis was done on these data, using the Genstat 5 programme, release 2.2. Data counts were not normally distributed and a Poisson distribution was used (i.e. data were skew, with non-constant variances). It was observed that this type of distribution would give a better approximation of data. A generalised linear model (GLM) was used to analyze the counts.

The predictions which were obtained from the regression model as well as the standard errors which were calculated are given in Table 2.5. Only the treatments which included BA were used as no regeneration was seen on any of the treatments containing only IAA.

The predictions (averages) calculated from the regression model were again represented in a histogram (Figure 2.11). Clear trends emerged from this. Although shoot buds developed on the explants placed on media which contained only BA, shoot bud regeneration was stimulated in the presence of an auxin supplement. The average number of shoot buds which developed on each explant increased with increasing IAA concentration up to a level of 0.5 mg l^{-1} IAA. At levels higher than this, the average number of shoot buds decreased.

BA was a requirement for the formation of shoot buds in the Hales Best 36 cultivar, as none developed in the absence of the cytokinin. More shoot buds developed at the lower concentrations of 1 mg l^{-1} BA and 2 mg l^{-1} BA than at the higher concentrations.

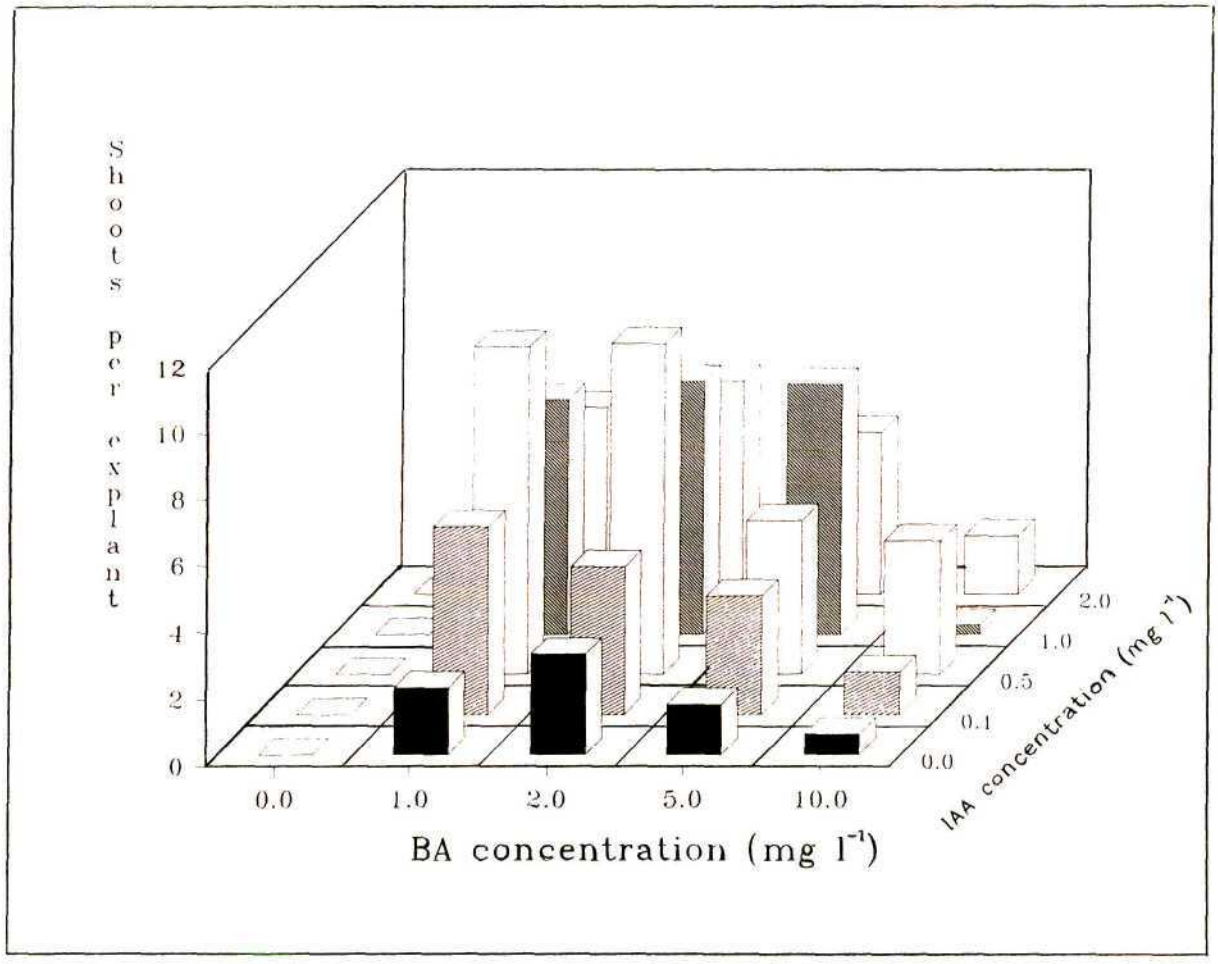


Figure 2.11. The average number of shoot buds that developed per cotyledonary explant of *Cucumis melo* cv Hales Best 36 (calculated from a regression model), on 25 different combinations of BA and IAA.

Table 2.5. Predictions and standard errors calculated from a regression analysis of the average number of shoot buds that regenerated from cotyledonary explants placed on different combinations of IAA and BA as outlined in Table 2.2. Standard errors are approximate, since the model is not linear.

IAA CONCENTRATION (mg l^{-1})	**BA CONCENTRATION (mg l^{-1})**				
	1	2	5	10	**Mean**
	x (s)	x (s)	x (s)	x (s)	
0.0	2.000 (0.809)	3.000 (0.992)	1.500 (0.696)	0.611 (1.470)	1.822 (0.392)
0.1	5.409 (1.270)	4.818 (1.198)	3.550 (1.079)	1.278 (0.682)	3.853 (0.552)
0.5	9.900 (1.802)	10.000 (1.727)	4.600 (1.228)	4.000 (1.204)	7.271 (0.772)
1.0	7.136 (1.458)	7.636 (1.509)	7.550 (1.574)	0.278 (0.317)	5.808 (0.678)
2.0	5.636 (1.296)	6.450 (1.454)	4.800 (1.255)	1.700 (0.746)	4.746 (0.622)
Mean	6.037 (0.612)	6.396 (0.627)	4.428 (0.540)	1.571 (0.334)	

An interaction existed between the two growth regulators used. The degree to which shoot bud regeneration was stimulated by BA, depended on the level of IAA that was used. The data suggest that the highest average number of shoot buds per explant can be obtained on combinations of BA and IAA where the BA concentration is equal to or lower than 2 mg l^{-1} and the IAA concentration is 0.5 mg l^{-1}.

Some root formation was seen on all the media which did not contain BA. The percentages of explants developing roots were 2 %, 13 %, 23 %, 18 % and 24 % for media 1, 6, 11, 18 and 21 respectively.

BOKELMAN *et al.*, (1991) tested media for shoot regeneration from protoplasts. Nine melon cultivars were tested. Levels of 0.2 to 5.0 mg l^{-1} BA were used alone or in combination with 0.01 to 2.5 mg l^{-1} IAA. Some of the cultivars responded better without an auxin supplement, but for others an IAA supplement of 0.1 mg l^{-1} improved the results.

CHEE (1991a) compared the organogenic response of cotyledonary explants of the melon cultivar Topmark on media containing 0 to 1 mg l^{-1} BA. Media were not supplemented with auxin. The best regeneration was found on the medium supplemented with 1 mg l^{-1} BA. This study supports the conclusion which was reached during the current study regarding the most suitable BA level to use for shoot regeneration, although no comparisons can be drawn between the two studies regarding the interaction between BA and IAA.

A number of other studies investigated the interaction between BA and IAA. In 1989, NIEDZ *et al.* tested four cultivars of *C. melo* for shoot regeneration response from cotyledonary explants. Three levels of BA (1, 2.5 and 7.5 mg l^{-1}) in combination with IAA (0, 0.9 and 1.5 mg l^{-1}) were examined. A combination of 1 mg l^{-1} BA and 0.9 mg l^{-1} IAA was found to give the best results. MORENO *et al.*, (1985) drew the opposite conclusion and found that IAA may not be essential for shoot regeneration. Nearly 70 % of calli derived from cotyledonary explants of the cultivar Amarillo Oro developed shoots on a medium containing 6 mg l^{-1} kinetin. Despite the lack of significant differences between the treatments with and without

an auxin supplement, both these research groups decided to include IAA in the regeneration media.

DIRKS and VAN BUGGENUM (1989) tested leaf explants of five genotypes of melon on combinations of 1 to 5 mg l^{-1} BA and 0 to 1 mg l^{-1} IAA. They reported that the best results were obtained on 1 mg l^{-1} BA without IAA. KATHAL *et al.*, (1988) also studied organogenesis from leaf explants, using the cultivar Pusa Sharbati, an Indian cultivar. Where growth regulators were tested individually, maximum response was obtained from media with 1 mg l^{-1} BA (91 %). Further increases in the concentration of the cytokinin was found to reduce the regeneration frequency. Only one combination of BA and IAA was tested, that of BA 0.7 mg l^{-1} and IAA 0.2 mg l^{-1}. Shoot regeneration was much lower when BA was used on its own. It can be deduced from these studies that the optimum BA level at which the cytokinin stimulates shoot regeneration from cotyledonary explants of melon is ± 1 mg l^{-1}. This conclusion is supported by the work reported here.

There is no consensus in the literature on the advisability of an auxin supplement for stimulation of shoot regeneration. It is however, clear from the work reported by DIRKS and VAN BUGGENUM (1989), NIEDZ *et al.*, (1989) and BOKELMAN *et al.*, (1991), that the influence of the genotype is often the parameter which will determine how a cultivar will react to growth regulator stimuli.

The use of different types of explants by different researchers make comparisons between reports difficult to assess. It is therefore necessary to test the response of specific explants from each cultivar to different levels of growth regulators, to determine optimum culture conditions for shoot regeneration.

In the study on the influence of different cytokinins on shoot bud regeneration, the average number of shoots that developed on each explant was calculated for each of the treatments. The average of the three treatments and the percentage of explants with shoot buds were calculated for each cytokinin (Table 2.6).

Table 2.6. The average number of shoot buds that developed per explant on all the media supplemented with one of three concentrations of the four cytokinins which were studied. The average number of shoots per explant and the percentage of explants forming shoot buds on all three concentrations of cytokinin is given for each cytokinin.

CYTOKININ	AVERAGE NUMBER OF SHOOT BUDS ON EACH CYTOKININ TREATMENT			COMBINED AVERAGE FOR EACH CYTOKININ	PERCENTAGE OF EXPLANTS RESPONDING
	1 mg l^{-1}	2 mg l^{-1}	5 mg l^{-1}		
BA	6.167	9.100	8.964	8.057	100
Kinetin	0.533	0.179	1.100	0.614	38.6
iP	0.567	0.200	0.143	0.307	16
Thidiazuron	0.133	0.633	0.233	0.333	29

The shoot regeneration response of cotyledonary explants in the presence of the cytokinins kinetin, iP and thidiazuron were significantly lower (5 % level), than the response in the presence of BA.

A regression analysis was done on the data obtained from the three BA treatments. A Poisson distribution was used and predictions and standard errors which were calculated for each of the BA treatments are given in Table 2.7. The variances are also given.

Table 2.7. Predictions, standard errors and variances which were obtained from a regression analysis of the data obtained from BA treatments in a comparative study of four different cytokinins.

BA CONCENTRATION (mg l^{-1})	PREDICTIONS	STANDARD ERROR	VARIANCE
1	6.17	1.21	11.13
2	9.10	1.47	27.15
5	8.96	1.51	53.98

The Bonferroni Least Significant Differences (LSD's) were calculated for the three BA treatments. No significant differences existed between the three treatments at the 5 % level.

CHEE (1991a) compared shoot organogenesis from melon cotyledonary explants on the kinetin containing medium developed by MORENO *et al.*, (1985) with that from BA containing media. The response from BA containing media was significantly better (1 % level). Four cytokinins, BA, kinetin, iP and zeatin, were compared by NIEDZ *et al.*, (1989) to determine an effective cytokinin for shoot bud induction from cotyledonary explants from four melon cultivars. IAA was added to all the media at a concentration of 0.8 mg l^{-1}. Best results were obtained with the cytokinin BA. Three cultivars failed to respond to iP treatments and one cultivar did not show a response on a kinetin containing medium. All cultivars responded to a 6.5 mg l^{-1} zeatin treatment, but no response was seen at lower concentrations of this cytokinin.

The results from these studies confirm the conclusion which was drawn from the current study; the cytokinin BA is the most suitable growth regulator for shoot bud induction in cotyledonary explants of *Cucumis melo*.

2.3.2.5 *Cucumis sativus* cv Ashley

Shoot buds were observed on explants on a number of media containing BA. The percentage of explants showing a regeneration response is shown in Figure 2.12. It is clear that a cytokinin supplement is essential for shoot bud development in this cultivar. The highest percentages of responding explants were seen on combinations of 1 to 5 mg l^{-1} BA and 0.5 to 2.0 mg l^{-1} IAA. On these media, 3 to 4 of the 24 explants cultured showed a response. It can also be seen that shoot development was nearly completely inhibited on the 10 mg l^{-1} BA treatments.

The average number of shoot buds that developed on each of the media was calculated and represented in the form of a histogram (Figure 2.13). The response on all the media was poor. The highest average number of shoot buds was seen on the 5 mg l^{-1} BA + 0.5 mg l^{-1} IAA treatment, with 1 shoot bud per explant. The regenerated shoots elongated well and root development was seen on MS medium. A number of the plantlets developed flowers and fruit. It was decided not to analyse the data further as the number of shoot buds formed was extremely low.

Root development occurred on a considerable number of treatments (Figure 2.14)

The use of other cytokinins did not lead to improved results. No shoot bud regeneration was seen on any of the kinetin or thidiazuron containing media. The average number of shoots that developed on each of the treatments containing BA or iP, as well as the variance is given in Table 2.8. Again, the number of shoot buds per explant was very low.

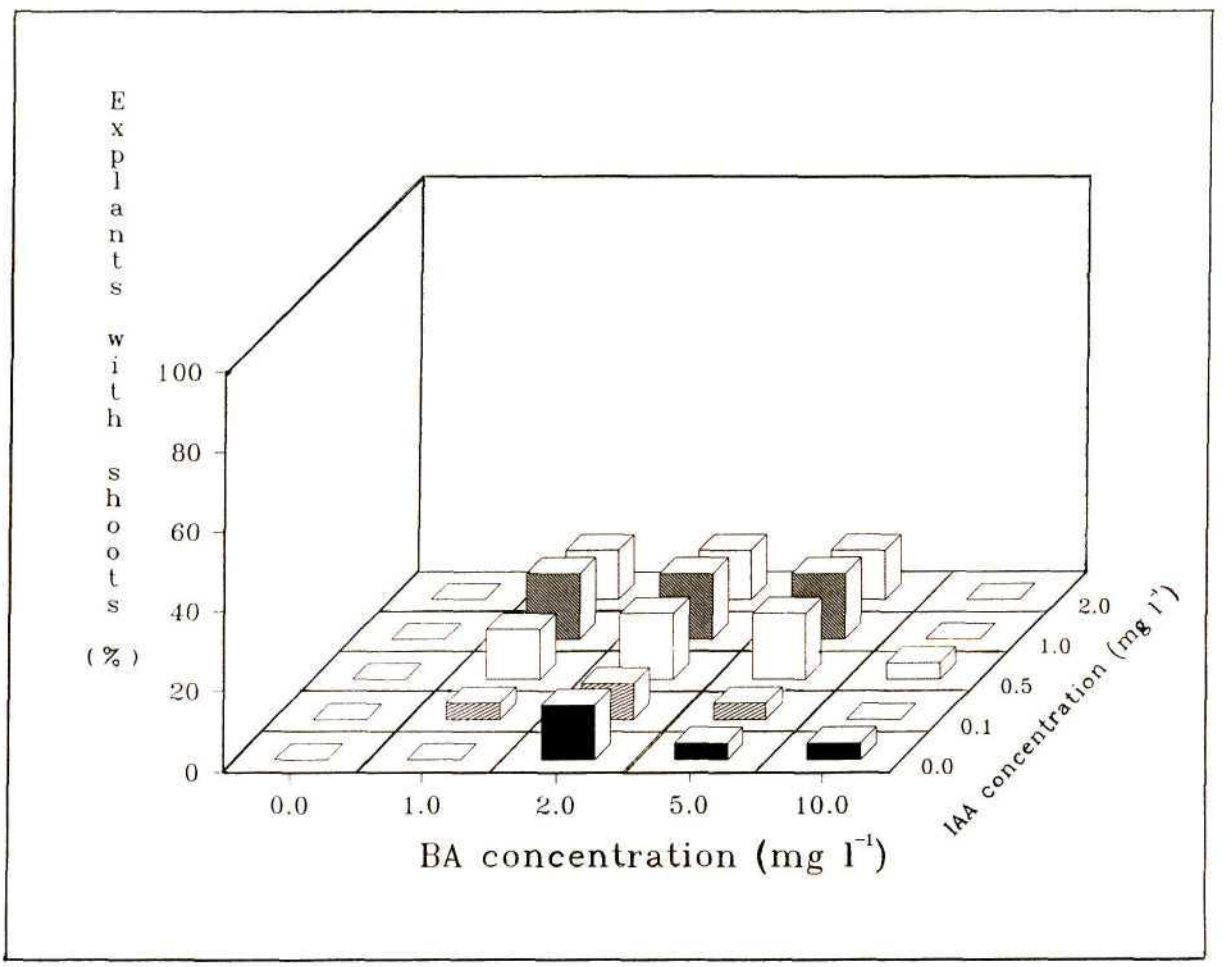


Figure 2.12. The percentage of cotyledonary explants of *Cucumis sativus* cv Ashley that formed shoot buds on combinations of the growth regulators BA and IAA.

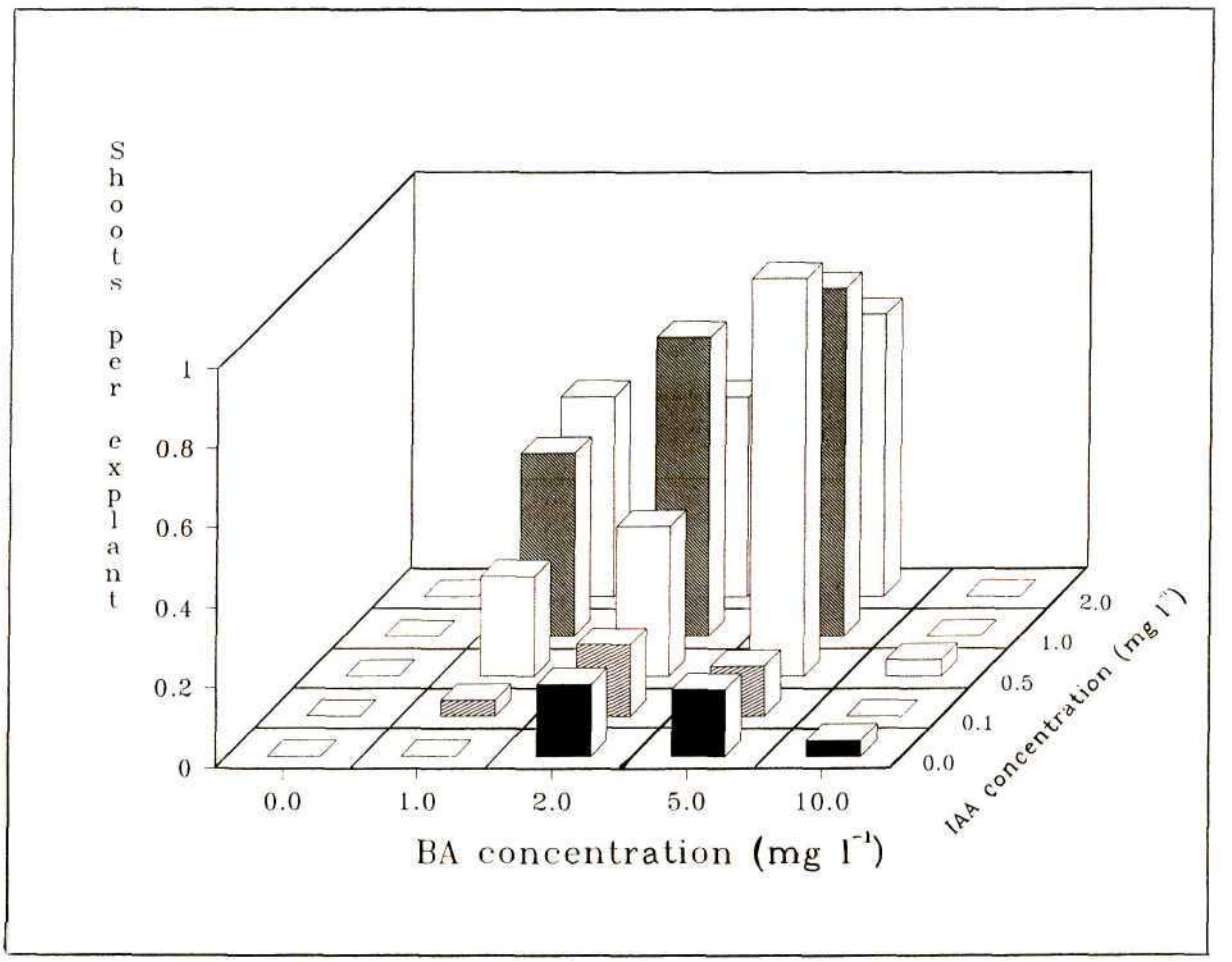


Figure 2.13. The average number of shoot buds that developed on each cotyledonary explant of *Cucumis sativus* cv Ashley using different concentrations of the growth regulators BA and IAA.

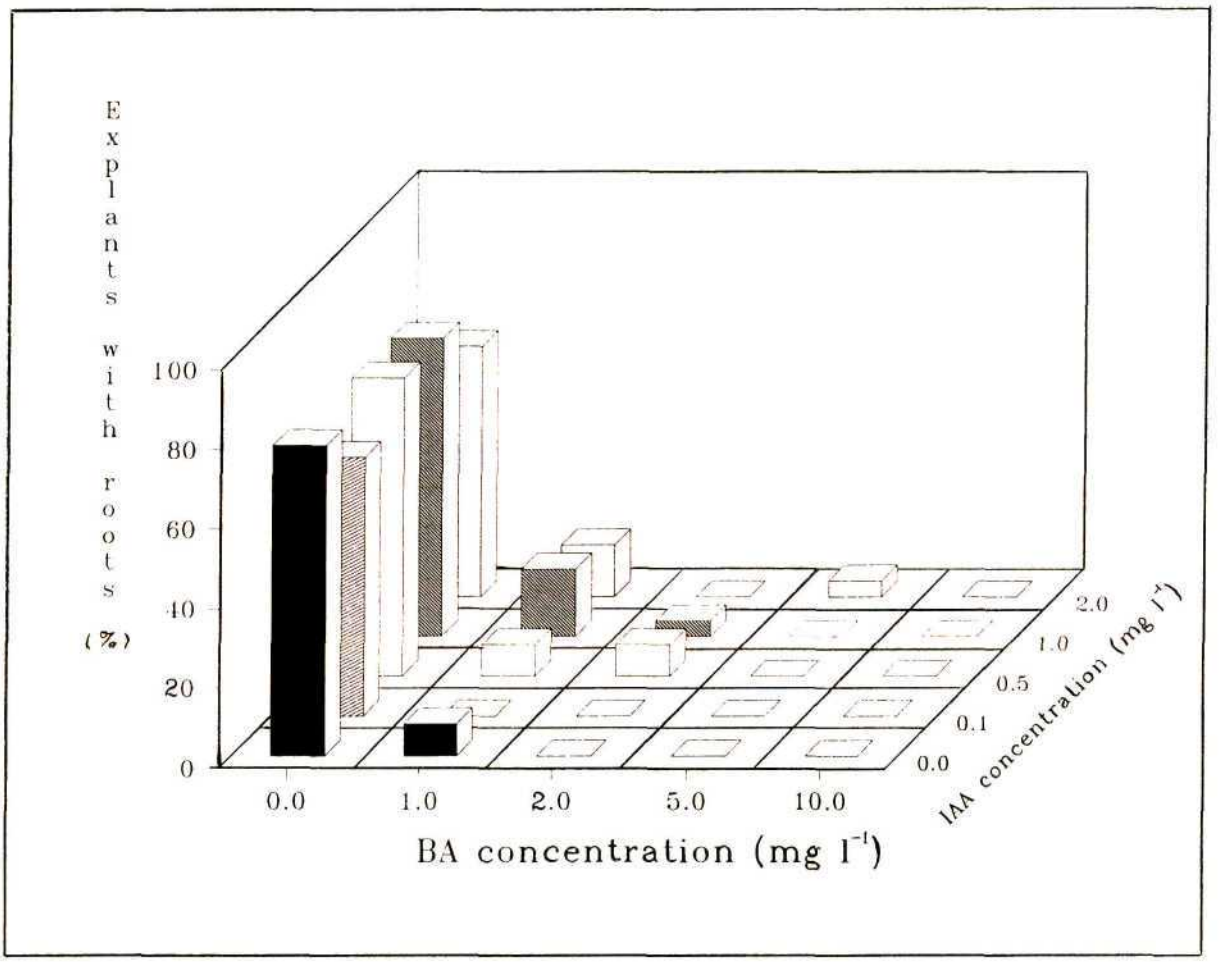


Figure 2.14. The percentage of cotyledonary explants of *Cucumis sativus* cv Ashley that formed roots on media containing combinations of the growth regulators BA and IAA.

Table 2.8. The average number of shoot buds that developed on media containing three different concentrations of the growth regulators BA and iP. All the media consisted of MS medium supplemented with 0.75 mg l^{-1} IAA.

CONCENTRATION (mg l^{-1})	CYTOKININ			
	BA		iP	
	Mean	Variance	Mean	Variance
1	0.66	1.41	0.46	1.55
2	0.63	0.73	0.33	0.274
5	0.30	0.45	1.30	4.10

The average number of buds obtained from the lower BA concentrations was higher than that of the 5 mg l^{-1} treatment. This is inconsistent with the results obtained previously. The highest number of shoot buds developed on 5 mg l^{-1} iP.

It was decided that no decision could be taken regarding the use of specific growth regulators for shoot regeneration in this cultivar due to the poor and variable response that was obtained. Further investigation into the requirements of this cultivar is needed before a shoot regeneration protocol can be established.

It is known from the literature that *Cucumis sativus* species show a varied shoot regeneration response. WEHNER and LOCY (1981) screened 85 cultivars and lines for their organogenetic response from hypocotyl and cotyledonary explants. Only 33 % of these formed shoots from cotyledon tissue and none formed shoots from hypocotyls.

Other reports have appeared describing regeneration from cucumber. A number of these (TRULSON and SHAHIN, 1986; CADE *et al.*, 1990; CHEE, 1990a;

LADYMAN and GIRARD, 1992) have focussed on somatic embryogenesis in cucumber. The media which were used in these studies were chosen with this in mind and the results are not pertinent to the current study.

Two reports, those of GAMBLEY and DODD (1990) and MSIKITA *et al.*, (1990) have described shoot organogenesis of cotyledonary explants of cucumber. GAMBLEY and DODD (1990) reported that 100 % of explants were capable of producing shoots on a number of media containing kinetin, BA, iP or a combination of cytokinin and IAA. The number of shoots per explant varied from 1 to 31 depending on the medium. In the report by MSIKITA *et al.*, (1990) 50 % of the explants regenerated shoots on media containing a combination of 2 or 4 mg l^{-1} BA and 0.3 mg l^{-1} NAA. These results indicate that a high regeneration rate is possible for cucumber if an appropriate genotype is used. This leads to the conclusion that the cultivar Ashley might give poor or inconsistent results even when the culture conditions have been optimised.

In this Chapter, a reliable protocol for the surface sterilisation of embryos of members of the Cucurbitaceae was established. In three of the cultivars tested, *Cucurbita maxima* cv A-line, *Cucurbita maxima* cv Chicago Warted and *Cucurbita pepo* cv Rolet, no shoot regeneration was seen in any of the treatments, although roots developed in several treatments. In several treatments, somatic embryos formed on *Cucurbita maxima* cv Chicago Warted explants. This work was not pursued, but further attempts to develop a regeneration protocol for *Cucurbita maxima* cv A-line are described in Chapter 3. Some shoot regeneration was observed in the cultivar *Cucumis sativus* cv Ashley, but abundant shoots developed on explants of *Cucumis melo* cv Hales Best 36. Subsequent experiments on these two cultivars are described in Chapter 4.

CHAPTER 3

REGENERATION OF *CUCURBITA MAXIMA* CV A-LINE *IN VITRO*

3.1 INTRODUCTION

Cucurbita maxima cv A-line, a well known vegetable crop, is also known as the Flat White Boer pumpkin. These are the most common pumpkins in South Africa and are planted in almost every district in the country where sufficient water is available during spring and early summer. They are well known for their extended lasting qualities and the fact that, because of its white colour, sunburn seldom occurs (BOSCH and OLIVIER, 1978).

No reports on the *in vitro* regeneration of this species could be found. A number of reports have appeared describing the regeneration of various pumpkins (JELASKA, 1972; JELASKA, 1974 and CHEE, 1991b). These cultivars all belong to the species *Cucurbita pepo*. Considerable confusion exists about the naming of members of the genus *Cucurbita*, but in South Africa members of *Cucurbita pepo* are generally known as squashes. The introduction of foreign genes that confer resistance to various diseases (such as powdery mildew and watermelon mosaic virus) is of great potential value in this cultivar. As the usefulness of any transformation system depends on the development of an efficient *in vitro* plant regeneration protocol, the regeneration potential of this cultivar was investigated.

3.2 MATERIALS AND METHODS

3.2.1 Investigation of the influence of different growth regulators on shoot regeneration

A number of attempts were made to develop a shoot regeneration protocol for *Cucurbita maxima* cv A-line. Seeds were obtained from the Genebank of the ARC-Roodeplaat. These seeds were used for all the experiments described in this chapter, except where it is otherwise specified. Embryos were excised and were surface sterilised according to the procedure developed in Chapter 2. These embryos were used to obtain explants for all the experiments described below. Where cotyledonary explants were used, four explants were obtained from each excised embryo. Embryos were cut with a scalpel blade to remove the distal and proximal ends of the embryo, as well as the two remaining edges. The cotyledons were separated and cut longitudinally into two explants of equal size.

All media consisted of MURASHIGE and SKOOG (1962) (MS) medium containing 3 % (w/v) sucrose and 0.75 % (w/v) agar. This medium was supplemented with growth regulators as specified for each experiment. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes.

Explants were cultured in 100 mm specimen tubes with a diameter of 25 mm or in 90 mm sterile disposable petri dishes. Culture vessels were sealed with Parafilm®. All explants were cultured in a growth room at 26 °C ± 2 °C.

3.2.1.1 **The influence of BA in combination with IAA on shoot regeneration**

Twenty five combinations of the cytokinin 6-benzyladenine (BA) and the auxin indole 3-acetic acid (IAA) were tested for their potential to stimulate the development of shoots from cotyledonary explants of the *Cucurbita maxima* A-line. The combinations that were used are outlined in Table 3.1. The growth regulators were supplements to the basic medium described above. The explants were prepared as described above.

Each treatment consisted of at least 20 specimen tubes containing one explant each. All explants were subcultured once after four weeks and results were recorded after eight to nine weeks. All explants were examined for the presence of shoot buds and roots. The amount of callus that formed on each explant was rated on a scale of 0 to 3. Explants rated 0 showed no callus development and explants rated 3 showed the most callus development.

3.2.1.2 **The influence of kinetin in combination with IAA on shoot regeneration**

The influence of twenty five different combinations of the growth regulators kinetin and indole 3-acetic acid (IAA) on the shoot regeneration ability of cotyledonary explants belonging to *Cucurbita maxima* A-line was investigated. The experiment was conducted in the same way that the influence of BA and IAA on shoot regeneration was investigated in the previous experiment. The MS medium was supplemented with the IAA concentrations as outlined in Table 3.1. The cytokinin kinetin was substituted for the concentrations of BA which were outlined in the same Table.

Table 3.1. Twenty five combinations of the cytokinin BA and auxin IAA that were tested for the ability to induce shoot regeneration in cotyledonary explants of *Cucurbita maxima* cv. A-line.

AUXIN CONCENTRATION (mg l^{-1})	CYTOKININ CONCENTRATION (mg l^{-1})				
	0.0	0.5	1.0	2.0	5.0
0.0	1	2	3	4	5
0.5	6	7	8	9	10
1.0	11	12	13	14	15
2.0	16	17	18	19	20
5.0	21	22	23	24	25

Each treatment consisted of 20 specimen tubes containing one explant per tube. Explants were subcultured once after four weeks. Results were again recorded after eight to nine weeks, but explants were only examined for the presence of shoot buds. Root formation and callus development were not recorded for this experiment.

3.2.1.3 **The influence of concentrated, liquid cytokinin pulses on shoot regeneration**

Cotyledonary explants of the *Cucurbita maxima* cv. A-line were subjected to liquid pulses of the cytokinin 6-benzyladenine (BA) for periods ranging from 30 minutes to 24 hours in order to stimulate shoot regeneration.

Explants were pulsed with autoclaved, liquid MS medium (pH 5.8) supplemented with one of the following cytokinin concentrations:

0.0 mg l^{-1} BA

1.0 mg l^{-1} BA

10.0 mg l^{-1} BA

100.0 mg l^{-1} BA.

The explants were soaked in these solutions for periods of 30 minutes, 2 hours, 6 hours or 24 hours; a total of 16 different treatments. Explants were removed from the solutions and cultured in petri dishes on MS medium. Each treatment consisted of four petri dishes each containing four explants. After four weeks all explants were subcultured onto fresh medium. Explants were examined for shoot regeneration four weeks later.

3.2.1.4 **Shoot regeneration from other explant types**

The shoot regeneration ability of *Cucurbita maxima* cv A-line was tested using different explant types. The failure of cotyledonary explants to demonstrate shoot regeneration on media supplemented with a range of growth regulators necessitated this investigation.

Excised embryos of *Cucurbita maxima* cv. A-line were sterilised according to the established procedure. These embryos were cultured on MS medium to provide leaf, hypocotyl, petiole and mature cotyledonary explants. Leaves were removed from the developing plants as soon as they were fully unfolded. Cuts were made diagonally across the surface of each leaf before they were placed on the culture media. Five petri dishes of each medium (see below), each holding four leaves were cultured.

Hypocotyl and petiole pieces were cut into segments of approximately 1 cm in length. Five petri dishes of each medium, each with at least five hypocotyl explants, were cultured. The same number of petiole explants were prepared and cultured. Mature cotyledons were removed from the developing plants after one week. Each cotyledon was divided into two explants, each with four wounded sides. Five petri dishes of each medium were prepared. Each of the petri dishes were planted with four explants.

All explants were cultured in petri dishes on MS medium containing 3 % (w/v) sucrose and 0.75 % (w/v) agar. No suitable growth regulator combination for shoot regeneration could be chosen from the previous experiments. Literature on shoot regeneration in the Cucurbitaceae was studied (WEHNER and LOCY, 1981; NIEDZ *et al.*, 1989; DIRKS and VAN BUGGENUM, 1989; CHEE, 1991 and TABEI *et al.*, 1991) and based on the information obtained, the decision was made to use media supplemented with 1 mg l^{-1} 6-benzyladenine (BA) or 1 mg l^{-1} BA + 1 mg l^{-1} indole 3-acetic acid (IAA). The latter combination of growth regulators was called BI-medium and the former was called B-medium. Explants were transferred to fresh medium after three weeks. Explants were inspected regularly for signs of regeneration and discarded when they had turned brown.

3.2.1.5 **The influence of light and dark treatments on shoot regeneration**

The possibility that a dark pre-treatment might stimulate shoot formation in *Cucurbita maxima* cv A-line was investigated. Embryos were placed in petri dishes containing MS medium, seven embryos per petri dish. Half of the petri dishes were

covered with foil, in order to allow seeds to germinate in darkness. All the petri dishes were placed together in the same growth room. After four days, explants were prepared from the expanded cotyledons and were placed in specimen tubes containing MS medium supplemented with 1 mg l^{-1} BA and 1 mg l^{-1} IAA. Half of the dark treated explants were transferred to the light (DL) and the second half were kept in the dark (DD). Explants that were obtained from light-treated seeds were also divided into two equal dark (LD) and light (LL) treatments. After ten days, all treatments were transferred to the light. Explants were transferred to fresh medium every three weeks.

Each treatment consisted of 20 specimen tubes each containing one explant. Explants were inspected regularly for signs of regeneration. After nine weeks, callus formation was rated on a scale of 0 to 3. Explants rated 0 showed no callus development and explants rated 3 the most callus development.

3.2.1.6 **The influence of the seed source on shoot regeneration**

The experiments described above were all conducted using seeds obtained from the Genebank of the ARC-Roodeplaat. A second batch of seeds was bought from Hygrotech Seed, a commercial seed supplier in Silverton, Pretoria. Investigation revealed that seeds from these two sources were obtained and treated in different ways (See Chapter 2 section 2.3.1 for details). The age of the seeds from the different sources also differed. Seeds from the Genebank were at least ten years old (STORK, P. personal communication 1992) and seeds from Hygrotech were harvested during the previous season (GROBLER, P. personal communication

1992). The regeneration response of cotyledonary explants that were prepared from seeds obtained from these two sources were compared.

Explants were placed on MS medium, supplemented with 1 mg l^{-1} BA, or a combination of 1 mg l^{-1} BA and 1 mg l^{-1} IAA. Six petri dishes of each medium were prepared for each of the seed sources and each petri dish contained five explants. Explants were transferred to fresh medium every three weeks. Explants were regularly examined for shoot regeneration and were only discarded when they had turned brown and no further changes were observed.

3.2.2 **Investigation into the use of different auxins to stimulate somatic embryogenesis**

Cotyledonary explants of *Cucurbita maxima* cv A-line were obtained as described above and placed on a number of auxin supplemented media in an attempt to stimulate somatic embryogenesis.

Explants were cultured on MS medium to which one of the following auxins had been added:

1 mg l^{-1} 2,4-dichlorophenoxyacetic acid
2 mg l^{-1} 2,4-dichlorophenoxyacetic acid
5 mg l^{-1} 2,4-dichlorophenoxyacetic acid
5 mg l^{-1} indole-3-butyric acid
5 mg l^{-1} indole 3-acetic acid.

The cytokinin BA was added to all the media at a concentration of 0.1 mg l^{-1}. A control treatment consisted of MS medium supplemented with 0.1 mg l^{-1} BA and no auxin. The pH of all the media was adjusted to 5.8 whereafter the media were autoclaved at 121 °C for 20 minutes.

Explants were cultured in petri dishes. Four explants were placed in each petri dish, of which five were prepared for each treatment. Explants were transferred to fresh medium every three weeks. The explants in two petri dishes from each treatment were transferred to MS medium with no growth regulators, six weeks after commencing with the experiment. Explants were regularly inspected for signs of embryogenesis. After nine weeks, all explants were inspected with the aid of a dissection microscope. Where necessary, some callus was cut away and sliced open to examine the tissue. Intact explants were left on the media and examined again after three weeks.

3.3 **RESULTS AND DISCUSSION**

3.3.1 **Investigation of the influence of different growth regulators on shoot regeneration**

No regeneration of shoots was observed on any of the treatments in any of the experiments. In all instances the explants were regularly inspected for signs of shoot regeneration. After eight to nine weeks all the explants were thoroughly examined. Where the appearance of the explant necessitated further investigation, explants were studied with the aid of a dissection microscope. Explants were kept until they

had turned brown and no further changes were noticeable. All the explants were again examined before they were destroyed. In some experiments the development of roots, callus or the size of the explants were recorded as described below.

3.3.1.1 **The influence of BA in combination with IAA on shoot regeneration**

No shoot development was seen on any of the treatments. However, root development was observed on a number of media supplemented with BA and IAA. Roots developed on all the media containing only IAA (Figure 3.1). Some root development was seen on media containing the lowest BA concentration, 0.5 mg l^{-1}. The percentage of explants with roots increased with increasing auxin concentration. At a level of 1 mg l^{-1} BA, roots developed only in the presence of a high auxin supplement of 10 mg l^{-1} IAA.

Callus development was also observed with all the treatments. Callus development was not dependent on an auxin supplement. BA supplemented media visibly stimulated more callus formation, when compared to the media with only an auxin supplement or to MS medium without any growth regulator supplement. The most callus formation was observed on media supplemented with the highest concentrations of BA and IAA.

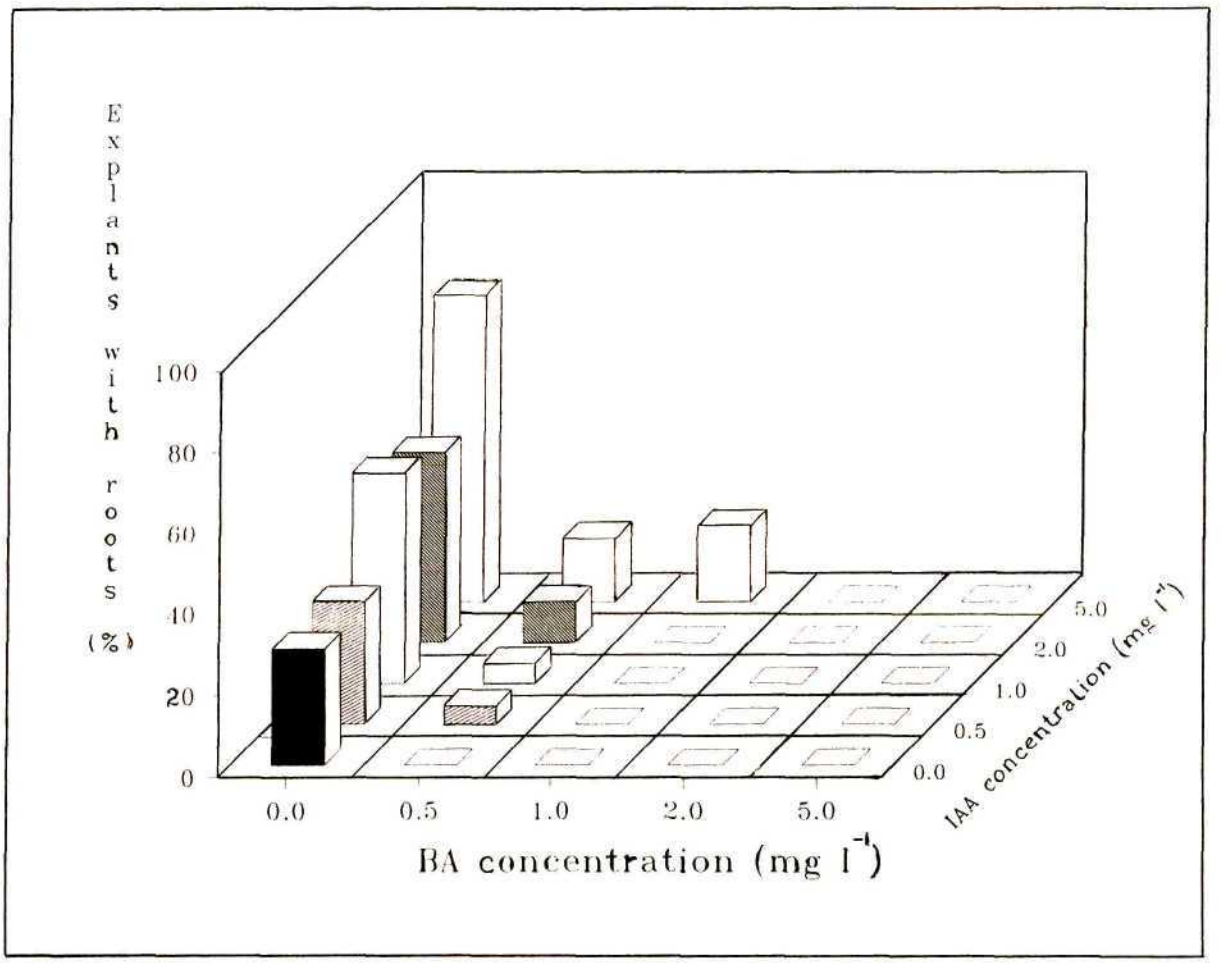


Figure 3.1. The percentage of cotyledonary explants of *Cucurbita maxima* cv A-line that formed roots using combinations of the growth regulators BA and IAA.

3.3.1.2 **The influence of light and dark treatments on shoot regeneration**

Again, no shoot regeneration was seen on any of the explants, but callus formation was observed on all the treatments. Unfortunately the information that was gathered on the root formation and callus development of cotyledonary explants of *Cucurbita maxima* cv A-line, in both the above mentioned experiments, did not lead to a better understanding of the reasons for the inability of the explants to regenerate shoots. It was therefore decided not to record these traits for the rest of the experiments.

3.3.2 **Investigation into the use of different auxins to stimulate somatic embryogenesis**

No embryogenesis was observed on any of the treatments. Roots and callus developed on all the treatments.

3.4 **CONCLUSION**

Although no reports have appeared on the shoot regeneration capacity of this species, the shoot regeneration of a number of the members of the Cucurbitaceae has been described (Chapter 1, Tables 1.1 to 1.4). Most of the successfully regenerated cultivars showed a response on one of the media that was tested in the current study (including the media tested in Chapter 2). The growth room conditions that were used did not differ significantly from conditions described by the majority of researchers. The possibility that shoot regeneration in this cultivar can only be achieved on an explant other than cotyledonary explants, was shown not to be true.

Two different seed sources reacted in the same way. These observations lead to the conclusion that the likelihood of this cultivar regenerating shoots on another medium or under different conditions is very slim.

According to Ms A. VAN DER SPOEL from the Department of Botany at the University of the Orange Free State, experiments to stimulate *in vitro* regeneration were conducted in that Department on this same cultivar (personal communication, 1992). A range of growth regulators, including BA, kinetin, and zeatin as well as IAA, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), were tested for the induction of shoot regeneration and/or embryogenesis, but no positive results were recorded. The decision not to continue with the study was made on the basis of this information as well as the results from the current study.

The most likely reason for the inability of the cultivar to exhibit regeneration is that the cultivar lack the genetic capacity. In maize it has been shown that a cross between an inbred with high shoot regeneration capacity and a non-regenerable inbred could lead to the development of regenerable inbreds through dominant inheritance of the trait (FAHEY, *et al.*, 1986). A genetic difference in competence for somatic embryogenesis was reported for alfalfa (BROWN, 1988), cucumber (PUNJA *et al.*, 1990) and other species (AMMIRATO, 1989). ORIDATE *et al.*, (1992) demonstrated that the somatic embryogenesis capacity in melon is inherited as a dominant trait in some cultivars, but in some cases the cytoplasm had some influence on the inheritance of somatic embryogenesis. It is very likely that *C. maxima* cv A-line can not be regenerated using our current understanding of the science of plant tissue cultures and that manipulation of the culture medium and conditions will not yield better results.

CHAPTER 4

IN VITRO REGENERATION OF *CUCUMIS MELO* AND *CUCUMIS SATIVUS*

4.1 INTRODUCTION

One of the possible problems with the use of an *Agrobacterium tumefaciens* transformation system is a reduction in regeneration efficiency of tissues after co-cultivation. It is therefore essential to optimise the regeneration protocol before transformation is attempted.

The *Cucumis melo* cultivar Hales Best 36 had a relatively high regeneration ability (Chapter 2). A number of parameters that were likely to influence the regeneration and growth of cultures were investigated further. The influence of casein hydrolysate on shoot regeneration *in vitro* was determined. Casein hydrolysate is an ill defined mixture of at least 18 different amino acids and vitamins that is often added to media to compensate for possible deficiencies (DODDS and ROBERTS, 1985). Explants were cultured in three types of container. The type of container can influence explant growth through factors such as the volume of air, movement and accumulation of gasses, amount of nutrient medium available per explant and drying out of the medium (PIERIK, 1987). These factors are difficult to measure and regulate, but the combined influence of these container related factors on the shoot regeneration *in vitro* can be evaluated. Other aspects that need to be taken into account when a container is chosen for experimental purposes are the opportunities

for contamination associated with handling of the container, ease of use for handling large numbers of cultures and the amount of growth room space taken up by a given container.

A number of plant factors can influence regeneration of shoots *in vitro*. The regeneration ability of different types of explant can differ significantly. The shoot regeneration ability of cotyledons, leaves, hypocotyls and petioles of this cultivar were investigated. Most of the differentiated shoots that were regenerated from cotyledonary explants remained small and did not develop into shoots long enough to be rooted. Attempts were made to improve the elongation of shoots through medium manipulation. Finally, the influence of growth regulators on the rooting of elongated shoots was determined.

A very low regeneration frequency was obtained from cotyledonary explants of the cucumber *Cucumis melo* cv Ashley in the regeneration experiments described in Chapter 2. The influence of a number of the growth regulators that were used in these experiments was tested again and compared to the influence of the plant hormone zeatin on regeneration. The shoot regeneration ability of cotyledons, leaves, hypocotyls and petioles was investigated.

4.2 **MATERIALS AND METHODS**

4.2.1 **The influence of casein hydrolysate on shoot regeneration of *Cucumis melo* cv Hales Best 36**

Embryos were manually excised from seeds of *Cucumis melo* cv Hales Best 36 and surface sterilised in 1 % (w/v) sodium hypochlorite solution (commercial Jik) for 20 minutes. Embryos were rinsed three times with sterile distilled water. Explants were obtained from these embryos by removing the proximal and distal ends of the embryo and separating the remaining cotyledons.

The basic medium that was used in all the experiments on *Cucumis melo* explants described in this chapter, consisted of MURASHIGE and SKOOG (1962) (MS) medium, supplemented with 3 % (w/v) sucrose, 0.75 % (w/v) agar, 0.5 mg l^{-1} indole-3-acetic acid (IAA) and 1 mg l^{-1} 6-benzyladenine (BA). The two treatments included additional supplements of 2 g l^{-1} and 4 g l^{-1} casein hydrolysate. The control had no additional supplements. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes. Explants were cultured in 90 mm sterile disposable petri dishes sealed with Parafilm ®, and placed in a growth room at 26 °C ± 2 °C. Each treatment consisted of 20 petri dishes each with two explants.

4.2.2 **The influence of the container on shoot regeneration of *Cucumis melo* cv Hales Best 36**

Explants were prepared and sterilised as described earlier (section 4.2.1). MS medium was prepared and supplemented as described for the previous experiment. Thirty ml of basic medium (4.2.1) was poured into culture bottles 75 mm in height and with a diameter of 55 mm before autoclaving. The volume of air above the medium was 110 ml. These bottles were closed with a screw cap. The second type of container that was used was a specimen tube 100 mm high with a diameter of 25 mm. Each tube contained 10 ml of medium with a 30 ml volume of air. Specimen

tubes were closed with a plastic cap that fitted tightly over the top and sealed with Parafilm ® that was wound around the tube one and a half times. The third type of container, 90 mm petri dishes, contained 20 ml medium with an air volume of 50 ml. Petri dishes were sealed with Parafilm ® that was wound around the container once with a short overlap of the edges. All cultures were placed together in one growth room and shifted around regularly to avoid microclimates affecting cultures in different shelf areas. All explants were subcultured every third week.

4.2.3 **Shoot regeneration from four types of explant of *Cucumis melo* cv Hales Best 36**

Embryos were excised and sterilised as described before (4.2.1). These embryos were placed in culture bottles (75 mm high, 55 mm diameter) on MS medium without growth regulators and allowed to germinate. Leaf explants were obtained from these plantlets as soon as leaves were fully expanded. The whole leaf was removed and cuts were made on the lamina prior to culture. Hypocotyl sections of 10 to 20 mm long were cut from these plantlets and used as explants. Petioles were removed from the plantlets and cut into 5 to 20 mm sections, depending on the length of the petiole. Cotyledonary explants were prepared as described in section 4.2.1. The basic medium that was described in section 4.2.1 was used throughout the experiment. Five petri dishes were prepared for each type of explant and each of the petri dishes contained at least five explants of the same type. Explants were subcultured every three weeks.

4.2.4 **The influence of the subculture medium on shoot elongation of regenerated shoots of *Cucumis melo* cv Hales Best 36**

Cotyledonary explants were prepared and sterilised as described in section 4.2.1. and placed on the basic regeneration medium. Three weeks after the experiment was initiated the explants were transferred to one of the following media:

1. Basic regeneration medium;
2. MS medium without growth regulators;
3. MS medium supplemented with 0.1 mg l^{-1} gibberellic acid;
4. MS medium with 1.0 mg l^{-1} gibberellic acid and
5. Half strength MS medium with no growth regulators.

Five petri dishes were prepared for each treatment, each containing five explants. Explants were subcultured onto the same medium a second time and results were assessed 8 weeks after the start of the experiment.

4.2.5 **The influence of growth regulators on rooting of regenerated shoots of *Cucumis melo* cv Hales Best 36**

Elongated shoots that were obtained in the previous study on MS medium with no growth regulators were removed from the explants on which they developed. The shoots were placed on one of the following media:

1. Basic regeneration medium;;
2. MS medium with no growth regulators;
3. MS medium supplemented with 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) and

4. MS medium supplemented with 2.0 mg l^{-1} indole-3-butyric acid (IBA).

Shoots were cultured in petri dishes containing the media listed above and inspected daily for root development. The experiment continued until at least 20 shoots were tested on each of the media.

4.2.6 **The influence of zeatin and previously tested cytokinins on shoot initiation in cotyledonary explants of *Cucumis sativus* cv Ashley**

Sterile explants were obtained from excised embryos of *Cucumis sativus* cv Ashley according to the procedure outlined for melon explants in section 4.2.1. A basic MS medium was prepared and supplemented with 3 % (w/v) sucrose and 0.75 % (w/v) agar. This medium was supplemented with 0.5 mg l^{-1} IAA in combination with the following growth regulators:

1. 5 mg l^{-1} kinetin;
2. 1 or 5 mg l^{-1} BA;
3. 1 or 5 mg l^{-1} iP and
4. 1, 2 or 5 mg l^{-1} zeatin.

Seven petri dishes, each containing four cotyledonary explants were prepared for each of these treatments. Explants were subcultured every three weeks and results were recorded after nine weeks.

4.2.7 **Shoot regeneration from four types of explant of *Cucumis sativus* cv Ashley**

Embryos were excised and sterilised as described for melon explants in section 4.2.1. These embryos were placed in culture bottles on MS medium without growth regulators and allowed to germinate. Leaf explants were obtained from these plantlets as soon as leaves were fully expanded. The whole leaf was removed and cuts were made on the lamina. Hypocotyls were cut into 10 to 20 mm sections and cultured. Petioles were removed from the plantlets and cut into 5 to 20 mm sections, depending on the length of the petiole. Cotyledonary explants were prepared as described in section 4.2.1. MS medium, supplemented with 3 % (w/v) sucrose, 0.75 % (w/v) agar, 0.5 mg l^{-1} IAA and 5 mg l^{-1} zeatin was used in this experiment. Five petri dishes were prepared for each type of explant and each of the petri dishes contained at least 5 explants of the same type. Explants were subcultured every three weeks.

4.3 **RESULTS AND DISCUSSION**

4.3.1 **The influence of casein hydrolysate on shoot regeneration of *Cucumis melo* cv Hales Best 36**

The number of shoots that developed on each explant decreased slightly with increasing casein hydrolysate concentration (Figure 4.1). However, the difference between the control and the two casein hydrolysate treatments was not significant. No other differences were observable between the treatments and the conclusion was reached that this supplement was an unnecessary addition. According to DODDS and ROBERTS (1985) casein hydrolysate can be added to media as a

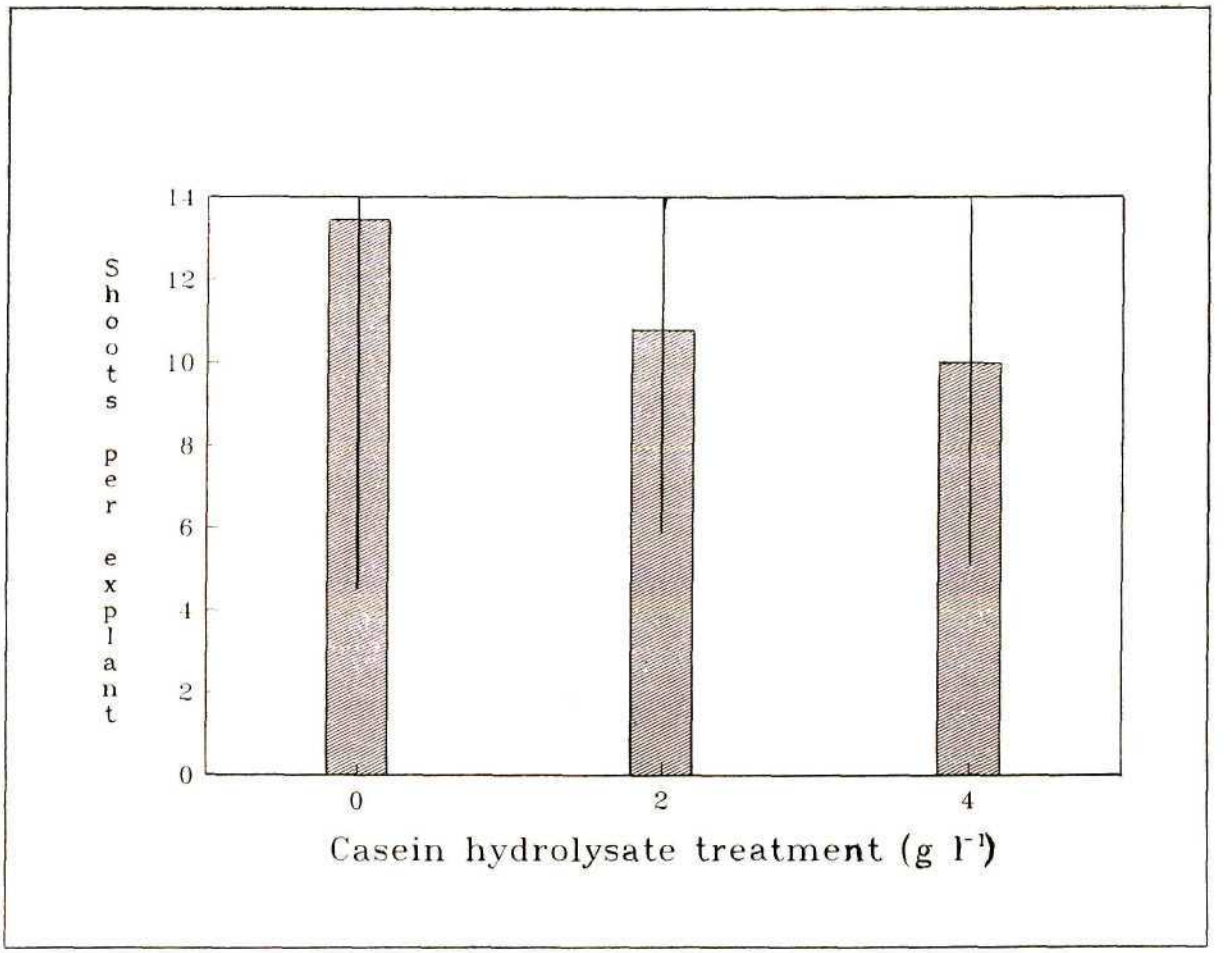


Figure 4.1. The influence of casein hydrolysate on the number of shoots that developed per cotyledonary explant of the cultivar Hales Best 36. The bars represent the standard errors.

source of organic nitrogen and vitamins, but growth inhibition is often observed, due to competitive interactions between the various amino acids. PIERIK (1987) advises against the use of undefined media such as casein hydrolysate, as the composition is largely unknown and variable. Furthermore, the use of amino acids as source of nitrogen is unnecessary where a proper NO_3^-/NH_4^+ balance in the medium fulfils the nitrogen requirements of the cultures.

4.3.2 **The influence of the container on shoot regeneration of *Cucumis melo* cv Hales Best 36**

There were no significant differences in the shoot regeneration in the different containers (Figure 4.2). This is the expected result where none of the factors associated with the shape and size of the container are limiting to a serious degree and where no gasses build up to levels that are visibly detrimental to growth. Most callus formation was seen in the bottles and least in the petri dishes. This could indicate a difference in the composition of gasses in the different types of container, although it is not possible to establish how such a difference would come about or which gasses are involved. The limited callus growth in petri dishes could also be ascribed to lesser amounts of nutrient medium that is available per explant (PIERIK, 1987), but this made it easier to obtain developed, uncallussed shoots. As the purpose of this experiment was mainly to establish a reliable regeneration protocol, these questions were not pursued.

The ease with which cultures were handled differed markedly between the containers. The absence of significant differences in the influence of the container

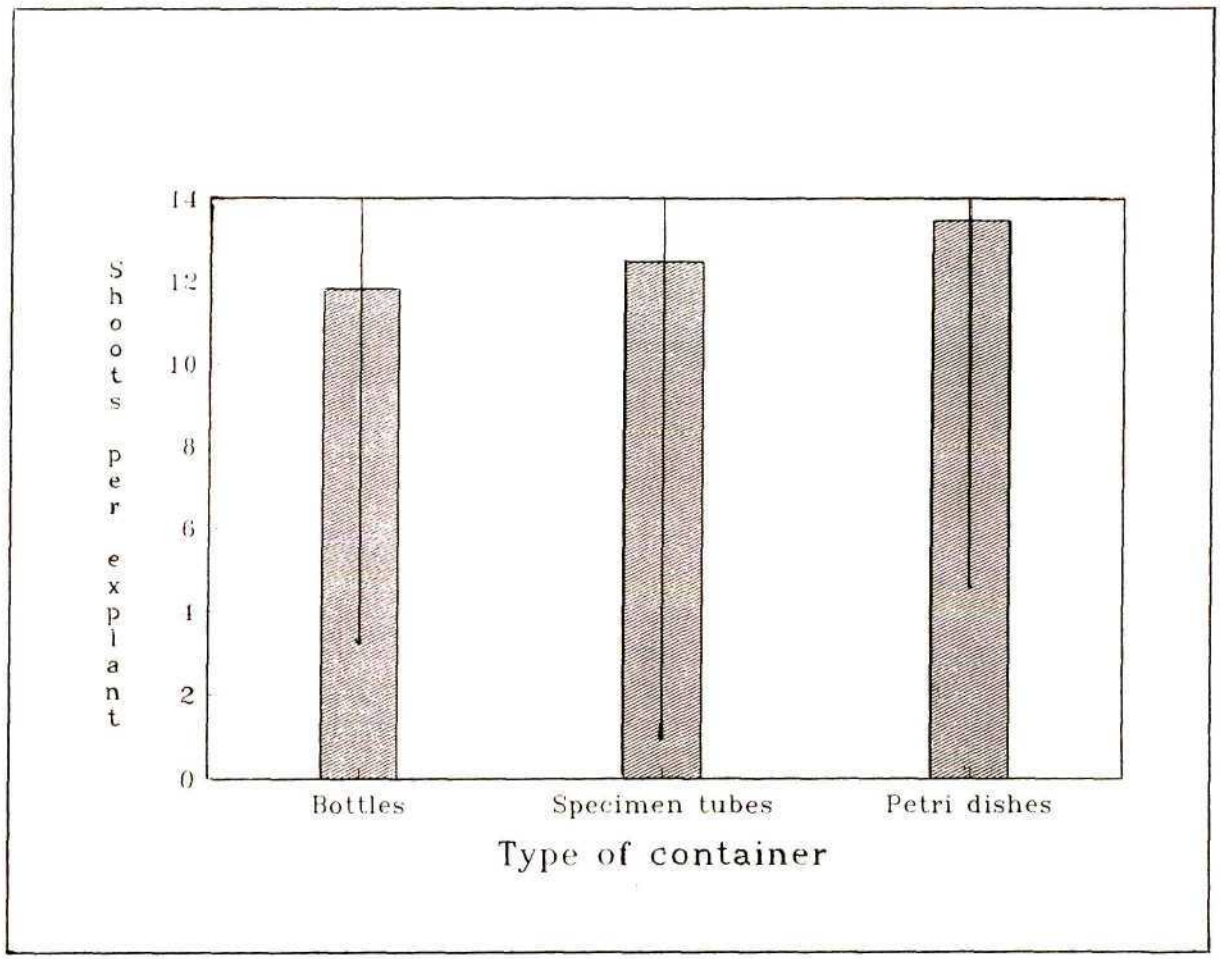


Figure 4.2. The influence of different containers on the number of shoots that developed per cotyledonary explant of the cultivar Hales Best 36. The bars represent the standard errors.

on shoot regeneration allows this to become a primary consideration in the choice of container. Petri dishes are the easiest to handle, can hold as many as four or five explants and have proven to be fairly contamination free. Bottles and specimen tubes are cumbersome to handle, especially on the laminar flow cabinet. Only one explant can be cultured in each specimen tube; two in bottles. Of the three types of container, bottles take up the most growth room space and although little contamination was observed, more opportunities for contamination were identified and compensated for during handling of explants. In some cases the explants in specimen tubes expanded to press against the sides of the tube, which made subculturing laborious. Petri dishes were used in all subsequent experiments.

4.3.3 **Shoot regeneration from four types of explant of *Cucumis melo* cv Hales Best 36**

Four shoots developed on one of the 27 leaf explants that were cultured. Very little callus development was seen on any of the explants and browning was visible after one week in culture. Large amounts of callus developed on hypocotyl sections, but only two shoots developed on one of the explants. The callus was light green with darker green islands. It is possible that these islands were embryogenic, but this possibility was not investigated. Callus development on petiole sections was equally vigorous and had a similar appearance to the callus on hypocotyl explants. No shoot development was seen on any of the petiole explants. Shoot development was observed on 92 % of cotyledonary explants. The number of shoots on cotyledonary explants were not counted, as the other explant types were clearly inferior for shoot regeneration *in vitro*.

MORENO *et al.*, (1985) reported a shoot initiation response on 96 % of cotyledonary explants of *Cucumis melo* in the presence of 1.5 mg l^{-1} IAA and 6 mg l^{-1} kinetin, but none was observed on hypocotyl explants on media supplemented with 0 to 6 mg l^{-1} IAA and 0 to 6 mg l^{-1} kinetin. This is in contrast to the report by TABEI *et al.*, (1991), where shoot regeneration in *Cucumis melo* was observed on up to 12 % and embryogenesis on 15 % of hypocotyl explants cultured on MS medium with 0.1 mg l^{-1} BA and 0.01 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). However, shoot regeneration was observed on more than 80 % of the cotyledonary explants cultured on the same medium.

Successful shoot regeneration from leaf explants of *Cucumis melo* was reported by KATHAL *et al.*, (1988) and DIRKS and VAN BUGGENUM (1989). In both reports 100 % of leaf explants could be induced to develop shoots through manipulation of the medium. According to DIRKS and VAN BUGGENUM (1989), cotyledonary explants produced double the number of shoots per explant that was recorded for leaf explants. TABEI *et al.*, (1991) described limited regeneration from leaf explants with more than 20 % of explants responding. The response of expanded cotyledons from seedlings and petiole explants was also tested, but only 12 % of explants regenerated shoots from these tissues.

It is apparent from these reports as well as from the current study, that cotyledonary explants consistently gave the best results in shoot regeneration studies of *Cucumis melo*. Leaf explants provide an alternative explant source, but if this option is explored for the cultivar Hales Best 36 in future, media supplements will have to be revised.

4.3.4 **The influence of the subculture medium on shoot elongation of regenerated shoots of *Cucumis melo* cv Hales Best 36**

The results that were obtained during this study were not quantifiable. The number of shoot buds that develop per explant during a regeneration experiment can only be counted properly if the explant is destroyed during counting. The proportion of shoots that elongate can therefore not be reliably determined. Visual observations did however, give some idea of the usefulness of the various subculture media.

Explants subcultured to regeneration medium produced larger quantities of callus than any of the other media. This can be ascribed to the presence of IAA in this medium. Callus formation made the removal of developed shoots difficult. Shoots remained small on half strength MS medium, but remained green longer than shoots on other media. Some shoot elongation was seen on the three other media, but visually no difference could be observed between them. NIEDZ *et al.*, (1989) also investigated the influence of gibberellic acid on shoot regeneration, but did not observe any benefits. Explants on the two gibberellic acid treatments had a higher incidence of hyperhydricity than those on the MS treatment alone. For elongation, it was decided to transfer cultures to MS medium without growth regulators in all subsequent experiments.

4.3.5 **The influence of growth regulators on rooting of regenerated shoots of *Cucumis melo* cv Hales Best 36**

No roots developed on the basic regeneration medium or the MS medium without growth regulators. Thirty five percent of shoots placed on the NAA supplemented

medium developed roots. Root development was visible after 12 days of culture. However, on this medium considerable callus development took place at the basal region of the shoot. CHEE (1991a) also observed callus formation at the shoot bases on medium containing 0.1 mg l^{-1} NAA, the highest concentration tested. Roots became callused if the plantlets were left on this medium. Ninety percent of shoots placed on IBA supplemented medium, rooted. The first roots developed after 7 days of culture and very little callus development was seen.

Regenerated shoots of *Cucumis melo* have been rooted on various media such as MS medium with 0.09 mg l^{-1} IAA (KATHAL *et al.*, 1988), MS medium with no growth regulator supplement (NIEDZ *et al.*, 1989) or half strength MS medium with 0.2 mg l^{-1} gibberellic acid (TABEI *et al.*, 1991). DIRKS and VAN BUGGENUM (1989) used MS medium with no growth regulators and with the sucrose concentration lowered to 20 g l^{-1}.

The growth regulator most commonly used to stimulate rooting in regenerated shoots of *Cucumis melo* is NAA. It has been used at levels of 0.01 mg l^{-1} (CHEE, 1991), 0.01 to 0.1 mg l^{-1} (MORENO *et al.*, 1985) and 0.03 mg l^{-1} (BOKELMAN *et al.*, 1991). Of the three reports, only the report by CHEE, (1991a) gave the percentage of shoots that developed roots as 42 %. It is possible that the concentration that was used in this study (0.5 mg l^{-1}) was too high and inhibited more effective rooting. However, the root formation that was observed on the medium containing 2 mg l^{-1} IBA was sufficiently high so that no further investigation of rooting media was required.

4.3.6 **The influence of zeatin and previously tested cytokinins on shoot initiation in cotyledonary explants of *Cucumis sativus* cv Ashley**

The highest shoot regeneration response was seen on the medium supplemented with 5 mg l^{-1} zeatin, where 50 % of explants responded (Figure 4.3). Significantly more explants responded on this medium than on the 1 and 5 mg l^{-1} iP, 1 and 5 mg l^{-1} BA or 1 mg l^{-1} zeatin treatments. On media containing 2 mg l^{-1} zeatin and 5 mg l^{-1} kinetin, 25 % of explants and on 5 mg l^{-1} iP, 21.4 % of explants responded. It is apparent that the response on zeatin containing media was better than for other cytokinins tested. These results are in contrast to those obtained previously (Chapter 2), where no response was seen on the kinetin containing media. The higher response on the medium containing 1 mg l^{-1} BA compared to the response on 5 mg l^{-1} BA, is in contrast to the results reported in Chapter 2, where the opposite was found. Only one report, that of TRULSON and SHAHIN (1986) exist describing the use of zeatin in the regeneration of cucumber from cotyledonary protoplasts. In most other cases, the cytokinins kinetin (HANDLEY and CHAMBLISS, 1979; GAMBLEY and DODD, 1990) or BA (WEHNER and LOCY 1981; KIM *et al.*, 1988; MSIKITA *et al.*, 1990) were used in the successful regeneration of cucumber. The medium containing 5 mg l^{-1} zeatin in combination with 0.5 mg l^{-1} IAA was used for subsequent experimentation.

4.3.7 **Shoot regeneration from four types of explant of *Cucumis sativus* cv Ashley**

No regeneration was seen on any of the explants. Very little callus developed on leaf and hypocotyl explants. More callus formed on petiole and cotyledonary explants,

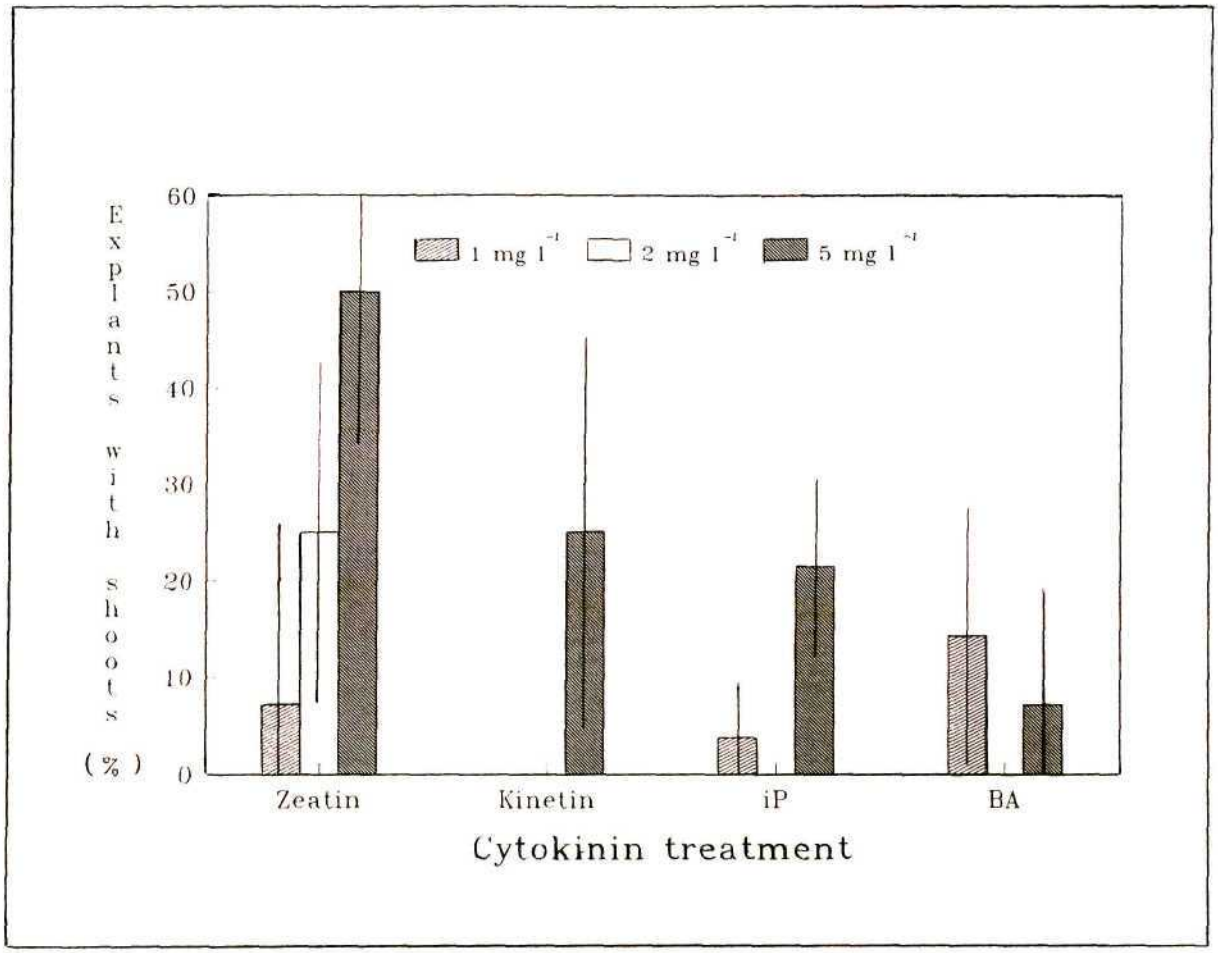


Figure 4.3. The influence of the cytokinins zeatin, kinetin, iP and BA on the percentage of explants that formed shoots. The bars represent the standard errors.

but this did not give rise to plant regeneration. This inconsistent, poor response is undesirable where transformation of tissues is the purpose of the study. According to POTRYKUS (1991) plant tissues are mixed populations of cells that are (a) competent for regeneration, (b) competent for transformation, (c) competent for regeneration and transformation, or (d) non-competent for both. It is therefore important to use tissues with high regeneration ability to increase the possibility of obtaining explants competent for both regeneration and transformation. This is even more important in the light of the inhibitory effect that co-cultivation with *Agrobacterium* has on regeneration of many species (POTRYKUS, 1991).

4.4 **CONCLUSION**

It was possible to obtain reliable, high levels of regeneration from cotyledonary explants of *Cucumis melo* cv Hales Best 36 on MS medium containing 0.5 mg l^{-1} IAA and 1.0mg l^{-1} BA. Initiated shoots could be elongated on MS medium containing no growth regulator supplement and could subsequently be rooted on MS medium containing 2 mg l^{-1} indole-3-butyric acid. The protocol is best performed in petri dishes and the explants cultured in a growth room with a 16 hour/8 hour light/dark cycle at 26 °C ± 2° C. It was decided to utilise this cultivar for transformation studies and to obtain transformed plants using the above regeneration protocol.

The inconsistent response of the cultivar Ashley, coupled to the high cost of zeatin made the search for a regeneration protocol for this cultivar inappropriate. This research was therefore discontinued.

CHAPTER 5

BIOTRANSFORMATION OF *CUCUMIS MELO* cv HALES BEST 36

5.1 INTRODUCTION

One of the most effective means of gene transfer into plants is the system that utilises the soil bacterium *Agrobacterium tumefaciens* as a vector. This system has been used to transform and regenerate agronomically important crops such as tobacco (DE BLOCK *et al.*, 1984; HORSCH *et al.*, 1985), carrot (THOMAS *et al.*, 1989) and tomato (McCORMICK *et al.*, 1986). Reports have appeared describing the regeneration of stably transformed plants of the family Cucurbitaceae (TRULSON *et al.*, 1986; CHEE, 1990b; FANG and GRUMET, 1990; DONG *et al.*,1991; CHEE and SLIGHTOM, 1991; CHOI *et al.*, 1994; GONSALVES *et al.*, 1994). The development of a reliable system for the transfer and stable integration of genetic material into the genome of cucurbit species is potentially useful in the transfer of traits such as virus resistance or herbicide resistance, which are difficult or impossible to transfer via conventional breeding techniques.

In this study an *Agrobacterium tumefaciens* transformation system was tested to establish some of the conditions necessary for the transformation and regeneration of plantlets from cotyledonary tissue. The *Agrobacterium* strain LBA4404, containing the plasmid pBI121 was used. This plasmid was introduced into *Agrobacterium tumefaciens* from the *E. coli* strain HB101 via a triparental mating. The plasmid contains two genes that are used for the detection of transformed

plants, the selectable neomycin phosphotransferase II (NPT II) gene that confers kanamycin resistance to transformed tissue (HERRERA-ESTRELLA *et al.*, 1983) and the ß-glucuronidase gene, a sensitive reporter gene that makes the identification of transformed tissue easy (JEFFERSON *et al.*, 1987).

The influence of the antibiotics kanamycin and cefotaxime on the shoot regeneration ability of cotyledonary explants of *Cucumis melo* cv. Hales Best 36 was determined before transformation was attempted. The level at which kanamycin completely inhibits shoot regeneration in this cultivar was determined to ensure that untransformed tissue is unable to grow on media containing the chosen concentration of the antibiotic. Cotyledonary explants were also cultivated on regeneration medium containing 250 $\mu g\ ml^{-1}$ cefotaxime to determine whether this antibiotic influences the regeneration ability of explants. This antibiotic is commonly used to destroy the *Agrobacterium tumefaciens* cells after co-cultivation without causing harm to the explant (MATHIAS and BOYD, 1986).

The influence of different co-cultivation and inoculation times on transformation were tested. In a further experiment the influence of the wound factor acetosyringone on transformation was investigated. This phenolic compound is actively secreted from wounded cells and is responsible for the induction of *vir* gene expression (STACHEL *et al.*, 1986a).

Plantlets that developed and rooted on the selection media were tested for the presence of ß-glucuronidase (GUS, EC 3.2.1.31) using fluorometric and histochemical assays. The fluorescence assay detects ß-glucuronidase activity when the substrate 4-methyl umbelliferyl glucuronide (MUG) is cleaved by ß-glucuronidase to release the fluorescent product 4-methyl umbelliferone (MU)(7-

hydroxy-4-methyl coumarin). The histochemical assay utilises the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc). This substrate produces a blue precipitate at the site of enzyme activity.

5.2 **MATERIALS AND METHODS**

5.2.1 **The influence of kanamycin and cefotaxime on explant development**

Embryos were excised from seeds obtained from Hygrotech Seeds and surface sterilised in a 1% (w/v) sodium hypochlorite solution for 20 minutes as described in Chapter 2. MS medium containing 3% (w/v) sucrose and 0.75% (w/v) agar and supplemented with 1 mg l^{-1} 6-benzyladenine (BA) and 0.5 mg l^{-1} indole 3-acetic acid (IAA) was used as the basic regeneration medium. Four concentrations of kanamycin were added to this medium; 50 µg ml^{-1}, 100 µg ml^{-1}, 200 mg ml^{-1} or 300 mg ml^{-1}. Cefotaxime was tested at a concentration of 250 mg ml^{-1}. A control treatment that consisted of the basic regeneration medium with no further supplements was included in the experiment.

Five petri-dishes were prepared for each of the five treatments as well as the control treatment and planted with two explants each. Explants were transferred to fresh medium once a week. The percentage of explants that developed shoots were recorded after one month. The appearance of explants was also recorded.

5.2.2 **Conjugation of recombinant plasmids into *Agrobacterium***

The conjugations that are required to transfer the plasmid of interest from *Escherichia coli* to *Agrobacterium* were carried out as a triparental mating (VAN HAUTE *et al.*, 1983). Triparental matings with some modifications, were performed according to the procedure described by ARMITAGE *et al.*, (1988).

Cultures of *Escherichia coli* strain HB101 containing the plasmid pBI121, *Escherichia coli* with the helper plasmid pRK2013 and recipient *Agrobacterium tumefaciens* strain LBA4404 were prepared. A single colony of each of these strains was picked from actively growing plates with a sterile inoculating needle and suspended in Erlenmyer flasks containing 10 ml Luria broth. The *Agrobacterium* medium was supplemented with 100 μg ml^{-1} rifampicin and the media of the *Escherichia coli* strains with 50 μg ml^{-1} kanamycin for the helper plasmid, and 100 μg ml^{-1} kanamycin for the donor strain respectively. *Escherichia coli* cells were incubated overnight at 37 °C, while the *Agrobacterium* cells were cultured at 28 °C for 48 hours. Cultures were incubated on an orbital shaker.

A volume of 100 μl was removed from each culture and pipetted onto a single Luria agar plate. The samples were spread out using a glass rod and incubated at 28 °C. Colonies were taken from the lawn that developed on the plate after 24 to 36 hours and streaked out on Luria agar selection plates containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin. These plates were incubated for 48 hours before colonies were streaked out again, to make sure cultures were transformed. Overnight bacterial cultures were prepared from colonies picked from these plates. The cultures were grown in Erlenmyer flasks containing 10 ml Luria broth supplemented

with the selective antibiotics. Colonies were tested for transformation prior to the inoculation of the overnight cultures and cultures were again tested prior to use.

Transformation was confirmed using a qualitative ß-glucuronidase (GUS) fluorometric assay (JEFFERSON, 1987). Fifty µl of GUS fluorometric assay buffer (Appendix B) was pipetted into each well of a microtitre plate. Ten to twenty µl of the culture was added to each of the wells. No *Agrobacterium* was added to control wells, which contained only buffer. A second control consisted of untransformed *Agrobacterium*. The plate was incubated at 37 °C for an hour. The plate was examined for fluorescence on a transilluminator.

Cultures were stored in screw cap cryo vials containing a cryopreservative liquid and 25 - 30 porous beads. Forty µl of the culture was pipetted aseptically into the vial. The vial was inverted four to five times to distribute the bacteria and allow them to adhere to the beads. Excess cryopreservative was removed aseptically. The vial was closed and stored at -70 °C until cultures were needed.

When fresh overnight cultures were required, one bead was removed from the vial under sterile conditions and streaked out on a fresh bacterial plate containing selective antibiotics. The plates were incubated under the appropriate conditions and colonies which grew on the plates were used to inoculate the overnight cultures. All cultures obtained in this way were retested on the appropriate antibiotics and assayed for GUS activity before use.

5.2.3 **The influence of co-cultivation time on transformation**

5.2.3.1 **Plant material**

Excised embryos of the melon cultivar Hales Best 36 were sterilised in a 1 % (w/v) solution of sodium hypochlorite for 20 minutes, as described in Chapter 2. Prior to inoculation the distal and proximal ends of the embryos were cut away with a dull scalpel blade to maximise wounding. The two cotyledons were separated and were placed on regeneration medium prior to inoculation. Each treatment consisted of seven petri-dishes with five explants in each petri-dish.

5.2.3.2 **Media**

The regeneration medium consisted of MS medium with 3 % (w/v) sucrose and 0.75 % (w/v) agar, supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} IAA. The selection medium was similar to the regeneration medium, but it contained the antibiotics kanamycin and cefotaxime at concentrations of 100 µg ml^{-1} and 250 µg ml respectively. Both these antibiotics were also added to the rooting medium which consisted of MS medium with 3 % (w/v) sucrose, 0.75 % (w/v) agar and 2 mg l^{-1} indole-3-butyric acid (IBA).

5.2.3.3 **Bacteria**

Agrobacterium tumefaciens strain LBA4404 containing the plasmid pBI121 was used for transformation. The plasmid was introduced into the *Agrobacterium* by a triparental mating as described previously. Fresh overnight cultures of the bacteria were prepared and decanted aseptically into sterile Oakridge bottles. Cells were collected by centrifugation at 3300 rpm at 4 ºC for 25 minutes. The supernatant was discarded and the pellet resuspended in 10 ml Luria broth. No antibiotics were added. The resuspended cells were assayed for ß-glucuronidase activity using a qualitative fluorometric test, before being used for the transformations. Cultures of untransformed *Agrobacterium tumefaciens* LBA4404 were prepared as well, and used for a negative transformation control.

5.2.3.4 **Inoculation and co-cultivation**

Explants were removed from the regeneration medium and placed in a petri-dish. Explants were inoculated by pipetting 40 µl of bacterial suspension onto each explant. This was an adequate volume to cover the explant completely. After five minutes explants were blotted dry with sterile filter paper and returned to the regeneration medium. Explants were co-cultivated with the *Agrobacterium* on regeneration medium for 0, 1, 3, 5 or 7 days respectively.

5.2.3.5 **Plant regeneration and selection**

After the co-cultivation period explants were placed on regeneration medium supplemented with 100 µl ml^{-1} kanamycin and 250 µl ml^{-1} cefotaxime. Explants were transferred to fresh selection medium weekly for one month. After one month

explants were transferred to MS medium containing both antibiotics, but no growth regulators. Elongated shoots were removed from the explants and placed on rooting medium. Plantlets with a well developed root system were transferred to a potting soil/vermiculite mixture and hardened off. Plantlets were covered with a clear plastic bag that was gradually removed over the next week, each day an hour longer than the previous day, until plantlets could survive without the covering.

5.2.3.6 **Controls**

A negative control was included for each treatment. Negative controls consisted of *Cucumis melo* cv Hales Best 36 cotyledonary explants, treated in exactly the same way as the test explants, except that they were inoculated with *Agrobacterium* harbouring the pBI121 plasmid. An untransformed *Agrobacterium tumefaciens* strain LBA4404 was used. One petri dish, containing five explants was prepared for each of the co-cultivation times tested.

A tobacco (*Nicotiana tabacum*) transformation was included as positive control for this experiment. Leaves of axenically grown tobacco plants were removed aseptically and cut numerous times across the surface. These leaves were dipped into an excess of *Agrobacterium* suspension for two minutes, blotted dry with sterile filter paper and placed on the regeneration medium. (This medium had been tested for tobacco shoot regeneration in a pilot study.) After two days of co-cultivation the explants were placed on the selection medium. Explants were transferred to fresh medium once a week.

A third control consisted of one petri dish with five explants that were not exposed to any *Agrobacterium* for each of the co-cultivation times tested.

5.2.3.7 **Determination of ß-glucuronidase enzyme activity using fluorometric and histochemical GUS-assays**

Qualitative fluorometric assay

The transgenic nature of rooted plantlets growing on kanamycin was verified by testing inoculated tissues for the presence of ß-glucuronidase, using a fluorometric assay adapted from the method described by JEFFERSON in 1987. Small sections of leaves and roots from these plants were removed and placed into the microtiter wells of microtiter plates. Plant material was punched numerous times with a sterile toothpick. Fifty µl GUS fluorometric assay buffer was pipetted into each well containing plant cuttings. Buffer was placed into a few wells containing no plants to serve as controls. The plate was incubated at 37 °C for at least one hour and then examined under ultra-violet light for fluorescence. Tissues from control explants and untransformed plants were tested as well.

Quantitative fluorometric assay

One of the melon plantlets from the three day treatment that developed roots on kanamycin containing rooting medium was tested using this assay. An untransformed melon plantlet, a tobacco plant from the positive control treatment and an untransformed tobacco plantlet were also tested for control purposes.

Plant extracts were prepared by adding 100 µl of GUS extraction buffer and 5 mg polyvinyl pyrollidone to 50 to 100 mg leaf tissue sample. The tissue was ground with a glass rod and the extract centrifuged for two minutes at full speed in an Eppendorf centrifuge. The supernatant was made up to 100 µl with extraction buffer. The protein content of the extracts were determined using the assay of BRADFORD (1976). Bovine serum albumin (BSA) was dissolved in GUS extraction buffer and used to prepare tubes containing 4 µl volumes of 0, 0.5, 1.0, 2.0, 3.0, 3.5, 4.0, 7.5 and 8.0 µg BSA. Sterile distilled water was used to bring the volume up to 400 µl. One hundred µl of Bio-Rad dye was added to each tube to give a final volume of 500 µl. The absorbance was measured at 595 nm and the O.D. readings were plotted on a graph of absorbance versus protein concentration in µg.

The absorbance of the plant extracts were obtained by adding 4 µl of plant extract to 396 µl of sterile distilled water, adding 100 µl of Bio-Rad dye and reading the absorbance at 595 nm in a Bausch and Lomb Spectronic 501 spectrophotometer.

A Hitachi F2000 fluorescence spectrophotometer was calibrated with 1 ml volumes of methylumbelliferone (7-hydroxy-4-methyl coumarin) solutions with the following concentrations: 0.0, 0.01, 0.02, 0.025, 0.03, 0.05, 0.075, 0.10, 0.125 and 0.2 µM. These were prepared from a 10 mM stock solution of methylumbelliferone (MU) (See Appendix B). Fluorescence was read at the exitation wavelength of 363 nm and emission 469 nm. The fluorescence readings were used to construct a standard curve.

Aliquots of 0.5 ml GUS reaction buffer containing the substrate 4-methyl, umbelliferyl, ß-D glucuronide (MUG), were prewarmed to 37 °C. This substrate is

cleaved by ß-glucuronidase to form the fluorescent product methylumbelliferone (MU). Aliquots (0.9 ml) of the stop buffer (See Appendix B) were prepared in microfuge tubes. Forty µl of the tissue extract was added to the 0.5 ml GUS reaction buffer, mixed thoroughly, and incubated for one minute from the time the extract was added to the buffer. One hundred µl of the reaction mixture was removed and added to 0.9 ml of the stop buffer. This stopped the reaction and constituted time = 0. The reaction was incubated at 37 °C and 100 µl aliquots were removed and added to stop buffer at five minute intervals, until the reaction had run for 20 minutes. Fluorescence of the stopped reaction samples were read at room temperature at exitation wavelength 363 nm and emission 469 nm. The production of methylumbelliferone in nmol MU/ mg protein was plotted against time.

Histochemical assay

This assay was performed under sterile conditions, because many common bacterial contaminants produce ß-glucuronidase (JEFFERSON, 1987). The substrate that was used for the assay, 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-gluc), makes the detection of ß-glucuronidase possible through the formation of a blue precipitate. The assay solution contained 0.05 % (w/v) X-gluc, 0.1 % (v/v) Triton X-100 and 100 mM $NaPO_4$ pH 7.0. Tissue sections, cut free-hand, or whole pieces of leaf, stem and root were soaked overnight in 100 µl of the X-gluc assay solution at 37 °C in Eppendorf tubes. Untransformed tissue was assayed simultaneously as a negative control. After incubation, tissues were washed in 70 % (v/v) ethanol and mounted in distilled water on slides for viewing. ß-glucuronidase was detected in cells by the formation of a blue precipitate. Photographs of the free-hand sections were taken with an Nikon Microphot FX photomicroscope.

5.2.4 **The influence of inoculation time on transformation**

The influence of inoculation time on transformation of *Cucumis melo* cv Hales Best 36 was investigated. The plant material, media, bacteria and regeneration and selection protocols were similar to those used previously. Tobacco was again used as positive control and negative controls consisted of cotyledonary explants inoculated in untransformed *Agrobacterium* LBA4404.

In this experiment, five inoculation times were tested; 2, 5, 10, 30 and 60 minutes respectively. Explants were co-cultivated with the *Agrobacterium* strain for two days before being transferred to selection medium. Selection and rooting were accomplished as described previously, and potential transformants were tested using the qualitative fluorometric GUS assay.

5.2.5 **The influence of acetosyringone on transformation**

Wounded plant tissue release compounds which results in the transcription of the *vir* region of the Ti plasmid when it is brought into contact with *Agrobacterium*. It is thought that the *virA* gene product recognises and interacts with these wound factors, resulting in the activation of the rest of the *vir* genes. One chemical which is highly active in this regard is acetosyringone (STACHEL *et al.*, 1986a). The influence of this chemical on transformation of cotyledonary explants of *Cucumis melo* cv Hales Best 36 was tested. The experiment was repeated twice.

5.2.5.1 **Plant material and media**

Plant material was prepared as described in Chapter 2 and placed on regeneration medium prior to inoculation. The same media that were used previously (section 5.2.3.2), were used again for regeneration, selection and rooting.

5.2.5.2 **Bacteria**

Two overnight suspensions of *Agrobacterium* were prepared as described previously. Both were transferred aseptically to sterile Oakridge bottles and spun down at 3300 rpm for 25 minutes at 4°C. The supernatant was discarded aseptically and one of the pellets was resuspended in 10 ml Luria broth containing 50 µM acetosyringone. The second pellet was resuspended in 10 ml Luria broth with no additions. Untransformed *Agrobacterium* LBA4404 was also prepared using the 50 µM acetosyringone supplemented Luria broth for resuspension of the cells.

5.2.5.3 **Inoculation and co-cultivation**

Explants were removed from the regeneration medium, placed in a petri-dish and inoculated for five minutes as described before. Explants were blotted dry with sterile filter paper and returned to the regeneration medium. Explants were co-cultivated with *Agrobacterium* for three days before being transferred to selection medium. Ten petri-dishes each containing five explants were inoculated with acetosyringone containing bacterial suspension and the same number of explants were inoculated with *Agrobacterium* without the acetosyringone.

Three petri-dishes each containing five explants were inoculated with untransformed bacteria to serve as negative control. A second negative control consisted of three petri-dishes of uninoculated explants. Tobacco leaves were inoculated with bacterial suspensions with and without acetosyringone added. These served as positive control of transformation.

5.3 **RESULTS AND DISCUSSION**

5.3.1 **Influence of kanamycin and cefotaxime on explant development**

No shoots developed on any of the media containing kanamycin. The explants remained small and did not expand in size. None of the explants on the 100 $\mu g\ ml^{-1}$, 200 $\mu g\ ml^{-1}$ and 300 $\mu g\ ml^{-1}$ kanamycin treatments turned green, but on the 50 $\mu g\ ml^{-1}$ kanamycin treatment, 50 % of the explants had visible pigment development.

All the explants placed on 250 $\mu g\ ml^{-1}$ cefotaxime doubled in size within 48 to 72 hours after commencing with the experiment, turned green within a week and developed shoots within two to three weeks. These developments were similar to those seen in the control treatment. In the control treatment, one explant failed to show shoot regeneration. Shoots that developed on the cefotaxime treatment appeared to develop faster, showed more vigorous growth and elongated better than those on the control medium. This observation was not quantified. The results are summarised in Table 5.1.

Table 5.1. The influence of the antibiotics kanamycin (Km) and cefotaxime (Cx) on the shoot regeneration ability of cotyledonary explants of *Cucumis melo* cv Hales Best 36.

Treatment	Explants with shoots (%)	Green explants (%)	Expanded explants (%)*
50 μg ml^{-1} Km	0	50	0
100 μg ml^{-1} Km	0	0	0
200 μg ml^{-1} Km	0	0	0
300 μg ml^{-1} Km	0	0	0
250 μg ml^{-1} Cx	100	100	100
Control	90	100	100

* Expanded explants were defined as explants that at least trebled in size.

Kanamycin acts as an inhibitor of protein biosynthesis by producing a misreading of the 30s-ribosome (Sigma Cell Culture catalogue, 1993). Severe inhibition of growth and development of sensitive tissues can be expected. According to DRAPER *et al.*, (1988), kanamycin resistance has been used successfully as a selectable marker in the transformation of several plant species. However, it is occasionally ineffective in some plant species, for reasons such as high tolerance of the plant tissues or supersensitivity. The cotyledonary tissues tested in this study did not show either high tolerance or the mass necrosis associated with supersensitivity. It was decided to include the antibiotic in selection media at a concentration of 100 μg ml^{-1}, the lowest concentration tested that completely inhibited explant development.

Cefotaxime is classified as a cephalosporin antibiotic with low eucaryote toxicity. It interferes with normal bacterial cell division at low doses. MATHIAS and BOYD (1986) reported that media supplemented with 60 or 100 μg ml^{-1} cefotaxime stimulated callus growth, embryogenesis and shoot regeneration in wheat (*Triticum*

aestivum L EM.THELL). A similar stimulatory effect was observed in studies on plant regeneration from passionfruit protoplasts (D'UTRA VAZ *et al.*, 1993). MATHIAS and BOYD (1986) suggested that the metabolism of cefotaxime by plant esterases may produce metabolites with growth regulator-like activity. The visual observations that were made in this study suggest that cotyledonary explants of *Cucumis melo* demonstrate a similar effect. These results show that 250 μg ml^{-1} cefotaxime can safely be used to inhibit *Agrobacterium* growth without negatively interfering with plant regeneration.

5.3.2 **Conjugation of recombinant plasmids into *Agrobacterium***

Small *Agrobacterium* colonies grew on selective plates containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin and were tested for transformation, using a GUS fluorometric assay. Transformed colonies showed bright fluorescence when the microtiter plate was placed on a transilluminator. Only colonies that showed substantial fluorescence was used to inoculate overnight cultures. The overnight cultures were tested again, using the same method. The cultures were stored in cryo vials at -70 °C and used to obtain fresh cultures when needed. These were always retested before use.

5.3.3 **The influence of co-cultivation time on transformation**

Agrobacterium growth was visible on all the explants, except for the 0 day treatment. This was expected, as the cefotaxime in the selection medium inhibits the growth of the bacteria within a very short time. No bacterial growth was visible on

explants on any of the treatments 16 hours after being transferred to selection medium.

Explants placed on the 0 and 1 day co-cultivation treatments remained relatively small and white, although some enlargement of the explants was observed. Callus formed on 11.4 % of the explants of the 1 day treatment, but no shoot regeneration was seen on any of the explants.

Shoots developed on explants co-cultivated with *Agrobacterium* for three days or more. The percentage of explants that showed regeneration were 47.6 %, 82.8 % and 88.5 % for the 3, 5 and 7 day treatments respectively (Figure 5.1). The number of shoots that developed per explant increased with increasing co-cultivation time, with 4.7 (± 7.2), 11.6 (±11.7) and 19.4 (± 11.8) shoots developing on the 3 day, 5 day and 7 day treatments respectively (Figure 5.2). The influence of co-cultivation time on transformation of melon was investigated previously by FANG and GRUMET (1990). These authors reported that callus and shoot production was achieved on treatments of 0 to 7 days co-cultivation. The highest number of explants with callus and developed shoots was observed for the three day co-cultivation treatment. This reduction in shoot production in longer co-cultivation times (5 and 7 days) was not observed in the current experiments.

Two other reports, those of CHEE (1990b) and CHEE and SLIGHTOM (1991) have appeared describing co-cultivation periods used during the transformation of Cucurbitaceae. In both these reports, a co-cultivation time of four days was successfully used in the transformation of cucumber.

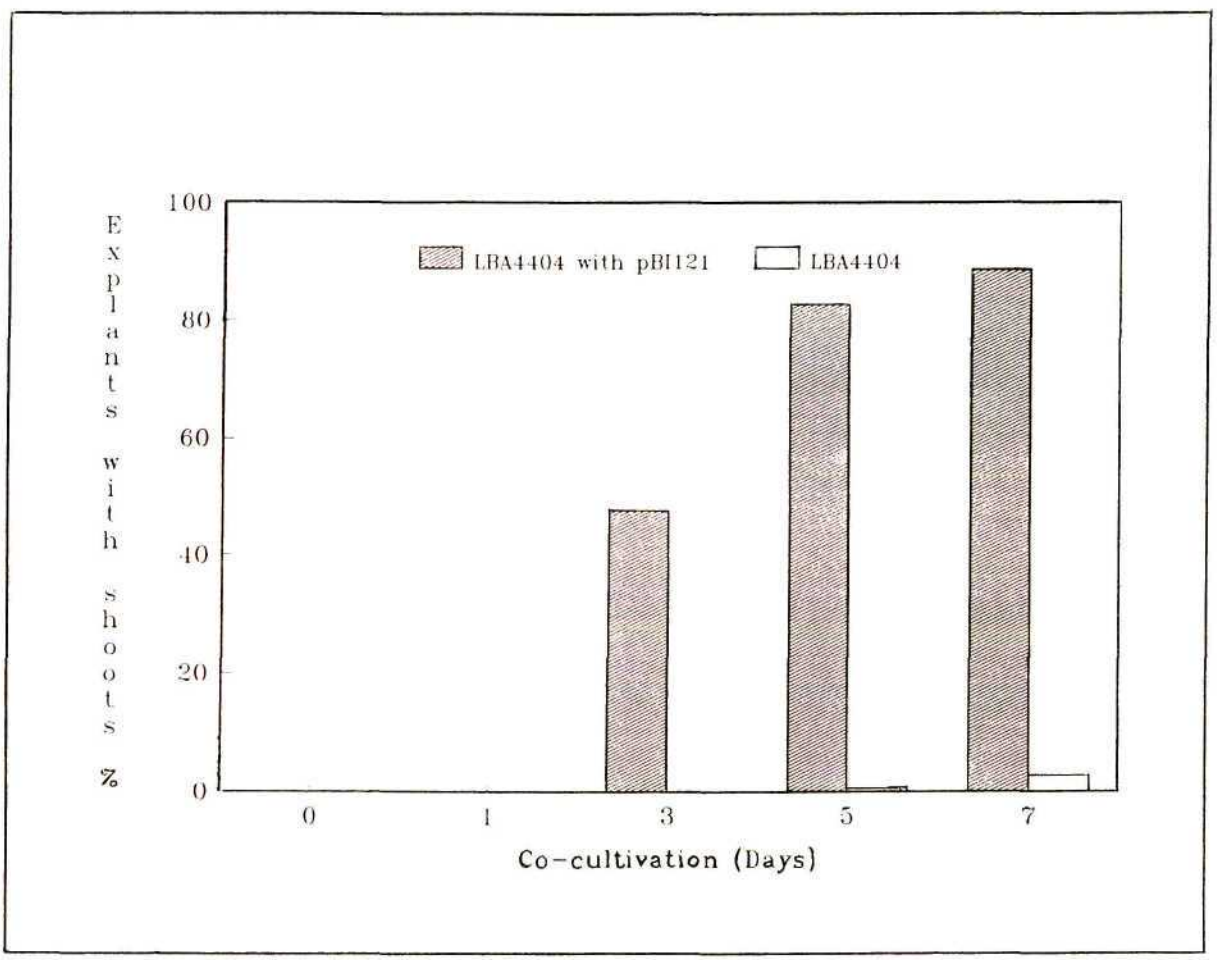


Figure 5.1. The influence of co-cultivation time on the percentage of explants showing shoot development. Explants were inoculated with the *Agrobacterium* strain LBA4404, containing the plasmid pBI121 (experiment), or with *Agrobacterium* LBA4404 without the plasmid (control).

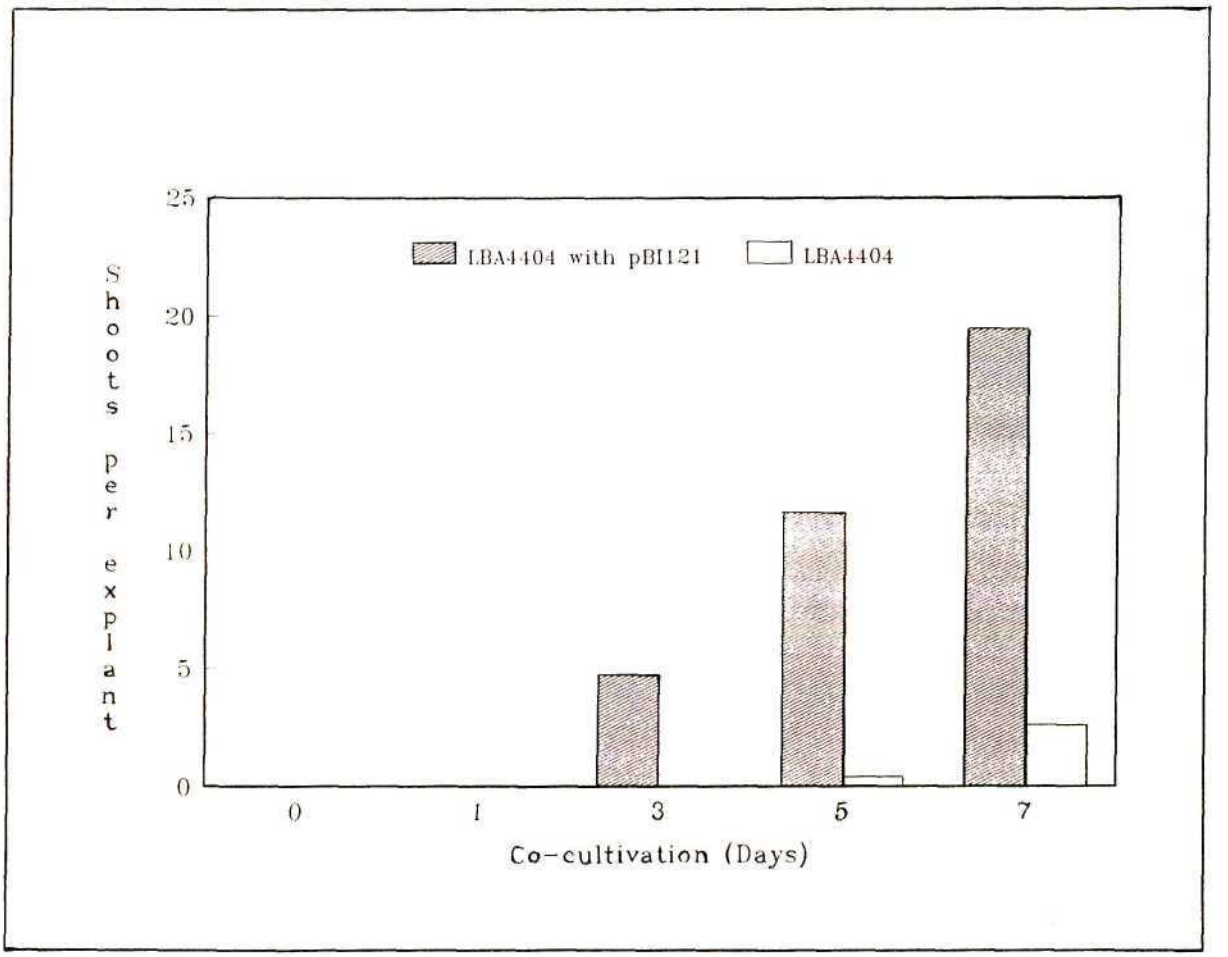


Figure 5.2. The influence of co-cultivation time on the number of shoots that developed on each explant. Explants were inoculated with the *Agrobacterium* strain LBA4404, containing the plasmid pBI121 (experiment), or with *Agrobacterium* LBA4404 without the plasmid (control).

Some of the shoots elongated when the explants were subcultured onto media without the growth regulator supplement with the sub-culture period extended to 3 weeks. These shoots were removed from the explants and placed on rooting medium containing the two antibiotics. On this medium, most of the shoots turned white and died. In previous regeneration experiments (Chapter 2), tissue death was usually associated with browning, not with bleaching. This is evidently the result of kanamycin inhibition of protein biosynthesis. Most shoots had to be cultured for at least two months and up to three months after the experiment was initiated, before these tissues died. In the 5 day and 7 day co-cultivation treatments, some bacterial growth became visible again between successive subcultures. A number of the shoots were tested for transformation, using the quantitative fluorometric GUS assay, but none of the shoots that eventually died showed signs of fluorescence. Only two shoots, both from the 3 day co-cultivation treatment were successfully rooted (Figure 5.3). Roots formed within ten days on selection media and rooted plantlets could be repeatedly subcultured. These plantlets were successfully transferred to soil (Figure 5.4). These two shoots gave positive results when they were tested for the presence of the GUS gene using both the fluorometric and the histochemical assays.

The negative control explants, co-cultivated with untransformed *Agrobacterium*, showed very little shoot regeneration. A few shoots developed on the 5 and 7 day treatments, but the number of shoots per explant was very low; 0.4 and 2.6 for the two treatments respectively (Figure 5.2). The shoots all turned white. This is characteristic for untransformed explants when cultured on kanamycin containing media. The uninoculated control explants also showed a low shoot regeneration response on the 5 and 7 day treatments, with 1.2 and 1.8 shoots per explant developing on these treatments. These shoots also bleached and died.

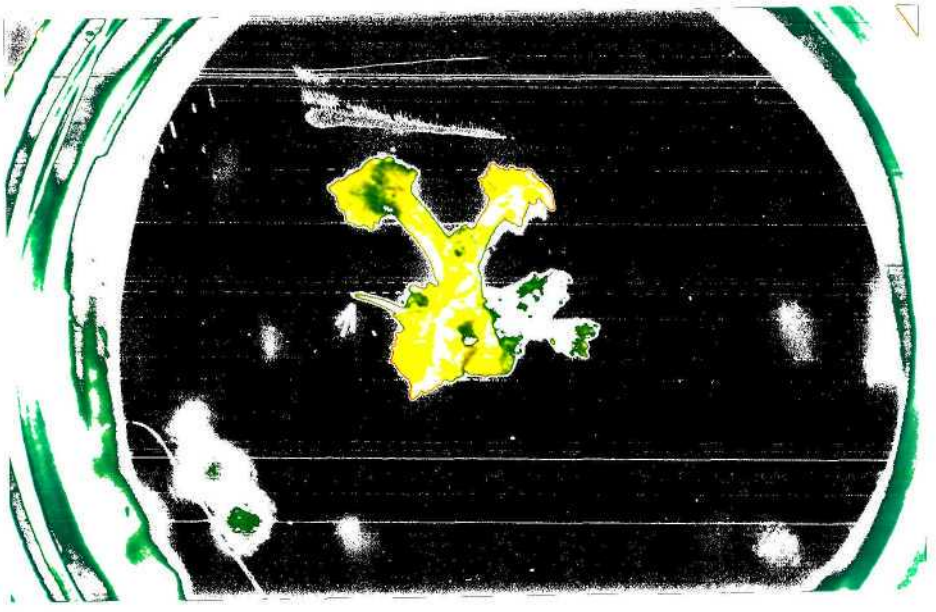


Figure 5.5. Root development on a regenerated shoot that was co-cultivated with *A. tumefaciens* LBA4404 (pBI121) for three days, cultured on MS medium supplemented with 2 mg l^{-1} BA, 250 µg ml^{-1} cefotaxime and 100 µg ml^{-1} kanamycin.

Figure 5.6. A hardened off, Rules Dec. 36 plant that has been transformed with *Agrobacterium tumefaciens* LBA 4404 containing the plasmid p121.

Numerous plantlets developed on the tobacco leaves used for the positive control treatment. The plantlets were not counted, as the purpose of this treatment was only to verify the ability of the *Agrobacterium* culture to effect transformation. Most of the shoots that developed were rooted with great ease on MS medium supplemented with both antibiotics. The roots and shoots of these plantlets tested positive for transformation using the GUS quantitative fluorometric assay.

Results from the fluorometric assay were photographed using a Sony UP-850 video graphic printer (Figure 5.5). The microtiter wells A1, B2, C3 and D4 contained only buffer and did not show fluorescence. Fluorescence was seen in wells B12 and C2, that contained positive *Agrobacterium*, A3 and A4 (positive control tobacco leaf and root) and wells containing tissues from rooted melon plants (B3 and C4, leaf tissue, B4 and D5, stem tissue, and C5, root tissue). Only one well with roots from a selected plant did not show fluorescence. Non-transformed tobacco (A4, leaf, and A5, root) and melon tissues (D1 and D2, leaf, and D3 root) did not show any fluorescence.

The characteristic blue precipitate associated with incubation of ß-glucuronidase with X-gluc buffer, formed in tissues from selected, rooted plantlets. The site of enzyme activity could be identified in sections of plant tissues. Expression was not uniform through the whole plant and even different roots from the same plant did not show the same pattern of expression. Expression could be localised in the epidermal and cortical cells and root hairs (Figure 5.6A and 5.6B), with no expression in the transport tissues, or strong expression could be seen in the xylem and phloem, with little expression in the cortex and none in the epidermis, including the root hairs (Figure 5.7A and 5.7B). In a longitudinal section (Figure 5.8), the GUS activity in the meristem cells of the developing secondary root can be clearly seen.

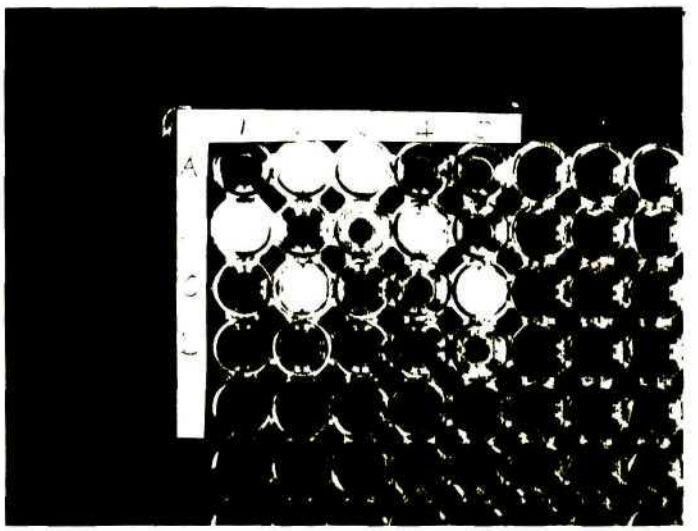


Figure 5.5. GUS qualitative fluorometric assay.

Well	Contents
A1, B2, C3, D4	Assay buffer (blank).
A2	Tobbacco (positive control) leaf
A3	Tobbacco (positive control) root
A4	Non transformed tobbacco leaf
A5	Non transformed tobbacco root
B1, C2	*Agrobacterium* LBA4404 containing pBI121
C1	*Agrobacterium* LBA4404
D1, D2	Non transformed melon leaf
D3	Non transformed melon root
B3	Transformed Hales Best 36 leaf
B4	Transformed Hales Best 36 stem
B5	Transformed Hales Best 36 root
C4	Transformed Hales Best 36 leaf
C5	Transformed Hales Best 36 root
D5	Transformed Hales Best 36 stem

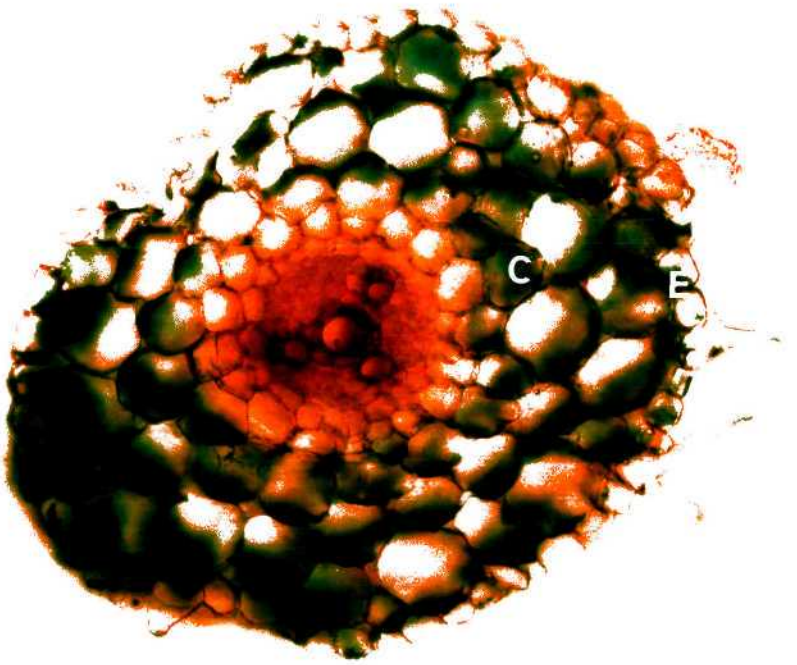


Figure 5.6A. Micrograph of a cross-section of Hales Best 36 root tissue that was inoculated with *A. tumefaciens* LBA4404 (pBI121). The root was incubated overnight in X-gluc buffer. The blue precipitate, located mainly in the epidermal (E) and cortical (C) cells, indicates sites of ß-glucuronidase enzyme activity.

Figure 5.6B. Micrograph of a longitudinal view of Hales Best 36 root tissue that was inoculated with *A. tumefaciens* LBA4404 (pBI121). The root was incubated overnight in X-gluc buffer. A blue precipitate indicates sites of ß-glucuronidase enzyme activity. The pattern of GUS expression is similar to that in Figure 5.6A.

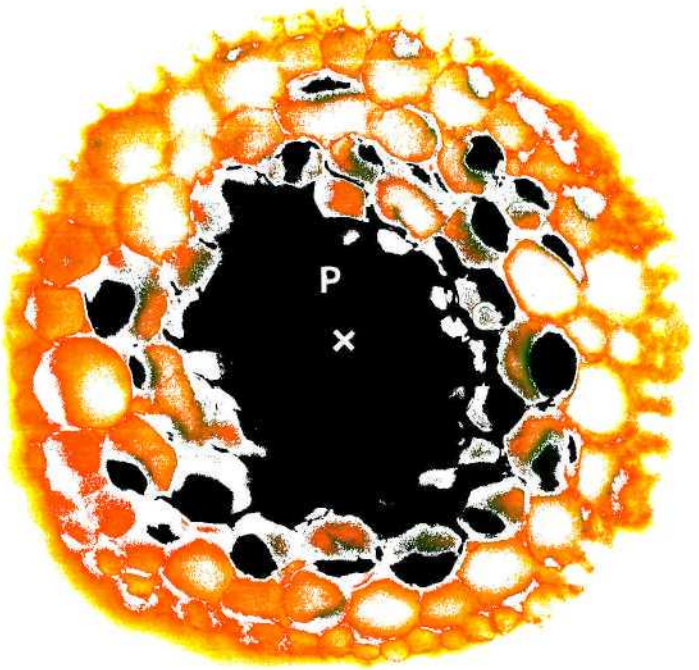


Figure 5.7A. Micrograph of a cross-section of Hales Best 36 root tissue that was inoculated with *A. tumefaciens* LBA4404 (pBI121). The root was incubated overnight in X-gluc buffer. ß-glucuronidase enzyme activity is visible in the cortex (C) and transport tissues (X-xylem, P-phloem).

Figure 5.7B. Micrograph of a longitudinal view of Hales Best 36 root tissue that was inoculated with *A. tumefaciens* LBA4404 (pBI121). The root was incubated overnight in X-gluc buffer. ß-glucuronidase enzyme activity is located mainly in the transport tissues (T), with some expression in the cortical (C) cells.

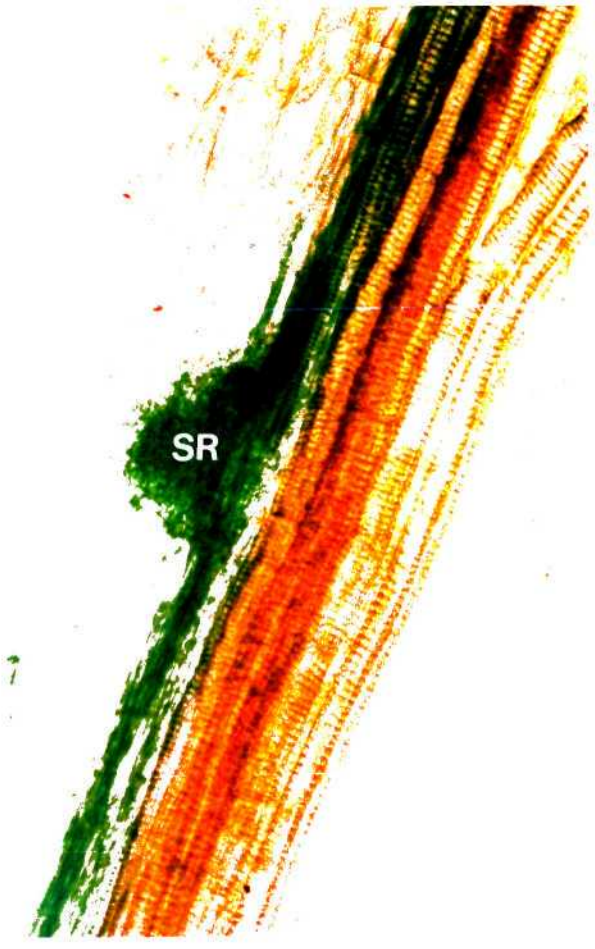


Figure 5.8. Longitudinal section of Hales Best 36 root tissue that was inoculated with *A. tumefaciens* LBA4404 (pBI121). The root was incubated overnight with X-gluc. A developing secondary root (SR) can be seen.

The precipitate in the xylem can be attributed to export from the strongly expressing phloem, as the cleaved substrate is soluble. The formation of the blue precipitate in leaf tissue was sometimes confined to the tip of the leaf (Figure 5.9), or could be seen distributed throughout the leaf in epidermal, trichomal and parenchyma tissues. In stem sections, ß-glucuronidase was expressed in all the tissues, resulting in uniform blue sections. Sections through the positive control tobacco stems incubated in X-gluc, stained blue in the epidermal, cortical and phloem tissues, but not in the xylem. No expression was ever observed in non-transformed stems, leaves or roots (Figure 5.10).

The quality of the histochemical localization is dependent on numerous variables such as the preparation of the tissue, physiological state of tissues, permeability of the tissues to the buffer and fixation of tissues (JEFFERSON *et al.*, 1987). Furthermore, it has been shown that kanamycin resistant potato transformants did not have detectable GUS activity, despite the presence of at least three copy numbers of the gene, determined through Southern blots (OTTAVIANI and HÄNISCH TEN CATE, 1991). This might be due to the specific methylation of the CaMV35S/GUS gene. PEACH and VELTEN, (1991) reported intra-clonal variability in transformed tobacco callus lines and came to a similar conclusion regarding methylation. All these factors might play a role in the variability of expression that was observed in the tissue sections.

The increase in the percentage of explants with shoot buds as well as the increase in the number of shoots per explant could be attributed to shoot initiation prior to the introduction of kanamycin in the medium. The longer the time that the explant was cultured on regeneration medium without selective antibiotics, the more shoot buds could be initiated in the explant. When explants were tested to investigate

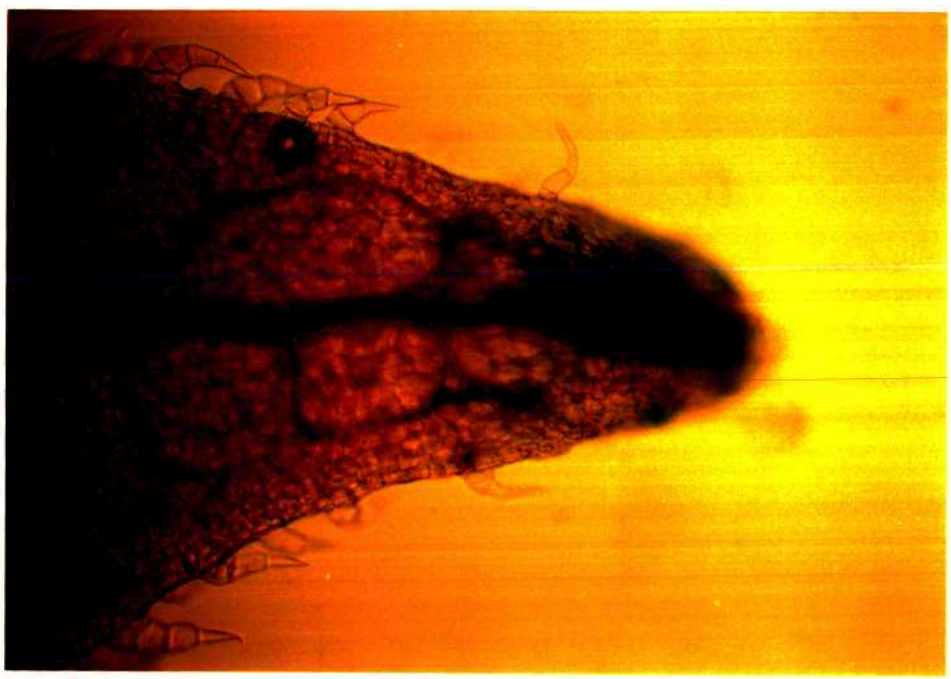


Figure 5.9. Adaxial view of a Hales Best 36 leaf, showing GUS enzyme activity in the tip of the leaf in the transport tissues.

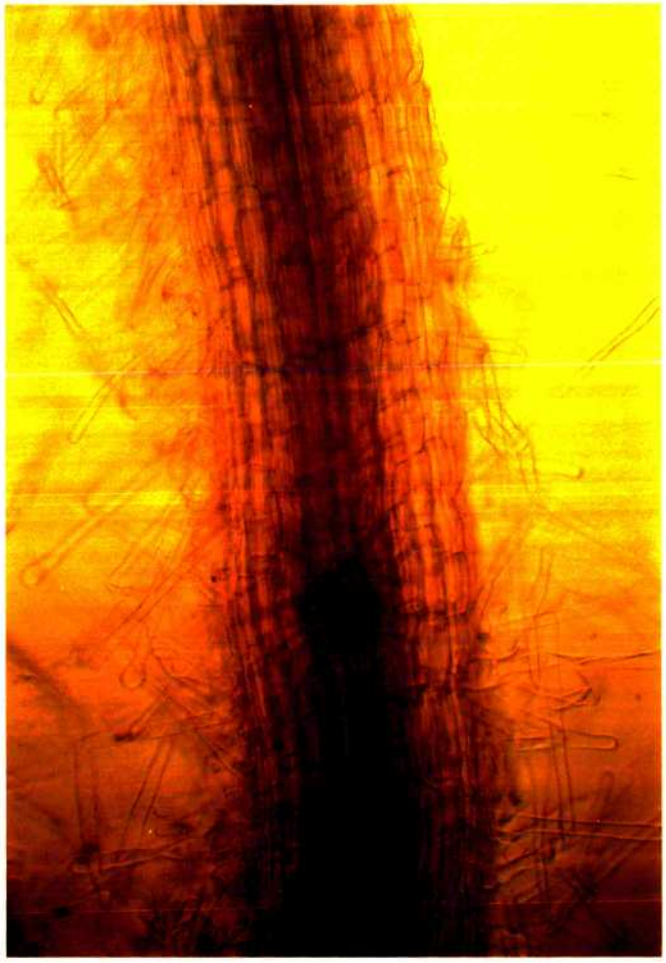


Figure 5.10. Longitudinal view of an un-inoculated root that was incubated overnight in X-gluc.

the influence of kanamycin on shoot regeneration, no allowance was made for the possible effects of co-cultivation times. It is clear that kanamycin inhibits the development of shoots, but once the shoots are initiated, this influence is reduced.

Another possible reason for the increase in shoot formation could be that more of the explant tissue was transformed, the longer explants were co-cultivated with *Agrobacterium*, but that this transformation was transient, or that no regeneration occurred in transformed tissue. In both cases, the concentration of kanamycin in the explant as a whole would be reduced when transformed cells act as feeders. This would allow untransformed tissue to grow, but as soon as shoots were detached from the explant and cultivated separately, the untransformed tissue would die. According to PEACH and VELTEN (1991) many plant cells receiving new DNA may express introduced genes at very low levels. Expression of the neomycin phosphotransferase II gene at low levels would also lead to a lowering of the kanamycin concentration in the explant, allowing more shoots to develop, but the concentration might be too low to allow long term survival of transformed tissues.

The evidence from the negative control and uninoculated control explants would support an explanation of improved transformation with limited regeneration. Very little shoot development occurred in these explants. If the effects of kanamycin on initiated shoot buds were reduced compared to the effects of kanamycin on a newly cultured cotyledonary explant, the same number of shoots should initially form on negative controls during the co-cultivation period. These should then die at the same rate as untransformed shoots from the treated tissues. This clearly did not happen.

The number of shoots that developed on explants in the 5 and 7 day treatments was higher compared to the number of shoots that developed on explants during the

regeneration experiments described in Chapter 2. When the experiments are compared, the differences in the ways explants were treated are significant and would explain this result. It is possible that shoot development is inhibited when explants are subcultured every third to fourth week due to depletion of the medium. The explants in the transformation experiment were subcultured once a week, providing a more constant source of nutrients and growth regulators. A second explanation may be the stimulatory influence of cefotaxime that was mentioned above. Lastly, the explants in the transformation experiment were wounded deliberately during preparation. This resulted in the breaking up of a large number of the explants. Shoots were removed from the explant as soon as they were long enough to detach without damage. This would remove the apical dominance effect a developing shoot might exert over the surrounding axillary buds (PIERIK, 1987).

The non-uniform distribution of GUS activity that could be visualised in the histochemical test and the absence of a positive reaction of some plant parts using the qualitative fluorometric test, could be attributed to a number of factors: a) The availability of the precursors necessary for the manufacture of the enzyme might vary and lead to heterogenous expression. b) Inhibitors of transcription and translation could have reduced expression. c) Inhibitors of enzyme activity might be present in certain cells. d) A position effect (SPOFFORD, 1976) could cause genes to be active in some cells and not in others, when the action of a gene is dependent on its position with respect to neighbouring genes. e) The activity of the CaMV 35S promotor has been shown to be particularly high during the S phase of the cell cycle (NAGATA *et al.*, 1987). This would explain the prominent expression that was observed in the developing secondary root (Figure 5.8).

The results from this experiment indicate that the co-cultivation period has a marked influence on explant and shoot development during transformation experiments in Hales Best 36. The low transformation frequency that was obtained makes differences between treatments difficult to assess, but a number of deductions can be made. Explants should be co-cultivated with *Agrobacterium* for at least three days after inoculation. If the co-cultivation time is extended further, the number of shoots that develop increases, but non-transformed shoots have to be cultured for extended periods before this can be established. Problems with *Agrobacterium* contamination may be experienced.

In the light of the above, two distinct approaches can be followed regarding the co-cultivation time. Explants can be co-cultivated for the minimum period necessary to achieve transformation. The benefit of this approach will be the smaller number of untransformed shoots that has to be selected against. On the other hand, if the co-cultivation period is extended, the possibility of transformation taking place is increased as well. The greater number of untransformed shoots can be dealt with if shoots are placed on rooting medium as soon as possible, tested for the GUS gene expression and discarded if no root development takes place within 15 days.

5.3.4 **The influence of inoculation time on transformation**

Agrobacterium growth was visible on all the treatments, but growth was noticeably less on the 2 minute treatment than on the other treatments. Expansion of explants was seen on all the treatments. Shoot development was observed for all five inoculation treatments (Figure 5.11), with the highest percentage of explants responding to the 10 minute inoculation treatment and the lowest percentage

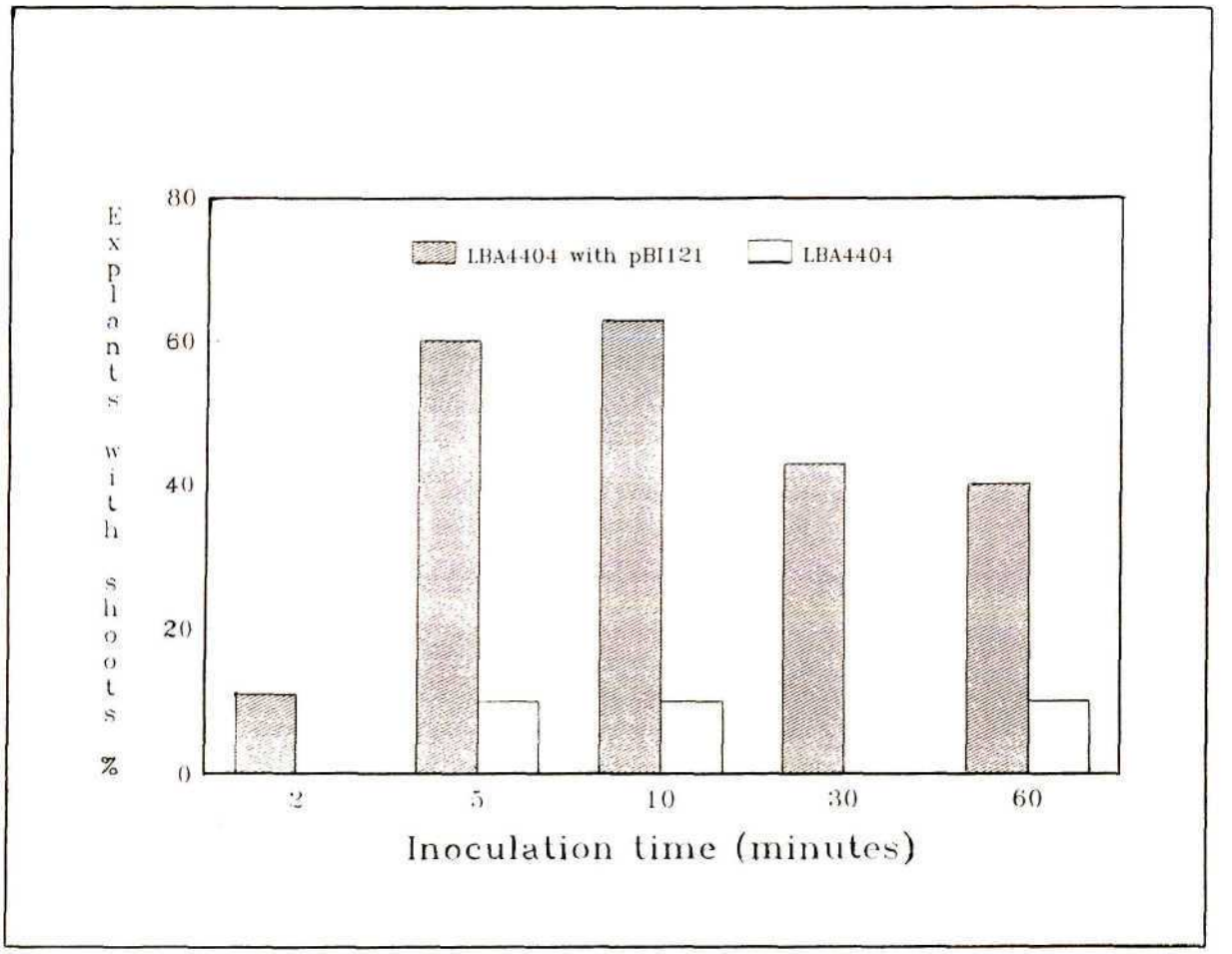


Figure 5.11. The influence of inoculation time on the percentage of explants with shoot development. Explants were inoculated with *Agrobacterium* strain LBA4404, containing the plasmid pBI121 (experiment) or with *Agrobacterium* LBA4404 without the plasmid.

responding to the 2 minute treatment. The number of shoots per explant was highest in the 5 minute treatment (Figure 5.12). None of the observed differences were significant, but in view of the general trend, an inoculation time of between 5 and 10 minutes could be suitable for transformation of the Cucurbitaceae. Most of the shoots bleached in a very short time and did not develop further. Only two shoots could be rooted on the kanamycin containing rooting medium, one from the 2 minute treatment, and one from the 5 minute treatment. Again, this low transformation frequency makes it difficult to make reliable decisions regarding the length of inoculation.

5.3.5 **The effect of acetosyringone on transformation**

Both times this experiment was done, no differences could be seen between the explants inoculated with *Agrobacterium* cultures exposed to acetosyringone and those cultures that were not. In this experiment, explants were destroyed as soon as they became severely bleached, therefore the number of shoots per explant was not determined. Two developed shoots could be rooted on selection rooting medium containing kanamycin, but both of these developed on explants inoculated without acetosyringone. To date, the experience in our laboratory has been that no benefits are derived from the use of acetosyringone in *Agrobacterium* transformation systems (MURRAY, S. and DE VILLIERS, S.M. personal communication 1993). This is true for transformation of tobacco, potato and *Ornithogalum* sp.

5.3.6 **Determination of ß-glucuronidase activity using a quantitative GUS-fluorometric assay**

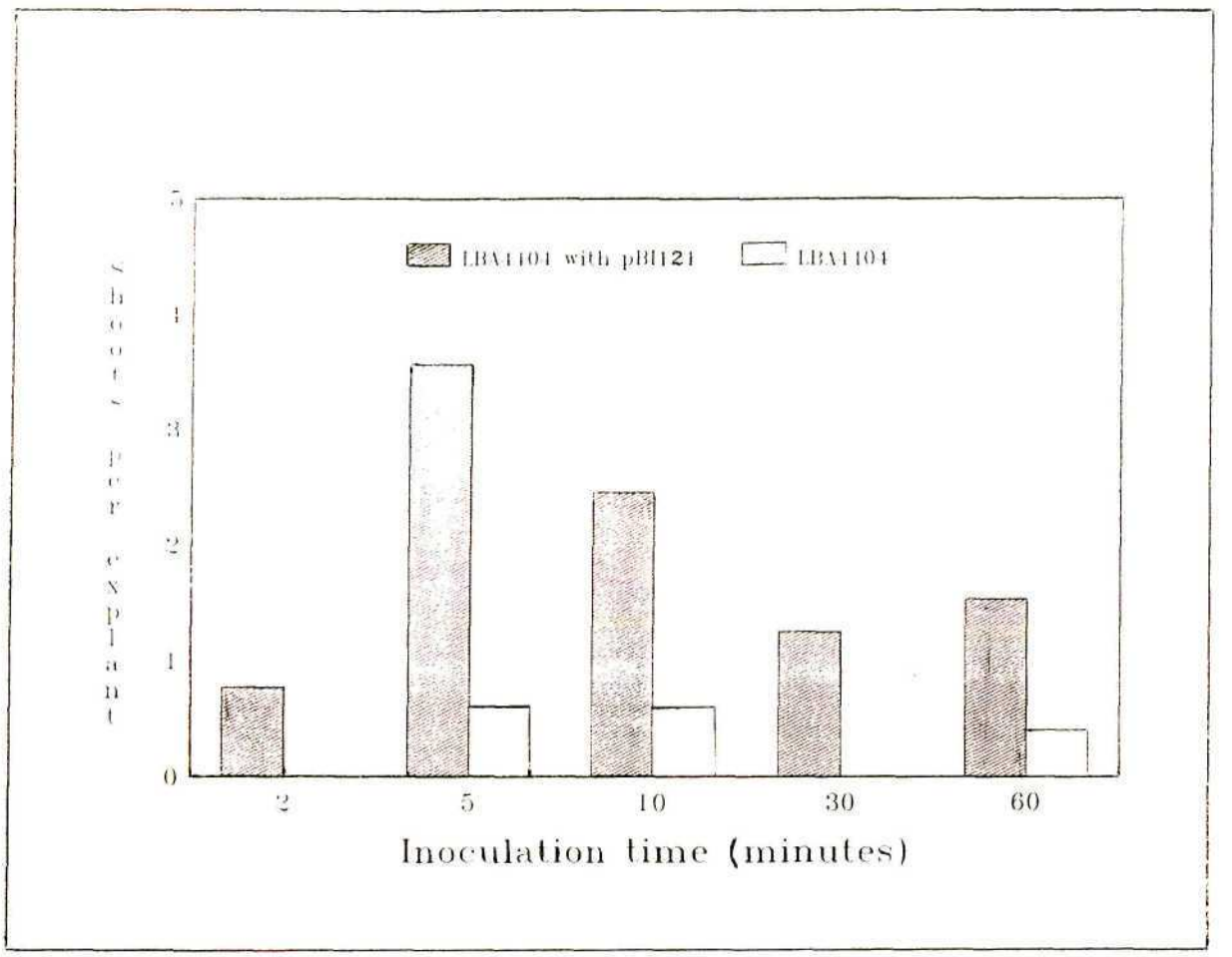


Figure 5.12. The influence of the inoculation time on the number of shoots that developed on each explant. Explants were inoculated with *Agrobacterium* strain LBA4404, containing the plasmid pBI121 (experiment) or with *Agrobacterium* LBA4404 without the plasmid.

The protein content of the tissue extracts were obtained from the standard curve (Figure 5.13). The MU standard curve that was obtained is shown in Figure 5.14 and was used to calibrate the fluorometer. The treated Hales Best 36 and tobacco tissues both produced the fluorescing product methyl umbelliferone (MU) from the substrate 4-methyl umbelliferryl ß-D-glucuronic acid (MUG). Figure 5.15 and Figure 5.16 illustrates the production of MU by Hales Best 36 and tobacco extracts respectively. In both these tissues the amount of MU that was produced increased linearly over time. The uninoculated Hales Best 36 and tobacco tissues did not produce measurable fluorescence. This is an indication that these tissues lack endogenous ß-glucuronidase activity. Contrary to the report by JEFFERSON *et al.*, (1987), HODAL *et al.*, (1992) found detectable ß-glucuronidase activity in a number of non-transgenic plants, but in this study this was not observed.

5.4 **CONCLUSION**

Transformation of *Cucumis melo* cv Hales Best 36 via an *Agrobacterium tumefaciens* protocol was achieved, but the frequency of transformation was very low. The co-cultivation period proved to have a significant effect on the outcome of the transformation. The inoculation times that were investigated were tested using a short co-cultivation period and the shoot regeneration was lowered accordingly. This made it very difficult to draw conclusions regarding the influence of this parameter on transformation. Addition of acetosyringone in the inoculation bacterial suspension did not improve the transformation frequency measurably. All three the GUS assays that were used showed GUS activity only in inoculated tissues. The qualitative and histochemical assays were easy to use and gave rapid results.

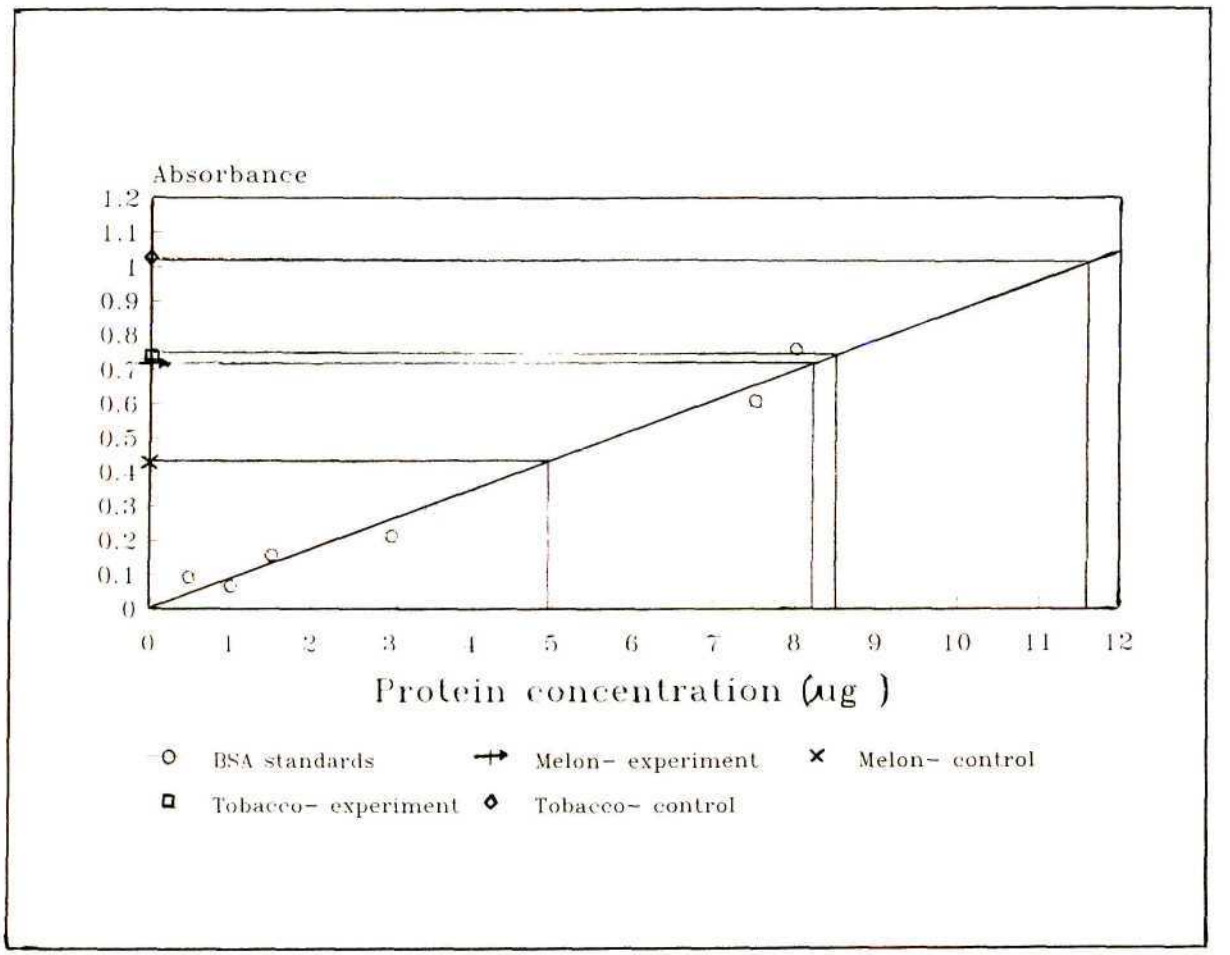


Figure 5.13. Standard protein concentration curve that was used to determine the protein concentration of plant extracts. The absorbance was measured at 595 nm.

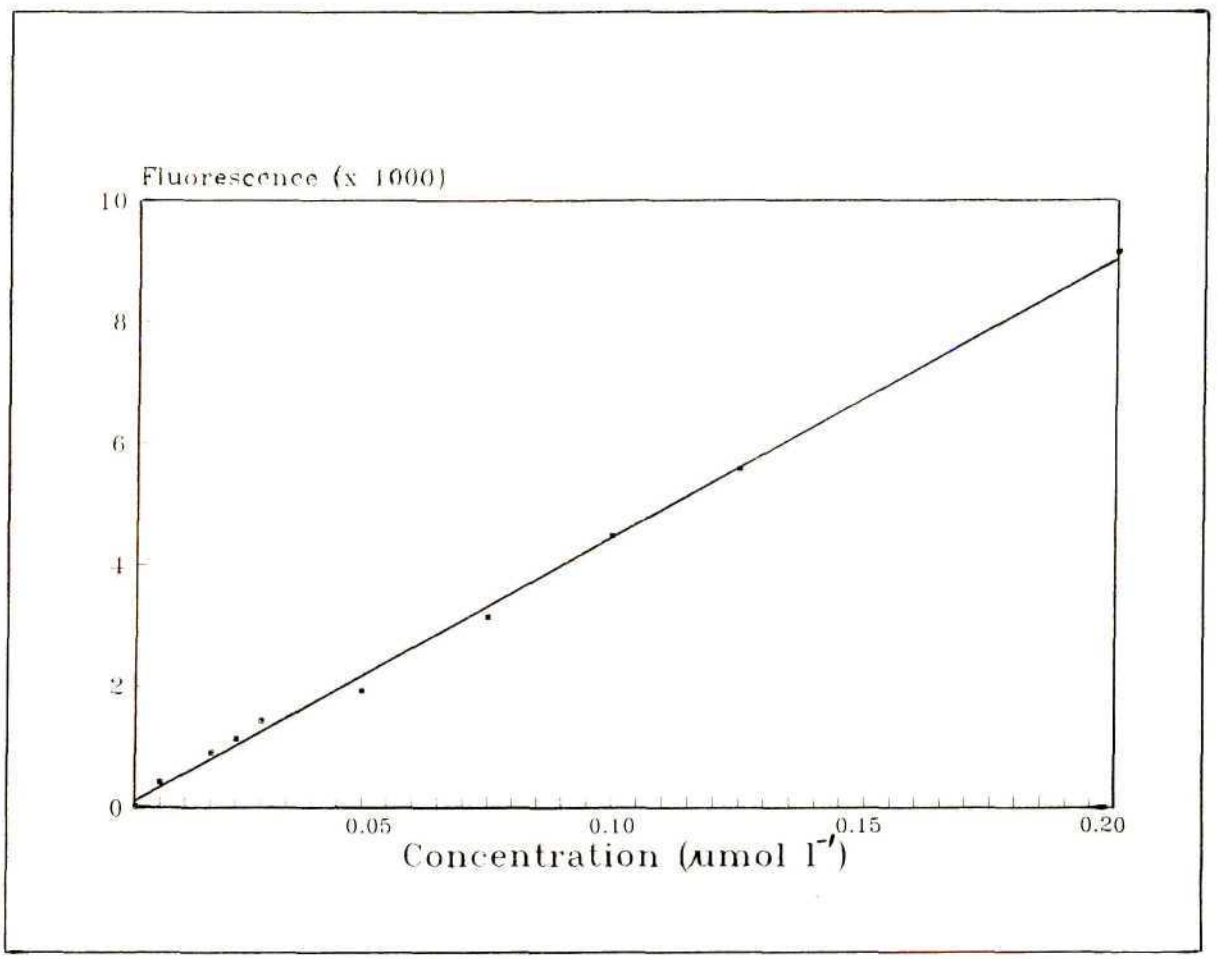


Figure 5.14. Fluorescence of standard concentrations of methyl umbelliferone.

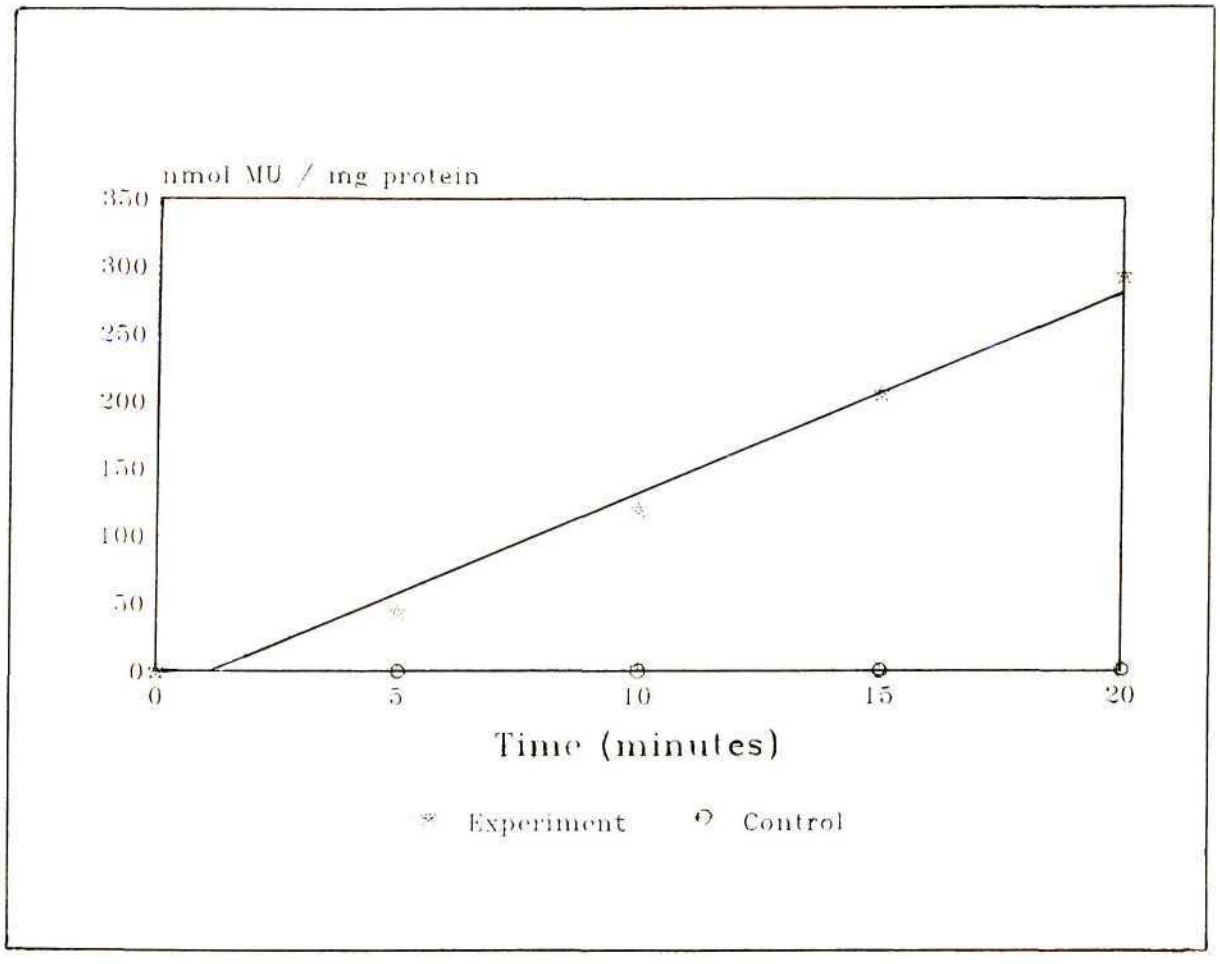


Figure 5.15. GUS activity in a leaf of transformed melon Hales Best 36.

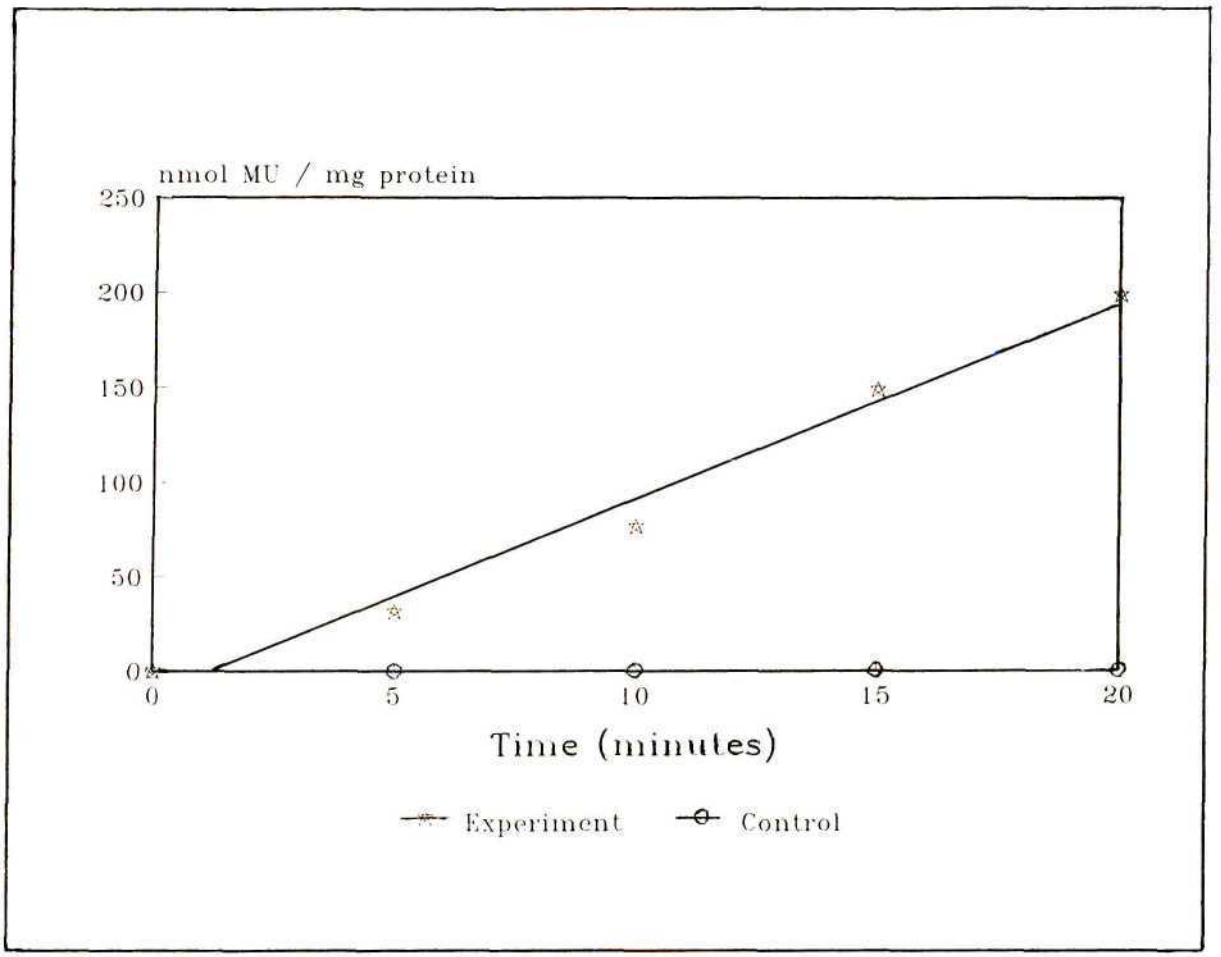


Figure 5.16. GUS activity in a leaf of transformed tobacco (positive control).

CHAPTER 6

BIOTRANSFORMATION OF *CUCUMIS MELO* CV HALES BEST 36 USING THE *PAT* GENE

6.1 INTRODUCTION

Herbicides are an indispensable part of modern agriculture and allows for rapid, efficient and economical weed control. A number of new herbicides such as the herbicide Ignite®, combine high effectivity with non-toxicity to mammals and rapid degradation in soil. However, the non-selectivity of the herbicide limits use to pre-emergence applications (DE BLOCK *et al.* 1987, DE GREEF *et al.* 1989). The development of herbicide resistant crops will expand the possible applications of this chemical to include post-emergence utilisation for crop protection.

The established *Agrobacterium tumefaciens* mediated transformation protocol was used to transform *Cucumis melo* cv Hales Best 36 with the *pat* gene. As before, the *Agrobacterium* strain LBA 4404, was used. The plasmid pBI101 containing a cassette with the neomycin phosphotransferase (NPT II) gene, driven by a NOS promoter and terminator, and a promotorless glucuronidase (GUS) gene was introduced into the strain. The *pat* gene is under control of the cauliflower mosaic virus 35S promotor. This casette is illustrated in Chapter 1 Figure 1.2.

A phosphinothricin selection medium was developed for cotyledonary explant tissues. In the transformation experiments that followed, plantlets were placed on

this medium to select transformants. Five lines were recovered. The plants were acclimatized and herbicide resistance was confirmed in glasshouse trials. The detection of the *pat* gene in the genomes of these lines confirmed the successful transformation of these plants and is the subject of Chapter 7.

Several reports have appeared describing the occurrence of various degrees of polyploidy and aneuploidy in cell and tissue cultures of members of the Cucurbitaceae (FASSULIOTUS, 1990; ADELBERG *et al.*, 1994; EZURA and OOSAWA, 1994). Although tetraploids are rare and sought after by breeders, this variation is not desirable in transformants. In this study the ploidy level of the lines that were obtained was deduced from indirect evidence of micro- and macroscopic characteristics that had been shown to have a correlation with the chromosome number (FASSULIOTIS and NELSON, 1992; NUGENT and RAY, 1992).

6.2 **MATERIALS AND METHODS**

6.2.1 **Determination of selection levels for phosphinothricin**

Cotyledonary explants of the cultivar Hales Best 36 were prepared as described in Chapter 2. It was cultivated on regeneration medium, consisting of MURASHIGE and SKOOG (1962) medium with 3 % (w/v) sucrose, 0.75 % (w/v) agar, 1 mg l^{-1} 6-benzylaminopurine (BA) and 0.5 mg l^{-1} indole 3-acetic acid (IAA). The explants were subcultured to regeneration media containing 0, 1, 5, 10, 50 or 100 mg l^{-1} phosphinothricin. Each treatment consisted of six petri dishes with six explants in each dish. Explants were regularly examined for emergence of shoots. Results were

noted after eight weeks of culture. In a second experiment, five Hales Best 36 seeds were germinated on MS medium and subcultured twice to MS medium. Half of the resulting explants were then transferred to MS medium supplemented with 10 mg l^{-1} phosphinothricin.

6.2.2 **Transformation and regeneration**

The *Agrobacterium tumefaciens* transformation protocol described in Chapter 5 was adapted for the introduction of the *pat* gene.

6.2.2.1 **Bacteria**

The *Agrobacterium* strain LBA4404 was used for the transformation as described in Chapter 5, but it contained a different plasmid, pBI101/*pat* with the *pat* gene as illustrated in Figure1.2 B. This plasmid allows for the selection of transformed plants on kanamycin sulphate containing medium, but the GUS gene in this plasmid is non-functional in plants. However, the *pat* gene serves as a second selectable marker. Overnight bacterial cultures were prepared from colonies picked from selective Luria agar plates. The cultures were grown in Erlenmyer flasks containing 10 ml Luria broth supplemented with 100 µg ml^{-1} rifampicin and 100 µg ml^{-1} kanamycin. Colonies were tested for transformation prior to use. The GUS gene is expressed in the bacteria and the cultures were assayed using a qualitative GUS fluorometric assay (JEFFERSON, 1987) as described in Chapter 5.

6.2.2.2 **Plant material and media**

Explants were prepared as described previously and placed on regeneration medium immediately prior to inoculation. In the third experiment, explants were precultured for 48 hours prior to inoculation. In the first experiment, 175 explants were inoculated, in the second experiment 120 were inoculated and in the third experiment 90.

The regeneration medium consisted of MURASHIGE and SKOOG (1962) medium with 3 % (w/v) sucrose, 0.75 % (w/v) agar, 1 mg l^{-1} BA and 0.5 mg l^{-1} IAA. The selection medium was similar to the regeneration medium, but it was supplemented with the antibiotics kanamycin and cefotaxime at concentrations of 200 µg ml^{-1} and 250 µg ml^{-1} respectively. This was double the concentration of kanamycin that was used previously (Chapter 5) to allow for more stringent selection. Plants were maintained in screw-top bottles on MS medium containing no growth regulators, as prolonged exposure to BA appeared to cause hyperhydricity and IAA induced callus formation. Initially, both kanamycin and cefotaxime were added to the rooting medium which consisted of MS medium with 3 % (w/v) sucrose, 0.75 % (w/v) agar and 2 mg l^{-1} 3-indolebutyric acid (IBA). Later, as *Agrobacterium* contamination disappeared, these antibiotics were excluded from all the media and 10 mg l^{-1} phosphinothricin was included as selection agent.

6.2.2.3 **Inoculation and co-cultivation**

Explants were inoculated by pipetting 40 µl bacterial suspension onto each explant. This was an adequate volume to cover the explant completely. After five minutes

explants were blotted dry with sterile filter paper and returned to the regeneration medium. The second and third times the experiment was repeated, the explants were placed under partial vacuum for 20 minutes before they were blotted dry. Explants were co-cultivated with the *Agrobacterium* on regeneration medium for six days in the case of the first experiment, and four days for the second and third experiments. The co-cultivation period depended on the growth of *Agrobacterium* on the explants.

6.2.2.4 **Plant regeneration and selection**

After the co-cultivation period, explants were placed on regeneration medium and subcultured weekly for six weeks. Thereafter, explants were subcultured once a month. The second and third experiments were only subcultured after three to four weeks, as it was observed that this prevented the occurrence of hyperhydricity to some extent. Developing shoot buds were transferred to maintainance medium. Elongated shoots were removed from the explants and placed on rooting medium.

6.2.2.5 **Controls**

A tobacco (*Nicotiana tabacum*) leaf disk transformation was included as positive control for all three experiments. The leaf disks were inoculated with the culture containing the plasmid and co-cultivated for two days prior to selection. A negative control was included for each treatment. Negative controls consisted of *Cucumis melo* cv Hales Best 36 cotyledonary explants and tobacco leaf disks, treated in exactly the same way as the test explants, except that they were inoculated with

Agrobacterium LBA4404 without the plasmid. A third control consisted of melon explants not exposed to any *Agrobacterium*.

6.2.3 **Acclimatizing**

Initially, plantlets with a well developed root system were transferred to bottles containing sterilised vermiculite soaked in liquid MS containing 2 mg l^{-1} IBA. Plants were kept in the growth room, allowed to develop a good root system and transplanted to pots containing sterilised soil. Plants were then acclimatized in the glasshouse. The plantlets were covered with a clear plastic bag and over the next week, the bags were punctured progressively every day to allow the plants to adapt to the lower humidity. Subsequently, the low survival rate and the discovery of malformed stomata led to the development of an improved protocol. Plantlets still growing in maintenance medium were placed in the glasshouse until new leaves formed. The plantlets were then transferred to bottles containing sterile vermiculite and halfstrength MURASHIGE and SKOOG (1962) medium, supplemented with 2 mg l^{-1} IBA and 2 (w/v) % sucrose. The bottles were returned to the glasshouse until new root and shoot growth could be observed. The lids were then gradually opened, a little bit every day, until the plants could survive in completely opened bottles. Only when two to four new leaves had formed under greenhouse conditions were the plants transferred to sterile soil in pots.

6.2.4 **Estimation of ploidy level of regenerated lines**

Leaf disks were collected from *in vitro* grown plants. Small pieces were exised, stained with KI stain, covered with a coverslip and examined under the microscope.

The dark staining chloroplasts within each pair of guard cells were counted and recorded. The count in 25 pairs were recorded for each line as well as the control plants. The counts were conducted on five different leaves collected from *in vitro* grown plants, five stomata per leaf. A Student's t-test was performed on the data, using the programme Quattro Pro version 6.0. In the test, the data from each of the five lines were compared to the data from control plants that were established from seed.

Pollen shape was determined by microscopic examination after staining with 2 % (w/v) acetocarmine stain. Three flowers of each line were examined for the presence of pollen that had non-triangular shapes. The presence of these pollen grains were noted, but the prevalence was not quantified.

The diameter of the corollas of newly opened male and female flowers were measured at their widest point. The flowers were measured on the vines and were not removed. Measurements started simultaneously on all lines and continued daily until the diameter of ten male and ten female flowers were obtained for each line. Only one female flower was ever observed for Line 3 and that developed prior to the start of the measurements. The data was subjected to a Student's t-test, using the programme Quattro Pro version 6.0.

Observations were made on the appearance of the leaf edges, the blossom-end scar of the fruit and the appearance of the seed. These subjective characteristics were not quantified.

6.2.5 **Greenhouse spray tests**

Acclimatized plants of untransformed Hales Best 36 and Lines 1 to 5 were subjected to greenhouse spray tests. Four vines, from the same or different individual plants were selected for each line and sprayed with solutions of 0.1 %, 0.5 %, and 2.0 % (v/v) Ignite® (200 mg ml^{-1} active ingredient). In the case of Line 1, only three vines were available to be sprayed and the 2 % spray was omittted. The solutions were applied with a handheld spray can. The vines were sprayed overall until completely wet. The vines were examined on a daily basis and results were noted after seven days. The results were confirmed after 14 days and new growth was noted. The spray test was repeated a second time later in the season, on newly grown vines.

6.2.6 **Recovery and testing of progeny**

Initially, due to the problems with hyperhydricity, only plants from Line 2 were successfully acclimitized. Later, all the lines were established in the greenhouse. Female flowers, borne on the secondary branches, were hand pollinated with pollen obtained from male flowers from the same plant. The corollas of the male flower were peeled away, the anthers exposed and the pollen smeared directly in the pistil. Fruit that developed were allowed to ripen on the vine and was collected only when it became detached from the plant. Seed was collected from the fruit, washed, dried and stored in bottles.

Twenty five embryos collected from one of the fruits from Line 2 were exised, sterilised according to the protocol described previously (Chapter 2) and placed on MS medium for germination. Five Hales Best 36, untransformed embryos were also included for control purposes. The *in vitro* germination rate was 100 % and the 25 progeny lines and controls were subcultured twice. With the third subculture, half

the plantlets of each line were placed on MS medium and the other half were placed on MS medium supplemented with 10 mg l^{-1} phosphinothricin.

Seed from Lines 1, 2, 4 and 5 were later sterilised according to the established protocol, placed on MS medium for germination and transferred to MS medium supplemented with 10 mg l^{-1} phosphinothricin. The lack of female flowers in Line 3 prevented pollination and recovery of progeny. The number of plantlets that survived on each of the treatments was recorded.

6.3 **RESULTS AND DISCUSSION**

6.3.1 **Determination of selection levels for phosphinothricin**

In this experiment no shoot regeneration was seen on any explants cultured on media containing 5 mg l^{-1} or more phosphinothricin (Figure 6.1). Shoot regeneration was inhibited in explants cultured on 1 mg l^{-1} phosphinothricin, with shoot buds observed on 91.6 % of explants, compared to 100 % of the control explants. The explants were smaller than those of the control and grew less vigorously. In the higher phosphinothricin applications, explants expanded, but became chlorotic and died without any indication of shoot development. A concentration of 5 mg l^{-1} phosphinothricin was sufficient to prevent shoot regeneration, but it was decided to use a concentration of 10 mg l^{-1} to make the selection more stringent.

In the second experiment, all shoots placed on the 10 mg l^{-1} phosphinothricin treatment died within five to seven days of subculturing to the selection medium.

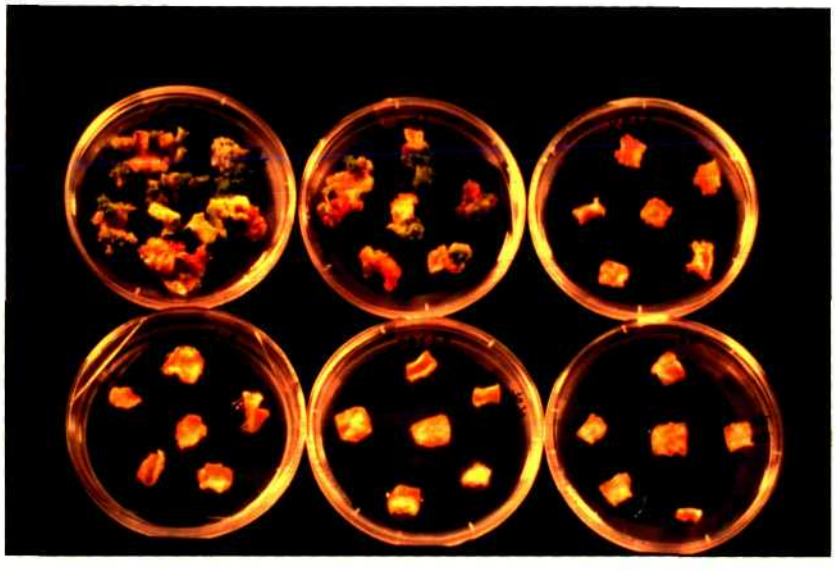


Figure 6.1 Determination of selection levels for phophinothricin (PPT). Left to right, top row,: Control, 1 mg l^{-1}, 5 mg l^{-1}, bottom row: 10 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1}.
No shoot regeneration was observed on phosphinothricin levels of 5 mg l^{-1} or more.

The explants that remained on the unsupplemented MS medium all survived. These data indicate that phosphinothricin is a potent selection agent for melon transformation with the *pat* gene. The selection can be applied directly following *Agrobacterium* co-cultivation, or it can be applied to regenerated shoots.

Several researchers have developed selection systems using phosphinothricin. Tobacco leaf protoplasts were transformed with the *bar* gene (DE BLOCK *et al.*, 1987) and the regenerated callus was placed on various concentrations of phosphinothricin. Initially lower concentrations were used, but subsequently, calli were placed on medium containing 500 or 1000 mg l^{-1} phosphinothricin. Some calli died on the highest concentration; 85 % of the calli did, however, survive. AKAMA *et al.*, (1995) used concentrations of 20 or 50 mg l^{-1} phosphinothricin for *in vitro* selection of *Arabidopsis* transformants. Concentrations of up to 2 mg l^{-1} allowed some development of shoots in *Arabidopsis*, but no development was seen on concentrations of 20 mg l^{-1} or higher. No concentrations between 2 and 20 mg l^{-1} were tested in this study. Phosphinothricin did not significantly influence the percentage regenerated transformants, suggesting that the number of shoots escaping selection was minimal and that transformants could withstand high doses of the herbicide. In the case of barley callus, transformed by biolistic particle bombardment (STIFF *et al* ., 1995), non-transformed calli could survive on medium containing 100 mg l^{-1} phosphinothricin. These calli could not, however, survive long term culture on the selective medium. From these reports it is clear that the levels of phosphinothricin that are used for selection in different species and different transformation systems vary greatly and that a reliable selection protocol must be developed for each species.

6.3.2 **Transformation and regeneration**

Plants have been obtained from all the transformation experiments, although the transformation frequency was very low. In the first experiment, four plantlets and fourteen buds were able to develop roots on selective media. The developing plants were able to root on 200 mg l^{-1} kanamycin. It was decided to double the concentration of the kanamycin as non-transformants were sometimes able to grow on media with kanamycin levels of 100 mg l^{-1} for as long as three months before death of the plantlet. One of the plantlets and all the buds did not survive, mostly due to severe hyperhydricity. The three surviving lines were maintained and used for futher testing. In the second and third experiments, only two plantlets were recovered, one from each experiment. The partial vacuum treatment in the second and third experiments could have had deterimental effects on the explants or the bacteria, decreasing the transformation frequency. It was also observed that the *Agrobacterium* cultures were drawn into the medium and only a thin dry film of the bacterial culture remained on the surface of the explant. *Agrobacterium* growth did occur, but appeared to be less prolific than the growth in the first experiment.

6.3.2.1 **Controls**

In all the experiments, the control treatments reacted appropriately. No shoots developed on explants inoculated with *Agrobacterium* strain LBA4404 without the plasmid nor on uninoculated explants, confirming that the selection medium was effective. Numerous shoots developed on the positive control tobacco explants and were rooted on the selection media.

6.3.2.2 **Maintenance of cultures**

Problems were experienced with both shoot tip necrosis and hyperhydricity. Plants that were kept on maintenance medium were often only subcultured at six-weekly intervals. This was especially the case during the winter months when the growth was noticeably slower and cultures remained small for longer periods. Under these circumstances, the apical buds died, followed by spreading of the necrotic tissue from the tip down. Although a number of factors such as calcium deficiency, rapid water loss, or gaseous pollutants can lead to necrosis (GEORGE, 1996), the reasons for necrosis in these cultures were never established. The problem was alleviated with a shorter subculture interval of four weeks.

6.3.2.3 **Acclimatization**

Hyperhydricity has been observed on the cultures throughout the study, but became a serious problem when the propagation and acclimatization of putative transgenics were inhibited. Plantlets often appeared to be normal, but could not be acclimatized. When the leaves were examined to estimate the ploidy level of the lines, it was discovered that some of the plants had malformed stomata (Figure 6.2a). The stomata were small, permanently open, with fused or improperly formed guard cells. No chloroplasts were visible in the guard cells. These abnormalities are typical of the changes observed in hyperhydric plants (PâQUES and BOXUS, 1987; DEBERGH, *et al.*, 1992). It can be assumed that changes in the anatomy, morphology and physiology of plants start as soon as the plant is placed in culture (DEBERGH *et al.*, 1992), but that visual symptoms of these changes will only appear after a certain period of time and under certain conditions. According to

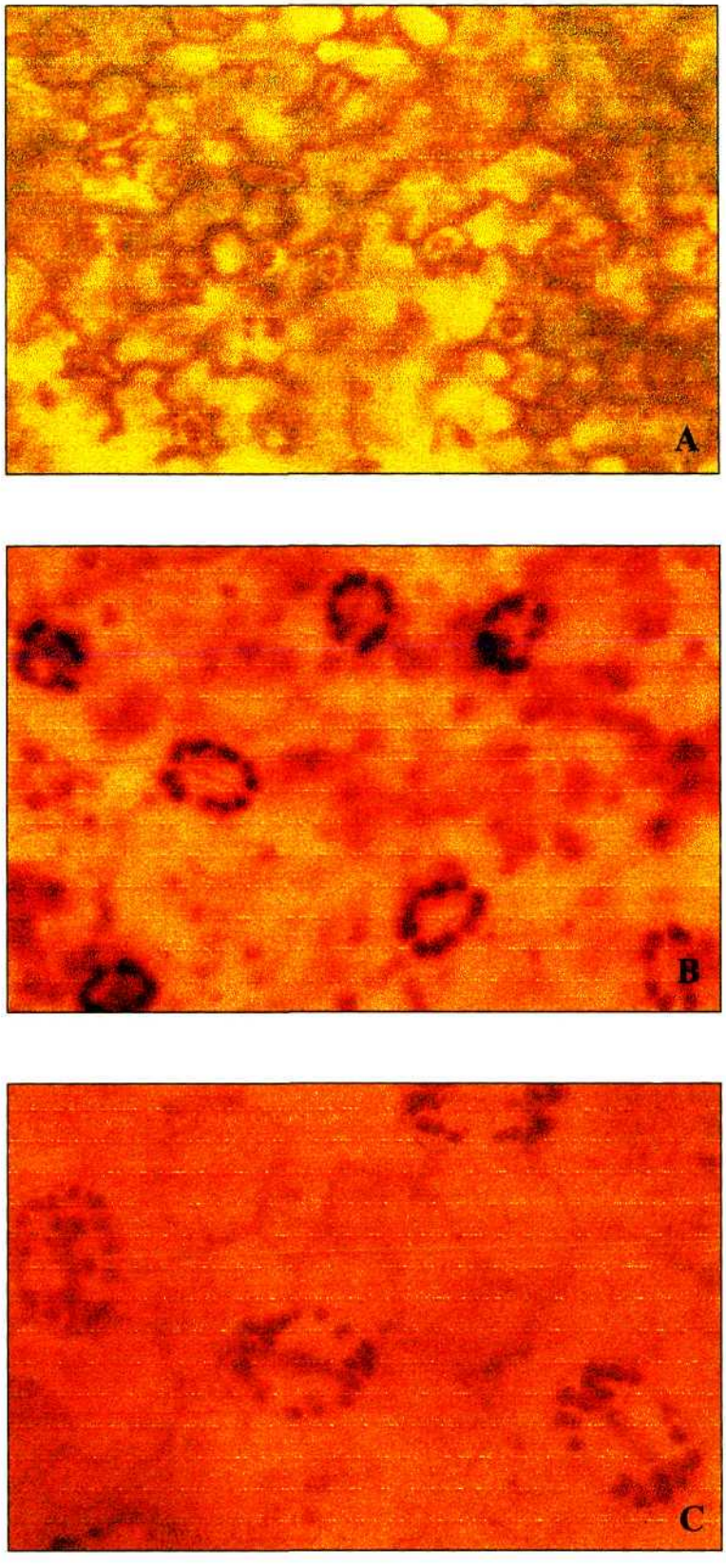


Figure 6.2 Micrographs of the leaf surface of regenerated lines of *Cucumis melo* Hales Best 36. A) Malformed stomata. B) Normal stomata from a diploid plant. C) Normal stomata from a tetraploid line.

DEBERGH *et al.* (1992), contributing factors can be related to the medium, the container, the environment or the explant itself.

A number of changes were made to the culture system in an attempt to address the problem. The agar concentration was slightly raised from 0.75 % to 0.8 % or 0.85 % (w/v). An increased agar concentration was used successfully to decrease hyperhydricity in *Picea abies*, but the growth rate was also reduced (VON ARNOLD and ERIKSSON, 1984). It was argued that the agar concentration changed the matrix potential of the medium, influencing the free water in the culture. In the current study, the increase in concentration was too slight to lead to a decrease in growth rate, but it did appear to reduce the hyperhydricity. A further change was that the screw-cap jars that were used were not closed as tightly as before. The caps were placed on top of the jars and given a half twist; just enough to prevent the lids from falling off during handling. This caused noticable drying of the medium and a decrease in the condensation on the inside of the lids.

During the regeneration and selection stages, the explants were cultured in petri-dishes and initially subcultured weekly. A number of rooted buds were lost in the first experiment when the buds became succulent and brittle; these are severe signs of hyperhydricity. The subculturing interval in the second and third experiments was increased to three weeks on these media, which allowed the media to dry slightly. It can also be assumed that the growth regulator concentrations decreased during this longer subculture. Although only one plantlet was obtained in each experiment, the extreme hyperhydricity was not observed in these cultures. Growth regulators were omitted from the medium as soon as the developing plantlets were starting to elongate. Benzyladenine has been identified as a cause of hyperhydricity in melon (LESHEM, *et al.*, 1988) and apple cultures (PHAN, 1991). The cultures were also

placed in the greenhouse prior to acclimatization to increase the light intensity, as this influence the transpiration rate in leaves (MAENE and DEBERGH, 1987). It is unlikely that any one of these changes caused the decrease in the occurrence of hyperhydricity and led to the improved acclimatization. It is more likely that it was the combination of all these changes that improved the success rate for the recovery of plants.

6.3.3 **Estimation of ploidy level of regenerated lines**

The Cucurbitaceae is composed entirely of diploid species with a noticable lack of polyploid species in the family (WHITAKER and DAVIS, 1962). The spontaneous occurrence of tetraploids in melon is extremely rare and during 21 years of observation at the U.S. Vegetable Laboratory only three tetraploids were identified (NUGENT and RAY, 1992). Although EZURA and OOSAWA (1994) have shown that the ability of cells to form somatic embryos or adventitious shoots and subsequently to regenerate into plantlets decreased with increasing ploidy levels, tetraploids occur frequently in melon cultures. These variations can be useful to plant breeders, but as the objective of transformation is to obtain a single well defined characteristic such as herbicide resistance, it is not desirable for this purpose.

The interest that is shown in these plants by breeders is due to the possibility of improving the flesh quality and shelf life. The high occurrence of tetraploidy in melon tissue cultures is therefore of great interest to plant breeders. ADELBERG *et al.* (1993) has shown that the development of the tetraploids occurs in the first few cell divisions in cotyledonary explants and that they are not produced in axillary

buds during long term culture. It is not clear what, if any, influence the growth regulators or other media components have on the occurrence of tetraploids. It has been suggested that more tetraploids will develop from immature cotyledons than from mature, fully expanded cotyledons. This needs further investigation in the case of Hales Best 36 transformants, as this aspect was not specifically investigated.

FASSULIOTIS and NELSON (1992) and NUGENT and RAY (1992) reported that tetraploid melons have distinct characteristics that makes it possible to identify them at a culture stage, or later in the greenhouse. These include dark green leathery leaves with prominent serrations of the leaf margin, more trichomes, larger flowers, pollen, stomata and seed, changes in pollen shape, double the number of chloroplasts in the guard cells of stomata, and smaller fruit with a large blossom-end scar. Both these groups did chromosome counts to confirm their results. The proven reliability of these methods in the abovementioned studies and in studies on other crops (see later), coupled with the unambiguous results obtained in the current study, motivated the decision not to continue with chromosome counts, but to accept the morphological data for determination of ploidy level. Two of these characteristics were chosen as early indicators of ploidy of regenerants. The first characteristic was the number of chloroplasts in the guard cells of stomata (Figure 6.2 B and C) and the second the shape of the pollen grain (Figure 6.3 A, B and C). These data were also supported by other ploidy dependent characteristics (Figure 6.3 D, E,and F). The observed characteristics are summarised in Tables 6.1 and 6.2.

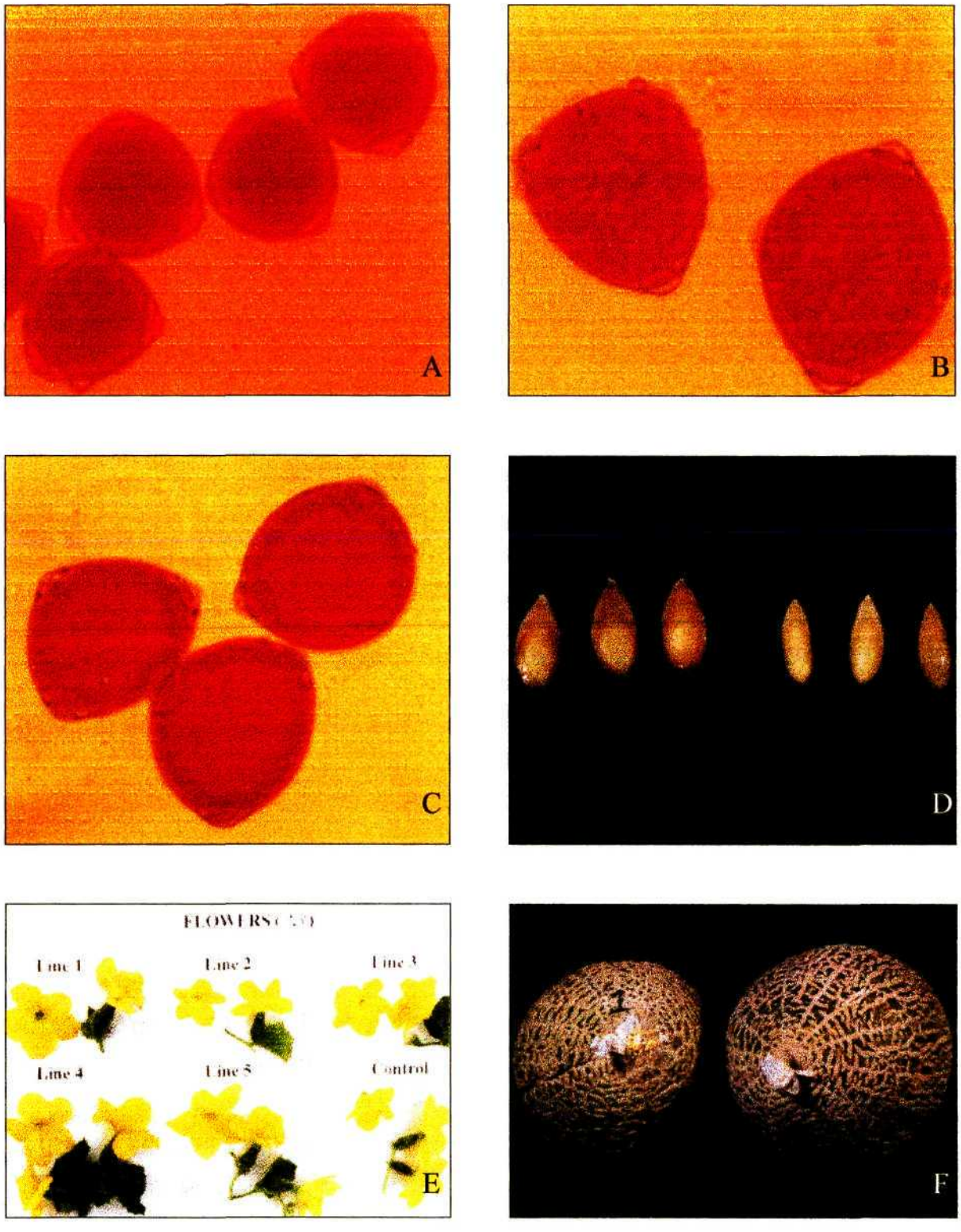


Figure 6.3 Morphological indicators of ploidy level in *Cucumis melo* Hales Best 36. A) Triangular diploid pollen. B) and C) Pollen from tetraploid lines. D) Seed from tetraploid (left) and diploid (right) lines. E) Male and female flowers from the regenerated lines. F) Tetraploid (left) and diploid fruit, showing the blossom-end scars.

Table 6.1 Characteristics of regenerated Hales Best 36 lines that can give an indication of ploidy levels.

	Control	Line 1	Line 2	Line 3	Line 4	Line 5
Chloroplasts per pair of guard cells	10.79 σ 1.10	20.71 σ 1.16	10.46 σ 1.25	11.21 σ 0.78	19.92 σ1.79	10.33 σ1.05
Pollen shape	∇*	∇, □* and other.	∇	∇	∇, □ and other.	∇
Blossom-end scar	Small	Large	Small	N.a.	Large	Small
Seed shape	Thin	Thick, plump	Thin	N.a.	Thick, plump	Thin

*∇- triangular

* □-square

The small size of the *in vitro* grown leaves made it very difficult to remove the epidermis for chloroplast observations. Fortunately it proved to be unnecessary, as the chloroplasts are clearly visible despite the thickness of the leaf. The number of chloroplasts in pairs of intact guard cells were counted. A Student's t- test was performed on the data, using Quattro Pro,Version 6.01 for Windows. Three of the regenerated lines, Lines 2, 3 and 5 had an average chloroplast number that did not significantly differ from that of the control plants (Figure 6.2B). Two of the lines however, had approximately double the number of chloroplasts per pair of guard cells, Line 1 with 20.70 and Line 4 with 19.92 chloroplasts per pair of guard cells (Figure 6.2C), which is significantly different to that of the control at the 1 % significance level.

Several reports have appeared recently that correlated the number of chloroplasts per pair of guard cells to the ploidy level of regenerated plants. These include a number of crop species such as tomato (JACOBS and YODER, 1989), tamarillo and pepino (STANDRING *et al.*, 1990), potato (SINGSIT and VEILLEUX, 1991) peanut (SINGSIT and OZIAS-AKINS, 1992), melon (FASSULIOTIS and NELSON, 1992; ADELBERG *et al.*, 1993) and pepper (QIN and ROTINO, 1995). These reports have shown it to be a very reliable parameter in predicting ploidy levels in these crops. Although indicators relating to cell size have been found to be extremely reliable in predicting ploidy (STANDRING *et al.*, 1990), QIN and ROTINO (1995) found chloroplast number to be a more consistent indicator of ploidy than stomatal guard cell length, as size can be influenced by age and leaf position. Chloroplasts can be counted in any green tissue, but guard cells are preferable because of their ease of preparation and measurement, as well as the inherent lack of endopolyploidy in these cells (STANDRING *et al.*, 1990). In all these reports, the chloroplast number for tetraploids was double that of diploids. Haploid plants had half the chromosome number and hybrids had a mean chloroplast number intermediate between the parental values. It is therefore concluded that the regenerated Lines 2, 3 and 5 from the current study are diploids and Lines 1 and 4 are tetraploids. No plants with intermediate chloroplast numbers were obtained, indicating that no aneuploid plants developed.

The appearance of the pollen grains was noted for three flowers of each line and the control. Pollen of three of the lines and the control plants were consistently only equilateral triangular, appearing uniform in size (Figure 6.3A). In two of the lines, Line 1 and Line 4, other pollen shapes were seen (Figure 6.3B and C). These included squares, elliptical-round, and non-equilateral triangles. FASSULIOTIS (1990), FASSULIOTIS and NELSON (1992), ADELBERG *et al.*, (1993) and

ADELBERG *et al.*, (1994) described the occurrence of pollen of different shapes in tetraploid melon. In all these studies, the diploid pollen were described as 100 % triangular and of uniform size, while the tetraploid pollen was described as four sided, square appearing, elliptical-round, and varying in size. The report by NUGENT and RAY (1992) on spontaneous tetraploid melons also indicated that the pollen was bigger than that of diploid plants, but no mention was made of the shape of the pollen. These data also support the deduction that two of the regenerated lines in the current study, Line 1 and Line 4, are tetraploids.

Several other characteristics supporting the deduction that Lines 1 and 4 are tetraploids were noted. These characteristics were all described by FASSULIOTIS and NELSON (1992) as typical of tetraploid plants. Fruit of both Line 1 and Line 4 had a larger scar on the blossom-end, compared to the control and other lines. In Figure 6.3F, the relative larger size of the blossom-end scar of Line 1 fruit is compared to that of Line 5. In Figure 6.3D, the seeds obtained from Line 4 fruit on the left, are compared to the seed from a control plant on the right. Both Line 1 and Line 4 displayed these larger seeds.

The size of the male and female flowers of the regenerated lines are shown in Figure 6.3E. The diameter of both male and female flowers were measured (Figure 6.3e). In the case of Line 3, no female flowers could be recorded, as only one was ever seen on these plants. The results of the Student's t-test, as calculated using Quattro Pro Version 6.01 for Windows, are summarised in Table 6.2. The female flowers of Lines 1 and 4 were significantly larger than the flowers of the control plants (P (T<=t) 6.31×10^{-5} and 10^{-4} respectively). Two of the lines, Line 1 and Line 4 had significantly larger male flowers than the control plants. The male flowers of Line 5 just failed to differ at the 1 % level (P (T<=t) 0.011). However, this result was

significant at the 5 % level and a more sensitive test, the Bonferroni multiple comparison test was done, using the programme Genstat 5 Release 3.2. The results are summarised in Table 6.3. In this test, while Line 5 was comparable to Lines 1 and 4, it was confirmed that the male flower diameter of control plants and Line 5 did not difffer significantly (5 % level). The bigger mean size of the Line 5 male flowers, although not significant, could possibly be due to environmental factors in the glasshouse. Although both ADELBERG *et al.*, (1994) and FASSULIOTIS and NELSON (1992) mention the larger size of tetraploid flowers, no measurements were given to support their statements. It might be added that the largest individual flowers, both male and female, were found in Lines 1 and 4. These flowers are more noticable than smaller ones and even casual observation will tend to favour a description of larger flowers.

Table 6.2. Results of the statistical analysis of the diameter of male and female flowers of the regenerated Lines of Hales best 36.

	Diameter of male flowers	**Diameter of female flowers**	**Student's t-test P(T<=t):** Comparison of each line with the control.	
			Male flowers	**Female flowers**
Control	37.6 ± 4.27	38.8 ± 5.90	N.a.	N.a.
Line 1	47.5 ± 2.01	50.8 ± 3.61	$P = 1.64 \times 10^{-5}$*	$P = 6.31 \times 10^{-5}$*
Line 2	38.1 ± 4.62	40.7 ± 3.95	P = 0.804	P = 0.409
Line 3	35.7 ± 5.86	N.a.	P = 0.420	N.a.
Line 4	45.6 ± 5.50	49.9 ± 3.18	P = 0.002*	P = 0.0001*
Line 5	42.6 ± 3.47	44.8 ± 4.64	P = 0.011*	P = 0.021

*** Significant at the 1 % level.**

All the data on the ploidy levels in the five regenerated lines in this study indicate that three of the lines, Lines 2, 3 and 5 are diploid. Two lines, Line 1 and 4, exhibited the distinctive morphological characteristics that are typical of tetraploid melon plants.

Table 6.3 The results of the Bonferroni multiple comparison test for the analysis of the diameter of the male flowers from the 5 regenerated lines.

Line	Means in ascending order (comparisons).
Line 3:	35.7 (a)
Control:	37.6 (a, b)
Line 2:	38.1 (a, b)
Line 5:	42.6 (b, c)
Line 4:	45.6 (c)
Line 1:	47.5 (c)

6.3.4 **Greenhouse spray tests**

The first spray test was conducted when the vines were still less than a metre in length, and most of the plant surface was sprayed. In contrast to the control plants, the test plants all survived. In both the first and the second tests, all the untransformed Hales Best 36 plants turned brown and died when sprayed with Ignite®, at all the concentrations used (Figure 6.4a). The plants were allowed to grow out again and sprayed for a second time. Only one of the selected lines showed slight damage when sprayed with a 0.1 % solution of Ignite®. At higher

concentrations, all the vines were damaged to a lesser or greater extent, but even at the highest concentration of 2 % Ignite®, the vines recovered and continued to grow. The type of damage that was observed included leaf injury and changes in the growth of internodes. The leaf damage included necrosis of the leaves, starting at the leaf margins and extending to the lamina, general yellowing and death of the leaf. The internodes often grew out very short, before the vine recovered and developed normal internodes. The damage did not extend to older or new growth and was always limited to the part of the vine that was directly in contact with the herbicide. Differences were seen in the way that the lines reacted to the treatments. Of the 5 lines that were tested, Lines 1, 2, 3 and 4 had the least visible damage (Figure 6.4b) and reacted similarly, while Line 5 appeared to have the most damage (Figure 6.4c). In this line, even the 1.0 % and 2.0 % treatments, that appeared to be dead, recovered and resprouted. The damage that was observed on the five selected lines is summarised in Table 6.4 below.

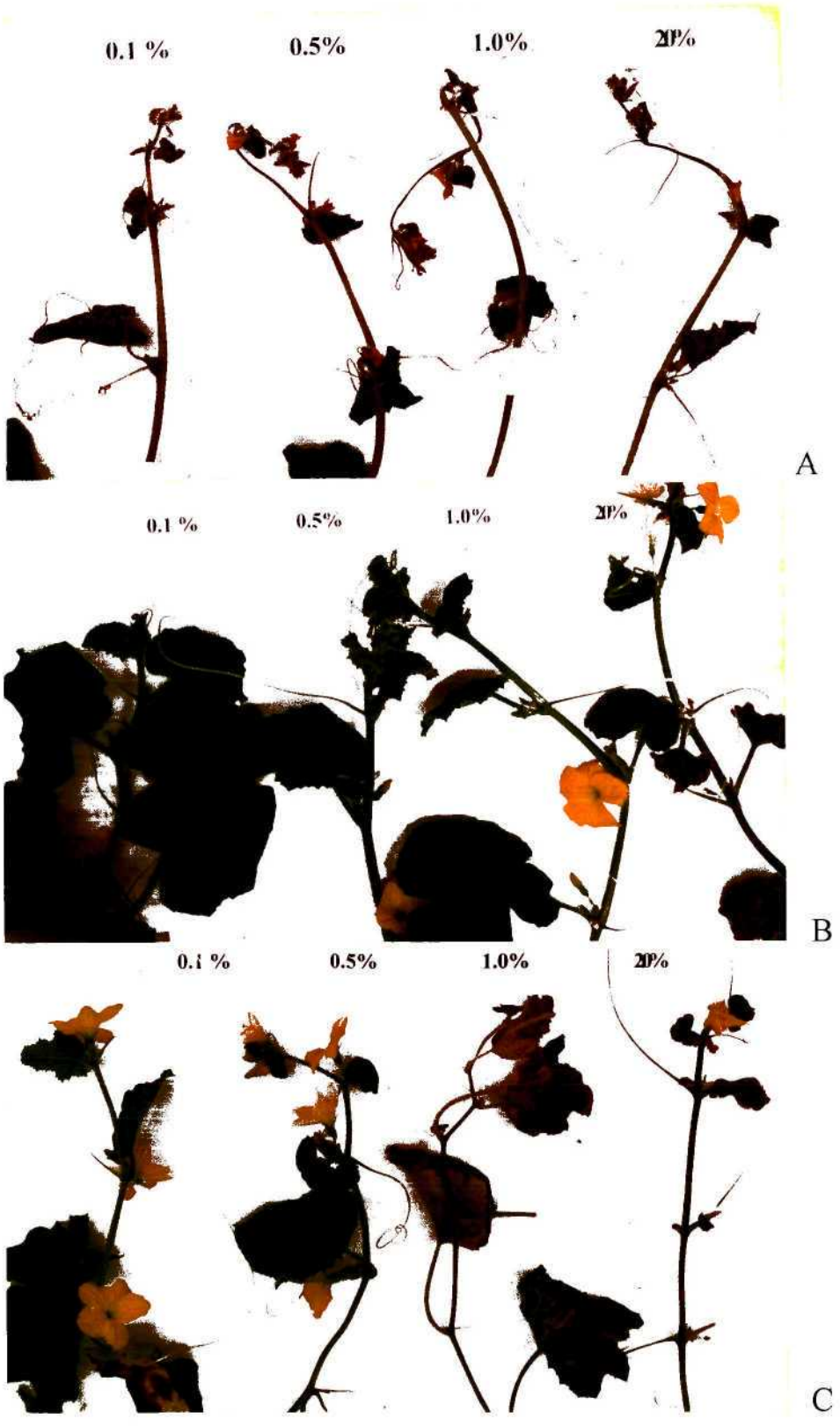


Figure 6.4 Results of greenhouse spray tests. A) The control Hales Best 36 plants, B) Line 3 and C) Line 5. The plants were sprayed with 0.1 %. 0.5 %, 1.0 % and 2.0 % (v/v) solutions of Ignite® (200 mg ml^{-1} active ingredient) respectively.

Table 6.4 Damage observed on vines of the putatively transformed Hales Best 36 lines one week after spraying with concentrations of 0.1 %, 0.5 %, 1.0 % and 2.0 % (v/v) Ignite® respectively. Observations from two spray experiments were combined. (The results from the control plants have been excluded, as all the plants turned brown and died within a week after spraying.)

Concentration of Ignite®	**Line 1**	**Line 2**	**Line 3**	**Line 4**	**Line 5**
0.1 %	Slight damage to leaf margin.	None.	None.	None.	Slight yellowing.
0.5 %	Leaves dead, or small, very short interrnodes.	Yellowing, short internodes, small leaves.	No leaf damage, short internodes.	Slight yellowing, leaf margins brown.	Leaf margins damaged, slight yellowing, small leaves.
1.0 %	Leaves dead or damaged , very short internodes.	Leaves dead, or damaged, short internodes, small leaves.	Short internodes, small leaves.	Leaves dead, or severe yellowing, short internodes.	Leaves dead, or severe yellowing.
2.0 %	N.a.	Leaves dead, very short internodes.	Leaves dead, short internodes.	Leaves dead, short internodes.	Leaves dead.

The commercial formulation of Ignite® contains 200 mg ml $^{-1}$ phosphinothricin. The prescribed application rate for this formulation is 0.5 % to 1.0 % (v/v), depending on the crop. It must be noted that the information is applicable to pre-emergence applications only, and has not been established for transgenic crops. Equivalent formulations have been used to test transformation in other crops. In the original paper on phosphinothricin resistance in transgenic plants, DE BLOCK *et al.* (1987) described the influence of Basta ® and Herbiace ® on transgenic tobacco containing

the *bar* gene. Basta ® and Ignite® are identical formulations and Herbiace ® is the commercial formulation of bialaphos. It differs from glufosinate in that the phosphinothricin in bialaphos only becomes active once two alanine residues are cleaved off (TACHIBANA *e al.*, 1986). All control plants were killed at 2 litres ha^{-1} Basta ® and 8 litres ha^{-1} or 20 litres ha^{-1} Herbiace ® while the transformants were fully resistant. GORDONN-KAMM *et al.* (1990) painted a 2 % (v/v) solution of Basta TX ® onto the leaves of transformed maize plants. Photographs of the leaves taken six days after application show that damage was limited to the non-transformed controls. HARTMAN *et al.* (1994) transformed turfgrass using a vector containing both *bar* and the GUS gene. The transformants could be selected with the herbicide Herbiace ®, applied with a paint brush at two concentrations of 0.15 % and 0.2 % (v/v). Escapes were able to survive the lower concentration, but not the 0.2 % application, while transformants were unaffected. D'HALLUIN *et al.* (1992) sprayed a 1 % (v/v) solution of Basta ® on sugarbeet containing the *bar* gene. The solution was appplied to *in vitro* plants at a rate of 8 litres ha^{-1}, as well as glasshouse grown and field grown plants (the latter at 4 litres ha^{-1}). They also found that the transformants were able to survive without damage. *Arabidopsis* transformants survived Basta ® applications of between 0.002 % and 0.2 % (v/v), although plants sprayed with the highest concentration showed damage and growth inhibition (AKAMA *et al.*, 1995). These plants recovered within a few weeks.

Although direct comparisons are difficult to make due to the different methods of application described in the reports, the damage appear to be highly crop specific. Herbicide resistant transgenic crops can only be utilised if the normal application rate needed to destroy the weeds associated with the crop does not harm the plants. These levels will need to be established in field trials, as the information for crops such as melon is not available. The results that were obtained in the melon spray

trails described above indicated that the selected lines could survive up to a 2 % application in glasshouse conditions. The plants were all able to continue to grow despite the damage that was caused by the herbicide. However, it remains to be seen whether the plants would survive in a field trail. It must also be borne in mind that the unsprayed parts of the plant could have provided enough support to ensure the survival of the vine. Even if the plants can survive in a field trial, the damage that the herbicide causes on the leaves of the plant might be unacceptable to the farmer, preventing commercial application of the technology.

6.3.5 *In vitro* progeny trails

In the first herbicide selection trail on progeny of transformants, embryos of Line 2 were placed on MS medium for germination. The *in vitro* germination rate was 100 % and half the plantlets of each of the 25 lines that were obtained were subcultured to MS medium and half to MS medium supplemented with 10 mg l^{-1} phosphinothricin.

Twenty four (96 %) of the lines on the phosphinothricin treatment survived and continued to grow. Only one line turned chlorotic and died within a week. None of the control plants survived the phosphinothricin treatment.This result indicates that the *pat* gene was transferred to progeny through self pollination. The high percentage of lines that survived selection could be attributed to a high copy number of the gene in the parental line. If only one copy of the gene was present, a ratio of 3:1 would be expected in the next generation. The low sample number could, however, explain the result even if the parental line had a single copy number of the gene.

In the second trail, the germination percentage was 93.5 % and 100 % for control seed and the diploid lines respectively. The germination of seed from the tetraploid lines were inhibited. The seeds from these lines often appeared to be well formed, but when the seed coat was removed, the embryo was absent. These seeds were discarded and only apparently well formed seeds were used. Of these, only 16.1 % of Line 1 and 69.5 % of Line 4 seed germinated. ADELBERG *et al.*, (1993) also reported the occurrence of non-viable seed in tetraploid plants. In hybrid crosses with diploid plants, only up to 33 % of seeds were viable, with a resulting progeny of sterile triploid plants.

Several plants were lost through contamination, due to a faulty laminar flow bench. When the surviving plants were exposed to the phosphinothricin treatment, 100 % of the control plants were noticably chlorotic after three days and dead after seven days. In Line 1 only two plants were recovered and both grew well on the treatment. In Line 2, 100 % of 24 plants survived, Line 4 had 88.25 % survival of 17 plants and in Line 5, 96.15 % of the 26 plants survived. The germination problems, coupled to the contamination that was experienced, made further statistical analysis difficult, but it was clear that the herbicide resistance trait was transferred to the F1 generation through self pollination. Again, it would appear that it is unlikely that single copies of the gene were present in Lines 2 and 5, but these results can only be clarified using the data from genomic Southern blots.

6.4 **CONCLUSION**

The transformation protocol that was established in Chapter 5 was successfully used to obtain five herbicide resistant lines of *Cucumis melo* cv Hales Best 36. An *in*

vitro selection protocol was developed using media containing phosphinothricin, the active ingredient of the herbicide Ignite®. The putative transgenic lines were resistant to *in vitro* selection. In greenshouse spray tests, the selected lines were able to survive the normal application rates of Ignite®. The herbicide resistance trait was transferred to the progeny of these plants, as indicated in *in vitro* selection experiments. Two of the lines, however, were shown to be tetraploid, using morphological indicators. Final verification of the integrative transformation will be Southern DNA hybridisation analysis of the transformants, using a probe for the introduced gene as described in Chapter 7.

CHAPTER 7

MOLECULAR ANALYSIS OF *CUCUMIS MELO* CV HALES BEST 36 LINES TRANSFORMED WITH THE *PAT* GENE

7.1 INTRODUCTION

The five Hales Best 36 transgenic lines that were generated in the transformation experiments described in Chapter 6 were subjected to molecular analysis. A polymerase chain reaction (PCR) was performed to show the presence of the *pat* gene in DNA isolated from leaf tissues of the putative transformants. Agarose gel electrophoresis demonstrated that the correct size fragment was obtained. The PCR product was transferred from the gel to a nylon membrane and the non-radioactive Boehringer Mannheim DIG system for nucleic acid analysis was used to verify that this fragment was indeed the *pat* gene. Stable gene insertion into the plant genome was verified by hybridising a radioactively labelled ^{32}P probe specific for the *pat* gene to a Southern blot of restriction digested total genomic DNA (SOUTHERN, 1975).

7.2 MATERIALS AND METHODS

The composition of all solutions and buffers described in this chapter are shown in Appendix C. Buffer compositions are not given in the text.

7.2.1. **DNA Extraction and quantification**

Initially, DNA extractions for PCR were performed on leaves obtained from *in vitro* grown plants, using the method described by EDWARDS *et al.* (1991). When acclimatized plants became available, extractions for PCR and Southern analysis were performed on leaf material collected from greenhouse plants, weighed and frozen at -70 °C, using an extraction method adapted from DELLAPORTA *et al.* (1983).

7.2.1.1 **DNA extraction for PCR analysis**

DNA was extracted from plant material using an adaptation of the method developed by EDWARDS *et al.* (1991). Leaf disks, the size of the top of an Eppendorf tube, were ground in "Super-quick" extraction buffer and carborundum and incubated at 60 °C for 10 minutes. DNA was extracted with chloroform:isoamyl alcohol (24:1) and alcohol precipitated in 96 % ethanol with 0.1 volume 3 M sodium acetate, pH 5.2. The DNA pellet was collected by centrifugation, washed in 70 % ethanol and resuspended in 100 µl TE buffer (10mM Tris HCl, pH 8.0, 1 mM EDTA).

DNA concentrations were determined using a Sequoia-Turner fluorometer using an adaptation of the method described by LABARCA and PAIGEN (1980). The dye binds specifically to DNA and the concentration of the sample can be determined by comparison to samples with known DNA concentrations. The fluorometer was set to zero using 2 ml TNE buffer containing 0.2 µl ml^{-1} Hoechst dye 33258. A 2 µl sample of 100 ng μl^{-1} calf thymus DNA was added to the solution and used to calibrate the fluorometer to give a reading of 100. The fluorometer reading from 2

µl samples of plant DNA, in 2 ml of the dye solution were used as an indication of the DNA concentration of the samples.

7.2.1.2 **DNA extraction for Southern analysis**

DNA was extracted essentially as described by DELLAPORTA *et al.* (1983), but with certain modifications. The key step of this method is the simultaneous precipitation of proteins and polysaccharides by high concentrations of potassium acetate in the presence of the detergent sodium dodecyl sulfate (SDS). The DNA obtained from the basic protocol was viscous and usually green or brown in colour. One extraction was performed with the concentration of the antioxidant β-mercaptoethanol in the extraction buffer increased from 10 mM to 140 mM, but a concentration of 10 mM was used for all future experiments. When the DNA was separated on an 0.8 % agarose gel to examine how intact the DNA was, a bright smear was seen on the gel, below the band of high molecular weight DNA. This is usually associated with RNA contamination and the protocol was adapted to include a step for removal of RNA using RNAse. Initially the basic protocol did not include a phenol extraction step, but the protocol was adapted further to include this step to remove protein contaminants.

The extraction was performed under sterile conditions. The protocol, including the additional RNAse and phenol steps, was performed as follows:

1. Grind 10 g frozen or fresh plant material to a fine powder using liquid nitrogen and a mortar and pestle. Do not allow the plant material to thaw.
2. Transfer the powder as fast as possible to 60 ml prewarmed (65 °C) DNA extraction buffer in a capped polypropylene tube.

3. Add 8 ml 20 % SDS and mix thoroughly but gently.

4. Incubate at 65 °C for 30 minutes in a water bath, mixing every 10 minutes.

5. Add 20 ml 5 M potassium acetate and mix thoroughly.

6. Incubate on ice (0 °C) for 20 minutes. In this step, the proteins and polysaccharides are precipitated together with insoluble potassium dodecyl sulphate, leaving the nucleic acids in solution.

7. Centrifuge at 4 °C, 12 000 rpm for 20 minutes in a Beckman centrifuge.

8. Filter the supernatant through cheesecloth to remove any floating particulate material.

9. Precipitate the nucleic acids with the addition of 0.7 vol (40 ml) ice-cold iso-propanol.

10. Incubate at -20 °C for 30 minutes to overnight.

11. Centrifuge at 12 000 rpm for 20 minutes at 4 °C to pellet the DNA.

12. Invert and dry on a paper towel for 10 minutes.

13. Redissolve the DNA in 3 ml TE5 buffer. Transfer the DNA to three 2.2 ml Eppendorf tubes.

14. Add 150 µl of a 10 mg ml^{-1} solution RNAse to each tube.

15. Incubate for 30 minutes at 37 °C to allow the breakdown of contaminating RNA.

16. Prepare a fresh solution of 25:24:1 phenol:chloroform:isoamyl alcohol, with phenol saturated with TE buffer. Add 1 ml of this mixture to each tube.

17. Mix by inversion, collect the upper phase in a clean Eppendorf tube, taking care not to collect any of the lower phase solution.

18. Repeat steps 16 and 17.

19. Add 1 ml chloroform:isoamyl alcohol 24:1 to the upper phase, mix thoroughly, centrifuge and discard the lower phase.

20. Determine the volume of the samples. Add an equal volume of iso-propanol and 1/10 volume of 3 M $NaClO_4$ to each tube.

21. Incubate at -70 °C for 10 minutes.

22. Pellet the DNA through centrifugation at 12 000 rpm for 10 minutes.

22. Discard the supernatant, invert and dry the pellet, taking care that it does not slip down the side of the tube.

23. Wash with 70 % ethanol, centrifuge again and dry the pellet.

24. Resuspend the pellet in 150 µl TE buffer (or more, depending on the solubility) and quantify the DNA.

The DNA obtained from this protocol was still very viscous, most probably due to polysaccharide contamination. It did not dissolve completely in TE buffer and could not be used for Southern analysis. The DNA isolation protocol was modified again to include a high salt / ethanol precipitation as described by FANG *et al.* (1992). This step was added directly after the phenol extraction. The protocol was the same as above, up to, and including step 19, whereafter it was changed as follows:

20. Pool the 3 tubes of DNA from each line and precipitate with two volumes of 96 % ethanol.

21. Redissolve the resulting DNA pellet as far as possible in 1 ml TE buffer. If necessary, heat (to about 50 °C) in a heating block to dissolve.

22. Add 0.45 µl of a 5 M NaCl solution, to give a final concentration of 1.5 M NaCl and mix thoroughly.

23. Precipitate again using 2 volumes 96 % ethanol. In the presence of the salt, polysaccharides remain in solution with the ethanol, rather than precipitating with the DNA (FANG *et al.*1992).

24.Wash twice with 70 % ethanol, dissolve the pellet in 2 ml TE buffer and quantify the DNA. In this study, the DNA was quantified using a TKO 100 fluorometer from Hoefer Scientific Instruments, as described in 7.2.1.1.

7.2.2 **PCR analysis**

A PCR analysis was performed in order to show the presence of the *pat* gene in the tissues of plants selected on medium containing 10 mg l^{-1} phosphinothricin. PCR was conducted on approximately 30 µg genomic DNA templates in a PCR mix containing the following ingredients per reaction:

10 mM Tris-HCl (pH 9.0 at 25°C),

50 mM KCl,

0.1% Triton® X-100 (as part of the Promega 10X buffer),

1.5 mM $MgCl_2$,

0.2 mM of each dNTP,

0.5 µM of each of the primers and

0.5 units *Taq* DNA polymerase per 10 µl reaction volume (Supplied by Promega).

The *pat* specific primers were synthesised by the Biochemistry Department, University of Cape Town. The sequence of the left primer was (5' - GTCTCCGGAGAGGAGACCAG-3' and that of the right primer (5' - CCTAACTGGCCTTGGAGGAG-3'). The reactions were prepared in a laminar flow cabinet, using only sterile solutions and working with fresh gloves. Only pipette tips with sterile filters were used for this work. A working solution was prepared containing all the reagents except the DNA templates and the DNA polymerase, to reduce inaccuracies in the pipetting of very small volumes. The appropriate volume of reagent mix was then aliquotted into PCR tubes, followed by double distilled water to provide the correct dilution, the DNA templates and lastly the DNA polymerase. An overlay of 40 µl sterile mineral oil was added to each of the reaction tubes to prevent evaporation during PCR.

A negative control was included for each experiment. This so-called water control contained all the reagents but no DNA and the preparation was completed prior to the addition of the DNA templates to the other reaction tubes. Initially a second negative control was included consisting of untransformed potato DNA, but this was discontinued.

A positive control was prepared for each experiment, using 1 ng plasmid DNA isolated from the *Agrobacterium tumefaciens* LBA4404 strain containing the plasmid pBI101/*pat*. This plasmid was developed as a collaboration between the Department of Microbiology, University of the Witwatersrand and the ARC-Roodeplaat (VILJOEN *et al*. 1993). It contains the T-DNA region of the plasmid pBI101 that is illustrated in Chapter 1, Figure 1.2. The *pat* gene was ligated into the polycloning site of pBI101 between the *Hind* III and *Xba* I sites. The DNA template for the positive control was added after the other reactions were completed. This preparation sequence allowed the water control to indicate DNA contamination of the reagents and prevented aerosol contamination of the test reactions with positive control DNA.

The PCR reactions were performed in a PTC 100 Programmable Thermal Controller or a PTC 200 Peltier Thermal Cycler, from MJ Research Inc. The PCR program that was used is shown in Table 7.1. The annealing temperature was deduced from the simplified equation used by THEIN and WALLACE (1986):

$$T_m = 4°C(G+C) + 2°C(A+T),$$

where T_m is the melting temperature of primer and annealing site. In the case of the left primer T_m was 66 °C and for the right primer the T_m was 64° C. Usually the annealing temperature is set 5 °C below the melting temperature, in this case T_m -5 °C = 60 °C.

Table 7.1 The PCR programme that was designed for the amplification of the *pat* gene from genomic DNA isolated from putative transgenic plants.

	Temperature	Time	Number of cycles
Initial denaturation	94 °C	30 seconds.	1
Denaturation **Annealing** **Elongation**	94 °C 60 °C 72 °C	30 seconds 30 seconds 45 seconds	30
Final elongation	72 °C	5 minutes	1

The PCR products were resolved in a 1.0 % agarose gel, visualised by ethidium bromide staining and sized in comparison to the Boehringer Mannheim Molecular Weight Marker III. This marker, obtained from λ-DNA cleaved with *Eco* RI and *Hind* III contains 13 fragments of 21 226, 5 148, 4 973, 4 268, 3 530, 2 027, 1 904, 1 584, 1 375, 947, 831, 564 and 125 base pairs for the molecular weight determination of double stranded DNA.

7.2.3 **Southern analysis**

7.2.3.1 **Nonradioactive detection of the *pat* gene in a PCR blot**

The Boehringer Mannheim DIG system for nucleic acid analysis was used for the detection of the *pat* gene in Southern blots of the resolved PCR products of the putative transgenic lines and plasmid DNA. Several preparations were completed before Southern hybridisations could be performed. The *pat* specific DNA probe

was labelled by incorporation of Digoxigenin-11-dUTP (DIG-11-dUTP) during PCR. The efficiency of two types of positively charged nylon membranes were tested and the detection system was tested for it's sensitivity in detecting the presence of the *pat* gene. Amplification fragments obtained by PCR were separated by agarose gel electrophoresis and transferred to a nylon membrane. Hybridisation of the *pat* specific probe to the membrane bound DNA was detected using a colorimetric protocol, or the chemiluminescent CDP-Star ™.

7.2.3.1.1 **PCR labelling of a *pat* specific probe using DIG**

A ratio of 65 % dTTP to 35 % digoxigenin-11-dUTP (2:1) has been shown to produce a probe that contains one digoxigenin molecule per 20 - 25 nucleotides (BOEHRINGER MANNHEIM, 1995a). In a laboratory discussion with OELOFSE and BERGER (personal communication, 1996) it was speculated that lower ratios will produce probes that are theoretically less sensitive, but that will exhibit less non-specific background hybridisation. Probes with high ratios of modified nucleotides to normal nucleotides might possess poor hybridisation efficiency due to interference by the modified nucleotides. It is also possible that high ratios can interfere with the efficiency and yield of the PCR reaction. Initially, three different mixes of dTTP:DIG-11-dUTP were prepared and used to produce the PCR labelled probe. It was later decided to use only the probe prepared from a nucleotide mix with a dTTP:DIG-11-dUTP ratio of 7:1.

The reaction mix consisted of the following components in a total volume of 10 µl:

10 mM Tris-HCl,

50 mM KCl,

0.1% Triton® X-100 (as part of the Promega 10X buffer),

1.5 mM $MgCl_2$,

0.2 mM of each of the dNTPs: dATP, dCTP and dGTP,

0.2 mM of a dTTP:DIG-11-dUTP mix in a 3:1, 7:1 or 12:1 ratio,

0.5 µM of each of the primers,

0.5 units *Taq* DNA polymerase per 10 µl reaction volume (Supplied by Promega).

1 ng template DNA.

The template DNA consisted of isolated plasmid pBI101/*pat* and was used for the labelling reactions. An unlabelled control reaction prepared with an unlabelled dNTP mix was included. A water control was prepared as described in 7.2.2. Unlabelled dNTP mix was used for the water control and no DNA was added. The PCR was conducted as described in 7.2.2 and Table 7.1.

7.2.3.1.2 **Testing of different nylon membranes**

A preliminary test was conducted to compare the suitability of different nylon membranes used for Southern blotting. The Magna Charge Nylon membrane from Micron Separations Inc. (MSI) and Nylon Membranes, (positively charged) from Boehringer Mannheim, in the form of sheets of unknown age, or a newly bought roll were used. The membranes were cut into pieces of roughly 25 mm x 45 mm; three pieces of each type were used. The membranes were blotted in 20x SSC buffer for 4 hours to mimic the standard hybridisation protocol. One milligram plasmid DNA, isolated from pBI101/*pat* was spotted in the one corner of each membrane. The membranes were baked at 80°C for two hours to fix the DNA to the membrane. The

membranes were then prehybridised in Boehringer Mannheim Easy Hyb for six hours, followed by an overnight hybridisation. The probe that was obtained from the 12: 1 dTTP:DIG-11-dUTP mix was used for the hybridisation. Chemiluminescent detection was performed on the membranes.

Chemiluminescent detection produces a light signal at the site of the hybridised probe that can be recorded on X-ray films. The detection protocol consisted of three steps. First, the membranes were blocked to prevent non-specific attraction of the antibody to the membrane. Then they were incubated in anti-Digoxigenin-Fab fragments, which are conjugated to alkaline phosphatase. Lastly, the antibody conjugate was allowed to react to a chemiluminescent substrate and exposed to an autoradiograph to record the signal. All incubations were performed at room temperature. The following protocol was used:

1. Equilibrate the membrane in washing buffer for 1 minute.
2. Block in blocking buffer for 1 hour.
3. Prepare the antibody solution near the end of the blocking time. Dilute anti-Digoxigenin-AP Fab fragments 1:20 000 in blocking solution and mix by inversion.
4. Incubate the membrane in the antibody solution for 30 minutes.
5. Wash the membrane twice in washing buffer, 15 minutes per wash.
6. Equilibrate the membrane in detection buffer for two minutes. Dilute CDP-Star™ 1:100 in detection buffer and apply this to the membrane that is placed between two acetate sheets for the detection. Drip the CDP-Star™ onto the membrane and lower the top acetate sheet to form a liquid film over the membrane. Incubate the membrane for 5 minutes, place between two new acetate sheets and seal.
7. The sealed membrane can now be exposed to an autoradiograph for varying lengths of time before it is developed.

7.2.3.1.3 **Southern blotting of PCR products**

The techniques that were used for the Southern blots were as described by BOEHRINGER MANNHEIM (1995b). Procedures were performed at room temperature unless specified differently.

Depurination

In this step the DNA is depurinated in a controlled acid treatment which will break the DNA during the subsequent alkaline denaturation. The gel was submerged in 250 mM HCl for 10 minutes with shaking at room temperature and rinsed with H_2O before proceeding.

Denaturation, neutralisation and blotting

The protocol was performed as follows:

1. Submerge the gel in denaturation solution for 15 minutes. Repeat.
2. Rinse with water.
3. Submerge the gel in neutralisation buffer for 15 minutes and repeat.
4. Blot the DNA from the gel to a nylon membrane by capillary transfer using 20x SSC buffer. Cut the membrane the same size as the gel and mark the upper side with pencil. Cut a filter paper to fit the length of a horizontal electrophoresis apparatus and bend it to dip into the wells. Fill the wells with buffer, in such a way that the edges of the filter paper will absorb the buffer from the wells. Place the gel on top of the filter paper, with the bottom side up. Place the nylon membrane on top of the gel, wet with 20x SSC buffer and remove all air bubbles by rolling a glass rod over the membrane. Prepare a Parafilm™ mask to fit around the gel to prevent buffer

from moving past the gel, instead of through the gel. Place four smaller filter papers and a wad of paper towels on top of the membrane and weigh it down with a water filled bottle. Allow the transfer to proceed overnight.

DNA fixation

The DNA was fixed to the membrane by baking at 80 °C for 2 hours. In this form the membrane can be stored or used for prehybridisation.

Prehybridisation and hybridisation

Prehybridisation blocks non-specific nucleic acid binding sites on the membrane. The protocol was performed as follows:

1. Transfer the membrane to a hybridisation bottle containing 20 ml DIG Easy Hyb and incubate at 38 °C for at least 3 hours. Pour off this solution.
2. Heat the DIG-labelled DNA probe in a boiling water bath for 5 minutes, chill the tube on ethanol containing ice, add to fresh Easy Hyb to a final concentration of 0.25 $\mu l\ ml^{-1}$ and pour over the membrane.
3. Allow the probe to hybridise overnight at 38 °C in a hybridisation oven.
4. Remove the probe and store at -20 °C for reuse.
5. Wash the membrane twice, 5 minutes per wash, in 2x washing solution to remove unbound probe.
6. Wash the membrane twice, 15 minutes per wash in 0.5x washing solution at 68 °C. The membrane is now ready for nucleic acid detection.

Detection

Chemiluminescent detection was performed on membranes using the protocol outlined in 7.2.3.1.2.

Colorimetric detection was performed on some of the membranes. The following protocol was used:

1. Equilibrate the membranes in washing buffer for 1 minute, block by gentle agitation in blocking solution for one hour and incubate in a freshly prepared antibody solution for 30 minutes. The antibody solution is prepared by diluting centrifuged Anti-Digoxigenin-AP Fab fragments 1:50 000 in blocking buffer followed by mixing.
2. Remove unbound antibody with washing buffer, using two 15 minute washes.
3. Equilibrate the membrane in detection buffer for two minutes.
4. Mix 45 µl NBT solution and 35 µl BCIP solution (both from Boehringer Mannheim) in 10 ml detection buffer. Pour off the detection buffer and replace with this colour substrate. Incubate in a sealed plastic bag in the dark.
5. Remove the colour substrate by washing with water once the bands are detected, to prevent over development.

7.2.3.2 **Radioactive detection of the *pat* gene in a genomic DNA blot**

It was decided to use a radioactive detection system for the verification of transformation of the putative transgenic lines with Southern hybridisation. Although there are additional safety hazards inherent in the use of radioactivity, experience

in this laboratory has shown this to be more sensitive than non-radioactive detection, with less need for optimisation than using non-radioactive detection.

7.2.3.2.1 **Restriction digestion of isolated genomic DNA**

In a trail digest, 2 µg of genomic DNA was restriction digested with the three enzymes *Eco* RI, *Hind* III and *Pst* I, obtained from Boehringer Mannheim. This digest was performed only to determine whether the isolated DNA was pure enough for restriction digestion. The digests were performed according to standard procedures at 37 °C, using 3 units of restriction enzyme per µg of DNA, the appropriate amount of DNA, 10x buffer appropriate to the specific enzyme and distilled water (SAMBROOK *et al.* 1989). The DNA cut by *Eco* RI produced a partial digest and did not produce a smear when it was resolved on a 0.8 % agarose gel, but produced a band similar to the undigested DNA. The other enzymes produced smears characteristic of a complete digest. Initially, each of the enzymes mentioned were used to digest 4 µg DNA at 37 °C overnight, in order to perform the Southern blots. The restriction enzymes were added in excess at a concentration of 3 units of enzyme per µg of DNA. A volume of 5 µl of a 0.1 M spermidine solution was added to the *Eco* RI digest to improve the efficiency of the reaction. The digested DNA was precipitated with 1.2 volumes of a precipitation solution containing 1 volume 5 M potassium acetate, 2 volumes water and 22 volumes iso-propanol, as described by FELICIELLO and CHINALI (1993). The pellet was collected through centrifugation at 10 000 rpm for 10 minutes and washed twice with 80 % ethanol. The pellet was redissolved in 20 µl TE buffer. This DNA was separated on a 0.8 % agarose gel, at 25 volt overnight at 4 °C and used to perform a Southern blot, but no results were obtained.

In the final digest, 20 µg of genomic DNA was digested, precipitated and redissolved as described above. The DNA from the digest was separated on a 0.8 % agarose gel at 80 volts at room temperature and the *pat* gene was detected using a Southern blotting procedure as described below.

7.2.3.2.2 **Southern blotting of genomic DNA**

The DNA agarose gels were depurinated, denaturated and neutralised as described in 7.2.2.3. The DNA was transferred to a Magna Charge nylon membrane using a Transphor electrophoretic blotting apparatus from Hoefer Scientific Instruments. The apparatus was filled with a 0.025 M phosphate buffer. The transfer was allowed to proceed overnight at 0.5 ampere in a cold room at 4 °C. The DNA was fixed to the membrane by baking at 80 °C for two hours.

7.2.3.2.3 **Radioactive labelling of a *pat* specific probe using random primer labelling**

The Prime-It® RmT Random primer labelling kit was obtained from Stratagene® and used for the generation of a *pat* specific probe. The probe was generated immediately prior to the hybridisation step. Template DNA for the labelling reaction was prepared using PCR. DNA from the plasmid pBI101/ *pat* was amplified using the PCR mix and conditions as described in section 7.2.2 and Table 7.1. Approximately 30 ng plasmid DNA was used for the reaction. The PCR products were resolved on a low-melting-temperature agarose gel, visualised by ethidium bromide staining and the appropriate 531 base pair band was identified in

comparison with a molecular weight marker. The band was cut out of the gel with as little extraneous agarose as possible and placed into a preweighed Eppendorf tube. The tube was weighed again to obtain the weight of the gel and 3 ml distilled water per gram of gel was added. The tube was heated to 65°C for 5 minutes to melt the agarose. This DNA solution was used as the template for the labelling reaction and 42 µl was added to one of the single-use reaction tubes provided in the kit. The reaction tube was heated in a boiling water bath for 5 minutes, centrifuged briefly to collect all the liquid and transferred to a waterbath at 37°C. A 5 µl volume of the radioactively labelled nucleotide α-^{32}P-dCTP at 3000 Ci mmol^{-1}, was added and the contents mixed thoroughly. Twelve units of magenta DNA polymerase was added and the reaction was incubated at 37 °C for 10 minutes. The reaction was stopped by adding 2 µl stop mix. A similar labelling reaction was followed using DNA of the Boehringer Mannheim Molecular Weight Marker III. This was used for the visualisation of the markers in the genomic Southern analysis.

The probe was purified from the sample mix on a Sephadex G-50® pasteur column that was equilibrated with TE buffer (SAMBROOK *et al.* 1989). The total volume of probe was added to the column and eluted with TE buffer. Fractions of 200 µl were recovered in Eppendorf tubes and the tubes were held up to a Geiger-Müller detector for an indication of the activity, as no scintillation counter was available at the time.

7.2.3.2.4 **Southern hybridisation and radioactive detection of the *pat* gene**

Both prehybridisation and hybridisation were performed in tubes in a hybridisation oven. The membrane was blocked for 3 hours at 40 °C in prehybridisation solution.

During this time the radioactively labelled probe was generated. The prehybridisation solution was poured off and replaced with hybridisation solution containing the prepared probe. Hybridisation was performed at 40 °C overnight. The following post hybridisation washes were performed: two washes of 15 minutes each in 2x SSC solution at room temperature, two washes of 15 minutes each in 2x SSC with 0.1 % SDS at 65 °C, and two washes of 15 minutes each in 0.1x SSC with 0.1 % SDS at 65 °C.

The membranes were removed from the hybridisation tubes and sealed in plastic bags. The membranes were exposed to Hyperfilm MP autoradiographs, obtained from Amersham Life Science and placed between two Hyperscreen intensifying screens. The casettes were placed at -70 °C and the film was developed after exposure times ranging from a few hours to two weeks.

7.3 **RESULTS AND DISCUSSION**

7.3.1 **DNA extraction and quantification**

Several problems may be encountered during the isolation and purification of high molecular weight DNA from plants. DNA degradation can occur if endogenous nucleases are present, lowering the yield and highly viscous polysaccharides may be co-isolated, rendering the handling of the samples difficult. Polyphenols or other secondary plant compounds can interfere with restriction enzymes and polymerases or damage the DNA (WEISING *et al.* 1995). It follows that success in DNA extraction can be measured by the DNA yield, the condition of the extract and the

utility, or ease of use with restriction or other enzymes (ROGERS and BENDICH, 1994).

In this study, DNA extraction proved to be the biggest stumbling block in the molecular analysis of the putative transgenic lines. Yields were generally low and where the method described by EDWARDS (*et al.* 1991) was used, the total yield was typically between 250 and 1250 ng. This was enough to be able to proceed with PCR, but was not enough to perform Southern blots, where microgram quantities are required for each restriction digest. The appearance of the DNA also suggested that unacceptable levels of contamination with cell components was present, as the DNA pellet was often greenish or brown. The brown colour is associated with oxidised polyphenols, that are in turn powerful oxidising agents that damage DNA (LOOMIS, 1974). No attempts were made to cut this DNA with restriction enzymes and no further purification was attempted, as this would have decreased the yield. Despite these problems, little plant material was needed and the crude DNA preparation could be used for PCR, often successfully amplifying the correct size band from selected *in vitro* plants.

Large quantities of plant material only became available for large scale DNA isolation when the problems that were experienced with acclimatisation, described in the previous chapter, were solved and greenhouse plants were available. It became imperative to do the isolation from a large quantity of material, as the DNA yield decreased with every purification step that had to be included to obtain good quality DNA. The isolation method outlined by DELLAPORTA *et al.* (1983) was chosen because in this laboratory, DNA has been successfully isolated from several species, using this protocol (OELOFSE, personal communication, 1996). This method has been used to isolate DNA from melon (FANG and GRUMET, 1990,

VALLéS and LASA, 1994), but no further details were given in these reports. CHEE and SLIGHTOM (1991) used the CTAB method (SAGHAI-MAROOF *et al.* 1984), also without elaborating on details and SARMENTO *et al.* (1992) isolated DNA using the protocol of MURRAY and THOMPSON (1980).

Several modifications had to be made to the protocol before the problems that were encountered with the DNA isolation were solved. The DNA obtained from the basic potassium acetate protocol was still green or brown and increasing the β-mercaptoethanol in the isolation buffer from 10 mM to 140 mM made no visible difference to the condition or measurable yield. The addition of a phenol extraction step successfully removed any green or brown colour from the extract. The contamination could clearly be seen in the interphase and careful extraction of the upper phase finally removed this from the isolate. The addition of the RNAse step effectively removed the RNA contamination and this was visible when the isolate was separated on a 0.8 % agarose gel. Previously, the high molecular weight DNA was visible as a thick band on the agarose gels, with a bright RNA smear below that. In the new isolates, the RNA smear almost disappeared.

Although these steps improved the purity of the DNA considerably, problems were still encountered with viscous DNA solutions. The DNA solution was on occasion so viscous, that it could be removed from the Eppendorf tube as a solid mass. This was true even after the addition of two millilitres of TE buffer to the pellet. It could not be quantified, or loaded into the wells of an electrophoresis gel, making the most basic procedures difficult to perform. FANG *et al.* (1992) described this as typical of isolated melon DNA and reported that DNA isolated from young melon leaves contained approximately 3 µg carbohydrate per µg DNA, when assayed by a phenol-sulfuric acid method. It is this carbohydrate contamination that makes the

resulting pellet extremely viscous, making pipetting and quantification of the DNA difficult and sometimes impossible.

The last modification that was made to the protocol was the addition of 1.5 M NaCl to DNA dissolved in TE buffer, followed by an ethanol precipitation. The pellet that was recovered dissolved readily in TE buffer and was used to produce a genomic Southern blot. The total DNA yield of a single isolation from approximately 10 g of frozen leaf tissue was between 150 and 380 mg, with an average of 221 mg. This was enough for several restriction digests of 20 mg DNA each. This NaCl purification protocol was developed by FANG *et al.* (1992) who demonstrated that NaCl concentrations of 1.0 to 2.5 M NaCl can be used to remove polysaccharide contamination. When the DNA is precipitated with ethanol, the polysaccharides remain in solution, rather than precipitating out. As much as 90 % of the dissolved solids can be removed from the solution in a single precipitation step.

7.3.2 **PCR analysis**

The PCR reaction was used as an early indication of the transformed nature of selected plants, but is not reliable enough to serve as proof of integrative transformation, due to high numbers of false negatives and the inability of the method to distinguish between transformants and *Agrobacterium* contamination of the sample. The polymerase chain reaction is used to synthesise defined sequences of DNA using two oligonucleotide primers that bind to opposite sides of the DNA and bordering the DNA strand that is to be amplified (MULLIS *et al.*, 1986). The *pat* gene is 558 base pairs in length and the PCR primers were designed to amplify a 531 base pair section of this. The PCR products were separated and visualised on

agarose gels and amplified sections could be identified in comparison to the 564 base pair band in the Boehringer Mannheim Molecular Weight Marker III (MWMIII) that migrates only a little bit slower. The thermostable *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, catalyses the elongation of the primers (SAIKI *et al.* 1988). Exponential amplification is achieved by repetition of a cycle that includes a high temperature denaturation of the DNA, followed by primer annealing and extension of the annealed primers by the polymerase enzyme (MULLIS *et al.*, 1986). It is theoretically possible to amplify the target DNA region by a million-fold (2^{20}) in twenty cycles.

The PCR protocol that was used proved to be reliable despite initial problems related to inexperience and the quality of the DNA template. A number of times, the selected plants did not yield the expected size band, but on re-testing these proved to be false negatives. Although very little DNA is needed for PCR and relatively crude DNA preparations can be used, it is possible that contaminants in the solution can inhibit the *Taq* polymerase (SLIGHTOM *et al.* 1995). Contaminants interfered with the measurement of DNA concentrations with the result that the quantity of DNA template in the PCR reactions was variable. This had an influence on the amplification and could be seen on the agarose gels as bands with widely differing intensities, from barely visible to very strong. VALLéS and LASA (1994) also reported that the intensity of bands varied in the PCR of a fragment of the NPT II gene that was transferred to the *Cucumis melo* cultivar Amarillo Oro. In some cases, a faint smear was visible from the slot to the band, possibly due to degraded DNA. These problems were alleviated with the development of improved DNA isolation protocols as described previously.

Initially, two negative DNA controls were included with every PCR reaction; an untransformed Hales Best 36 control and a control of untransformed potato DNA. Later, the potato control was omitted. These PCR reactions did not produce the 531 base pair band expected when the *pat* gene is present. This indicated that false positives were unlikely unless the DNA templates were contaminated. The same was true for the water controls, indicating that the reagents were free from contamination. The positive control was derived from the plasmid pBI101/*pat* and never failed to amplify the expected band of 531 base pairs. Occasionally, well contamination during gel electrophoresis was encountered when the PCR products were separated on an agarose gel with the positive control in the lane next to one of the negative controls (Figure 7.1A). It was usually a very faint band and misinterpretation could be prevented by leaving a well open next to the positive control. When this happened, the agarose gel electrophoresis was repeated if some of the product was still available, or the PCR was repeated using the same reagents.

The results of a typical PCR experiment are shown in Figure 7.1A. This example was chosen because the *pat* gene in the putative transgenic lines are all sufficiently amplified to produce clear bands. In the first lane the MWMIII is loaded, followed by the five putative transgenic plants described in Chapter 6 in lanes two to six. The water control is next to this, followed by the untransformed Hales Best 36 control and the positive control. The expected 531 base pair fragment was amplified in all the putative transgenic lines, as well as in the positive control. The water control did not produce the band. A very faint band can be seen in the negative melon control. A Southern analysis was performed on this gel and the faint band hybridised to the *pat* probe. It was deduced that this was well contamination during electrophoresis, because the band was very faint and it was not seen in subsequent tests of the same material using the same reagents.

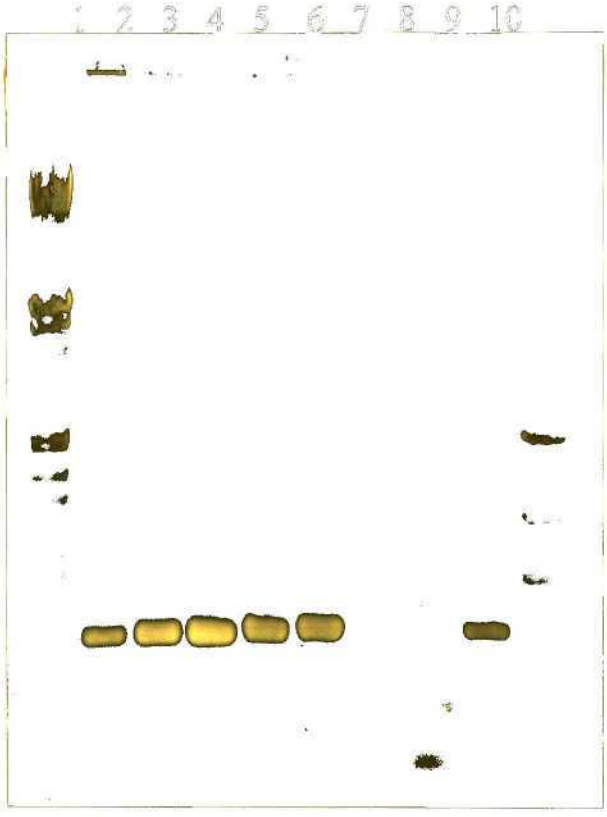


Figure V.1. Ethidium bromide stained agarose gel of the PCR amplified *pat* gene from five putative transformants of the melon cultivar Hales Best 36 (A) and Southern hybridisation (B) of the same gel, using the DIG system for detection of the *pat* gene. Lane 1, Molecular Weight Marker III; Lanes 2, 3, 4, 5 and 6, putative transgenic Line 1 to Line 5; Lane 7, water control; Lane 8, untransformed Hales Best 36 control; Lane 9, positive control pBI101/*pat*; Lane 10, molecular mass marker.

McGARVEY and KAPER (1991) reported that the small sample size required made PCR a useful method for screening putative transgenic tomatoes at a very early stage. Two reports have appeared using the PCR reaction as an indication of the transformed nature of cucurbits. GONSALVES *et al.* (1994) used it to detect the genes for cucumber mosaic virus -white leaf strain coat protein, NPT II and GUS in putative transgenics. The results were confirmed using Western analysis and phenotypic data. VALLéS and LASA (1994) used PCR to identify escapes on kanamycin selection medium. The expected fragment from the NPT II gene could not be amplified from the plants that were unable to root on the kanamycin containing medium. Although PCR analysis can be used as an indication of the transformed nature of plants, it cannot distinguish between a gene that is incorporated into the genome and artifacts such as latent *Agrobacterium tumefaciens* contamination (McGARVEY and KAPER, 1991; BIRCH, 1997). For this reason, it is essential to show incorporation of the gene into the plant genome using Southern hybridisation.

7.3.3 **Southern analysis**

7.3.3.1 **Non-radioactive detection of the *pat* gene in a PCR blot**

The digoxigenin-anti-digoxigenin labelling system is based on the use of Digoxigenin (DIG) that is isolated from *Digitalis* plants (KARCHER, 1994). Standard labelling protocols, such as nick-translation, random prime labelling or PCR based labelling can be used to incorporate a nucleotide triphosphate analogue containing digoxigenin into probe DNA. The probe is subsequently detected by

enzyme-linked immunoassay using an antibody to DIG, to which alkaline phosphatase has been conjugated. Chemiluminescent or colorimetric substrates can be used to detect the DIG-labelled probe.

7.3.3.1.1 **PCR labelling of a *pat* specific probe using DIG**

An aliquot of each of the DIG labelled, PCR amplified probes was analysed by agarose gel electrophoresis. Each probe produced a single product band after ethidium bromide staining (Figure 7.2). As predicted, the unlabelled control reaction produced a band slightly smaller than the MWMIII 546 base pair band. The labelled products produced significantly larger bands, all located above the band of the control reaction on the agarose gel, due to the incorporation of heavier DIG-dUTP residues. The largest band was produced by the 3:1 dTTP:DIG-11-dUTP reaction. The probes derived from the 3:1 and 7:1 dTTP:DIG-11-dUTP reactions were used to confirm the identity of the fragments obtained from PCR analysis of the putative transgenic plants.

7.3.3.1.2 **Testing of different nylon membranes**

Clear differences were seen between the Boehringer Mannheim membranes and the Magna Charge membrane after hybridisation with the DIG labelled probe. The amount of non-specific background that was produced on the Magna Charge membrane was consistently less than the background produced on the Boehringer Mannheim membranes. The plasmid DNA dot was visible on all the membranes where shorter exposure times were used, but with longer exposure times, the

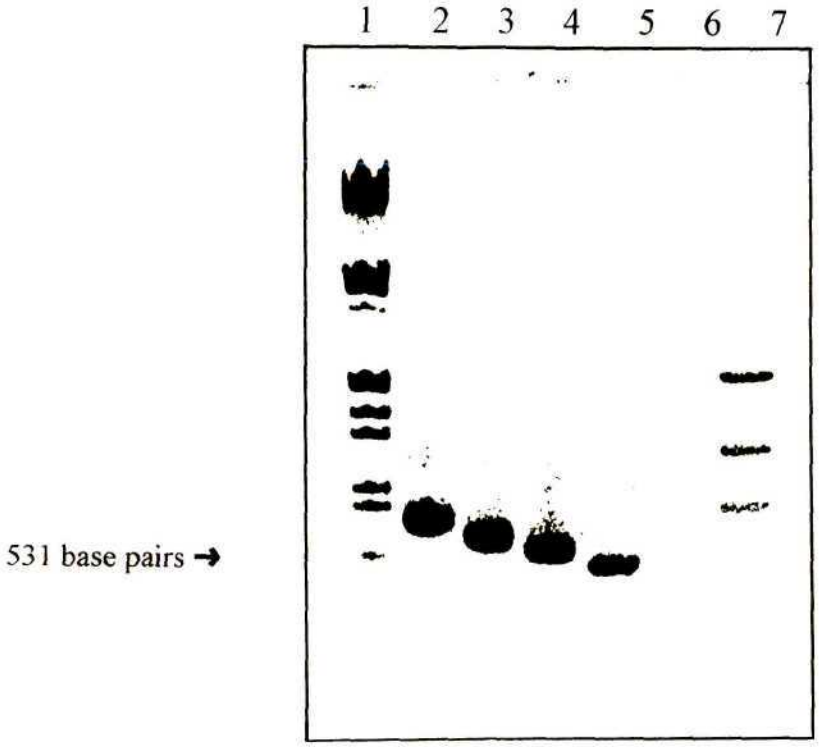


Figure 7.2 Ethidium bromide stained agarose gel of PCR amplified *pat* specific probes labelled with different ratios of dTTP: DIG-11-dUTP. Lane 1, Molecular Weight Marker III. Lane 2, 3:1 dTTP:DIG-11-dUTP; Lane 3, 7:1 dTTP: DIG-11dUTP; Lane 4, 12:1 dTTP:DIG-11-dUTP; Lane 5, unlabelled dNTP control; Lane 6, unlabelled dNTP water control; Lane 7, DNA mass marker.

background on the Boehringer Mannheim membranes was dark enough to mask weaker signals. It was decided to use the 7:1 dTTP:DIG-11-dUTP probe for future experiments, in combination with the Magna Charge membrane, to produce stronger signals without increasing the non-specific background.

7.3.3.1.3 **Southern blotting of PCR products**

The chemiluminescent and colorimetric detection protocols were both successful in locating the expected bands on the Southern blots of the PCR products. A PCR amplifications of the *pat* gene was performed on the first three selected lines that were produced. The positive tobacco control plants, the three putative transgenic plants and the positive control plasmid DNA, hybridised to the probe and could be seen as a brown stain on the membrane (Figure 7.3). The lanes containing the water control and the untransformed potato DNA did not produce this signal. The pattern of the signal corresponded to the pattern that was seen on the agarose gel after ethidium bromide staining. This made it unnecessary to use a probe for the molecular weight marker. Non-specific background hybridisation was seen on the membrane, but the bands could easily be distinguished from this.

The chemiluminescent detection protocol was used for the detection of the *pat* gene in the PCR amplified products from the five putative transgenic lines shown in Figure 7.1A. All the bands that are visible on the agarose gel after ethidium bromide staining, were detected in the corresponding position on the autoradiograph, again eliminating the need for a marker probe (Figure 7.1B). The bands that produced the signal were the amplified products from the DNA of the five putative transgenic lines and the positive control plasmid DNA. A faint band could be detected in the

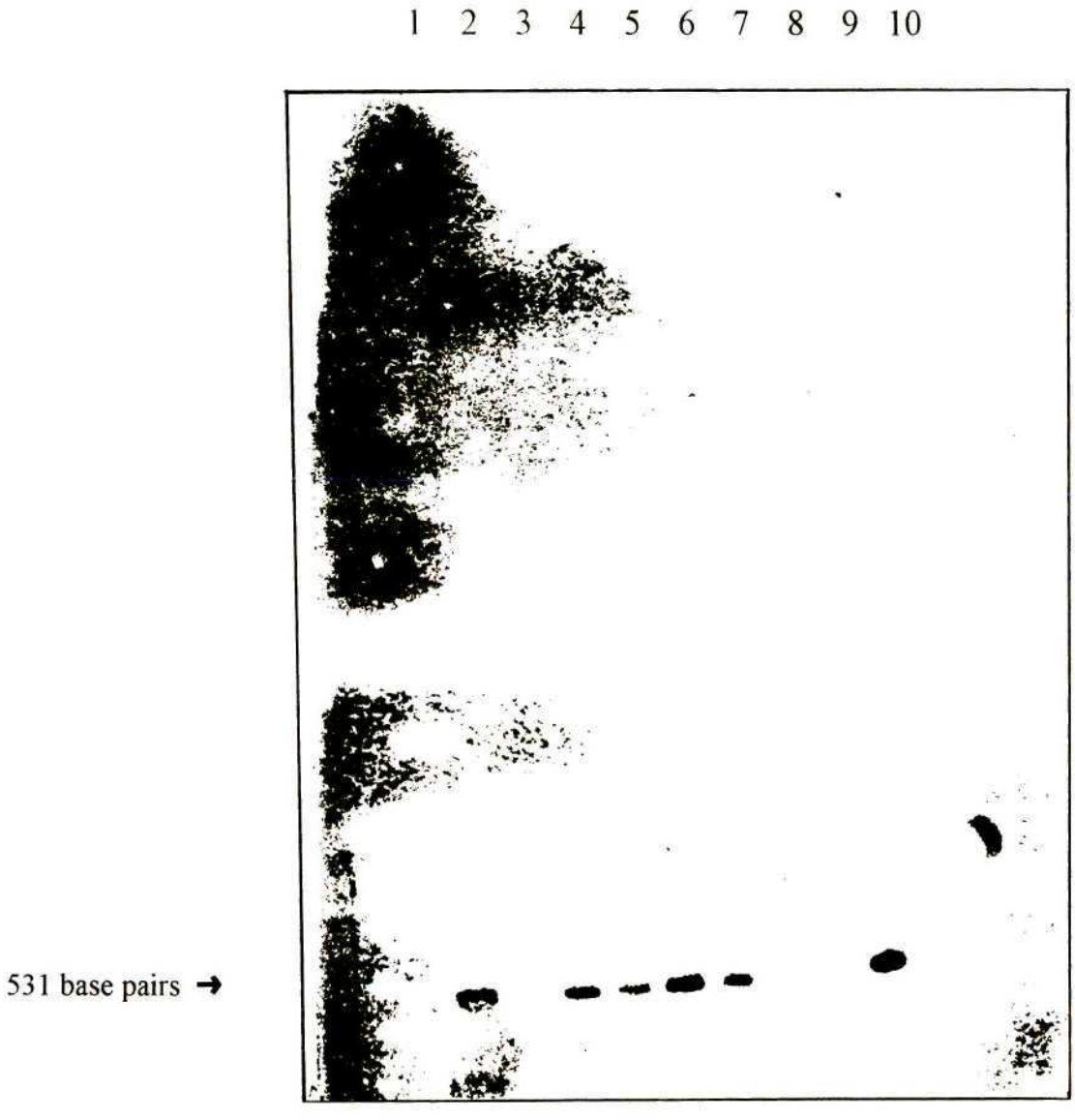


Figure 7.3 Colorimetric detection of the products of a PCR amplification of the *pat* gene in DNA isolated from putative Hales Best 36 transgenic plants. The PCR products were blotted into a nylon membrane and hybridised to a *pat* specific probe. Lane 1, Molecular Weight Marker III; Lane 2, transformed tobacco; Lane 3, untransformed Hales Best 36 control; Lane 4 and 7, Line 1 putative transformant; Lane 5, Line 2 putative transformant; Lane 6, Line 3 putative transformant; Lane 8, untransformed potato DNA control; Lane 9, water control; Lane 10, positive control pBI101/*pat*.

negative control, but as described before, this was most probably well contamination that occurred during gel electrophoresis, as it was not seen in previous or further experiments. This result confirmed the assumption that the bands that were generated in the PCR reaction are the section from the *pat* gene and that the gene was present in DNA isolated from the putative transformant lines. Although this result is a confirmation of the results from the PCR experiment, it is not an acceptable substitute for Southern hybridisation analysis, because it is difficult to exclude the possibility of artifacts in such data (BIRCH, 1997).

Although the PCR products were detected satisfactorily with the DIG system, some experiments had to be repeated a number of times to reduce the non-specific background. A number of possible reasons exist for this (BOEHRINGER MANNHEIM, 1995b) and several aspects of the protocol can be optimised. It was, however, decided to use a radioactive detection protocol for the Southern hybridisation analysis, as it has proven to be less labourious and more reliable in this laboratory. The signal that is generated in a Southern analysis is likely to be fainter than the PCR generated bands and the increased sensitivity of a radioactive detection system will improve the probability of obtaining a successful result.

7.3.3.2 **Radioactive detection of the *pat* gene in a genomic DNA blot**

7.3.3.2.1 **Radioactive labelling of a *pat* specific probe using random primer labelling**

Random primer labelling (FEINBERG and VOGELSTEIN, 1983) was used for the labelling of the *pat* specific probe. According to the Stratagene Instruction Manual

that is provided with the Prime-It® Random Primer Labelling Kit, a random 9-mer oligonucleotide is used as primer for the DNA syntheses along the length of a single stranded DNA template. The magenta polymerase enzyme in the kit incorporates nucleotides at the 3' -OH group provided by the primer. The radioactive nucleotide (α-^{32}P)dCTP was substituted for one of the nucleotides in the reaction mixture, with the result that the newly synthesised DNA was also radioactively labelled (WEISING *et al.* 1995).

The removal of unincorporated labelled nucleotides prevent non-specific background hybridisation and protect against unnecessary exposure to radioactivity (WEISING *et al.* 1995). The technique is based on gel filtration to separate the molecules according to size. DNA molecules larger than 80 base pairs do not enter the pores of the beads and run from the column very fast, through the inter bead spaces (SAMBROOK *et al.* 1989). Small molecules enter the bead pores and are retained in the column. The first few Eppendorf tubes containing individual 200 µl fractions did not display more radioactivity than the background. A clear peak could be distinguished and the three most active tubes were pooled in a single tube. This DNA was used as a hybridisation probe for probing a genomic Southern blot of the putative transgenic lines. A second peak, consisting of the unincorporated labelled nucleotides was eluted later and discarded as radioactive waste.

7.3.3.2.2 **Restriction digestion and Southern analyses of isolated genomic DNA**

A restriction digest was performed on 4 mg genomic DNA from each of the lines and untransformed Hales Best 36 control DNA. The digested DNA was separated with agarose gel electrophoresis, Southern blotted, hybridised to the radioactively

labelled probe and exposed to autoradiographs. In these blots, the positive control, consisting of undigested plasmid DNA produced a smear on the autoradiograph, indicating that the hybridisation was successful and that the probe functioned satisfactorily. No hybridisation bands were seen in any of the digested samples. The undigested samples produced an ambiguous smear on the autoradiograph that could have been non-specific background. The failure of the experiment indicated that the DNA sample was possibly too small to produce detectable bands and that a larger DNA digest might be successful.

A digest of 20 µg of DNA was prepared for each of the lines and the untransformed Hales Best 36 control DNA, using the restriction enzymes *Eco* RI, *Hind* III and *Pst* I. The digested DNA and an equal amount of uncut DNA was precipitated and redissolved, as the restriction enzymes had to be removed and the volume of the digests reduced to fit into the wells of an agarose gel. The concentration of the resultant DNA solution was very high and unexpectedly, when the gel loading buffer was added, the solution became very viscous, similar to the crude DNA preparations described previously. The volume of TE buffer had to be increased again and the preparations were heated to 65 °C. This increased the solubility of the DNA, but some of the samples were still viscous. More loading buffer had to be added to increase the density of the samples, as the diluted samples did not drop evenly into the wells. Two gels were prepared, the first contained the undigested DNA, the *Eco* RI digests, a marker and a positive control. The *Hind* III digests, the *Pst* I digests, a marker and positive control were loaded onto the second gel. When the agarose gels were loaded, the volume of these samples were larger than the volume of the wells and the wells were simply loaded until full. Although it was impossible to accurately determine the amount of DNA that was loaded in each well, about a

quarter of the total volume of each sample was left, indicating that approximately 15 µg of the samples were loaded.

The DNA was visualised in the gel with ethidium bromide stain prior to the Southern transfer. The *Hind* III and *Eco* RI digests were complete digests and produced the characteristic smear on the gel. Even though the *Pst* I digest was incomplete it was used, as it was on the same gel as the *Hind* III digest. Most of the undigested DNA did not migrate far in the lanes and could be seen in close proximity to the wells, although some breakdown of the DNA could be identified as a smear in the gel. A substantial amount of DNA did not migrate out of the wells and could be visualised there. This was possibly due to the problems of DNA viscosity. The lanes containing the molecular weight markers were cut from the gel and Southern blotted separately. This made it possible to hybridise the marker to a separate probe prepared from marker DNA. The gel was again stained after transfer of the DNA to the membrane and the DNA could be seen very faintly in the gel. This indicated that the transfer was incomplete.

After hybridisation, the membranes were sealed in polyethylene bags and placed in casettes with autoradiographs for detection of the hybridised probe. The autoradiographs were developed after four hours, three days, one week or two weeks. The signal from the positive control DNA could be detected within four hours, but the DNA banding pattern from the putative transgenic lines only became visible after two weeks. On these autoradiographs, the positive controls gave a very strong signal that prevented the identification of bands in the lanes next to it. In the *Eco* RI and *Pst* I digested DNA, bands were only detected for Line 1, Line 3 and Line 4, but the *Hind* III digested DNA yielded bands for all the transgenic lines. Very little non-specific background could be seen on any of the autoradiographs.

This result confirms that the radioactive detection method with ^{32}P can provide very sensitive detection, although it is far more time consuming than the non-radioactive methods and results are only available after weeks, not hours as is the case with the DIG-system. The autoradiographs were scanned into a desktop publishing programme. The images of the digested blots, the positive control and the markers that originated from the same gel, but that were developed on separate autoradiographs, were combined in composite images. One image contained results from the undigested DNA from the five lines, the *Eco* RI digested DNA, the positive control and the molecular weight marker that were all run on the same gel (Figure 7.4). The second image represented the *Hind* III digest and the *Pst* I digest, with the positive control and the molecular weight marker that were included in the second gel (Figure 7.5).

No band was visible in the lane containing uncut control DNA, but bands could be seen in all the lanes containing uncut DNA isolated from plants of Lines 1 to 5 (Figure 7.4). These bands were larger than the 21 226 base pair molecular weight marker, consisting of large fragments of intact DNA. The DNA of Line 4 was damaged and produced a slight smear, but even this was larger than the 5148 base pair marker band.

Bands were produced in the *Eco* RI digested DNA but only for Lines 1, 3 and 4 (Figure 7.4). No band could be identified in the control DNA from untransformed Hales Best 36. Line 1 and Line 3 produced one band each, both similar in size and considerably smaller than expected. A band of 1 300 base pairs should be produced in a complete digest of DNA containing the casette that was used for the transformation, as this restriction site flanks the promotor and the terminator, with the *pat* gene in between. Both these bands were not as distinct as the others that

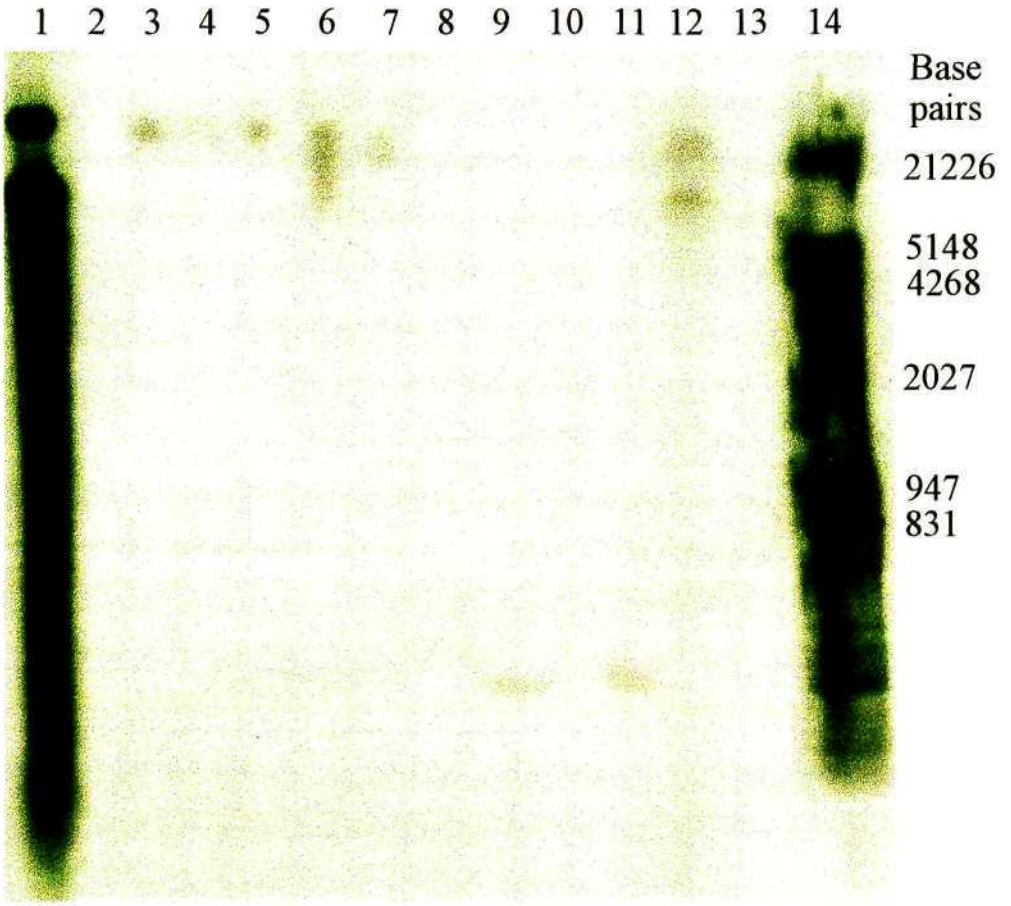


Figure 7.4. Southern hybridisation analysis of genomic DNA isolated from transformed Hales Best 36 plants. Lane 1, positive control pBI101/*pat*; Lane 2,untransformed Hales Best 36 DNA, Lanes 3 - 7, uncut DNA from Line 1 to Line 5 respectively; Lane 8, *Eco* RI digested DNA from untransformed Hales Best 36, Lanes 9 - 13, *Eco* RI digests of DNA from Line 1 to Line 5 respectively; Lane 14, MWMIII.

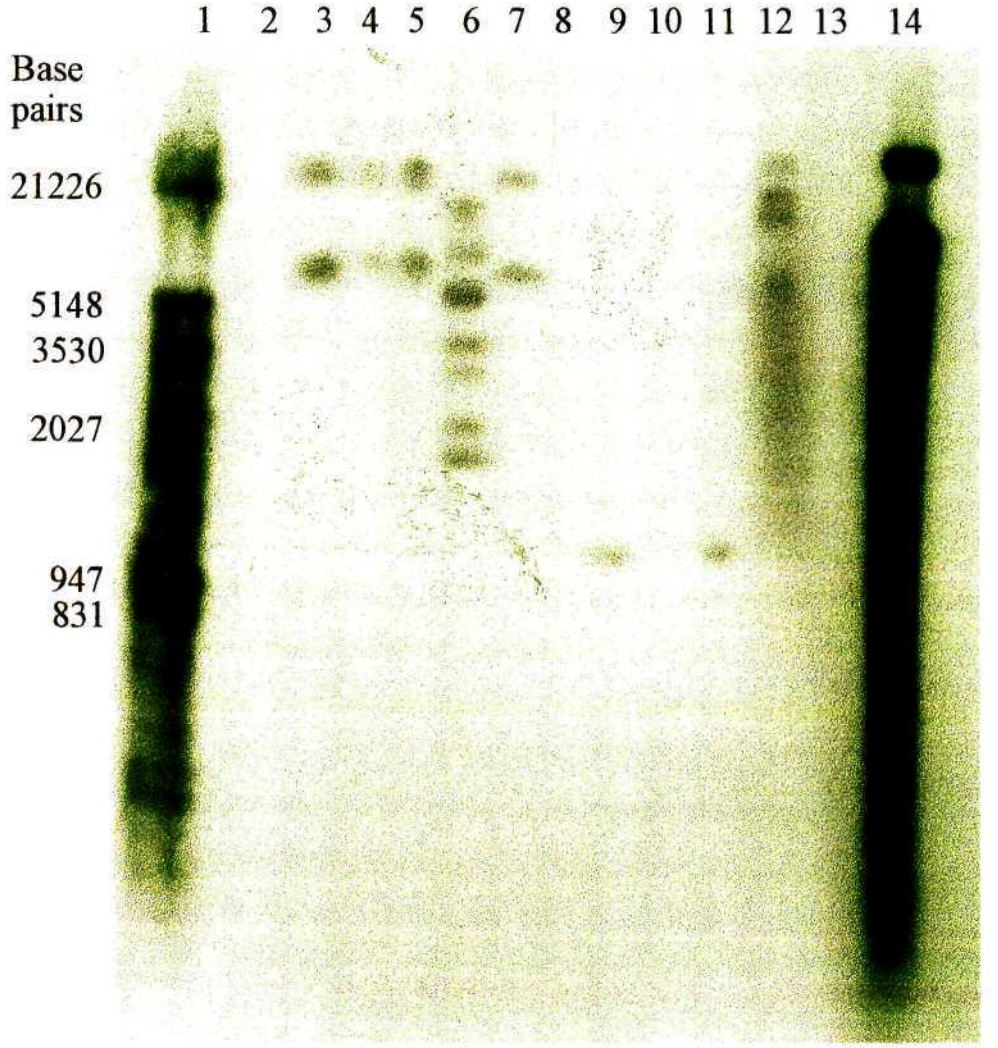


Figure 7.5. Southern hybridisation analysis of genomic DNA isolated from transformed Hales Best 36 plants, demonstrating integration of *pat* into the genome of transformants. Lane 1, MWM III; Lane 2, *Hind* III digest of untransformed Hales Best 36 DNA; Lanes 3 -7, *Hind* III digests of DNA from Line 1 to Line 5 respectively.; Line 8, *Pst* I digest of untransformed Hales Best 36 DNA; Lanes 9 -13, *Pst* I digests of DNA from Line 1 to Line 5 respectively; Lane 14, positive control pBI101/*pat*.

were produced and appeared to exceed the lane boundaries. For these reasons, these bands should be regarded as possible artifacts. The bands that were produced by the DNA from Line 4 also does not conform to the expected size of 1 300 base pairs expected for a complete digest, but produced at least two bands; one greater than 21 226 base pairs and one smaller than that, but larger than the 5 148 base pair marker. The digest could have been incomplete, or it is possible that the restriction site was methylated. Although the site recognised by the *Eco* RI restriction enzyme is not listed as sensitive to methylation, it does contain a cytosine base (RAZIN, 1988).

No band could be seen in the control DNA digested with the *Pst* I enzyme (Figure 7.5). The *Pst* I digested DNA of the selected plants yielded distinct bands for Lines 1, 3 and 4. In the casette that was used, a complete *Pst* I digest will yield one hybridising band of 213 base pairs (VILJOEN, 1994), or a band of approximately 3 000 base pairs, consisting of most of 1 300 base pair *pat* gene and 1 940 base pairs from the neomycin phosphotransferase (NPT II) gene and *nos* terminator. The bands produced for Line 1 and Line 3 fall between the 947 and the 1 904 base pair markers. In Line 4, the four bands that were produced were all larger than the 5 149 base pair marker. The restriction enzyme *Pst* I is known to be sensitive to methylation of one or both of the cytosine bases in the restriction site CTGCAG. The modified 5-methylcytosine prevents the digestion of the DNA sequence at this site (RAZIN, 1988). In this case, the DNA will only be digested at non-methylated *Pst* I sites. Any sequence resulting from this digest that contains the transferred DNA will hybridise with the probe, regardless of whether the sites bordering the fragment were both in the plant genome or whether one of the known sites in the transferred sequence was cleaved. From this, it would appear that the DNA was incorporated into at least four different sites in the genome.

The *Hind* III digest produced very clear bands in all the selected lines, but no band could be seen in the control DNA (Figure 7.5). This result confirms the transformed nature of the selected plants. The banding pattern is shown in Figure 7.5. Each of the lines produced at least two visible bands and in the case of Line 4, multiple bands could be identified. Some of the bands were close together and it was difficult to determine whether there were one or two, but at least eight could be identified clearly. The resolution of the gel was not sufficient to draw conclusions about the sizes of the bands. Some resolution was also lost in the reproduction, but in the original, the bands produced by the lines were clearly of different sizes. Since the *Hind* III enzyme cleaves the DNA only on one side of the *pat* gene, the size of the hybridising fragments is indicative of the location of the nearest *Hind* III site derived from the melon genome that borders the opposite side of the *pat* gene. In Chapter 6, the results from the *in vitro* progeny trails suggested that it was unlikely that Line 2 and Line 5 had single integrations of the *pat* gene into the genome. A lack of data prevented the same type of prediction for the rest of the lines. This result confirms that the gene was integrated into more than one site in the genome of all five lines, as each of the bands represents a separate T-DNA insertion. The size of these bands also provides the final indication that the hybridised gene is not derived from latent *Agrobacterium tumefaciens* contamination, although this is highly unlikely to be present in the plants after numerous subcultures. The pBI101/*pat* plasmid provides a 13 500 base fragment when cut with *Hind* III (VILJOEN, 1994) and this band was not present in any of the lines.

Four reports have appeared describing *Agrobacterium tumefaciens* mediated transformation of *Cucumis melo*, but in two of these, DONG *et al.* (1991) and GONZALVES *et al.* (1994), transformants were only identified by the expression of the transferred genes, PCR or the expression of the transferred gene in the

progeny. The reports of FANG and GRUMET (1990) and VALLéS and LASA (1994) described Southern analysis of the transformants. In the former report, the NPT II marker gene was transferred to the plants and in the latter report, this gene as well as the GUS gene were present in the T-DNA. Very little emphasis was given to the protocols that were followed for the DNA isolation or the Southern analysis. In both reports, restriction enzymes were chosen that could distinguish the number of integrations of T-DNA into the genome. FANG and GRUMET (1990) reported that two distinct bands were observed in the Southern blot of one of the 25 lines that was tested. All the other plants displayed only one band in the Southern analysis, which indicates a single insert. Although VALLéS and LASA (1994) recovered six kanamycin resistant shoots, only two of these could be rooted on kanamycin containing medium. These plants yielded the expected band in PCR and hybridised to the NPT II and GUS probes. Both produced single bands indicative of a single integration. These reports are in contrast to this study where all the transformed plants have multiple insertions. It has been reported that several plants with multiple insertions were identified in *Brassica napus* (FRY *et al.* 1987) and tobacco transformation studies (HEBERLE-BORS *et al.* 1988). It has been shown that the recovery of plants with multiple insertions is influenced by factors such as the explant type and age (GREVELDING *et al.* 1993), the regeneration conditions and even small differences in experimental technique used by different researchers (FRY *et al.* 1987). It is possible that the stringent selection that was used in this study selected against single insertions and that only plants with multiple insertions were able to survive on the 10 mg l^{-1} phosphinothricin selection medium.

It is possible that further treatment of the DNA and an improved protocol for the Southern blotting of the DNA will lead to the identification of even more bands, but

the current data has provided sufficient information to satisfy the purpose of this investigation.

7.4 **CONCLUSION**

In this Chapter, DNA was successfully isolated from leaf material of the lines developed in Chapter 6. Several modifications in the protocol improved the condition and utility of the isolated DNA considerably by removing RNA, protein and polysaccharide contamination. A PCR was performed on the DNA from all the lines and included several controls to differentiate between artifacts and transformed plants. The PCR amplified the expected band from the DNA of the putative transformants and positive control. This served as an indication of the transformed nature of the five selected lines, but although PCR has been used as evidence for transformation (McGARVEY and KAPER, 1991), it is not considered to be sufficient proof of integrative transformation (BIRCH, 1997). For this reason, Southern analysis was performed on the DNA. Little information was obtained from the DNA cut with the restriction enzymes *Eco* RI and *Pst* I, but the DNA that was cleaved with *Hind* III provided a clear banding pattern that indicated that the *pat* gene was incorporated into multiple sites in the genome of the melon Hales Best 36.

This information, combined with that obtained in the previous Chapter, satisfy the criteria described by POTRYKUS (1991) and BIRCH (1997) considered to be essential to conclusively demonstrate integrative transformation. The controls that were included for the transformation experiments and the analyses of the transformants consistently distinguished between putative transformants and untransformed plants. Tight correlations were observed between the treatments and

the predicted results in the recovery of the transformants and in the analysis of these plants. The results from the phenotypic analysis and the Southern analysis were mutually supportive. The phenotypic data showed sustained expression of the introduced gene exclusively in the plant lines that were positive for the gene. The Southern analysis of restriction digested DNA generated restriction fragments of different length, indicating the existence of different integration sites, as was demonstrated with the use of the enzyme *Hind* III. This result was predicted from the phenotypic analysis of the segregation of the gene in the progeny.

CHAPTER 8

CONCLUSION

The incompatibility barriers that exist between some of the members of the Cucurbitaceae, coupled to the difficulty in transferring traits such as insect, herbicide and virus resistance to crops using conventional breeding techniques, necessitated the development of a transformation protocol for these crops. The aim of this study was primarily to establish a model for the regeneration, transformation and molecular analysis of cucurbits.

In future, the applicability of this protocol to other members of the Cucurbitaceae could be tested. It became clear that the regeneration of plants from explants remains one of the most significant problems in the development of a transformation protocol. The experience gained during this study has shown that this aspect is likely to remain genotype specific. The investigation into the regeneration potential of other commercially important members of this family should be a priority in a future program of research. The potential of somatic embryogenesis as a regeneration strategy should also be investigated, especially for *Cucurbita maxima*.

Although the transformation efficiency of Hales Best 36 remains low, the protocol developed in Chapter 5 could be utilised to develop herbicide resistant lines of *Cucumis melo* Hales Best 36. The trait was transferred to the progeny, making it possible to use these plants in a breeding programme. To date, the transformation protocol has not been tested on other members of the Cucurbitaceae. The broad

applicability of molecular analysis techniques suggest that the protocol developed in this study is likely to be applicable to other cucurbits. The protocol can also be used to transfer other useful genes into the genome of the melon Hales Best 36. The choice of which beneficial genes should be introduced should be considered carefully. A gene that confer virus resistance has been isolated and cloned into transformation vectors at the ARC-Roodeplaat, but the benefits of transgenic plants with this characteristic need to be evaluated thoroughly against their potential detrimental environmental impact.

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APPENDIX A

MS medium (MURASHIGE and SKOOG, 1962)

Salts	**Concentration (mg l^{-1})**
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$ZnSO_4.7H_2O$	8.6
$MnSO_4.H_2O$	22.3
$CuSO_4.5H_2O$	0.025
$CaCl_2$	440
KI	0.83
$CoCl_2.6H_2O$	0.025
KH_2PO_4	170
H_3BO_3	6.2
$NaMoO_4.2H_2O$	0.25
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
Organics	
Inositol	100
Thiamine.HCl	0.1
Nicotinic acid	0.5
Pyridoxine.HCl	0.1
Glycine	2
Sucrose	
Agar	

APPENDIX B

Buffers for ß-glucuronidase assays (JEFFERSON, 1987)

GUS extraction buffer	**Concentration**	**Grams per 100 ml distilled water**
$NaPO_4$	50 mM	0.59 g
EDTA	10 mM	0.0292 g
Triton X-100	0.1 %	100 µl
ß-mercaptoethanol	10 mM	70 µl

GUS reaction buffer

(1 mM MUG in extraction buffer)

Dissolve 18 mg 4-methyl umbelliferyl ß-D-glucuronide in 50 ml GUS extraction buffer.

Stop buffer

(0.2 M Na_2CO_3)

Dissolve 21.2 g Na_2CO_3 in distilled water and make up to 1 litre.

APPENDIX C

1 GENERAL SOLUTIONS

1.1 Solutions for electrophoresis

TAE buffer
40 mM Tris-acetate
1 mM EDTA
Adjust the pH to 8.0 with glacial acetic acid.

Gel loading buffer
0.25 % bromophenol blue
0.25 % xylene cyanol
30 % glycerol

2 SOLUTIONS FOR DNA EXTRACTION

2.1 DNA extraction for PCR analysis, using the method of EDWARDS *et al.* 1991

"Super-quick" extraction buffer
200 mM Tris HCl (pH 7.5)
25 mM EDTA
250 mM NaCl
0.5 % SDS (sodium dodecyl sulfate)
Autoclave and add 1% β-mercathoethanol.

TE buffer
10mM Tris HCl, pH 8.0
1 mM EDTA

2.2 DNA quantification

TNE buffer
100 mM Tris HCl pH 7.4
10 mM EDTA
1 mM NaCl

Fluorometer solutions

Stock solution: 50 ml TNE Buffer and 10 µl Hoechst dye 33258 (1 mg ml^{-1}). Filter sterilised.
Use 2 ml stock solution to 'zero' the fluorometer.
Use 2 ml stock solution and 2 µl of a 100 ng μl^{-1} calf thymus DNA solution to as a standard.
Use 2 ml of the solution and 2 µl plant DNA to determine the concentration of the sample.

2.3 DNA isolation for Southern hybridisation, using the method of DELLAPORTA *et al.* 1983

DNA extraction buffer

100 mM Tris HCl (pH 8.0)
50 mM EDTA
500 mM NaCl
Autoclave and add 10 mM β-mercathoethanol.

TE5 buffer

50 mM Tris HCl pH, 8.0
10 mM EDTA

3 SOLUTIONS FOR SOUTHERN HYBRIDISATIONS

3.1 Solutions for the DIG system for filter hybridisation

Denaturation solution

0.5 N NaOH
1.5 M NaCl

Neutralisation buffer

0.5 M Tris -HCl pH 7.5
3 M NaCl

20x SSC

3 M NaCl
300 mM sodium citrate, pH 7.0

2x (and 0.5x) washing solutions

2x (or 0.5x) SSC (prepared from 20x SSC)
0.1 % SDS (sodium dodecyl sulphate)

Washing buffer
0,1 M Tris (pH 7.5)
0,15 M NaCl

Blocking buffer
Buffer 1 + 1 % blocking reagent
Leave approximately 1 hour (or overnight) at 68°C until dissolved

Detection buffer
100 mM Tris (pH 9.5)
100 mM NaCl
50 mM $MgCl_2$

NBT and BCIP solutions
NBT: 75 mg/ml nitroblue tetrazolium salt in 70 % (v/v) dimethylformamide
BCIP: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 % dimethylformamide

Prehybridisation and hybridisation buffers
Easy Hyb (Boehringer Mannheim) without or with probe

3.2 Solutions for Southern hybridisations with radioactive detection

Phosphate buffer
Prepare a 0.1 M Na_2PO_4 phosphate buffer stock from 46.3 ml of a 1 M Na_2HPO_4 solution and 53.7 ml of a 1 M NaH_2PO_4 solution in 1000 ml H_2O.
Prepare the 25 mM Na_2PO_4 phosphate buffer, pH6.5, from this.

Prehybridisation and hybridisation buffers
30 ml 20x SSC
5 ml 100x Denhards solution
5 ml 10 % SDS
10 ml H_2O
50 ml formamide
For the prehybridisation buffer: Add 1 % of a 100 µg $µl^{-1}$ solution of herring sperm DNA
For the hybridisation buffer: Add the freshly prepared probe

100x Denhards solution
2 % Ficol 400, 2 % polyvinylpyrrolidone, 2 % bovine serum albumin, Fraction 5