CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Micropropagation of Begonia and a study of genome stability in Begonia rex
Authors	Morrish, Fionnuala
Publication date	1985
Original Citation	Morrish, F. 1985. Micropropagation of Begonia and a study of genome stability in Begonia rex. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Link to publisher's version	http://library.ucc.ie/record=b1124210~S0
Rights	© 1985, Fionnuala Morrish - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2026-11-06 23:12:39
Item downloaded from	https://hdl.handle.net/10468/1658



UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

DP 1985 MORR (REF)
338391

MICROPROPAGATION OF *BEGONIA* AND A STUDY OF GENOME STABILITY IN *BEGONIA REX*

by

Fionnuala Morrish, BSc

Thesis submitted for the degree of
Doctor of Philosophy
of the National University of Ireland

Supervisor: Professor Alan Cassells

Department of Plant Science, University College, Cork, Ireland

October 1985

UNIVERSITY COLLEGE
Library
CORK

ABSTRACT

The development of procedures and media for the micropropagation of *B. rex* are described. Media for the production of plantlets from a number of other *Begonia* hybrids are also provided.

Growth analysis data is given for plants produced *in vivo* from leaf cuttings and *in vitro* from mature leaf petioles and immature leaves derived from singly and multiply recycled axenic plantlets. No significant difference was found in phenotype or quantitative vegetative characters for any of the populations assessed.

The results presented from studies on the development of broad spectrum media for the propagation of a number of *B. rex* cultivars using axenic leaf explants on factorial combinations of hormones illustrate the major influence played by the genotype on explant response *in vitro* and suggest media on which a range of *B. rex* cultivars may be propagated.

Procedures for *in vitro* irradiation and colchicine treatments to destabilize the *B. rex* genome have also been described. Variants produced from these treatments indicate the utility of *in vitro* procedures for the expression of induced somatic variation. Colour variants produced from irradiation treatment have been cultured and prove stable. Polyploids produced as variants from irradiation treatment have been subcultured but prove unstable.

Media for the induction and proliferation of callus are outlined. The influence of callus subculture and aging on the stability of the *B. rex* genome is assessed by chromosomal analysis of cells, *in vitro* and in regenerants. The *B. rex* genome is destabilized in callus culture but attenuation of variation occurs on regeneration. Diploid cell lines are maintained in callus subcultures and supplementation of regenerative media with high cytokinin concentrations, casein hydrolysate or adenine failed to produce variants. Callus aging however resulted in the production of polyploids.

The presence and expression of pre-existing somatic variation in *B. rex* pith and root tissue is assessed and polyploids have been produced from pith tissues cultured *in vitro*.

The stability of the *B. rex* genome and the application of tissue culture to micropropagation and breeding of *B. rex* are discussed.

ACKNOWLEDGEMENTS

To my supervisor and Professor, Alan Cassells, I wish to say a sincere thank you for encouragement and guidance throughout the course of this work.

I am also grateful to all the staff and postgraduates of the Botany Department UCC for their willingness to help when necessary. Special thanks are due to Don Kelleher, Dave White and John Mullane for technical assistance and to Mairéad Kiely for care of my plants. I would also like to thank Dr Peter Jones for helpful discussion on cytological work.

The begonias used in the study were kindly identified by Dr Doorenbos, to whom I am also grateful for a visit to the *Begonia* collection in Wageningen.

My thanks to Olwyn Mielke for sharing her time and talent to illustrate the tissue culture of *B. rex*. The excellent typing of this thesis by Laura O Mahony is also greatly appreciated.

Finally, I am deeply grateful and indebted to my brothers and sisters for their interest, patience and tolerance. To my parents and friends Mairin and Noel I pay special tribute for their moral support and generosity in giving me the time and encouragement needed to complete this work.

PUBLICATIONS

Cassells, A.C. and Morrish, F.M. (1985). Growth measurements of *Begonia rex* Putz. plants regenerated from leaf cuttings and *in vitro* from leaf petioles, axenic leaves, recycled axenic leaves and callus. Scientia Horticulturae (in press).

Cassells, A.C. and Morrish, F.M. (1985). Investigations for the presence of common regenerative media requirements in axenic leaf explants of *Begonia rex* Putz. cultivars. (In preparation).

LIST OF ABBREVIATIONS

APM	axenic plantlet medium
B	buds
BAP	benzyl amino purine
C	callus
CM	callus induction medium
DMSO	dimethyl sulphoxide
EM	Elatior medium
GA	gibberellic acid
IAA	indole acetic acid
LSD	least significant difference
NAA	naphthalene acetic acid
P	plantlets
S	shoots
SDM	shoot development medium
SIM	shoot induction medium
TIBA	2,3,5-triiodobenzoic acid
BR5	'Jubilium Weinstephan'
BR22	'Bodnant'
BR26	'Regia'
BR27	'Silver Corkscrew'
BR28	'Inimitable'
BR30	'Lucille Closon'

TABLE OF CONTENTS

Page

ABSTRACT i

ACKNOWLEDGEMENTS ii

PUBLICATIONS iii

LIST OF ABBREVIATIONS iv

TABLE OF CONTENTS v

SECTION A. INTRODUCTION 1

A.1. General introduction and objectives 2

SECTION B. LITERATURE REVIEW 6

B.1. The genus Begonia: origins and methods of propagation 7

1.1. Introduction 7

1.1.1. Rhizomatous begonias 8

1.1.2. Begonia x hiemalis 9

1.1.3. Begonia x tuberhybrida 10

1.2. Cytogenetics of Begonia 11

1.2.1. Introduction 11

1.2.2. Karyotype polymorphism 14

1.2.3. Implications for tissue culture 15

B.2. Plant propagation using tissue culture with particular reference to Begonia 16

2.1. General introduction 16

2.1.1. Plant micropropagation procedures 17

2.1.2. Selection of explant and mode of regeneration 17

2.1.3. Establishment of sterile cultures 20

2.1.4. Development of plantlets 21

2.1.5. Rooting and establishment of plantlets 22

2.1.6. Progeny plant assessment 23

Page

B.2.2. *Factors influencing morphogenic responses of explants in vitro* 24

2.2.1. *Introduction* 24

2.2.2. *Genotype influences on morphogenic responses of explants in vitro* 25

2.2.3. *Effect of media constituents on morphogenic responses of explants in vitro* 27

2.2.4. *Influence of the physical state of the medium on morphogenic responses of explants in vitro* 31

2.2.5. *Influence of explant origin, size and orientation on morphogenic responses in vitro* 32

2.2.6. *Influence of the donor plant on morphogenic responses of explants in vitro* 35

2.2.7. *Influence of the in vitro culture environment on morphogenic responses of explants* 39

2.3. *In vitro stock maintenance using cold storage* 42

2.4. *Continuous culture of axenic organ explants for stock multiplication and maintenance* 43

B.3. *Tissue culture as an adjuvant to conventional plant breeding* 46

3.1. *General introduction* 46

3.2. *Genetic variation in somatic plant cells: causitive mechanisms and the influence of tissue culture on the expression and induction of somatic variation in cultured cells and regenerated plants* 47

3.2.1. *Introduction* 47

3.2.2. *Somatic cell variation in vivo: causitive mechanisms and consequences* 49

3.2.3. *Mechanisms which give rise to somatic polyploidy* ... 52

3.2.4. *Mitotic cycle abnormalities, mutation and chromosome rearrangements* 57

3.2.5. *The influence of variation in the nuclear DNA of individual cells on the genetic architecture of plants and the production of phenotypic variants* ... 61

3.3. *Somatic cell variation in vitro: Speculations on origins and factors controlling development* 66

3.3.1. *Introduction* 66

3.3.2. *The influence of media constituents, subculture duration and level of organization on chromosomal variation in cultured cells* 74

3.3.3. *Possible explanations for the occurrence of genetic drifts in cultured cells* 80

Page

B.3.4. Tissue culture and somaclonal variation: factors controlling expression and stability 82

3.4.1. Introduction 82

3.4.2. Explant source 82

3.4.3. Genotype 83

3.4.4. Epigenetic effects 84

3.4.5. Regeneration procedures 85

3.4.6. Latent and transient somaclonal variation 93

3.4.7. Loss of morphogenic competence on subculture 94

3.5. Tissue culture in the expression and propagation of variants induced by treatments with mutagens or colchicine 96

3.5.1. Introduction 96

3.5.2. Mutation breeding 97

3.5.3. Induction of polyploids and other variants using colchicine 100

3.6. Generation and expression of somatic variation: general conclusions 105

SECTION C. GENERAL MATERIALS AND METHODS 109

C.1. Stock plants: sources and growth conditions 110

C.2. Tissue culture methods 110

2.1. Explant culture 110

2.2. Axenic plantlets: cultural procedures and storage 111

2.3. Development and rooting of adventitious shoots 111

2.4. Establishment of plants 111

C.3. Callus culture 112

3.1. Induction and proliferation of callus 112

3.2. Regeneration of plants 112

C.4. In vitro gamma irradiation 112

4.1. Preparation of material 112

4.2. Radiation treatment 112

4.3. Culture and regeneration from irradiated explants 113

C.5. Cytology 113

5.1. Fixing hydrolysis and staining 113

5.2. Chromosome analysis 114

Page

C.6. Light microscopy 114

6.1. Hand sectioning 114

6.2. Estimation of chloroplast numbers 114

6.3. Staining with fluorescein diacetate 114

C.7. Evaluation of leaf colour variants 115

SECTION D. EXPERIMENTAL WORK I 117

D.1. Micropropagation of Begonia 118

1.1. Introduction 118

D.2. Responses of explants from a number of Begonia species hybrids to exogenous auxin and cytokinin 118

2.1. Introduction 118

2.2. Material and method 119

2.2.1. Begonia rex Putz cv. Lucille Closon 119

2.2.2. Begonia x hiemalis 121

2.2.3. Begonia x tuberhybrida 'Crispa Margenata' 121

2.2.4. Begonia bowerii 121

2.2.5. In vitro derived explants 121

2.2.6. Development of plantlets 121

2.3. Results 121

2.4. Discussion 127

D.3. Optimization of explant source for micropropagation of B. rex 128

3.1. Introduction 128

3.2. Effect of petiole ontogeny on morphogenic responses of B. rex in vitro 129

3.2.1. Material and method 129

3.2.2. Results 129

3.3. Explant zonation influence on morphogenesis in B. rex petiole explants 129

3.3.1. Material and method 129

3.3.2. Results 129

3.4. Axenic leaf size influence on regeneration 133

3.4.1. Material and method 133

3.4.2. Results 133

3.5. Discussion 133

Page

D.4. Comparative analysis of B. rex plants propagated from leaf cuttings and from tissue cultures of mature petiole and axenic leaves 134

4.1. Introduction 134

4.2. Material and method 135

4.2.1. Leaf cuttings 135

4.2.2. Petiole explants 135

4.2.3. Axenic leaf explants 136

4.2.4. Growth analysis 136

4.3. Results 136

4.4. Discussion 141

D.5. Evaluation of the application of continuous culture to the propagation of B. rex 143

5.1. Introduction 143

5.2. Material and method 143

5.2.1. An analysis of the productivity and survival of axenic leaf explants in vitro 143

5.2.2. Comparative analysis of progeny from leaves of axenic plantlets and leaves of multiply recycled axenic leaves 144

5.2.3. Indirect assessment of changes in endogenous hormone levels during subculture 144

5.3. Results 145

5.4. Discussion 152

D.6. Assessment of the regenerative responses of a number of B. rex cultivars in vivo and in vitro 153

6.1. Introduction 153

6.2. Material and method 153

6.2.1. Cuttings 153

6.2.2. Petiole explants 154

6.2.3. Axenic leaf culture 154

6.3. Results 154

6.4. Discussion 161

D.7. In vitro storage of Begonia 162

7.1. Introduction 162

7.2. Material and method 163

7.2.1. Plantlet development on media in the presence and and absence of glycerol 163

7.2.2. Storage of budded petiole explants of B. rex by delayed growth at low temperature 163

Page

D. 7.2.3. *Storage by delayed growth of budded leaf explants of B. x hiemalis* 164

7.3. *Results* 164

7.3.1. *Plantlet storage using glycerol treatment* 164

7.3.2. *Plantlet storage by delayed growth in B. rex* 164

7.3.3. *Storage by delayed growth of buds on leaf explants of B. x hiemalis* 166

7.4. *Discussion* 166

SECTION E. EXPERIMENTAL WORK II 167

E.1. *The use of tissue culture in the production of variants from B. rex* 168

1.1. *Introduction* 168

E.2. *Mutation breeding of B. rex using in vitro culture of axenic leaves* 170

2.1. *Introduction* 170

2.2. *Material and method* 171

2.3. *Results* 171

2.4. *Discussion* 187

E.3. *The effect of colchicine treatment on plantlet regeneration from tissue culture of B. rex by the adventitious bud technique* 190

3.1. *Introduction* 190

3.2. *Material and method* 190

3.3. *Results* 191

3.4. *Discussion* 195

E.4. *Callus culture of B. rex* 197

4.1. *Introduction* 197

E.5. *Induction, proliferation and regeneration of callus from B. rex* 198

5.1. *Introduction* 198

5.2. *Material and method* 198

5.2.1. *Callus induction* 198

5.2.2. *Callus proliferation* 199

5.2.3. *Regeneration of callus* 199

5.3. *Results* 199

5.4. *Discussion* 204

Page

E.6. Assessment of the regenerative capacity and plants produced from primary and subcultured calli of B. rex 205

6.1. Introduction 205

6.2. Material and method 205

6.3. Results 206

6.4. Discussion 210

E.7. Callus aging and plantlet regeneration in B. rex 211

7.1. Introduction 211

7.2. Material and method 211

7.3. Results 213

7.4. Discussion 215

E.8. Cytogenetic analysis of callus and regenerated plants in B. rex 216

8.1. Introduction 216

8.2. Material and method 217

8.3. Results 217

8.4. Discussion 226

E.9. The influence of supplementing regenerative media with casein hydrolysate, adenine or high concentrations of cytokinins on variant production from B. rex callus 228

9.1. Introduction 228

9.2. Material and method 228

9.2.1. Media supplementation with kinetin and BAP 228

9.2.2. Media supplementation with casein hydrolysate and adenine 229

9.3. Results 229

9.3.1. Adenine and casein hydrolysate treatments 229

9.4. Discussion 231

E.10. Callus induction on colchicine and its influence on regenerated plants of B. rex 231

10.1. Introduction 231

10.2. Material and method 232

10.3. Results 232

10.4. Discussion 233

E.11. Studies on the presence of somatic cell variation in pith and root explants of B. rex and expression in in vitro culture 233

11.1. Introduction 233

Page

E.11.2. Material and method 234
11.2.1. Explants 234
11.2.2. Pre-culture of explants for cell division prior to chromosome analysis 234
11.2.3. Callus culture 235
11.3. Results 235
11.3.1. Cytological studies of root and pith explants and callus 235
11.3.2. Pith culture 235
11.3.3. Root culture 239
11.4. Discussion 239

SECTION F. GENERAL DISCUSSION 242

F.1. General discussion 243

F.2. Proposals for future work 254

SECTION G. GLOSSARY 257

SECTION H. BIBLIOGRAPHY 260

SECTION I. APPENDIX 301

I.1. Chloroplast counts and chromosome counts from plants produced from colchicine treatments 302
1.1. Results and discussion 302

SECTION A. INTRODUCTION

A.1. General introduction and objectives

Tissue culture has gained a reputation as a useful method for large-scale clonal plant propagation and plant improvement (Reinert and Bajaj, 1977) and is also acknowledged as providing a means of short or longterm storage of plant tissues or cells (Henshaw, 1982). To fulfil this role however, regeneration from plant tissues or cells *in vitro* must result in the production of high numbers of vigorously growing plants which will compete successfully with their conventionally propagated counterparts. While a high multiplication rate has been recorded for cultures *in vitro* (e.g. Takayama and Misawa, 1982a) and novel techniques have been developed for plant improvement (see Barton and Britt, 1983; Owens, 1983; Sybenga, 1983; Gustafson, 1984), the practical application of these techniques for large-scale clonal propagation and plant improvement has been impeded by a number of major bottlenecks in the regeneration of plant tissues *in vitro*. These include tissue recalcitrance, low numbers of surviving plants and genome destabilization. Hence, the development of culture procedures which overcome these problems and result in the production of large numbers of plants which do not show phenotypic variation from the parent (i.e. are true to type) are of prime importance both to the clonal propagation of existing genotypes as well as new genotypes which may be produced in plant breeding programmes.

Low levels of morphogenic competence and variation in explant response can be attributed in many cases to the routine use of glasshouse grown donor plants as explant material for tissue cultures. The responses of plants *in vitro* being significantly influenced by the physiological and ontological state of the donor plant (i.e. epigenotype) (e.g. Cassells *et al.*, 1982). The use of glasshouse grown donor plants also has two other drawbacks, its expense and the constant threat of disease.

Tissue culture methods have been developed for the elimination of disease (see Ingram and Helgeson, 1980) and hence *in vitro* plantlets provide a source of disease indexed stock which may be used in plant propagation. Presently, disease indexed plants are grown under disease free conditions and then used as donor plants to establish stock (e.g. Barnhill Jones, 1979).

An alternative approach which would reduce labour costs and allow both storage and large-scale multiplication of plant stocks *in vitro* involves the development of continuous subculture systems (see Section G.) using axenic organs as explants (Lê and Collet, 1981). In view of the induction of variants as a result of subculture however (see Cassells and Plunkett, 1986) the practical application of this procedure to a given plant requires extensive testing for its influence on genome stability and plant vigour. An alternative procedure for storage which would ensure genome stability is the maintenance of plantlets at low temperatures (Henshaw, 1982). While these procedures have as yet only been evaluated for a limited range of plants these methods would appear to be applicable to many plants if properly researched.

Different plant genotypes when cultured *in vitro* on a given medium have been shown to differ in their morphogenic responses (e.g. Welander, 1977). In many cases these differences have been due to the combined influence of epigenotype (see Section G.) and genotype. The development of similar micropropagation media and protocols for tissue culture of a number of different genotypes is desirable in view of the subsequent reduced expenditure on media and labour. While it is possible that overlap may occur in responses of different genotype if tested on a range of media, especially if epigenotype influences were reduced by the use of axenic explants, no studies of this nature have been reported in the literature.

The major attribute of plant tissue culture which makes it a useful adjuvant to conventional plant breeding techniques is the increase in the number of variant cells which may undergo organogenesis, such as those induced by mutation breeding (Roest *et al.*, 1981), colchicine treatment (Heinz and Mee, 1970) or those pre-existing in the explant (Hermsen *et al.*, 1981). The production of large numbers of phenotypic variants (see Section G.) from protoplast culture of potato (Shepard *et al.*, 1980) and cells of sugarcane (Heinz and Mee, 1971) suggest that variants may also be induced in culture as a result of the destabilizing influence of *in vitro* conditions. These variants which have been defined as somaclonal variants (see Section G.) (Larkin and Scowcroft, 1981) may have traits not readily achieved through conventional breeding techniques and hence provide a useful source of plant material for plant breeders.

While cell or protoplast culture appear to provide a potentially useful source of variation, many of the variants induced in tissue culture have been found to breakdown on subsequent culture (e.g. Larkin and Scowcroft, 1983b) and variants identified at the cell level have been found to loose their variant traits on regeneration (e.g. Dix, 1977). Hence variation induced *in vitro* may not be stable and may have an epigenetic or physiological base (Cassells, 1985). In order to overcome these problems and utilize tissue culture more efficiently in the production of useful stable variants we require a fuller understanding of factors controlling genome stability/instability *in vitro* and the subsequent regeneration of stable variants. Developments in this field require studies which encompass cytogenetic evaluation of source tissue, cultured tissue and regenerants with emphasis on the evaluation of the effect of different culture conditions and regeneration protocols on the genotype and phenotype of regenerants. In all these studies it will be essential to be able to regenerate a large population of plants for assessment.

The present study on *B. rex* has been undertaken to develop an effective micropropagation procedure which could then be used to assess the potential for the production of variants and to study genome stability under different culture conditions. Studies on *in vitro* storage and the development of broad spectrum media for the propagation of other *Begonia* genotypes have also been undertaken. The objectives of this study are outlined below:

(1) The development of efficient micropropagation procedures for *B. rex* and the screening of media for the culture of a number of *Begonia* hybrids.

(2) The assessment of continuous and discontinuous culture procedures (see Section G.) at the explant level with emphasis on the number of shoots produced and the efficiency of the protocol, with subsequent evaluation at the plant level to assess the vigour and stability of the plants produced.

(3) Elucidation of the influence of plant tissue culture on the improvement of propagation in a number of *B. rex* genotypes with a concurrent examination of the potential for the development of common media for their micropropagation.

(4) To evaluate the potential use of tissue culture as an adjuvant to conventional plant breeding in the production of variants from *B. rex*, firstly, by treating explants with gamma irradiation and colchicine, secondly, by producing plants from callus and, thirdly, by inducing the expression of inherent somatic variation in *B. rex* tissues.

(5) To assess the level of genome destabilization induced in *B. rex* at the cellular level and subsequent assessment of the effect of destabilization on regenerants as a result of callus induction, subculture, aging and treatment with colchicine and other media supplements.

(6) To determine whether quantitative nuclear somatic variation exists in the root or pith tissue of *B. rex* and develop procedures which would allow the expression of this variation, if present, as regenerated plants.

SECTION B. LITERATURE REVIEW

B. Literature Review

In relation to the objectives outlined above (Section *A.1.*) the literature review is divided into three sections. The first section is a general review of the genus *Begonia* with special reference to propagation and cytogenetics. The second reviews micropropagation and factors influencing organogenesis in explant culture. Finally, the use of tissue culture as an adjuvant to plant breeding is reviewed.

Nomenclature: To avoid any misunderstanding in interpretation of the present work a glossary of terms has been provided to indicate the meanings ascribed here to individual terms (see Section G.).

B.1. The genus Begonia: origins and methods of propagation

B.1.1. Introduction

The genus *Begonia* is a member of the family *Begoniaceae* in which there are two other genera, *Hillebrandi* and *Symbegonia*, of the three genera plants from the genus *Begonia* are the most widely found in cultivation.

The name *Begonia* was first ascribed to the genus by Charles Plumier a Franciscan monk and botanist who went to the Antilles Islands in the West Indies in 1690 to collect rare plants. He discovered 6 new species after which he named the genus *Begonia* in honour of Michael Begon the then "Intendant" of the French Antilles. The first live specimen of *Begonia* reached Kew in 1777 when *Begonia nitida* Dryand was sent from Jamaica. Through extensive hybridization between members a wide range of begonias have been produced. Buxton's 1939 check list of begonias contains about 7,500 names of species and cultivars. Details of their development have been outlined by Haegemann (1979) and Thompson and Thompson (1981).

Begonias are characterized by the winged ovary of the female flower and appear in the most divergent forms and include; herbaceous plants (e.g. *B. semperflorens*); plants with rhizomes (e.g. *B. rex*); plants with tubers (e.g. *B. tuberhybrida*); plants with creeping, climbing and hanging stems and even small trees (up to 4 m high) (for further examples see Thompson and Thompson, 1981).

Many of the more exotic begonias are unsuitable as house plants and are only found in Botanic gardens or private collections. A number of plants in this genus, however, have been successfully

commercialized. In Europe commercially grown begonias include; *B. semperflorens*, *B.* x *tuberhybrida*, *B.* x *hiemalis* Fotsch. and the rhizomatous begonias (e.g. *B. rex*). A brief outline of the origin and methods of propagation of some commercial begonias is included here. For a wider review see Thompson and Thompson (1981).

B.1.1.1. Rhizomatous begonia

Rhizomatous begonias are divided into two geographical groups American and Asiatic. Rex begonias, which are grown mainly for their colourful foliage, have been derived from the latter group. The original *B. rex* Putz. species are natives of the Himalayas where they grow in humid shady places. Jean Linden introduced the original *B. rex* Putz. in 1858 having bought it among orchids at a London auction (Bedson, 1954 *loc. cit.*). Since then extensive hybridization and the production of spontaneous and induced mutations has led to the production of plants with a diversity of growth forms and colour patterns, a more detailed account of which is provided by Thompson and Thompson (1981). Countless hybrids and cultivars of *B. rex* exist which has led to confusion regarding their nomenclature as many are unnamed, and some old varieties have been re-issued under new names.

Members of the Rex begonias readily produce plantlets from leaf cuttings. The earliest report of plantlet production in *B. rex* Putz. was made by Regel (1876) and histological studies were subsequently conducted by Hartsema (1926) and Prevot (1948) who concluded that buds arise exclusively from the epidermis and roots from the internal tissue. Studies by Prevot (1948), Bigot (1971) and Chlyah and Tran Thanh van (1975, 1984) demonstrate that in some cultivars buds arise specifically from zones surrounding glandular hairs. Bigot (1971) found the site of explantation of leaf discs from the leaf influenced the number and vigour of plants produced and cuttings taken from near the petiole and including a portion of the main vein proved optimal (Bigot, 1971). Photoperiod has also been shown to influence regenerative capacity and cuttings taken from plants grown under short days gave the highest percentage of plantlet formation (Bigot, 1971). Short day treatments however limit plant growth and *B. rex* plants are preferentially grown in long days (Bigot, 1971). Bud production in *B. rex* cuttings is polar, this polarity can be overcome by exogenous treatments with cytokinins which induce the production

of multiple buds over the total surface of a leaf cutting (Bigot, 1971). However maximal induction and development is always found near main veins which may act as a source of endogenous hormones and nutrients (Bigot, 1971; Chlyah, 1972). Studies by Bigot (1971) on cuttings from a number of different *B. rex* cultivars demonstrate that cultivars show distinctive differences in their regenerative responses which include; (1) sequence of morphogenesis (shoot → root, root → shoot); (2) site of organogenesis (adaxial or abaxial); (3) number of buds formed and (4) timescale of bud induction. The production of sports is also dependent on the cultivar and *B. rex* 'Winter Queen' is reported by Bigot (1971) to produce a range of off-types from cuttings. Bigot (1971) suggests that the differences between cultivars in regenerative potential may be due to genetic differences as a result of their interspecific (Prevot, 1939) and intraspecific (Villerts, 1938) hybridization.

The American rhizomatous begonias generally have smaller leaves than the Asiatic forms and include plants with peltate leaves, spiral leaves and leaves with crested edges or furry leaves. Most produce panicles of small pink or white flowers on erect stems above the leaves in early spring (see Thompson and Thompson, 1981).

In commerce the most widely grown member of this group are the *B. boweri* varieties. The species *B. boweri* was found by McDougall in Mexico in 1948 and has been hybridized extensively by Rudolf Ziesenhenne (Bedson, 1954 *loc. cit.*). Members of this group are propagated either by leaf petiole cuttings or by subdivision of rhizomes.

B.1.1.2. *Begonia* x *hiemalis*

The group of winter flowering begonia hybrids known as Elatior begonias (*Begonia* x *hiemalis* Fotsch.) have been developed by crossing various tetraploid (2n = 52) tuberous begonias species hybrids with the diploid (2n = 28) winter flowering species *B. socotrana* (reviewed by Arends, 1970). These hybrids have a compact growth habit and the bright flower colour of the tuberous parent, but do not form a tuber, nor do they become dormant in the autumn.

The name Elatior was derived from the varietal name of a hybrid introduced by Veitch in 1906 (Arends, 1970 *loc. cit.*). *Begonia elatior* hybrids are also called Hiemalis begonias from the name *B.* x *hiemalis* Fotsch. proposed for the group by Fotsch (1933). A further addition to the development of this group began in 1955 on the introduction

of the first hybrids of O. Rieger at Nurtingen in Germany which led to the development of the Rieger begonias. An extensive array of cultivars exists and these have been developed from both spontaneous and induced mutations.

In commerce *B.* x *hiemalis* plants are propagated vegetatively from leaf petiole cuttings. Commercial propagation of these plants has revealed both seasonal and cultivar variation in regenerative ability (Cohl and Moser, 1976).

The effect of environmental conditions on the budding potential in *B.* x *cheimantha* 'Everett', another winter flowering begonia, has been extensively studied (e.g. Heide, 1964, 1965a, 1965b; Zieslin *et al.*, 1984). Results of these studies suggest that the seasonal changes observed in regenerative capacity of *Begonia* leaves (e.g. Cohl and Moser, 1976) are the result of complex interactions between temperature, daylight energy and day length on the level of endogenous hormones (Heide, 1964). Clarification of the nature of change in endogenous hormones awaits the development of suitable methods for the routine quantitative assessment of cytokinin and auxin levels in plant tissue. Presently seasonal variation is reduced by growing mother stock plants in short days and treating cuttings with long days on the basis of the hypothesis that short days stimulate the initial bud formation while long days stimulate subsequent elongation into shoots (Cohl and Moser, 1976).

B.1.1.3. *Begonia* x *tuberhybrida*

Tuberous begonia hybrids (*Begonia* x *tuberhybrida* Voss) are widely grown as ornamental plants. They are characterized by their flowers which due to their large size, richness of colour and variation of shapes, make them highly valued as bedding plants. The origins of this group have been thoroughly discussed by Haegemann (1979).

The diploid species mentioned as ancestors of *B.* x *tuberhybrida*, cross readily to form fertile hybrids which facilitated the rapid expansion of this group once the initial crosses had been made and many of these plants are commercially grown from seed. Sterility, however, also occurs among such hybrids as the "multiflora group" and some large flowered double begonias and these are propagated by stem cuttings. Problems with virus disease and labour costs however have led to a decline in the production of these varieties (cf. Samyn *et al.*, 1984).

B.1.2. Cytogenetics of Begonia

B.1.2.1. Introduction

The genus *Begonia* includes plants with a diverse range of morphological characters (see Thompson and Thompson, 1981). This diversity is reflected in the differences in chromosome number and size recorded for different *Begonia* species hybrids and cultivars (e.g. Piton, 1962; Legros and Doorenbos, 1969, 1971, 1973; Legros and Haegemann, 1971). The number of chromosomes recorded in *Begonia* has been shown to range from 16-156 (Legros and Doorenbos, 1973).

Begonia chromosomes are in general very small, ranging from 0.5-2.8 µm and are thus difficult to study. One of the major problems encountered has been the slow growth of *Begonia* roots which give relatively low numbers of cells for analysis. Arends (1970) estimated that over 1,000 root tips had to be squashed in order to obtain 250 cells for analysis in *B.* x *hiemalis* cultivars. In view of these difficulties it is surprising that no attempts have been made to use young leaf tissue or buds as a source of tissue for analysis. These tissues should provide more actively dividing meristematic cells and are easier to handle than slender root tips, the cells of which may include heavy cytoplasmic contents which decrease the staining and visibility of the chromosomes (Sharma and Bhattacharyya, 1957). Other difficulties have been reported in chromosome staining.

Studies by Legros and Doorenbos (1969) demonstrate that Feulgen staining is only successful if tissues are treated overnight instead of the standard 2 hours required for many other plant tissues (see Sharma and Sharma, 1981). Even then chromosomes can only be observed clearly using phase contrast microscopy (Legros and Doorenbos, 1969; Arends, 1970). Efforts made by Arends (1970) to improve staining by alterations in the stains and the time and temperature of the treatments or the use of different fixatives, were unsuccessful. The small size of the chromosomes has also prevented the construction of karyotypes for many species as they do not show sufficiently defined morphological features (Legros and Doorenbos, 1973).

The most serious complication found in chromosome counting in *Begonia* is the variation in chromosome number found within individual plant tissues (Piton, 1962; Sharma and Bhattacharyya, 1957, 1961; Legros and Doorenbos, 1968). Because of these variations, when counting *Begonia* chromosomes the number present in the highest

frequency is regarded as the norm (Sharma and Bhattacharyya, 1957).

The variation in somatic chromosome numbers recorded for *Begonia* occurs in too high a frequency, 30-40 percent of cells in some cases (Sharma and Bhattacharyya, 1957) for it to be ignored. In *B. barbata*, for example, chromosome numbers of 25, 26, 34, 35 and 36 have been recorded from a single individual (Sharma and Bhattacharyya, 1957).

This phenomenon is not unusual in plants, conspicuous inconstancy in chromosome number in somatic tissues was first reported by Duncan (1945) in root tips of *Paphiopedium wardii* where chromosome numbers ranging from 41 to 45 were recorded. The term aneusomaty (see Section G.) was suggested by him to characterize this phenomenon. Thereafter many investigators have reported aneusomaty in a wide range of plants (see Fukumoto, 1962; John and Lewis, 1968; Jones, 1978). On the basis of observations on mitosis in plants in which aneusomaty occurs there have been a number of different proposals put forward to explain the underlying events which cause this phenomenon and briefly they include:

(1) Fragmentation and elimination of chromosomes.
(2) Structural changes i.e. Robertsonian fusion (see Section G.).
(3) Separate plate or double plate metaphase.
(4) Nuclear abnormalities such as lobation or cleavage.
(5) Mitotic abnormalities including; multipolar spindles, straying chromosomes at anaphase and non disjunction. (See also Figure B.1. and Dyer, 1976).

While loss or gain of individual chromosomes may occur, discrepencies in chromosome counts in somatic tissues may also be due to the difficulty involved in counting small chromosomes. Furthermore, asynchronous condensation of small and large chromosomes in hybrid genomes, as found in *B.* x *hiemalis* (Arends, 1970) may prevent accurate chromosome counting. It has also been suggested (Arends, 1970) that asynchrony may lead to chromosome elimination as found in *Hordeum vulgare* x *Hordeum bulbosum* (e.g. Finch and Bennett, 1983).

The presence of B-chromosomes (see Section G.) has been noted in some begonias e.g. *B. ampla* (de Wilde and Arends, 1980) and it is possible that inconstancy in chromosome number in *Begonia* may be due to the presence of Bs which have been shown to vary in number within tissues of some plants (Jones, 1975). Bs have already been acknowledged by Darlington (1973) as a source of discrepancies in chromosome counts.

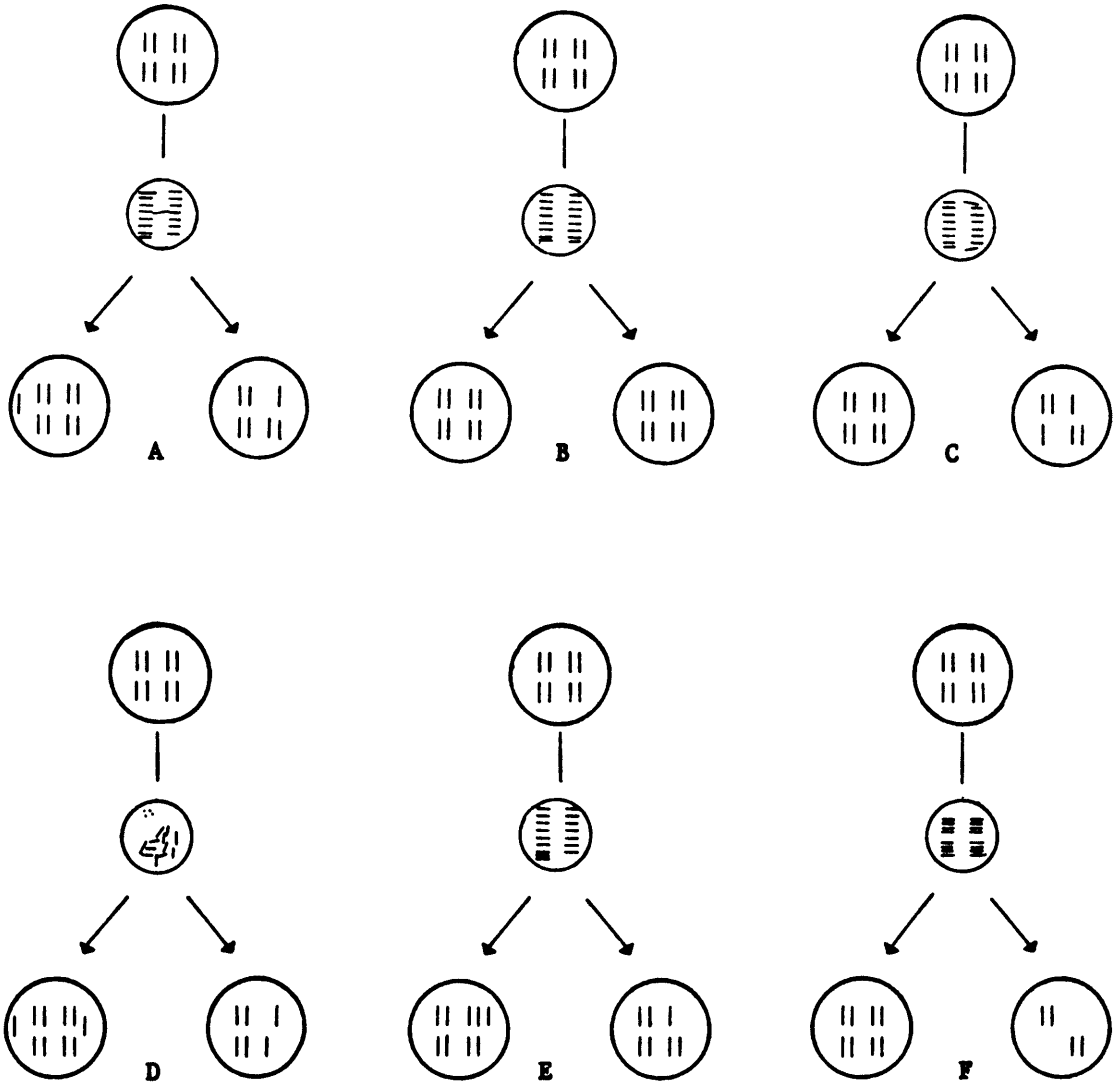


Figure B.1. Schematic representation of pathways of chromosome loss or gain during mitosis leading to aneusomaty in plant cells. The products of mitosis illustrated here are only examples and other cell karyotypes may equally be produced. Here in A, B, C, E and F represent anaphase while the condition represented in D is metaphase.

A. Anaphase bridge which may lead to continuous loss and gain of chromosomes or chromosome fragments through breakage fusion bridge cycles (McClintock, 1951).

B. Normal mitosis.

C. Laggards at anaphase which may result in chromosomal elimination.

D. Asynchronous division of chromosomes, due to a timing imbalance, which may result in chromosome loss.

E. Non-disjunction.

F. Twin spindles resulting in somatic reduction with the level of genome segregation depending on the nature of pairing of homologues (Huskins, 1948).

Bs are unstable at mitosis and succumb to loss through anaphase lagging and mitotic non-disjunction. The latter event leads to numerical variation within the individual and is associated with a net gain or loss of Bs depending on the stage of development at which it occurs and the way in which the dividing cells are subsequently distributed (cf. Jones, 1975). While it has been claimed that B-chromosomes are inert (cf. Dyer, 1976) in several of the 150 or so species in which they have been observed direct phenotypic effects of their presence have been recorded (cf. Dyer, 1976). There is for example an additive effect on pericarp pigmentation in *Haplopappus* (Jackson and Newmark, 1960) and increased chromosome breakage in *Trillium* (Rutishauser, 1956).

B.1.2.2. Karyotype polymorphism

In *B. semperflorens* Matsura and Okuno (1943) report the occurrence of cultivars with 33, 36, 60 and 66 chromosomes. In *B. rex* the same authors studied cultivars with 32, 33, 34, 42, 43 and 44 chromosomes. These *B. rex* plants showed distinct phenotypic differences and were referred to as cytotypes (see Section G.) in view of their postulated origins from aneusomatic tissue in *B. rex* (Sharma and Bhattacharyya, 1957, 1961). These plants are believed to arise through the asexual reproductive cycle by the division of cells with altered chromosome complements which subsequently form adventitious buds. Plants produced from these buds if they maintain the altered genomic constitution will behave as individuals with different phenotypes (Sharma and Bhattacharyya, 1961).

In *Begonia* cytotypes it is difficult to understand how deficiencies or gains involving several chromosomes can allow the survival of an individual even though reproduction may be purely vegetative. Sharma and Bhattacharyya (1961) suggest that if such chromosomes contain the essential genes responsible for the maintenance of viability and normal metabolism of the individual then survival is bound to be affected. On the other hand if they contain non-essential genes, that is, are heterochromatic or of an accessory type (Bs), then their loss or gain will not affect viability but discrepancies in their number may influence minor phenotypic characters such as leaf colour and texture as found in cytotypes of *B. rex* (Matsuura and Okuno, 1943). This type of chromosome behaviour and method of speciation has been suggested as a general feature in many vegetatively propagated plants

(Sharma and Sharma, 1959; Whitham and Stobodchikoff, 1981) (see Section *B.3.2.5.*). Thus the occurrence of aneusomaty in somatic tissues may be imperative, particularly in sterile plants, if the plant is to maintain its ability to produce new variants. Sharma and Bahttacharyya (1957) also suggest that the reason why a range of euploid and aneuploid chromosome complements survive in *Begonia* is because the variant chromosome complement arising in the somatic tissues do not have to pass the survival test of sexual reproduction and similar explanation may explain the presence and survival of aneusomaty in other plant species e.g. *Rubus* (Britton and Hull, 1957).

Through the widespread presence of inconstancy in chromosome number in somatic tissue the germinal line has also been affected and many *Begonia* species and varieties are characterized by meiotic irregularities (Matsuura and Okuno, 1943). Infertility resulting from non-functional gametes may be due to the accumulation of abnormalities in the somatic tissues which would otherwise have been eliminated during selection.

B.1.2.3. Implications for tissue culture

The variation of somatic chromosome number present in *Begonia* tissues would suggest that in tissue culture, the production of large numbers of adventitious buds from explants would result in the regeneration of a wide range of variant phenotypes. This assumption is not supported by reports on plants regenerated from *B.* x *hiemalis* cultivars (e.g. Mikkelson and Sink, 1978; Bigot, 1982) where the level of variation was not excessively higher than the normal rate of bud sport production from cuttings. Moreover any of the changes reported were generally flower colour changes and studies on colour bud sports of *B.* x *hiemalis* by Arends (1970) suggest that these are caused by changes in chromosome structure rather than number. These reports however are based on results obtained from small populations of plants (58-300). The full implications of the effect of somatic variation in tissues of *Begonia* on tissue culture progeny awaits further study. These studies should involve regeneration and subsequent morphological and chromosomal evaluation of large populations of tissue cultured plants. In interpreting the results of these experiments it must however be recognized that records of somatic chromosome number in *Begonia* have been obtained from root tissue and the extent of chromosomal variation in other somatic tissues of the plant remains to be determined.

B.2. Plant propagation using tissue culture with particular reference to Begonia

B.2.1. General introduction

The concept of totipotency in plant cells as proposed by Haberlandt (1902) suggests that many plant cells are capable of giving rise to new plants if provided with an appropriate stimulus and a suitable environment for growth. This concept and the successful development of tissue culture procedures for the propagation of *Cymbidium* by Morel (1964) have been largely responsible for the interest developed in the use of tissue culture for plant propagation. The extensive developments in tissue culture research since the initial studies of Morel (1964) are evident in the large number of reviews available on micropropagation (e.g. Murashige, 1974, 1977; Holdgate, 1977; Hussey, 1978; Hartmann and Kester, 1975; Conger, 1981; Hughes, 1981; Zimmerman, 1983; George and Sherrington, 1984).

While tissue culture has proven applicable to plantlet regeneration in a diverse range of plants (e.g. Murashige, 1974, 1977) it has yet to be established as the most effective method for plant propagation. The cost of tissue culture is high, systemic infection of donor plants and tissue recalcitrance is a problem and there are studies which indicate that genome stability and plant vigour are affected by culture *in vitro* (see Section *B.3.*). This situation exists due to the paucity of our knowledge of factors influencing genome stability *in vitro* and major limitations in the present micropropagation procedures including:

(1) Its labour intensive nature.

(2) Variation between genotypes in morphogenic responses which prevent the use of common media for regeneration.

(3) Variation between explants in morphogenic responses and recalcitrance due to the influence of the epigenotype (see Section G.).

(4) The requirement for elaborate acclimatization procedures for the establishment of tissue culture plants *in vivo*.

(5) The absence of criteria other than direct assessment of progeny for the evaluation of procedures for the propagation of plants with a complex genetic architecture (see Section G.).

Increases in the cost effectiveness of micropropagation and its application to a wider range of plants will depend on the development of more efficient protocols which will overcome these limitations.

The present review will examine how factors such as the physiological state of the explant, regeneration procedures and culture conditions can influence the successful and efficient regeneration, development and survival of true to type plants produced from tissue culture and the research which seeks to overcome present limitations on the clonal propagation of plants *in vitro*.

While many of the examples cited here will relate to tissue culture of *Begonia*, examples from other plants and studies not exclusively devoted to clonal propagation, but which provide relevant results, will also be included where appropriate.

B.2.1.1. Plant micropropagation procedures

The successful clonal propagation of plants *in vitro* depends on the initiation of aseptic and genetically stable cultures from which large numbers of plantlets can be successfully established and grown to maturity.

Procedures for the establishment of such cultures have been outlined by Murashige (1974) and Debergh and Maene (1981). The major steps involved include:

(1) Establishment of aseptic cultures.

(2) Induction and development of plantlets.

(3) Plant establishment.

The development of a successful cloning procedure for a particular plant is dependent on the selection of a suitable tissue for culture (explant) and an assessment of optimal conditions for growth and development during each of these stages. Finally, plants produced should be compared at maturity with a population produced from conventional propagation procedure to ensure they retain the phenotype and vigour of the original parent.

B.2.1.2. Selection of explant and mode of regeneration

In tissue culture the choice of explant and mode of regeneration are of prime importance in the establishment of clonal propagation procedures. The type of explant available may be limited by the presence of systemic infection (Debergh and Maene, 1981), recalcitrance (Street, 1979) or a low morphogenic capacity (see Murashige, 1974). Also the regeneration protocol employed will depend on the genetic architecture of the plant as this will influence the phenotype of the plants produced (Cassells, 1984, 1985). The consequence of using

inappropriate protocols for regeneration from genetically heterogenous tissues has been outlined (Section *B.3.6.5.*) and will only be briefly mentioned here.

Tissue culture involves the manipulation of asexual methods of plant regeneration which depend on the formation of shoot meristems (cf. Hussey, 1978). These arise in three ways. Firstly, axillary meristems may be formed in the axil of each leaf. Secondly, adventitious meristems can arise at other sites, either spontaneously or as a result of isolation and treatment of the organ as a cutting. Thirdly, adventitious meristems can develop in, or as embryos arising from, a callus tissue which forms as a wound reaction at cut surfaces (Yeomen, 1970). *In vivo* the proliferation of axillary buds is inhibited by apical dominance (Wareing and Phillips, 1978). In tissue culture axillary shoot proliferation is achieved by culturing shoot tips in the presence of cytokinin and auxins. By continued subdivision and subculture of axillaries high multiplication rates can be achieved. This is the most commonly used commercial method of multiplication (Murashige, 1977) and is believed to be the most genetically conserved (Lawrence, 1981) (but see Section *B.3.4.5.*). Examples of plants cultured in this way include strawberry (Boxus, 1974), carnation (Earle and Langhans, 1975) and Asparagus (Murashige *et al.*, 1972). Multiplication rates are high and in strawberry a 20-fold increase is obtainable every 2 months (cf. Hussey, 1978).

In nature, adventitious shoots are formed on many different organs and are usually derived from shoot apices formed superficially from the epidermis or hypodermis. Broertjes *et al.* (1968) have listed 350 species in which adventitious shoots are obtainable from leaves. Using tissue culture, adventitious shoots can be produced in much higher numbers and from a wider range of species. Small fragments of tissue weighing as little as 20-50 mg have been shown to respond in culture (cf. Hussey, 1978). This method has proved highly successful in the propagation of a wide range of plants and is one of the principle methods used in the micropropagation of begonias (e.g. Mikkelson and Sink, 1978; Bigot, 1981; Takayama and Misawa, 1981). In some plants however the multicellular nature of adventitious buds (Broertjes and Keen, 1981) and the presence of a minor callusing stage has resulted in the production of variants e.g. Potato (Hermsen *et al.*, 1981). For these plants regeneration from preformed buds via

axillary buds, aseptic nodal cuttings or secondary nodal cuttings (see Cassells, 1984) proves a preferable node of propagation and has been advocated for the clonal propagation of true to type progeny from plants which may be chimeral or which show high levels of somatic mutation or genetic instability e.g. Potato (Hussey and Stacy, 1981) (but see Section *B.3.4.5.*). These procedures can also result in a high multiplication rate (MR), nodal cuttings of potato for example have been shown to have an MR rate of x8-x10 per month (Hussey and Stacy, 1981).

While not widely used due to the increased risk of variant production (see Section *B.3.4.*) plants may also be regenerated from adventitious shoots produced from callus cultures. Callus is induced *in vivo* on some plants as a result of wounding which stimulates cell division by the increase in concentration of endogenous growth factors particularly auxin (cf. Hussey, 1978). *In vitro* the presence of the auxin NAA or 2,4-D sustains callus growth and can induce callus on tissues which would normally not undergo this form of morphogenesis (Yeoman, 1970). Most calli typically contain large vacuolated cells irregularly interspersed with areas of small meristematic cells (Yeoman, 1970). When large enough calli may be cut up and subdivided onto fresh media and this process may in theory be continued indefinitely. Transfer of callus to media containing low auxins and high cytokinins allow the formation of plantlets. In callus a variety of structures are formed with varying degrees of resemblance to zygotic embryos, both superficially and within the callus. These structures are thought to derive mainly from the division of one or a few totipotent cells (cf. Yeoman, 1970). Callus can be readily bulked up by subculture and hence extends the potential for adventitious bud production otherwise limited by the amount of parent tissue available as organ explants. While this procedure has proved applicable to the maintenance and propagation of a number of plants e.g. celery (Williams and Collins, 1976), *Lilium* (Sheridan, 1974) and daylily (*Hemerocallus*) (Krikorian and Kann, 1981) its use may be confined to stable genotypes and it is not generally advocated as a method of plant propagation due to loss of regenerative capacity and variant production in many genotypes during subculture (see Section *B.3.4.*).

In *Begonia*, callus production has been reported for a number of hybrids e.g. *B. rex* (Arora *et al.*, 1970; Bigot, 1971), *B.* x *hiemalis* (Mikkelson and Sink, 1978; Margara and Piollat, 1982, 1983, 1984) and *B. semperflorens* (Sehgal, 1975). Reports on callus subculture are limited to *B. rex* (Bigot, 1971) and *B.* x *hiemalis* (Mikkelson and Sink, 1978; Margara and Piollat, 1982) and organogensis has been achieved in *B.* x *hiemalis* where petals, leaves and roots were produced from petal callus (Margara and Piollat, 1984) and in *B. rex* callus where Bigot (1971) produced plantlets from callus subcultured for 2 years. No details are available however on the trueness to type of plantlets produced.

Adventitious meristems may also arise from callus as somatic or asexual embryos which involves the formation of bipolar structures bearing roots and shoots. Embryos continue to develop in a manner analogous to germination and subsequently develop into whole plants. This phenomenon, which was first confirmed *in vitro* by Reinert (1958) and Steward *et al.* (1958) working independently on carrot, has been extensively reviewed (Wetherell, 1978; Street, 1979; Tisserat *et al.*, 1979; Sharp *et al.*, 1980; Ammirato, 1983a, b; Vasil, 1983, 1985). Tisserat *et al.* (1979) reported 132 Angiosperm species which show somatic embryogenesis (both naturally and *in vitro*).

The advantages of an embryogenic protocol of regeneration include the high multiplication rate, the elimination of a rooting step and steps to separate individual plants and the potential for automation (cf. Lawerence, 1981). The most recently reported multiplication rate, based on a preliminary study on *Pennisetum purpureum* Schum., suggests that a single leaf explant can be used to produce 24 x 10^3 plants within 26 weeks (Vasil, 1985). The application of embryo culture to large-scale micropropagation however will require more synchronous production, higher rates of germination and encapsulation methods which would allow the use of mechanized procedures (Lawerence, 1981). Due to its commercial importance the development of such a system is being extensively researched (e.g. Ammirato, 1983a, b; Vasil, 1983, 1985).

B.2.1.3. Establishment of sterile cultures

Tissues from disease free donor plants are readily introduced into culture and surface sterilization procedures and reagents routinely used for sterilization have been outlined by a number of authors (e.g. De Fossard, 1976; Thorpe, 1981).

Tissue culture studies have highlighted the widespread presence of systemic and latent contamination in plants (e.g. Debergh and Maene, 1981). In these plants, meristems may provide the only available disease free material and may be used to produce stocks which after appropriate disease indexing (see Barnhill Jones, 1979; Smith and Oglevee-O'Donovan, 1979) act as mother stock plants which can be used for large-scale micropropagation. This procedure has been used in *B.* x *hiemalis* for the elimination of *Xanthomonas begoniae* (Reuther and Bandari, 1981; Hakkaart and Versluijs, 1983b) and in *B.* x *tuberhybrida* to produce virus free stock (Walvaert *et al.*, 1980).

The incorporation of antibiotics in the medium has also been advocated as a means of eliminating contamination, and while it was initially anticipated that this procedure would be detrimental to survival (Hussey, 1978), various publications have mentioned the beneficial effects of antibiotic compounds in the establishment of aseptic cultures (Venis, 1967; Watts and King, 1973; Phillips *et al.*, 1981). These reported bacteriostatic effects however only occurred in short term cultures and procedures have yet to be developed which can ensure routine elimination of systemic or latent contamination. This may be achieved by more widespread testing of a range of antibiotics such as those conducted by Bastiaens *et al.* (1983). However, as these authors point out, the antibiotics presently available are developed for human pathogens and as such may not be suitable for the elimination of plant pathogens and specific antibiotic formulations which incorporate both a bacterioside and a fungicide suitable for plant pathogens need to be developed.

B.2.1.4. Development of plantlets

While high levels of hormones are required in many cases to initiate morphogenesis their continued presence in the medium may limit shoot development. In *B.* x *hiemalis* and other plants a transfer step is routinely used to overcome this effect. This can involve subdivision of explants and transfer to solid media as used for example in *B.* x *hiemalis* (Mikkelson and Sink, 1978) and *Saintpaulia* (Harney and Knap, 1979) or to liquid culture e.g. *B.* x *hiemalis* (Takayama and Misawa, 1981). The development media may contain no hormones (Harney and Knap, 1979) or lower levels of auxin and cytokinin as used in *B.* x *hiemalis* (Takayama and Misawa, 1981). In some cases GA_3 is added at this stage to enhance shoot development e.g. potato

(Jarret *et al.*, 1981). This subdivision step adds to the cost of micropropagation and also increases the risk of loss due to contamination. At present the development of one step procedures for plantlet development are being investigated for a number of plants. Batch culture systems have been used by Takayama and Misawa (1982a) for the propagation of *B.* x *hiemalis*, but a number of problems including injury to plantlets must be overcome before this system can be applied as a routine method of propagation. Explant homogenization has also been investigated for propagation of *Davallia* and *Platycerum* (Cook, 1979) and the productivity of this method at the plantlet stage shows some promise. For all these new developments however, an assessment of overall productivity and the vigour, uniformity and trueness to type of the plants produced will be required before they can be employed in the large-scale clonal propagation of plants.

B.2.1.5. Rooting and establishment of plantlets

After development shoots are generally rooted *in vitro* on modified rooting media. Concentrations of mineral salts and sugar are normally reduced to half strength, cytokinin is omitted and the auxin concentration is often adjusted to higher levels e.g. *B.* x *hiemalis* (Takayama and Misawa, 1981). Supplements such as activated charcoal e.g. *B.* x *hiemalis* (Bigot, 1981a) may be added to enhance rooting. This *in vitro* rooting step however caused delays in planting out and increased cost due to the need for separate media and the separation of propagules under aseptic conditions. In *Lilium* for example it is responsible for 76 percent of the total micropropagation cost (Anderson and Meagher, 1978). Hence the elimination of this stage is desirable for the development of cost effective micropropagation protocols. In some plants media can be developed on which plantlets will self root at the shoot development stage e.g. potato (Hussey and Stacey, 1981). In other plants the *in vivo* rooting step developed by Debergh and Maene (1981) for *B.* x *tuberhybrida* may prove effective. This step involves the pretreatment of shoots with a solution of liquid IAA for 10 days prior to planting out. Other plants may self root when transferred from culture e.g. *Saintpaulia* (Cassells and Plunkett, 1984).

The poor survival rate of plantlets on transfer from *in vitro* conditions to soil environments (e.g. Earle and Langhans, 1985; Roest *et al.*, 1981) have considerably reduced the overall productivity of

micropropagation. A number of investigations have shown that the cause of these losses may lie in the altered morphology of test tube plants (see also Section *B.2.2.4.*). When contrasted with greenhouse grown plants tissue culture progeny of some plants were found to have less wax on their leaf surface to inhibit water loss (Grout and Aston, 1977; Sutters and Langhans, 1979) smaller and fewer palisade cells and larger mesophyll air spaces (Brainerd and Fuchigami, 1981) and open unprotected stomata on the abaxial surface and periphery of leaves (Donnelly and Vidaver, 1984). These morphological features contribute to the rapid desiccation of transplanted plantlets if procedures for the gradual acclimatization of these plantlets to greenhouse conditions are not adopted. Methods used for the acclimatization of plants have been reviewed by Conner and Thomas (1981) and include the use of mist benches or humidified chambers (McCowan, 1980). In these environments the relative humidity is between 90 to 95 percent and plants are gradually acclimatized to lower relative humidity to ensure continued and vigorous growth. More recently Sutters and Hutzell (1984) studied the use of anti-transpirants on *Chrysanthemum* and *Dianthus* with a view to eliminating the use of humidity tents which are cumbersome and labour intensive, however these studies were not successful. As a result of the reduced epicuticular wax on tissue cultured plants antitranspirants penetrated the leaves and caused phytotoxic effects. Hence the use of humidity tents, although cumbersome and labour intensive in a commercial operation, remains the method of choice to ensure maximum growth of cultured plants after their transfer to the greenhouse. New developments in this area which would reduce the labour and time factor involved would greatly increase the efficiency and lower the cost of micropropagation.

B.2.1.6. Progeny plant assessment

The real proof of the success of a given micropropagation procedure lies in the establishment of large numbers of vigorous true to type plantlets which readily compete with plants produced by conventional propagation methods. An assessment of progeny plants therefore requires a comprehensive comparative evaluation of salient morphological features from both tissue cultured and conventionally propagated plants. Such detailed studies however are rarely undertaken, most authors limiting their assessment to a study of gross phenotypic variation e.g.

flower colour or leaf shape (e.g. Mikkelson and Sink, 1978; Bigot, 1982). There are however a number of recent reports of quantitative and qualitative comparative assessments of tissue culture progeny including those by Hwang *et al.* (1984) on banana, Tamura *et al.* (1984) on *Stevia*, Samyn *et al.* (1984) on *B.* x *tuberhybrida* and Cassells and Plunkett (1986) on *Saintpaulia*. In these studies plants produced from the first tissue culture cycle were all true to type and in all cases an increased uniformity was reported in tissue culture populations.

Studies on tissue culture progeny of *B.* x *hiemalis* which have not been supported with quantitative data on vegetative characteristics suggest that tissue culture increased variation in some cultivars. Bigot (1982) found 10 percent variation in regenerates from petiole explants and 80 percent in regenerates from stem explants of *B.* x *hiemalis* cultivar 'Nixe' and Mikkelson and Sink (1978) report the production of six similar flower colour variants in regenerates from *B.* x *hiemalis* cv. Schwabenland red. In contrast Hilding and Welander (1976) and Welander (1977) report no variation in plants produced from a number of different *B.* x *hiemalis* cultivars. In a number of other micropropagation studies on *B.* x *hiemalis* no data have been provided on the phenotypes of the plants produced (Khoder *et al.*, 1981; Takayama and Misawa, 1981).

B.2.2. Factors influencing morphogenic responses of explants in vitro

B.2.2.1. Introduction

The successful induction of plantlets from explants in culture is dependent on three major factors; (1) the genotype (Hughes, 1981); (2) the physiological and ontological state of the explant used (i.e. epigenotype) (e.g. Cassells *et al.*, 1982) and; (3) the provision of adequate nutrients, an energy source for growth and correct hormonal stimulus for morphogenesis (Murashige, 1974; Street, 1979; Hughes, 1981).

In most tissue culture studies attention has been focused predominantly on manipulating the third variable. More recent research however shows an increased awareness of the part played by the epigenotype on plant tissue responses *in vitro* (e.g. Cassells *et al.*, 1982), and future research in this area may result in improved tissue culture procedures.

B.2.2.2. Genotype influences on morphogenic responses of explants in vitro

In developing tissue culture procedures micropropagators would like to reduce costs by using similar media, culture conditions and transfer steps for a wide range of cultivars. The implementation of such procedures however has been prevented by the fact that plant genotype and epigenotype significantly influence the nature and extent of morphogenesis induced in cultured tissues (see Hughes, 1981).

Genotype would appear to influence growth *in vitro* more than any other factor and there are many examples in the literature of the need to alter media constituents to accommodate morphogenesis in different species, cultivars or varieties (reviewed by Hughes, 1981). In *Begonia* differences in responses have been reported by a number of authors (Welander, 1977; Mikkelson and Sink, 1978; Bigot, 1981a). In a study of petiole explants from 17 cultivars of *B.* x *hiemalis* Welander (1977) found that the percentage of explants forming organs and the degree of plantlet formation differed significantly between cultivars. An assessment of the vitamin requirements of four of these cultivars indicated that while most cultivars gave similar responses with or without vitamins one cultivar responded negatively when supplied with vitamins. Furthermore, alterations of the hormone concentration of the medium reduced the variation between cultivars. Welander (1977) suggests that the differences observed between different cultivars are due to sub-optimal growth conditions for some mother stock plants which are manifest as differences in hormonal requirements. The vitamin study however demonstrates that these cultivars may have distinct media requirements and optimization of the growing environment alone may not be sufficient to optimize regeneration.

Differences in responses between cultivars of *B.* x *hiemalis* have also been reported in meristem culture (Reuther, 1978) and differences between *Begonia* species were reported by Ringe and Nitsch (1968) and Bigot (1971).

Where cited, differential responses between cultivars or species have been subjectively assessed and no quantitative data is available regarding the differences in number, distribution or growth of organs or plantlets produced. As yet no determined effort has been made to reduce the variation between genotypes of *Begonia* by controlling the physiological and ontological state of explant source tissues. Nor has there been any study on the potential for the development of

broad spectrum media which could be used for the propagation of a number of different *Begonia* genotypes.

Assessment of the former approach is important in view of the differences reported by Welander (1978a) between individual donor plants of a given cultivar where differences of up to 50 percent were recorded for explant response. This variation will magnify or nullify any true differences which exist between cultivars and must be controlled. The major influence played by the epigenotype in the responses of explants from *B.* x *hiemalis* cultivars as demonstrated by Welander (1978a) may be reduced by the use of axenic tissue (Section *B.2.4.*). This approach however has yet to be examined in *Begonia*.

Other ornamental plants where genotypic differences have been reported in shoot regenerative capacity include; *Gerbera* (Murashige *et al.*, 1975), *Saintpaulia* (Cook, 1977) and *Dianthus* (Roest and Bokelman, 1981).

Genotypic differences in the ability to form callus and regenerate plants has been noted for several species including cauliflower (Buiatti and Bennci, 1974), corn (Green and Phillips, 1975), *Alfalfa* (Saunders and Bingham, 1972), potato (Webb *et al.*, 1983) and a range of legumes (Oelck and Schieder, 1983). The majority of these studies have been undertaken on single media. Screening of the response of genotypes on a range of media combinations has however been assessed in tomato (*Lycopersicon esculentum*). Ohki and Bigot (1976) found two tomato genotypes differed in their shoot regenerative capacity in response to fourteen different combinations of IAA and 2IP. Culture of twelve cultivars of tomato on 24 combinations of IAA and BAP (0-10 mg/l) studied by Kurtz and Lineberger (1983) indicate genotype influence on the ability to regenerate shoots and on the average numbers of shoots regenerated. Optimal shoot regeneration media were also found to vary with the cultivar. Other morphological responses were cultivar dependent and exhibited broad maxima over the range of media tested. In the latter study, mature tissues were used as explants and may have influenced explant responses. While axenic tissues have been used for screening genotype-media interactions in a number of plants screening in most cases has been limited to responses on a single medium. In *Cucumus sativus* of 85 lines tested only 28 formed shoots from cotyledons grown on media containing 1 mg/l each of BAP and NAA (Wehner and Locy, 1981), while in 100 genotypes of

Lycopersicon esculentum only one fourth of the monitored genotypes displayed a relatively high shooting response (Zelcer *et al.*, 1984).

The contribution of genetic factors to *in vitro* growth responses of cultured plant tissues has been noted (Baroncelli *et al.*, 1974) and a number of studies have been conducted to investigate the genetic factors determining the control of genotype responses *in vitro*. Studies on the quantitative evaluation of callus growth and the heritability of dedifferentiation in *Brassica oleracea* by Baroncelli *et al.* (1974) and Buiatti *et al.* (1974) indicate that caulogenesis is controlled by additive genetic systems. In maize (*Zea mays* L.) genetic analysis of callus growth indicates that this growth is controlled by two genetic systems one located in the nucleus the other in the cytoplasm (Nesticky *et al.*, 1983).

In *Petunia* Izhar and Powell (1977) suggest that only a few genes may be involved in genetic control of protoplast growth. While Skvirsky *et al.* (1984) in a study of two *Petunia hybrida* cultivars, to elucidate factors causing differences in cytokinin receptiveness of leaf and stem explants, suggest cytoplasmic genes may exert some effect. Results from studies on *Alfalfa* genotypes suggest that two genes may control regenerative capacity and this resulted in the development of a breeding programme in which clones with 12 percent shoot forming capacity were increased to 67 percent by two cycles of selection (McCoy and Binghams, 1977). These methods for improving genotype responses are however not feasible for the many ornamentals which are vegetatively propagated. In these plants our ability to reduce the differences in regenerative capacity between genotypes in tissue culture may be limited to reducing epigenotype effects.

B.2.2.3. *Effect of media constituents on morphogenic responses of explants in vitro*

For the successful initiation of morphogenesis from competent tissue, cells or protoplasts, tissue culture media must provide a suitable osmoticum, sufficient nutrients and energy for growth and a hormonal stimulus for the induction of division and differentiation.

Research on the development of basal nutrient media for culture of plant tissues or cells *in vitro* is extensive and a wide range of complex media have been developed. Reviews by Huang and Murashige (1977), De Fossard (1976), and Gamborg *et al.* (1976) summarize these media and their constituents.

The basal nutrient medium of Murashige and Skoog (1962) (MS medium) which is high in nitrates, potassium and ammonium has proved successful with many plant tissues and dilutions of the formula have been used in some taxa (Murashige, 1974). Salt susceptible plants such as *Begonia* perform better with a reduction in the macroelements to half strength which results in higher survival rates and faster growth e.g. *B.* x *hiemalis* (Reuther, 1978; Reuther and Bandari, 1981). For petiole explants of *B.* x *hiemalis* reducing the ammonium nitrate concentrations of the basal MS medium increased the number of plants produced by 70 percent (Khoder *et al.*, 1981).

The energy requirement of cells *in vitro* is generally satisfied by the incorporation of 2-3 percent sucrose. Extensive studies by Takayama and Misawa (1982a) on *B.* x *hiemalis* show 3 percent to be the optimal concentration for plantlet induction and development and similar levels of sucrose are used by other workers on *Begonia*. Sucrose may not alone act as an energy source but may also play a role in organogenesis through regulation of osmotic potential. The osmotic effects of sucrose in culture solutions is well demonstrated in tobacco callus by Brown *et al.* (1979) and Brown and Thorpe (1980).

The addition of compounds other than those required for growth has also been shown to influence morphogenesis *in vitro* (reviewed by Murashige 1974, 1977; Hughes, 1981). Vitamin additions to culture media may enhance callus growth and differentiation. Thiamine is the most often added followed by nicotinic acid and pyrodoxine (Yeoman, 1970). The addition of organic nitrogen is most often used during callus initiation. Casein hydrolysate (0.02-0.1%) is a most frequently used source and has also been shown to induce embryo production in *B. semperflorens* (Sehgal, 1975) while the compounds glutamine, asparagine, tyrosine and adenine are the most frequently used reduced nitrogen additives. Adenine has been found to stimulate budding in a number of *Begonia* species (Ringe and Nitsch, 1968). Activated charcoal is known to stimulate growth, organogenesis and embryogenesis in a wide range of species (cf. Murashige, 1974) and its effect has been attributed to absorption of inhibitory compounds.

The early studies by Skoog and Miller (1957) on tobacco indicated that exogenous auxin and cytokinin ratios determined the nature of organogenesis with a high auxin to cytokinin ratio stimulating root production and high cytokinin to auxin ratio inducing shoots. These

findings have greatly influenced subsequent investigations on hormonal requirements of plant tissue cultures. Unfortunately no universal ratio for root or shoot induction in plant tissue exists and considerable differences in responses between genotypes (see Section *B.2.2.2.*) necessitates that appropriate auxin and cytokinin levels for optimum shoot regeneration must be determined for each plant genotype introduced into culture.

Cytokinins and auxins are active over a wide range of concentrations from 0.01 mg/l^{-1} to 30 mg/l^{-1} (cf. Hussey, 1978). In establishing optimum hormone levels for regeneration it is usual to examine all combinations of a given cytokinin and auxin in concentration steps of x10.

The commonly used auxins are naphthaleneacetic acid (NAA), 3-indole acetic acid (IAA), 3-indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). NAA being the most widely used in studies on shoot initiation while 2,4-D is used in the production of callus. More recently studies have been conducted on a number of IAA conjugates (Pences and Caruso, 1984) and compounds with callus inducing ability such as the chlorophenoxyacetic acids (Irvine *et al.*, 1983) and 5-triazine compounds which induce callus in the *Graminaceae* (Conger *et al.*, 1982). These studies have been undertaken with a view to finding auxins which stimulate callus growth but do not cause the cytological instability induced by 2,4-D (e.g. Sunderland, 1977 *loc. cit.*) or IAA (e.g. Naylor *et al.*, 1956).

Cytokinins vary in their ability to induce organogenesis and different cytokinins show variation in shoot inducing capacity in different genotypes (Skvirsky *et al.*, 1982). In general 6-Benzylamine purine (BAP) has proved the least expensive and most potent and is widely used. Other cytokinins used in tissue culture include isopentyladenine (2-IP), 6-furfurlaminopurine (kinetin) and zeatin.

Gibberellin, while stimulating growth, generally supresses organ initiation processes (Murashige, 1974) and is not routinely included in the induction medium. In *B. rex*, GA_3 treatment in the initial 24 hours after explant excision has been found to inhibit bud initiation (Bigot, 1971) and it is suggested that this effect is caused by the influence of GA_3 on the accumulation of starch (Chlyah, 1972). GA_3 however is known to stimulate the development of organs that have already been initiated and for this reason it is included

in many shoot development media e.g. potato (Jarret *et al.*, 1981) (see Section *B.2.1.4.*).

While exogenous hormones supply a stimulus for morphogenesis it is their interaction with endogenous hormones which determines the nature and extent of morphogenesis (e.g. Cassells *et al.*, 1982). A number of other substances, which influence the level of endogenous hormones when included in the medium, have been reported to influence the effectiveness of exogenously supplied auxin and cytokinin. Certain phenolics enhance shoot formation (Lee and Skoog, 1965) and appear to stimulate auxin inactivation consequently raising the physiologically effective level of cytokinin. Other substances which enhance shoot formation include those which appear to competitively inhibit auxin action e.g. N-1-naphthylphthalmic acid (Feng and Linck, 1970). The role of 2,3,5-triiodobenzoic acid (TIBA) as an inhibitor of auxin transport has been well documented (Goldsmith, 1968; Depta *et al.*, 1983) and its inclusion in tissue culture media is known to cause increased and precocious shoot production as well as reducing apical dominance and callus polarity (Goldsmith, 1968; Halperin, 1978; Nyman and Arditti, 1984). In *B. rex* TIBA was found to delay rooting and had no apparent effect on budding which led Bigot (1971) to suggest that blockage of auxin had no real influence on bud induction. TIBA has been used successfully to increase shoot production in *Colocasia esculenta* callus where changes in the ratio or chemical nature of auxin and cytokinin proved inefficient (Nyman and Arditti, 1984) and in tomato the inclusion of TIBA in the medium has been shown to enhance shoot formation from winter grown donor plants (Cassells, 1979).

Anticytokinins like antiauxins and auxin inhibitors may act in the alteration of endogenous levels of hormones and thereby influence morphogenic responses. However as yet studies on the influence of anticytokinins have not demonstrated any beneficial effect and in general they antagonise the action of exogenously applied cytokinins (Tanimoto and Harada, 1982).

Morphactins cause changes in higher plants which indicate an interference in the complex effects of phytohormones (Schneider, 1970; Rucker, 1982). Studies by Rucker (1982) demonstrate their cytokinin like activity and treatment of *B. rex* leaves with chlorflurenol has been shown to induce an increase in adventitious budding (cf. Schneider,

1970). Morphactins have also been found to induce bud formation on intact undetached leaves of *Heloniopsis* in darkness where treatments with BAP failed to induce buds (Kato, 1978a). In the latter study morphactins were suggested as having a stimulatory influence on carbohydrate metabolism.

The influence of pH on morphogenesis *in vitro* is a factor which has been much neglected in tissue culture studies. *In vitro* growth occurs on media over the pH range 4 to 7.2 and detrimental effects usually relate to ion availability and nutrient uptake (cf. George and Sherrington, 1984). The usual procedure is to set the pH at a value within the range 5 to 6. However some plants may perform better on more acidic media, for example, *Begonia franconis* Liebm. (Berghoff and Bruinsma, 1979) which responded best when the pH was initially adjusted to between 4 to 5, and *Azalea* which gave optimum shoot regeneration at pH 5 (Economou and Reid, 1984).

B.2.2.4. Influence of the physical state of the medium on in vitro regeneration

While media constituents play a major role in the induction of morphogenesis their action is significantly influenced by the physical state of the medium which will affect hormone and nutrient availability and growth of regenerated organs. Solid media are routinely used in the initiation of morphogenesis and are generally solidified by adding agar at 0.7-1 percent. The concentration of agar used in tissue cultures has more recently been directly linked to the phenomenon of vitrification (Debergh, 1983). Vitrified plants have a waterlogged appearance and are characterized by leaves which are broad, thick and translucent, wrinkled and/or curled and easily breakable. Translucency and malformation apparently are due to chlorophyll deficiency and general hyperhydricity (cf. Werner and Boe, 1980). Vitrified plantlets present a major problem for tissue culturalists as they generally cannot survive transfer to greenhouse conditions due to loss of water and susceptibility to disease.

Vitrification occurs predominantly on media with low agar concentrations (Debergh *et al.*, 1981). Free water and the high humidity of the culture vessel (Aitken *et al.*, 1981) and the type of vessel closure (Hakkaart and Versluijs, 1983a) has also contributed to the formation of these shoots. While increasing the agar concentration reduces vitrification in some plants e.g. *Picea abies* (Romberger and

Tabor, 1971), *Dianthus* (Hakkaart and Versluijs, 1983b) and *Cynara scotymus* (Debergh, 1983) this procedure also reduces shoot proliferation and development and some workers (von Arnold and Eriksson, 1983) prefer to retain productivity and instead develop elaborate establishment procedures for vitrified shoots.

Notwithstanding the importance of the problem very few thorough studies on the phenomenon of vitrification have been undertaken. The work of Debergh *et al.* (1981) and Debergh (1983) being the first real examination of this phenomenon. More recent work suggests vitrification is a result of stress induced ethylene (Keevers *et al.*, 1984).

Changes in the physical form of media have been shown to enhance shoot multiplication and development in a number of plants during step 2 (see Section *B.2.1.4.*). The physiological basis for increased productivity with liquid media is not clear. Harris and Mason (1983) suggest that media mixing reduces accumulation of toxic substances and depletion of essential nutrients and hormones in the vicinity of shoot cultures. The total immersion of tissues and direct contact with hormones and nutrients is also likely to enhance the rate of multiplication and development.

The most effective use of liquid culture to obtain high rates of multiplication has been described in a series of papers by Takayama and Misawa (1981, 1982a, b, 1983) on plants capable of adventitious bud formation. In *B.* x *hiemalis*, *Saintpaulia* and *Gloxina* (1982b) they have described how leaf segments bearing newly initiated buds can be transferred to flasks of liquid on rotary shakers at 180 rpm with subsequent rapid shoot proliferation. The production of 150–200 buds per leaf explant was reported for *B.* x *hiemalis* (Takayama and Misawa, 1981). However, these authors do not report the number of plants surviving to maturity nor do they provide an assessment of the progeny produced which prevents a true evaluation of the application of this technique on a commercial scale.

B.2.2.5. Influence of explant origin size and orientation on in vitro responses

The selection of explants for tissue culture both in *Begonia* and other plants has in most cases been an arbitrary decision based on personal preference or availability of material. Very few studies look closely at the effect of position, size, age of explant or the epigenotype of the donor plant in relation to their effects on

regenerative capacity. This situation prevails despite the fact that it has long been recognized that these factors significantly influence plant tissue and organ responses in culture (see Murashige, 1974, 1977; Hughes, 1981).

A number of examples from tissue culture studies on *B.* x *hiemalis* demonstrate the effect of explant source on responses in petiole and leaf explants. Welander (1978a) found that explants taken indiscriminately from all leaves of the explant differed significantly in the percentage of explants responding and the subsequent induction and development of plantlets and suggests that this may be due to differences in endogenous hormone levels. This suggestion was made to accord with similar results provided by Hewett and Wareing (1973) for *Populus* where endogenous levels of hormones were found to vary in leaves of different ages. This variance in response between explants of different ages was also reported by Bigot (1981) who conducted a study to optimize explant source in *B.* x *hiemalis* cultivars and found maximum organogenesis occurred in petioles from the first and second leaves which had just finished growth. Studies by Khoder *et al.* (1981) on *B.* x *hiemalis* indicate that the source zone of the petiole from which explants were derived significantly influences the number of plantlets produced with those of the mid zone producing 50 percent more plantlets than those from the apical zone. In using leaf explants Takayama and Misawa (1982a) found old leaves of *B.* x *hiemalis* gave no response and routinely used young leaves of 3 cm in length. Age effects are evident in other plants where alterations can also occur in the nature of morphogenesis as found in *Echeveria elegans* (Raju and Mann, 1970) where young leaves produced only roots, older leaves shoots only and leaves of medium age produced both roots and shoots. In Bromeliads young axenic leaflets were found to produce twice as many shoots as old leaves (Hosoki and Ashari, 1981) and young axenic petals of *B.* x *hiemalis* are reported to have greater morphogenic capacity than adult petals (Margara and Piollat, 1984).

Many different tissues have been used as sources of explants (see Murashige, 1974; Hughes, 1981), few studies however report differences in productivity between different organs of the same plant when cultured *in vitro*. These studies are important to the development of effective micropropagation procedures however, as major differences may exist in regenerative capacity between different organs. In sweet

potato for example axenic petiole explants produced a greater number of plants when compared with roots which produced fewer plantlets and leaves which produced only callus (Hwang *et al.*, 1983).

The differences in morphogenic capacity reported above for different explants in tissue culture may be linked to changes which occur in plant cells during development which may alter their ability (competence) to respond to exogenous hormonal stimuli (see Wareing, 1978) or to physiological or ontological effects which alter endogenous hormone concentrations (see e.g. Letham *et al.*, 1978; Cassells *et al.*, 1982). The successful development of tissue culture procedures requires a full appreciation of the limitations and influences imposed by the above phenomenon and must be borne in mind when selecting an explant for routine use in micropropagation.

Because of gradients in endogenous hormones and the differential distribution of nutrients in plants, tissue size and orientation in culture also influence regenerative capacity. In *Heloniopsis orientalis* the critical size for bud regeneration in young leaves was found to be 1 x 1 mm and 3 x 3 mm for old leaves. Smaller segments produced roots but no vegetative buds (Kato, 1974). In *Alfalfa* callus tissue less than 105 µm in diameter failed to regenerate and it is suggested that a specific number of cells may be required for response to an inductive signal (Walker *et al.*, 1979). On the basis of their studies on floral bud differentiation in *Torreia* Tanimoto and Harada (1979) suggest that small explants may contain reduced levels of endogenous substances which would enhance the stimulatory action of exogenous hormones. In *Torreia*, segments 2-8 mm in length were capable of differentiating floral buds while 10-12 mm segments were not. Studies by Hilding and Welander (1976) on *B.* x *hiemalis* also suggest the influence of endogenous factors may be reduced in smaller explants. When petiole explants of different sizes were taken from long and short day treated mother stock 2 mm segments were found to show no difference in response while 4 mm segments produced fewer shoots when explanted from short day treated donor plants.

Explant anatomy may also influence explant response *in vitro* as different tissues have been shown to have differential competence *in vitro*. The epidermis of petiole explants of both *B. rex* (Bigot, 1971) and *B.* x *hiemalis* (Bigot, 1981a; Welander, 1981) produced buds while pith explants produced roots only when cultured on the same combination

of hormones. While some cells of the pith may have lost their competence to respond to exogenous hormones, for example highly endopolyploid cells (Brossard, 1974), other cells may require different combinations of hormones for shoot initiation to those required by the epidermis. It is also possible that other media constituents may require adjustment. In studies by Bilkey and Cocking (1981) on *Saintpaulia*, petiole explants without an epidermis were found to produce shoots only when cultured on Gamborg's B5 medium, this medium contains lower amounts of mineral salts than MS.

Explant orientation can significantly influence explant response *in vitro* by changes in the hormone gradient or the degree of tissue media contact. The productivity of *Lilium* bulbscales was reduced if explants were placed in an upright or inverted position as compared with those placed horizontally on the medium (Leshen *et al.*, 1982). Shoot production on *B.* x *hiemalis* is similarly affected (Bigot, 1981a; Welander, 1981). In sweet potato inversion of explants influenced the amount and polarity of callus production (Gunckel *et al.*, 1972) and in *Saintpaulia* leaf explants failed to respond unless their upper surface was in contact with the medium (see Plunkett, 1984). These responses indicate that more attention should be paid to explant orientation particularly in plants showing low morphogenic responses or recalcitrance.

The production of low numbers of well developed shoots confined to the base of the explant has been noted in a number of plants e.g. *Gloxinia* (Bigot, 1975) and *B.* x *hiemalis* (Bigot, 1981a). This effect is due to the inhibitory influence of the primary initiated shoot which becomes dominant and suppresses the development of other shoots hence significantly influencing the multiplication capacity of the explant. As this phenomenon may be caused by a hormone gradient in the plant, procedures to overcome this effect have included treatments with TIBA (e.g. Nyman and Arditti, 1984). Horizontal placement of shoots has also been found to increase shoot development in red clover and Campbell and Tomes (1983) suggest that this orientation allowed more rapid growth of normally quiescent axillary buds by releasing them from apical dominance.

B.2.2.6. Influence of the donor plant on tissue and organ responses in vitro

Many tissue culturists ignore the influence of the epigenotype on *in vitro* responses and rely on media manipulations to achieve the

desired tissue culture response (see Section *B.2.2.3.*). Within plants however the distribution and concentration of hormones is profoundly affected by light, temperature and stress factors such as waterlogging, nutrient deficiency or low pH (see Letham *et al.*, 1978). As responses *in vitro* are a function of endogenous exogenous interaction of hormones these factors will have a profound influence on the morphogenic capacity of an individual explant. Despite this, very little has been reported on the effects of plant growth conditions on subsequent responses *in vitro*. While there are some studies on light (photoperiod, intensity) (e.g. Cassells *et al.*, 1982) and temperature (e.g. Welander, 1977) little attention has been paid to the influence of stress and this area of research deserves attention in view of the wideranging influence stressful environments can mediate both on the level of endogenous hormones (see Letham *et al.*, 1978) and genome organization (see Walbot and Cullis, 1985).

Light effects may be divided into photoperiod and wavelength. Much of the work on the influence of photoperiod on bud morphogenic capacity, particularly in *Begonia*, has been conducted *in vivo* by Heide (1964, 1965a, b). In *B.* x *cheimantha* extension of daylength from 8 to 24 hours decreased bud production by 45 percent (Heide, 1964, 1965a). It was suggested (Heide, 1964) that this effect is mediated by changes in endogenous hormones, long days increasing the ratio of endogenous auxin to endogenous cytokinin. The effect of these changes in hormone levels on regenerative responses in leaf petiole cuttings has been discussed in detail by Heide (1972). *In vitro* Hilding and Welander (1976) found that explants from short day treated donor plants of *B.* x *hiemalis* did not differentiate while explants from plants under long day treatments (16 hr) produced buds. In contrast Bigot (1971) found that explants from long day treated donor plants of *B. rex* gave fewer buds than those from short days. Short duration (48 hr) dark pretreatment of donor plants has been shown to reduce the morphogenic potential of tobacco petiole explants *in vitro*. This reduction was found to be associated with lower levels of IAA (Cassells *et al.*, 1982).

Increased light irradiance is reported to reduce rooting in *Pelargonium* petiole explants (Welander, 1978b) and in tomato low irradiance was correlated with low levels of morphogenesis (Cassells, 1979). In *B.* x *hiemalis* (Appelgren, 1985) demonstrated differences in

responses between explants from donor plants grown under different irradiance levels. High pressure sodium lamps (5-15 Wm^{-2}) gave increased regeneration as compared with incandescent lamps (0.8 Wm^{-2}) and demonstrates clearly how a simple change in donor light source can influence productivity *in vitro*.

Temperature effects have been noted in *Streptocarpus* (Appelgren and Heide, 1977) and *Saintpaulia* (cf. Tran Thanh Van, 1980) where explants from donors grown at a constant temperature of 22°C rather than 24°C-16°C (day, night temperature) formed buds over a greater range of growthroom temperatures. In *B.* x *hiemalis* explants from donor plants grown at 15°C under short days gave a limited response in culture and this was attributed to the slow growth rate of plants under these conditions (Welander, 1977).

As well as influencing levels of endogenous hormones in donor plants (e.g. Cassells *et al.*, 1982) alterations in the photoperiod, temperature or light wavelength may influence the rate of photosynthesis and respiration and hence the carbohydrate content of individual tissues (Kato, 1978b). The contribution made by this variable to a tissues regenerative capacity is as yet undetermined but may play an important role. Starch accumulation has been correlated with bud regeneration in tobacco (Thorpe, 1974; Thorpe and Meir, 1972) and studies by Kato (1978a, b) suggest that carbohydrate availability may control bud production in leaf explants of *Heloniopsis*. In this plant preincubation of excised leaves in the dark reduced the level of starch present with a subsequent decrease in the number of buds induced when these explants were transferred to induction media in the light (Kato, 1978a). The inhibition of morphogenesis by GA_3 has also been linked to the absence of starch accumulation in *B. semperflorens* (Sehgal, 1975) and starch breakdown in *B. rex* (Chlyah, 1972). Note however that these are speculations on correlated events, as yet there is no direct proof that carbohydrate accumulation is essential to the initiation of morphogenesis.

The effects of temperature, photoperiod, light intensity and water stress in combination, create seasonal effects in plants. The influence of these variables on endogenous hormone levels are deemed responsible for the seasonal effects observed in *B.* x *hiemalis* (Appelgren, 1985), tomato (Cassells, 1979) and *Pelargonium* (Cassells *et al.*, 1980). Cassells (1979) obtained indirect evidence that internal auxin levels

may change in tomato stems at different times of the year when inclusion of TIBA was found to enhance shoot regeneration in explants of winter grown donor plants.

Recalcitrance or reduced morphogenic responses in many tissues may be due to a reduction in endogenous hormones, nutrient, protein and carbohydrate levels which are a consequence of senescence (see Letham *et al.*, 1978; Gahan, 1981). While the application of growth regulators and/or nutrients and sugars to donor plants may slow down or inhibit senescence little work has been focused on the influence of such pretreatments on explant responses *in vitro* although extensive work has been done on the role of light and growth regulators in preventing senescence in excised leaves (see Letham *et al.*, 1978). The influence of the growth regulator paclobutrazol on the partitioning of assimilates and its demonstrated influence on increasing chlorophyll, soluble proteins and mineral nutrients in leaf tissue of apple seedlings (Wang *et al.*, 1985) suggests that pretreatment of donor plants with growth regulators could perhaps improve explant responses.

Changes in endogenous hormone levels after treatments of whole plants with exogenous hormones have been discussed and reviewed (see Letham *et al.*, 1978). In the few cases where pretreatments of donor plants or explants have been studied these treatments were found to successfully increase morphogenesis. Shoot morphogenesis in *Cordyline terminalis* 'Celastine Queen' was significantly greater when donor plants were presprayed with high concentrations (500 mg/l) of BAP (Maene and Debergh, 1982) and pretreatment of tomato plantlets with chlormequat (CCC an antigibberellin) gave a significant increase in shoot formation from stem explants (De Langhe and De Bruijne, 1976). While this approach may allow the use of lower phytohormone levels in culture giving improved genetic stability (Thomas and Davey, 1975), it would not appear to apply to some plant genotypes as regenerates from *Cordyline* pretreated by Maene and Debergh (1982) showed a high level of phenotypic variation.

The studies cited here suggest that manipulation of the donor plant prior to explanting (e.g. Kato, 1978a) or specific pretreatments of the isolated explant (e.g. Pence and Caruso, 1984) may, by altering endogenous levels of hormones, partitioning of assimilates or inhibiting cell degeneration, enhance the receptivity of somatic cells

to external hormonal stimuli. This may allow the successful culture of plants which have proved recalcitrant and may also increase the intensity of response and the number of responding explants in other plants.

B.2.2.7. Influence of the in vitro culture environment on tissue culture responses

The major factors in the cultural environment influencing organ initiation and development are light quality, photoperiod, temperature, the gas phase and relative humidity.

Because of preoccupation with the complexities of the medium, little work has been carried out on determining optimal *in vitro* conditions for organogenesis. Most tissue culture work being carried out in growth rooms with fluorescent tubes giving intensities of 1000-5000 lx for 12-16 hr daily at constant temperatures of 20-25°C with a relative humidity (RH) generally in the 70 to 80 percent range (cf. George and Sherrington, 1984).

The influence of culture environment on the success of individual micropropagation schemes has been emphasized by Murashige (1974) and Hughes (1981). Variations in the photoperiodic treatments of cultures can significantly affect their development. In *B. rex* a 24 hr photoperiod caused explant death despite the extensive production of buds over the explant surface (Chlyah, 1972), while continuous darkness inhibited bud differentiation in *B.* x *hiemalis* (Takayama and Misawa, 1982a). A similar dark inhibition effect was reported in tobacco callus (cf. Murashige, 1977). Maximum bud formation from all these cultures occurred with a photoperiod of 15-16 hours and this photoperiod is generally found to be the optimal period of illumination for a wide range of species (Murashige, 1977). Some plants, however, which have a specific photoperiod response *in vivo* may have similar requirements *in vitro*. In grape, root initiation in stem sections was shown to be dependent on satisfaction of its short day requirement (Alleweldt and Radler, 1961).

Wavelength specific responses are common in the plant kingdom hence it is not surprising that they are observed *in vitro*. The spectral quality of the lamps used has significant effects on root and shoot initiation in tissue culture. The stimulation of adventitious roots in plant tissue culture appears to be promoted by red light. Letouze and Beauchesne (1969) found that the effective region of the

light spectrum for initiation of roots in *Helianthus tuberosum* tuber sections was 600 nm . The critical protion of the light spectrum for shoot induction has been shown to be in the blue region. Red light is evidently without effect (Weis and Jaffe, 1969). More precise studies by Seibert (1973) showed that the most effective monochromatic radiation was at 467 nm. Murashige (1977) advises the use of lamps with an adequate amount of blue and red light with tissue cultures intended for plant multiplication.

Light intensity has been shown to affect the type of growth in culture and modifications at individual stages in the tissue culture programme may enhance development. Reduction in light intensity from 80 $\mu Em^{-2}s^{-1}$ to 50 $\mu Em^{-2}s^{-1}$ was found to enhance development in *Pinus contorta* (Patel and Thorpe, 1984) and a direct correlation was found between light intensity of the culture environment and survival in Asparagus shoot tip culture where an increase in light intensity from 1000 lux to 10,000 lux prior to planting increased survival from 25% to 95% (Hasegawa *et al.*, 1973). Light intensity is also critical in the induction of shoots in *Kalanchoe* with optimum shoot induction at intensities between 105-115 $\mu Em^{-2}s^{-1}$. Lower or higher intensities significantly decreased the number of adventitious shoots produced (Schneider-Moldrickx, 1983).

The general practice is to maintain cultures at constant temperatures of 22-25°C and this range of temperatures would appear to fulfil the temperature requirements of a number of plants. However in some cases, this range may not give an optimal response and a subtle change in the temperature could significantly alter explant response. The effect of temperature on root and shoot development in *B.* x *hiemalis* petiole explants has been thoroughly studied by Fonnesbech (1974a, b) who found that at constant temperatures, the best morphogenic responses were obtained at 18 to 20°C. If the temperature was increased to 24°C fewer cultures survived and the number of roots and shoots were reduced. While pretreatments with 24°C inhibited growth the application of this temperature after shoot initiation at 18°C had a promotive effect on development. In *B. rex* the most favourable temperature for bud induction was 24°C but other temperatures (17 to 27°C) did not prevent budding (Chlyah, 1972).

Changes in temperature at particular stages of culture may enhance tissue culture responses or survival after transfer to the

greenhouse. Increases in temperature up to 37°C increased shoot production in meristem culture of grape (Barlass and Skene, 1982) and dormancy on transfer to greenhouse conditions is prevented in *Gladiolus hortulans* corms produced in tissue culture by their exposure to 2°C for a period of 4 to 6 weeks (Hildebrandt, 1971). As found *in vivo* (e.g Heide, 1965b) temperature fluctuations *in vitro* may alter endogenous hormone levels within explants. Levels of hormones within explants *in vitro* under different environmental conditions have not yet been studied however.

Variability in tissue culture responses may in part be due to the influences of the gas phase on tissues on organs in culture. The gas atmosphere above plant tissue cultures is known to contain various volatile compounds including ethylene (Thomas and Murashige, 1979). This growth regulator may either stimulate or inhibit morphogenesis *in vitro*. Huxter *et al.* (1981) demonstrated that ethylene treatment within a critical culture timescale stimulated shoot regeneration in tobacco callus. However, exogenous ethylene treatment of carnation explants (Mele *et al.*, 1982) reduced growth and caused yellowing of cultures. The build up of high concentrations of ethylene produced during growth in culture is generally detrimental to development and is known to be a contributory factor in vitrification of shoots (Keevers *et al.*, 1984). Its elevation to toxic levels can be avoided by the use of containers which allow gas exchange such as those suggested by Campbell and Tomes (1983).

While not generally discussed, a moist atmosphere within the culture vessel is important to prevent cultures drying out. While the relative humidity is generally 70% in growthrooms that inside the culture vessel needs to be higher (cf. George and Sherrington, 1984). Culturing at high shoot density can be an advantage, Lane (1982) noted growing 3 to 4 pear shoots/tube was effective in preventing shoot necrosis. A balance must be achieved however between desiccation and vitrification. Ziv *et al.* (1983) found RH greater than 98% in culture vessels induced vitrification in carnation. Condensation within the culture vessel can also decrease or inhibit growth. Wernicke *et al.* (1982) found shoot forming sorghum embryos died off when covered with condensed water.

B.2.3. In vitro stock maintenance using cold storage

Many commercially grown ornamentals are vegetatively propagated and seasonal stock production requires the continuous maintenance of healthy mother stock plants. This practice is expensive and risky due to the use of greenhouse space and the danger of disease outbreak.

Storage of plantlets or meristems *in vitro* at low temperatures either directly or after appropriate pretreatments (Henshaw, 1982) offers a cheap and simple method for the maintenance of mother stock and has the added advantage of eliminating the sterilization step.

The direct storage of cultures at low temperatures (4-5°C) has been successful for a number of plants including strawberries (6 years, Mullins and Schlegel, 1976), apple (12 months, Lundergan and Janick, 1979) and *Lotus corniculatus* (Tomes, 1979). In the latter study a contrast may be made between the amount of space required for storage of stocks *in vivo* and *in vitro*. *In vitro*, one hundred genotypes occupied just 0.24 m^2 of shelf space. In contrast these plants would require 44 m^2 of field space (Tomes, 1979).

The minimal growth storage approach outlined by Henshaw (1982) suggests ways by which plantlets or meristems may be stored through reducing the overall rate of growth by the manipulation of physical or nutrient conditions or the addition of growth retardants. Research to date in this area has concentrated on raising the osmolarity of the medium. This is achieved without causing toxicity problems by the addition of mannitol at concentrations of 0.2 M. These levels retard growth and allow storage of meristem cultures e.g. potato (Westcott, 1981). This treatment had the added effect of promoting the production of multiple shoots when cultures were transferred to standard media after storage (Westcott, 1981).

Cryoprotectants such as glycerol or Dimethyl sulfoxide (DMSO) and proline which act by depressing the freezing point of tissues have been recommended as pretreatments to improve survival during prolonged cold storage (e.g. Bajaj and Reinert, 1977; Withers and King, 1979). Proline levels of 1 to 10 mg/l have however been shown to have an inhibitory effect on growth after storage in some plants e.g. strawberry (Holmes *et al.*, 1982) and would suggest the need to screen plants for proline tolerance. With strawberry an alternative method of storage has been adopted and pretreatment with 5 to 10 percent glycerol or 1 to 2 percent DMSO allowed successful regrowth of plantlets stored at 4 to 5°C (Holmes *et al.*, 1982).

The utility of cold storage in the upkeep of mother stock plants as a means of preventing loss due to disease outbreak or weather conditions is clearly illustrated in the case of *Lotus corniculatus* where 34 genotypes were lost one winter in a field maintenance nursery but an *in vitro* shoot maintenance programme at 2 to 4°C ensured that these genotypes were preserved (Tomes, 1979).

The methods described above are only applicable to short-term storage for 1-2 years. Long-term storage however may be desirable for the maintenance of valuable new genotypes derived from tissue culture or other breeding programmes or as a means of maintaining germplasm stocks.

Freeze preservation has been advocated as a method for the long-term storage of plant cells and organs and has been widely reviewed (e.g. Bajaj and Reinert, 1977; Withers, 1980, 1983, 1984). The underlying principle of this approach is that at the low temperature of liquid nitrogen (-196°C) all the metabolic activities of living cells are at a standstill and plant material could thus be preserved for indefinite periods of time. However this technique has yet to establish its reliability as a safe method for the long-term storage of valuable germplasm. As remarked by Bajaj (1977) this highly sophisticated technique involving freezing-storage-thawing-culture is a complicated multiple step event and fault at any stage can result in lethal injury to plant material. At present methods for the storage of cells from over 30 species are available (see Withers, 1983, 1984). Further developments which introduce routine methods for all types of organs, as well as cells, will serve a useful purpose in the upkeep of valuable new genotypes in the future.

B.2.4. *Continuous culture of axenic organ explants for stock multiplication and maintenance*

Axenic plantlets produced *in vitro* provide a useful but not fully exploited source of explants for tissue culture. Grown under aseptic and controlled environmental conditions the problem of systemic infection is eliminated and the influence of the epigenotype may be minimized. Also, the fact that tissues have only recently finished active cell division means that organogenesis occurs rapidly when tissues are provided with the appropriate stimulus (see Chlyah, 1972; Margara and Piollat, 1984). For this reason the development of procedures which could allow the clonal multiplication of plants using

continuous culture of axenic explants is desirable. Such a scheme has been outlined by Lê and Collet (1981) who proposed the concept of a continuous culture system (mother stock independent) which is based on the recycling of explants from *in vitro* plantlets. In developing such a system it is important to recognize the necessity for genetic stability at all stages of subculture and the maintenance of vigour in plants produced from successive recycling steps.

While there have been few studies on continuous culture the medium and explant used during subcultures has been shown to significantly affect its success. Bigot (1981a) found that explant response decreased with successive subculture in *B.* x *hiemalis*. This may be due to the use of a suboptimal medium for axenic tissue, as Bigot used a medium optimized for adult explants, or the unsuitability of the explant. Bigot (1981a) found changing from petiole to leaf explant during the course of subculture significantly increased explant multiplication rate. The level of nutrients in the medium can also influence the response of subcultured explants as illustrated in the case of *Castanea sativa* plantlets, which degenerated into a succulent form with a reduction in regenerative capacity when subcultured on full strength MS (Vieitez and Vieitiez, 1983). Culture on ½MS eliminated this problem. Full strength MS is also known to cause degenerative growth on subculture in *Prunus* (Quorin and Lepoivre, 1977). Other plants where explant subculture procedures have been developed include *Lilium* hybrids (Novak and Petri, 1981), Blueberry (Lyrene, 1979) and *Actinidia chinensis* (Standardi, 1982) in the latter, comparisons of subculture after two years with those of two months indicate an increase in multiplication rate, however shoot size was found to decrease during subculture. In *B.* x *hiemalis* subculture procedures have also been developed by Reuther and Bandari (1981) and Westerhof *et al.* (1984).

An assessment of the effectiveness of the continuous subculture system requires an evaluation of the plants produced. Comparative trials of plants produced from subculture with conventionally propagated plants are limited in number. Bigot (1982) grew on 215 plants from subcultures of *B.* x *hiemalis* 'Schwabenland orange' all of which were deemed phenotypically normal on the basis of flower colour, petal shape and leaf shape. However, Bigot (1982) did not conduct a quantitative evaluation of vegetative characters as compared with a

control population hence the overall vigour and level of interpopulation variation present in the subculture population was not assessed. Similarly Westerhof *et al.* (1984) compared two *B.* x *hiemalis* clones for variation after 1, 2 and 3 subculture cycles and found an increase of 30 and 10 percent respectively for each of the two clones after 3 subcultures. These authors however also failed to provide quantitative comparative data on the vegetative characteristics of subculture and control populations. Hence as yet the overall effect of subculture on the phenotype of the plant population produced from continuous subculture of *Begonia* has not been fully assessed. Furthermore, neither Bigot (1981a) nor Westerhof *et al.* (1984) appear to have optimized media for axenic explants hence the overall productivity of this system and the influence of subculture cycles on the regenerative capacity of explants, which are important factors in its use as a method for the rapid propagation of plants, cannot be ascertained. Comprehensive studies of this nature are also absent in the literature for other plants.

Recently an analysis of the progeny produced from subcultured explants of African violet was undertaken by Cassells and Plunkett (1986). Quantitative comparative analysis of vegetative characters with a control population demonstrated the maintenance of plant habit and vigour through a total of five subcultures (approx. 1 year). However, 74 percent of the plants produced from subculture had a fluted leaf form as distinct from the smooth leaf form of the parent, these plants also showed a reluctance to flower. Cassells and Plunkett (1986) suggest that the phenotypic change arising during subculture may be particular to the cultivar, possibly an unstable gene locus. While other plant cultivars and species may not be unstable under subculture conditions, the results presented above demonstrate the importance of progeny plant assessment which is essential in the analysis of a scheme for *in vitro* stock maintenance. Especially as the fluted leaf type was only manifest when plants had been grown under glasshouse conditions for three months. Furthermore, it illustrates the need to understand more fully how *in vitro* conditions can effect such a high frequency change be it genetic or epigenetic (see Section *B.3.4.*).

B.3. Tissue culture as an adjuvant to conventional plant breeding

B.3.1. General introduction

The objective of any plant breeding programme is to utilize existing genetic variation or to obtain new genetic variation and to manipulate this variation to incorporate useful genes into the genotype of cultivated varieties. Conventional methods used for improving existing genotypes have been outlined by Sybenga (1983) and include:

(1) Large-scale segregation after recombination in progeny hybrids between selected parents.

(2) Introduction of specific new genes, achieved for example by mutation of existing alleles.

(3) Gene dose effects through multiplication of the entire genome.

Many plant breeding programmes depend on the use of the generative cycle both for the introduction (by hybridization) and for the manipulation (by recombination followed by selection) of genetic variation. The somatic phase has also provided a source of variation in the form of spontaneous or induced mutations (cf. Sybenga, 1983). With the development of *in vitro* techniques however the prospects of exploiting the somatic phase for the production of new genotypes have improved dramatically. Methods are available for (1) cellular selection for the recovery of genetic variants, (2) anther culture, (3) somatic hybridization for recombining genomes of sexually incompatible species and more recently, (4) the possibility of specific gene additions or modifications by recombinant DNA techniques. Various aspects of the development and utility of these procedures have been reviewed (see e.g. Thomas *et al.*, 1979; Kado and Kleinhofs, 1980; Chaleff, 1983; Lurquin and Kleinhofs, 1983; Sybenga, 1983; Negrutiu *et al.*, 1984 a, b). These approaches however have yet to make major contributions to genotype improvement in commercial species. Numerous bottlenecks to these techniques must be overcome before they can be used effectively in plant improvement (see e.g. Sybenga, 1983; Negrutiu, 1984b).

Tissue culture *per se* however has provided an unexpected source of novel genetic variability in the form of somaclonal variants (see Section G.) (Larkin and Scowcroft, 1981) which are derived after tissues or cells are cultured *in vitro*. These variants are providing breeders with new genotypes which in many cases have not been produced in conventional breeding programmes.

In many crops the somatic phase is used for the induction of mutants or for the production of polyploids by treatment with colchicine. For both these treatments progress is frequently limited by the time required to grow the plants and the number of personnel available. In the case of vegetatively propagated crops the selection and build up to point of sale can take 7 to 15 years (cf. Holdgate, 1977). During this period there is substantial risk of loss due to disease. By using tissue culture less tissue is required for treatment, a higher frequency of mutants or variants may be produced, plantlets may be maintained *in vitro* to prevent loss from disease (see Section *B.2.3.*) and the long and expensive period for stock build up may be reduced by rapid micropropagation of new genotypes allowing the breeder to introduce new varieties within 2-3 years from the final selection trials (cf. Holdgate, 1977).

The application of tissue culture to plant breeding has advanced considerably and has become established as a useful technique to add to the methods available for plant improvement. In the present review the potential for the practical application of these techniques to plant breeding are outlined along with the problems encountered in maintaining the dual function of tissue culture for both the production of variation and clonal propagation of plant genotypes.

B.3.2. Genetic variation in somatic plant cells: causative mechanisms and the influence of tissue culture on the expression and induction of somatic variation in cultured cells and regenerated plants.

B.3.2.1. Introduction

By removing the organizational influences of the plant and allowing the expression of totipotency in a greater number of cells the culture of plant tissues, cells or protoplasts *in vitro* has, in some cases, resulted in the production of a wide range of phenotypic variants in agricultural and horticultural crops (see e.g. Skirvin, 1978; Constantin, 1981). In a review in 1981 Larkin and Scowcroft introduced the useful term 'somaclonal variation' to denote this variation which arises within or from populations of cultured cells. This phenomenon is now recognized as providing a new source of variants which may be of commercial importance (Nabors, 1976; Skirvin, 1978; Larkin and Scowcroft, 1981, 1983a; Sybenga, 1983; Evans *et al.*, 1984; Lörz, 1984; Orton, 1984a). The potential for the selection of variants at the cell level using mutagens or

selective environmental stresses is also acknowledged (Thomas *et al.*, 1979; Chaleff, 1981, 1983; Carlson *et al.*, 1983; Maliga, 1984; Meins, 1983; Meredith, 1984; Negrutiu *et al.*, 1984a, b). Despite this optimism the production of somaclonal variants *in vitro* has not provided the boon to plant breeders that was initially anticipated (Skirvin, 1978). Some somaclonal variants have proven unstable (e.g. Larkin and Scowcroft, 1983b) and while extensive variation has been reported in cultured cells *in vitro* (Bayliss, 1980) attenuation occurs during regeneration (Orton, 1984a) limiting the range of variants produced. To fully exploit the variation which may be present in cells *in vitro* we require a fuller understanding of (1) the extent of variation present in somatic tissues *in vivo*, (2) the factors which govern the production and expression of genetic variation *in vitro* and, (3) the circumstances which control the expression of somatic variation present in culture as regenerates which show stable inheritance of altered phenotypic traits. Our present understanding of these events has been extrapolated from studies on a range of different genotypes under different experimental conditions. Therein lies the difficulty when attempting to make generalizations regarding events controlling the production and expression of somaclonal variation, as variation exists in the behaviour of cultured cells from different genotypes (e.g. Browers and Orton, 1982) and under different environmental conditions (e.g. Guo, 1972).

The phenomenon of somaclonal variation has been reviewed under a number of different headings including cytology (D'Amoto, 1975, 1977b; Sunderland, 1977; Bayliss, 1980; Constantin, 1981), extranuclear changes (Pring *et al.*, 1981), morphology/physiology of regenerated plants (Skirvin, 1978; Chaleff, 1981; Larkin and Scowcroft, 1981, 1983a; Lörz, 1984; Orton, 1984a; Scowcroft, 1984), genetic/epigenetic forms (Meins, 1983), biological significance and speculation as to causes (Chaleff, 1981; Larkin and Scowcroft, 1981, 1983a; Orton, 1984a, b), experimental approaches (Orton, 1983a) and explant type and culture mode (Scowcroft, 1984; Cassells, 1985). In the present review, current opinion on the sources and factors which control both the presence of variation (genetic or epigenetic) *in vitro* and the expression of this variation in regenerated plants will be discussed. This will involve an initial examination of the nature and extent of somatic variation which may be present in plant tissues *in vivo*

and the mechanisms which may be responsible for its production. This discussion is necessary as it illustrates the degree of somatic variation which may be inherent in the explant introduced into culture and also illustrates the means by which a given cell genome can become modified to produce somatic cell variants which, given the correct stimuli and environmental conditions, may be expressed and produce somaclonal variants.

While tissue culture may be used to produce variants it also provides a means of clonal propagation (see Section *B.2.*) and is particularly useful for vegetatively propagated crops (Murashige, 1974). Hence plant breeders using tissue culture require the development of two tissue culture systems. One which will optimize the level of somaclonal variation produced and a second which will allow the propagation of induced variants without interfering with their phenotypic traits. The approach adopted in this review by concentrating on the presence and control of the expression of somatic variation in plant cells will also emphasize the problems faced by tissue culturalists attempting to either produce variants and/or maintain stability in plants regenerated from tissues introduced into culture.

B.3.2.2. Somatic cell variation in vivo: causative mechanisms and their consequences

Many early geneticists and cytologists believed that the amount of DNA present in cells and the linear arrangement of genes was fixed during cell development and differentiation. This concept however has been radically modified with the discoveries of McClintock (1951) and others which demonstrate that genomic change can occur during ontogeny and in response to particular environmental conditions (reviewed by Shapiro and Cordell, 1982; Cullis, 1983; Walbot and Cullis, 1985) and that changes in DNA are a natural consequence of development and differentiation in many plants (reviewed by D'Amato, 1952, 1977a; Kunakh, 1978; Nagl, 1978, 1979).

Qualitative and quantitative changes which occur in nuclear DNA play an essential role in cell differentiation and plant evolution and various mechanisms exist which evoke these changes. The main mechanisms believed to be responsible for genome and karyotype evolution include; amplification (saltatory replication) of a non-coding sequence, its diversification and dispersal throughout the

genome, heterochromatin polymorphism and Robertsonian fusion, generative polyploidy and karyotype diversification, addition and loss of B-chromosomes (cf. Nagl, 1978).

During differentiation highly active cells may require a higher DNA content than that provided by the 2c value of a given species. This DNA content can be achieved by somatic polyploidization (including endomitotic polyploidization, endoreduplication, polyteny) and differential DNA replication (DNA amplification, endo-cycles with underreplication) (cf. Nagl, 1978) (see Section *B.3.2.2.*).

As well as the above there are several other mechanisms envisaged by some authors which may play a role in differentiation. These include the modification mutation mechanism as discussed by Holliday and Pugh (1975), the modification restriction mechanism (Riggs, 1975) and the concept of informative or cytoplasmic DNA (for a general review see Nagl, 1976).

Many of the mechanisms of genome modification outlined above are evoked by alterations or failures of stages in the mitotic cell cycle which is responsible for regular mitotic division. The present review will briefly outline some of these changes, for a more detailed discussion the reader is referred to reviews by D'Amato (1952, 1959, 1977a), Rees (1961), John and Lewis (1968), Darlington (1973), Dyer (1976), Nagl (1976, 1978, 1979, 1983), Jones (1978), Sharma (1983), Sharma and Sharma (1984).

The mitotic cell cycle is divided into a number of stages (G_1, S, G_2 and M) (see Figure B.2.). G_1 is termed the DNA presynthetic period, S the DNA replication period and G_2 the post synthetic period. During mitosis (M) several independently controlled events take place; chromosome condensation and decondensation, breakdown and reconstruction of the nuclear envelope, formation and disintegration of the spindle apparatus and often dissolution and reorganization of the nucelus (or nucleoli) (cf. Nagl, 1978). For an understanding of what follows it is essential to be aware that the mitotic cycle is not an either/or mechanism but is evidently controlled stage by stage (see Nagl, 1978). Each of these stages requires specific enzymes, proteins, RNAs and a source of carbon oxygen and a certain level of hydration to progress to the next cycle stage (cf. Rost, 1977). Absence of one or other of these requirements or the presence of certain chemicals or mutagenic agents may cause the inhibition or disruption of mitosis and may

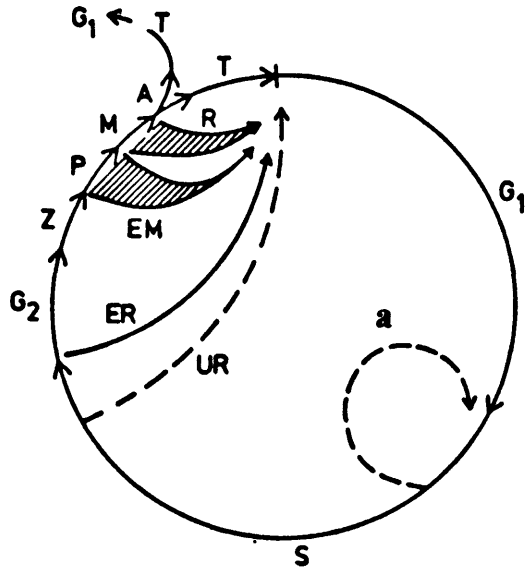


Figure B.2. Diagram illustrating the course of various cell cycles. The circle with the branch outward represents the mitotic cycle (G_1 = presynthetic or postmitotic period, S = DNA replication period, G_2 = postsynthetic or premitotic period, Z = dispersion phase, P = prophase, M = metaphase, A = anaphase, T = telophase; the branch indicates nuclear division). The hatched arrow marked with R indicates various possibilities of nuclear restitution cycles, that marked with EM the angiosperm-type endomitosis. ER shows the course of the endoreduplication cycle, UR an endocycle with underreplication of certain DNA sequences (evidently late-replicating portions, as heterochromatin and satellite DNAs are always affected) and a an amplification cycle (as the portion of the genome that is amplified may be either early or late replicating in the normal cell cycle, the location of the arrow is to be considered variable). From Nagl (1978).

result in mitotic aberrations, endocycles and changes in chromosome number or organization (e.g. D'Amato and Hoffman Ostenhoff, 1956; D'Amato, 1959; Kihlman, 1967; Van't Hof and Kovacs, 1972; Rost, 1977; Sharma and Sharma, 1981).

B.3.2.3. Mechanisms which give rise to somatic polyploidy

The most widely distributed change in nuclear organization can be ascribed to endo-cycles. This term is used to describe DNA replication within the nuclear membrane and without spindle formation (cf. Nagl, 1978). DNA synthesis in endo-cycles shows different possibilities. The events and results of division via endo-cycles are outlined in Figure B.3.

The endo-cycles which occur during tissue differentiation in many plants which results in the production of endopolyploid cells (i.e. with 4c, 8c, 12c etc. nuclei) include the endomitotic cell cycle (Geitler, 1939) and the endoreduplication cycle (Levan and Hauschke, 1953). Endomitosis consists of DNA synthesis and separation of chromosomes within the nuclear membrane but without spindle formation. The crucial difference between endomitosis and endoreduplication is chromosome polytenization which is caused by repeated duplication of chromosomes without separation within the nuclear membrane and without spindle formation. Of the two, endoreduplication is the most common process of endonuclear chromosome duplication found in plants (D'Amato, 1977a). Endopolyploid cells in plants are most commonly found in pith, root and embryo tissue (Nagl, 1978). In a given tissue the presence of endopolyploidy is not uniform and the meristematic cells of differentiated plant parts (pericycle, cambium, procambium) remain diploid (D'Amoto, 1952). Endopolyploidy has been found in 80 percent of the angiosperms so far studied (D'Amato, 1975) and is common in polysomatic (see Section G.) plants such as pea (e.g. Torrey, 1959), *Allium* (e.g. Carmona and Gutierrez, 1981) and *Nicotiana* species (e.g. Patau and Das, 1961; Nuti Ronchi *et al.*, 1973; Brossard, 1974; Martini and Nuti Ronchi, 1974). Non-polysomatic plants include for example *Crepis capillaris* (Brossard, 1978) and *Helianthus annus* (e.g. Butcher *et al.*, 1975).

The induction of endocycles is influenced by both environmental and nutritional factors (see Nagl, 1978). Hormones play an important role in controlling the progression of a cell through the cell cycle in plants (see e.g. Gould, 1984) and there is some evidence to suggest

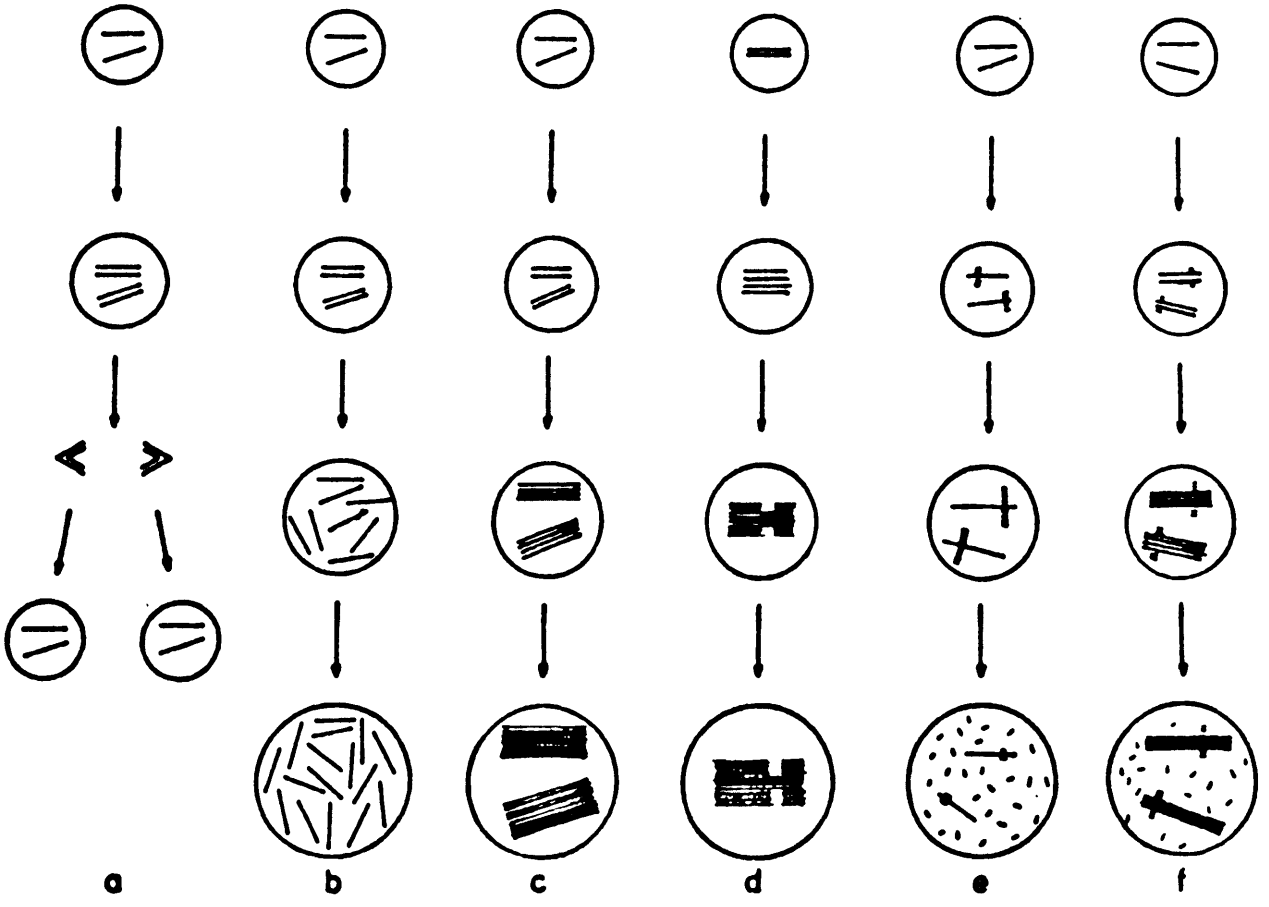


Figure B.3. The result of various cell and nuclear cycles occurring in higher organisms. Each long bar indicates a haploid genome, each short bar a certain gene (or DNA sequence).
(a) Mitotic cycle: the result is two nuclei which are genetically identical to the ancestor nucleus.
(b) Endomitotic cycle: the result is a nucleus with multiple genomes (increasing in geometric order); the nuclear structure is frequently 'polyploid' or 'reticular'.
(c) Endoreduplication (or polytenization) cycle: although the same number of genomes are present in the nucleus as in (b), the endochromosomes do not become separated through an endomitotic coiling process, and polyteny normally arises.
(d) Underreplication cycle: a certain DNA sequence is not replicated or is replicated less often (as shown here) than the rest of the genome. Underreplication may occur differentially in several sequences, and of course in nuclei with 'polyploid' structure also.
(e) Amplification cycle: only a certain gene (or a non-protein-coding sequence) is extrareplicated several times, resulting in a local polytenization. The amplified DNA becomes detached from the chromosome and is later degraded either within the nucleus or in the cytoplasm.
(f) Endoreduplication-amplification cycle: both types of cycle occur at the same period of development, but while the endopolyploid chromosome complement is stable, the extra DNA copies are not. (From Nagl, 1978).

that hormones are involved in the control of endomitosis (e.g. Libbenga and Torrey, 1973; Nagl and Rucker, 1974). Studies by Libbenga and Torrey (1973) on pea (*Pisum sativum*) root segments indicated that kinetin induced endoreduplication prior to mitosis during callus formation. Nagl (1978) suggests that the important point of the control of endo-cycles is the balance between growth regulators, that is mainly between auxins and cytokinins. Auxins generally stimulate DNA synthesis and DNA polymerase activity resulting in mitosis (Yeoman and Aitchinson, 1973) while cytokinin on the other hand acts as a trigger for mitosis in mature endo-polyploid cells (Torrey, 1965). In roots, differentiation and cell elongation are accompanied by endo-polyploidization (Torrey, 1966) and Barlow (1976) speculated that the presence of mitosis or endo-cycles in root cells is determined by the gradient of auxin and cytokinin which results in a specific ratio at any point in the root. Thus the supply of exogenous growth hormones changes the endogenous balance and therefore causes a change in the cell cycle pattern of cells within the root. The possible relationship between DNA synthesis, hormone action and gene expression in plants has been reviewed by Kessler (1973) and Nagl (1976).

The species specific and tissue specific occurrence of both endomitosis and of certain degree of endopolyploidy suggests a genetic control of this event. Butterfass (1965) has found that there are significant differences between related varieties of sugar beet (*Beta vulgaris*) in both the tendency for and the highest level of endo-polyploidy attained. This author demonstrated that trisomy for each chromosome of the complement influenced endopolyploidy in specific ways. Genetic disturbances such as chromosome addition led to increased endopolyploidy and this tendency was even more marked when chromosome loss occurred. Butterfass (1966) also demonstrated how the nutrition of the plant e.g. fertilizing, influenced the endo-polyploidy in sugar beet. Environmental changes due to season (day length, light quality) have also been suggested as leading to changes in the degree of endopolyploidy (cf. Nagl, 1978). In many cases the level of endopolyploidy is most likely the result of different balances of growth regulators within the plant (cf. Nagl, 1978). Other factors however may also be responsible. In their proposals of a 'principle control point hypothesis' Van't Hof and Kovacs (1972) demonstrate that the major control points in the cell cycle in plant

cells are the transition from G_1 to the S phase and the completion of G_2 leading to the onset and accomplishment of mitosis and these control points represent peaks in energy requirements which was demonstrated by the requirement for sucrose. Nagl (1978) suggests that other factors e.g. ions, enzyme co-factors, cAMP and other cyclic nucleotides would also provide controls. Signals from the cytoplasm which have been shown to be involved in the control of DNA replication (reviewed by Nagl, 1976) have also been suggested as regulators of DNA endoreduplication and endomitosis.

The role of endopolyploidy in differentiation has been a subject of discussion ever since its discovery (e.g. D'Amoto, 1952, 1977a; Torrey, 1966; Bennet, 1973; Nagl, 1978). The abolition of mitosis allows RNA synthesis, cell growth and differentiation to continue uninterrupted by nuclear and cell division processes. Continuous and increasing RNA synthesis increases the potential for protein synthesis in cells (Bennett, 1973; Clutter *et al.*, 1974). Hence endopolyploid cells may show a higher level of metabolism. Middelton and Gahan (1979) suggest a correlation between the high degree of polyploidy in the hepatocyte of mammalian liver cells and increased enzyme activity. Correlations have also been suggested between the high degree of polyploidy in the parenchyma cells of developing cotyledons of *Pisum sativum* and the high rate of RNA and protein synthesis (Scharpe and Van Parijs, 1973). The endopolyploid state also buffers the effect of chromosome changes which would have little chance of survival in diploid cells. This point has been highlighted by Gahan (1977) as an advantage of the endopolyploid state in mammalian liver cells where this condition may decrease the rate at which cells will be irreversibly damaged and require replacing. Polyploidy has been found to protect cells from damage by irradiation under certain conditions (discussed and reviewed by Von Wangenheim, 1976) and also to decrease sensitivity to alkylating mutagens (e.g. Evans, 1976).

Though variable from one plant to another about 60 percent of the genome of a "typical" plant is composed of repeat DNA. Most of the repeat sequence DNA is low or moderately repetitive with a reiteration frequency per sequence of less than 10^3. Some highly repetitive DNA (10^5-10^6 copies) also occurs in some plants and can comprise up to 70 percent of the total genome in maize (Sorenson, 1984). Considerable evidence has accumulated which substantiates the presence of

differential DNA amplification in plant cells during development (see Nagl, 1979). DNA sequence amplification or deamplification can lead to changes in the synthesis of a specific gene product or to perturbation in the timing of gene activity if the repeat sequences function in new chromosomal locations (cf. Scowcroft, 1984).

Differential DNA replication (i.e. semiconservative DNA replication) in eukaryotes involves precise chromatin replication which does not effect the whole genome. This may occur as underreplication where a cell becomes polyploid but certain portions of the genome remain diploid or as amplification where only a small portion of the genome (i.e. a certain gene or a repetitive DNA sequence) is replicated many times (see Figure B.3.). Amplified DNA may become degraded after some time (Nagl, 1979).

It has been suggested that the co-ordinated changes in growth patterns and characteristic physiological properties of the adult phase of *Hedera helix* may be linked to underreplication (cf. Nagl, 1979). Studies by Schaffner and Nagl (1979) indicate that heterochromatin comprises 35 percent of the juvenile phase nuclei but only 13 percent in adult phase nuclei and Nagl (1979) suggests that the underreplication induced by environmental stimuli leads to co-ordinate changes in growth pattern and physiological properties which are characteristic of the adult phase.

Amplification events occur in non-coding and coding sequences in somatic cells and has been associated with flower formation in tobacco (Wardell and Skoog, 1973). Similar increases in repetitive DNA during floral induction have also been reported in *Sambucus* (see Nagl, 1979). Other examples of specific developmentally related changes in the copy number and relative complexity of middle and highly repetitive DNA sequences have been reviewed by Nagl (1978, 1979).

It seems justifiable to conclude from these observations that mechanisms which incite genetic changes can be used by organisms during development as a means of generating clones of cells with desirable properties. How changes in repetitive DNA sequence, type, amount or arrangement mediate changes in chromosomal behaviour and gene expression is a current research priority.

Increases in DNA content may also occur as a result of a controlled short cut in the mitotic cell cycle between karyokinesis and cytokinesis which results in the production of bi- or polynucleate

cells (cf. Dyer, 1976). Increases also result from restitution cycles (Nagl, 1978). A mitosis which will not proceed normally but ends in nuclear restitution can be recognized by the overcontraction of the chromosomes, their enhanced stickiness and failure of an ordered arrangement due to misfunctioning of the spindle. Chromosomes do not complete anaphase but re-enter an interphase state within one and the same nucleus. Restitution mitosis can lead to polyploidy or aneuploidy. The restitution cycle is part of the normal development of certain tissues of many plants (see Nagl, 1978 for a review), it is not however under the same rigorous control as the endo-cycle. Hence this cycle may have the disadvantage of possible irregularities which would accompany a disturbed mitosis. Hormones and other factors such as stale products accumulating in tissue culture media have been suggested by Nagl (1978) as inducers of the restitution cycles which may occur in cell cultures (see Section *B.3.3.*).

B.3.2.4. Mitotic cycle abnormalities, mutations and chromosome rearrangements

While nuclear DNA content may be altered as a result of truncated mitotic cell cycles qualitative and quantitative changes in chromosomal DNA may also occur as a result of mitotic cycle abnormalities. These include for example; anaphase bridges, disorganized metaphases, lagging chromosomes at anaphase, multipolar or twin spindles (see e.g. Fukumoto, 1962; Dyer, 1976; Jones, 1978; see also Figure B.1.). These abnormalities result in the production of cells which may be aneuploid, polyploid, haploid, hyperdiploid or with rearranged karyotypes. The presence of one or a mixture of these cells in an individual plant will depend on its tolerance to somatic instability and in many plants such cells unless they have selective advantage are eliminated.

Mitotic instability is most frequently associated with recently introduced or high levels of polyploidy (4x-8x) for example *Rubus* (Britton and Hull, 1957), *Ribes* (Vaarma, 1949) and *Solanum* (Fukumoto, 1962). Recent hybrids also show mitotic instability e.g. *Gossypium* (Menzel and Brown, 1952) and sugarcane (Liu and Chen, 1979) but reports of its occurrence in diploids are rare (e.g. Sharma and Sharma, 1956).

The direct cause of mitotic instability is unknown. It has been suggested that it results from physiological disturbances caused by polyploidy or hybridity (Dyer, 1976) or the presence of mutations in

genes which may regulate cell division (e.g. Vaarma, 1949; Ogura, 1976, 1978; Orton, 1984b). Another cause of instability particularly in seedlings derived from aged seed, may be the accumulation of high levels of automutagens in aged somatic tissues which may induce high levels of spontaneous mutations or chromosome breakage (cf. D'Amato and Hoffman-Ostenhoff, 1956).

The combination of similar or dissimilar genomes in polyploid and hybrid cells respectively may give rise to mitotic abnormalities, especially if the chromosome sets are already unbalanced. In general the synchronization of different stages of cell division may be disturbed and can have a number of different consequences. Most of the divisional disturbances observed may be explained on the basis of an abnormal timing balance. Timing imbalance of the spindle is inferred from disturbances of the process of nuclear division such as, split and multipolar spindles (e.g. Vaarma, 1949; Fukumoto, 1962; Dyer, 1976). Timing differences in sister spindles occur most often as different contractions of chromosomes in different metaphase plates (Vaarma, 1949) (see Figure B.1.).

A large body of evidence exists to support the accumulation of genetic changes in tissues on aging (see D'Amato, 1977 for references). Among animals the best known case is that of the mammalian liver. In mice the frequency of chromosomal aberrations (deficiencies and translocations) steadily increased with age (Curtis, 1966, 1971). Loss of rRNA genes has also been correlated with the aging process in heart muscle of the dog (Johnson *et al.*, 1972).

The age associated genetic changes which occur in plant seeds has been reviewed by D'Amato and Hoffman-Ostenhoff (1956). The increase in chromosome aberrations and gene mutations observed in seedlings derived from aged seed e.g. *Lilium* (Kato, 1955) is believed to be the result of automutagenesis caused by the accumulations of mutagens, these include; nucleic acids, aldehydes, phenols and quinones (D'Amato and Hoffman-Ostenhoff, 1956). A number of studies also suggest that DNA lesions accumulate in aged seed as a result of poor DNA repair (see Osborne *et al.*, 1984 for a review).

Direct evidence for the accumulation of genetic mutations in aged seed is provided by the number of chromosome aberrations seen at the first mitotic division and morphological abnormalities in the established seedlings. Navashin (1933) reported that 80 percent of seed from 6-7

year stored stock of *Crepis* showed chromosomal aberrations in the root tip at germination compared with only 0.1 percent in 1 year old seed.

Poor DNA repair, which is believed to be linked to increases in chromosome aberrations, may be due to loss of function of specific repair enzymes, for example, those that recognise DNA base damage these include excision endonucleases, polymerases or specific repair ligases. Preliminary studies by Osborne *et al.* (1984, unpublished results) on rye have led to the suggestion that the impairment of DNA-ligase enzymes function may occur on aging.

The accumulation of DNA lesions in aged seeds can be seen as a potential source of heritable genetic change. The extent of modification of chromatin with aging and types of DNA damage that can arise is an area that remains to be fully explored.

A small number of studies have documented or strongly indicated the existence of mutations in genes which are involved in the control of the cell cycle. Such mutations may increase the frequency of mitotic errors which result in genetic variation (cf. Orton, 1984b). For example, a gene in maize "polymitotic" causes successive cytokinesis without intervening chromatid replication in pollen mitosis (Beadle, 1931), another gene in maize 'St' was found to cause chromosome breakage during mitosis and stickiness during meiosis (Beadle, 1933). Genetic tests by Ogura (1978) on chimeral tabacco plants regenerated from tissue culture suggest that mitotic instability was controlled by a single dominant gene.

The changes which may occur in the arrangement of genes during development or under stress (see Section G.) have recently been reviewed by Walbot and Cullis (1985). Mechanisms which these authors deemed responsible for genome rearrangement include transposable elements, chromosome imprinting and gene amplification.

Transposable elements were first recognized in maize but have now been found in many organisms. Recent moelcular and genetic analysis would suggest that the occurrence of transposable elements in eukaryotes may be the rule rather than the exception (Shapiro and Cardell, 1982). By virtue of their movement, transposable elements can inactivate structural genes, alter gene regulation, reactivate silent genes and generate duplications and deficiencies (cf. Larkin and Scowcroft, 1981). The structure and function of these elements in plants has recently been reviewed (Freeling, 1984). Transposable

elements may exert their effect at a specific stage in ontogony (McClintock, 1951). More recently McClintock (1978) speculated that the artificial initiation of breakage fusion bridge cycles (BFB) in maize embryos, which culminated in the rapid reorganization of the nuclear genome, constitutes a normal response to severe stress. Stress has also been implicated as a determining element in the heritable changes manifest in the progeny of flax genotrophs grown under different environmental conditions (reviewed by Cullis, 1983). The flax genotype is regarded as unstable (Durrant, 1981). Unstable genotypes are well known in cultivated plants and are characterized by genetic changes occurring in cells at frequencies well above the frequencies that are normally associated with gene mutations and are confined to a particular chromosome region. Genetic changes occurring in unstable genotypes are not due to classical gene mutation, deletions or base changes but to changes in gene expression which may be reversible or maintained for indefinite periods (cf. Durrant, 1981). The presence of an unstable gene is often first revealed by the occurrence of a mosaic of cells, or of colour flecking of a tissue. For example in *Antirrhinum majus* (Harrison and Fincham, 1964) an inactivated anthocyanin gene Pal → pal^{rec} gives white flowers instead of red. But pal^{rec} is unstable and is frequently reactivated pal^{rec} → Pal. Whenever this occurs red spots appear on the white petals, so that the instability of this allele is easily recognized and the frequency of reactivation assessed. The process of inactivation or reactivation is generally held to be due to changes in heterochromatin (e.g. Hagemann and Snoad, 1971) to the movement of controlling elements (e.g. McClintock, 1951) or to changes in the number of repeat DNA sequences (e.g. Brink *et al.*, 1968) but little is known about what controls the activation or inactivation process. It has been suggested that environmental conditions may play a role in view of the changes which occur in flax and a number of other plants when grown under different environmental conditions (see Durrant, 1981 for references).

DNA methylation may also be regarded as an influencing factor in genome diversification and may act by supressing or releasing the expression of a gene or genes which may have a phenotypic effect. This view is based on the growing evidence that undermethylation of DNA can result in heritable alterations in gene expression (reviewed by Razin *et al.*, 1985).

It is now known that the cytosine residues of DNA can be methylated and the resulting 5'methyl cytosine residues occur in specific sequences in the DNA most often adjacent to guanine residues (Razin and Riggs, 1980). There can be as high as 50 mol % in higher plants (cf. Doerfer, 1983). The pattern present in parental DNA strands is semiconservatively copied after replication so that the pattern is passed on to daughter cells. This is demonstrated using the cytidine analog 5-azacytidine (5-aza-Cr) which because it contains nitrogen instead of carbon in the 5 position, is resistant to enzyme modification and cannot be methylated (Jones and Taylor, 1980). Once incorporated into a DNA sequence 5-aza-Cr appears to be refactory to methylation and as a result, previously established patterns of DNA methylation are erased. Thus 5-aza-Cr has been found to induce stable phenotypic changes in cells that are epigenetic in nature, that is not true mutants but (rather) have genes silenced by methylation (e.g. Harris, 1982). Methylation changes therefore can masquerade as mutations and may be responsible for phenotypic changes occurring in cultures of both animal and plant cells. Aging may also result in a decrease in DNA methylation and there are reports of decreases in the level of 5-methylcytosine on aging *in vitro* of diploid fibroblasts from mice, hamsters and humans (Wilson and Jones, 1983). The decrease in methylation observed by these authors was suggested to account for aberrance in cultured cells for gene expression in aged cultures.

B.3.2.5. The influence of variation in nuclear DNA of individual cells on the genetic architecture of plants and the production of phenotypic variants

As a result of alterations in nuclear DNA plant tissues may contain cells with different amounts of DNA (polyploid, aneuploid, haploid) or with qualitative changes in DNA (mutated, rearranged karyotype). The distribution of these cells within a plant will significantly influence the likelihood of their expression as individual tissues or organs.

While endopolyploid cells appear to have a fixed distribution within plants (D'Amato, 1977a) other variant cells which occur as a result of random mitotic aberrations or spontaneous mutations may contribute in different ways to the composition of the tissue in which they occur. Sree Ramulu *et al.* (1984a) referred to the arrangement of somatic variation in tissues of plants as their genetic architecture (see Section G.), a term which will be used here with reference to plants with somatically variant cells.

One of the most interesting and widely studied forms of plant genetic architecture has been the chimera. These plants have been the subject of a large number of studies by Derman and co-workers (e.g. Derman and Bain, 1944; Derman, 1965; Stewart and Derman, 1975) and reviews by Neilson-Jones (1969), Stewart (1978) and Vaughn (1983).

True chimeras are defined as individuals composed of two or more genetically different tissues which differ in their chromosomal or plastid constituents (cf. Neilson-Jones, 1969). Horticulturally the most important type of chimera is the variegated leaf chimera in which the pale (yellow or white) leaf areas differ from the green leaf areas in a gene for chlorophyll, carotenoids or a chloroplast protein (Vaughn *et al.*, 1980). Chimeras involving traits other than variegation are also known an example being the thornless blackberry, a nuclear chimera where the epidermal tissue contains the genetic information for a thornless phenotype while the underlying tissue has the genetic potential to produce thorns (MacPheeters and Skirvin, 1981). Differences are noted between chimeras that are nuclear DNA mutants and those that are plastome (plastid DNA) mutants. Patterns of nuclear DNA mutants strictly follow cell lineages and hence produce patterns of wild type and mutant tissues, however patterns of variegation in plastome mutants are dependent on the sorting out of wild type and mutant plastids which results in a chequered leaf pattern (cv. Vaughn, 1983).

Chimeras are distinguished by the representation of their respective cell lines in a growing point of the shoot apex which is composed of three layers (LI, LII, LIII). Layer LI and LII are part of the tunica layer where cell division is mainly in the anticlinal plane while LIII makes up the corpus where cell divisions are both anticlinal and periclinal (cf. Broertjes and Van Harten, 1978).

Most chimeral plants originate as spontaneous somatic mutations and are maintained because of the undeterminate growth of their vegetative apical meristem. Chimeras may also be induced by irradiation and automatically occur if mutated and non-mutated cells contribute to adventitious bud production. If a non-lethal mutation is induced in a dividing cell of the apical meristem a lineage of mutated cells is produced, the dimensions of which depends on the position of the mutated cell, the total number of dividing apical cells and the fitness of the mutated cell (cf. Broertjes and Van Harten, 1978). A distinction

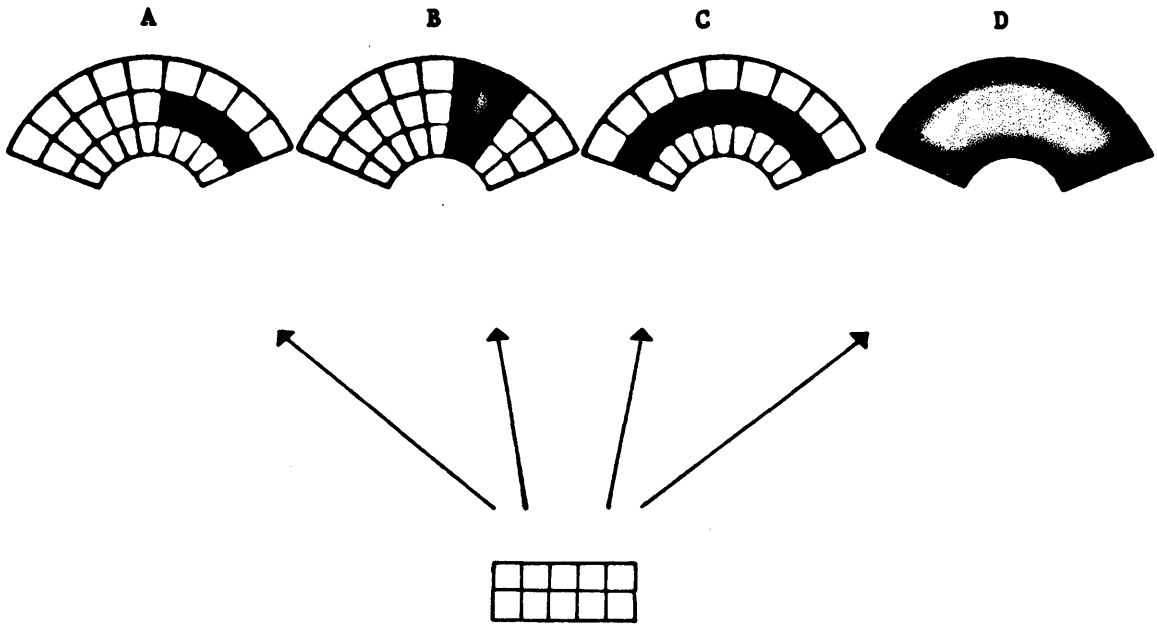


Figure B.4. Chimeral and solid mutant genetic architectures which may result after spontaneous or induced somatic mutation in multicellular plant tissue.

A = mericlinal chimera
B = sectorial chimera
C = periclinal chimera
D = solid mutant

is normally made between periclinal, mericlinal and sectorial chimeras (Figure B.4.). Shoot apex mutations may produce periclinal chimeras if mutations occur in one histogenic layer. If a full layer is not affected a mericlinal chimera results and where a mutated group of cells is represented in all histogenic cell layers and their derivatives are mutated, a sectorial chimera results (cf. Broertjes and Van Harten, 1978). Another source of chimeras are the vegetative organs derived from tissues treated with colchicine. Cells at different stages of the cell division cycle are differentially sensitive (Bayliss, 1976) hence in a treated apical meristem only a limited number of cells may be affected. If cells with both polyploid and diploid complements continue to divide a cytochimeral form may be established and persist (cf. Broertjes and Van Harten, 1978). A prime example of this phenomenona occurred in pear and apple which produced leaves with cells of different ploidy levels (Derman, 1965).

Shoot apex layers of chimeras are not fully stable and rearrangements of the cell layers through cell displacement or periclinal rather than anticlinal division can result in the reorganization of the cell layers and the synthesis of a new chimeral structure which may have a phenotypic effect (see D'Amoto, 1977a). Such rearrangements may be manifest as bud or leaf sports or changes in petal colour or shape (see Neilson-Jones, 1969). Single layers bearing a mutant cell genotype may also give rise to a solid sport if adventitious buds or leaves are formed solely from one layer. Variegated *Pelargonium* for example having either white or green bordered leaves when propagated by stem cuttings produced parental forms but when propagated from root cuttings gave wholly green or wholly albino plants according to the nature of the core tissue (LIII) (Baur, 1930). Other examples of root cuttings giving plants unlike the parent have been cited by Neilson-Jones (1969). In general mutations in the LI and LII are more frequently revealed due to the deep seated nature of the LIII layer.

With sectorial chimeras entire sections of the stem differ such that the LI, LII and LII layers on one side of the stem are genetically different from the same layer on the opposite side (Figure B.5.). The genetic composition of buds arising from sectorial chimeras is dependent on their position of origin in the stem. Buds arising wholly on one side (Figure B.5. A and C) are composed of totally normal wild type or totally mutated cells. Buds arising at the margins may be sectorial,

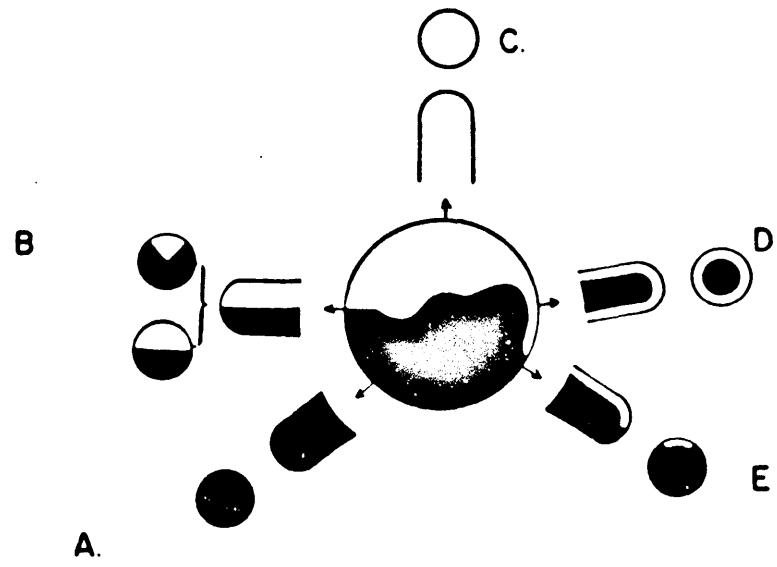


Figure B.5. Potential genetic architecture of adventitious buds produced from a sectorial chimera. (A, C = solid mutants, D = periclinal chimera, E = mericlinal chimera, B = sectorial chimera).

periclinal or mericlinal chimeras (Figure B.5. D and E respectively), each having its own pattern of arrangement of mutated and wild type cells (Neilson-Jones, 1969). Thus the induction of adventitious buds on chimeras may result in the production of another genotype. In nature, adventitious buds are formed after a plant suffers biological or physiological damage. Whitham and Stobodchikoff (1981) suggest that the derivation of these buds from different meristematic layers may be an adaptive response in that it presents an alternative genotype in a changing environment. Thus plants which are mosaics (i.e. contain mutated sectors of tissues) or chimeras can adjust to damage and have a greater chance of survival than plants which maintain a stable genotype.

The natural occurrence of bud sports in many plant genotypes have been used by horticulturists to develop new cultivars and many have become a useful source of commercially important fruit and ornamental plants. The introduction of plant tissues and isolated cells into tissue culture has revealed the presence of a far wider range of variation than was previously acknowledged to exist (see Section *B.3.4.*). The expression or control of this variation will require a greater awareness of the extent of variation present in individual plants i.e. a knowledge of plant genetic architecture and also of the factors which control its expression. This area of research is one which is under current review.

B.3.3. Somatic cell variation in vitro: speculations on origin and factors controlling development

B.3.3.1. Introduction

Plants regenerated from callus derived from protoplasts, single cells or explants are in many cases phenotypically and genotypically different from the donor plant from which the callus was derived. Regenerates include mixoploids (e.g. Lupi *et al.*, 1981), aneuploids (e.g. Novak, 1980), chimeras (e.g. Sree Ramulu *et al.*, 1976), true breeding (single gene) mutants (e.g. Evans and Sharp, 1983) and plants with rearranged karyotypes (e.g. Ogihara, 1981). There has been much speculation regarding the origin and cause of this variation (see D'Amoto, 1978; Larkin and Scowcroft, 1981, 1983a; Lörz, 1984; Orton, 1984a, b; Scowcroft, 1984). It is presently acknowledged however that there are two major sources of variation *in vitro*, firstly, genetic

variation may be inherent in the plant tissue or cells introduced into culture (see Section *B.3.2.5.*) and secondly, culture *in vitro* is also believed to selectively favour the growth of variant cells or induce variation *de novo* (Sunderland, 1977).

The genetic composition of the initial population of cells produced as a callus from cultured explants will depend on the explant source and the mode of callus growth. *In vitro*, cells of the explant may be induced to form a callus either via mitosis, endoreduplication followed by mitosis or nuclear fragmentation followed by mitosis (D'Amoto, 1978). Mitosis in non-polysomatic species will result in the production of diploid cells for example in *Crepis capillaris* (Brossard, 1979) and *Helianthus annuus* (Butcher *et al.*, 1975). While in polysomatic species, depending on the hormones and nutrients available (Torrey, 1965), both diploid and endopolyploid cells within the explant will be induced to divide forming a mixoploid population of cells with diploid and polyploid progeny (e.g. tetraploids starting from 2n-diplochromosome mitosis and octoploids starting from a 2n-quadruplochromosome mitosis). The best studied example of this phenomenon is the pea root where Torrey (1961) selectively induced division in endopolyploid cells by treatment with kinetin (see Section *B.3.3.2.*).

There is evidence that in species liable to chromosome endoreduplication some differentiated cells in the explant may be induced to endoreduplicate before entering mitosis. When tobacco (*Nicotiana tabacum* 2n = 48) stem pith which consists of cells with 2C, 4C, 8C and 16C nuclei is explanted *in vitro* in the presence of kinetin and IAA both diploid and endopolyploid nuclei are induced to divide. While some 4C nuclei undergo mitosis other 4C nuclei have been found to undergo further DNA replication and enter an 8C mitosis (Patel and Das, 1961). A similar behaviour has been observed in pea root segments following explantation *in vitro* (Matthysse and Torrey, 1967).

Nuclear fragmentation (amitosis) followed by mitosis in endopolyploid cells has also been frequently reported as a mode of callus growth and has resulted in the production of primary calli with a wide range of ploidies from *Phaseolus coccineus* embryos (Bennici *et al.*, 1976), *Vicia faba* cotyledons (Cionni *et al.*, 1978), *Triticum durum* mesocotyls (Bennici and D'Amoto, 1978) and *Cichorium intybus* roots (Caffaro *et al.*, 1982). Callus formation by amitosis has been

reported on media lacking kinetin or with unbalanced auxin cytokinin ratios (D'Amato *et al.*, 1980) and on high levels of sucrose (12 percent) with no hormones (Bennici *et al.*, 1976; Caffaro *et al.*, 1978). The absence of hormones indicates that the range of ploidies present in these latter studies are a direct result of division of pre-existing endopolyploid cells.

In vivo, the *Phaseolus coccineus* embryo supensor consists of diploid and endoreduplicated cells (up to 128C DNA content) (cf. D'Amato, 1975). The early dividing cell population produced by this explant consists for the great part (83%) of hypohaploid, haploid (11 chromosomes), hypodiploid and diploid cells, the remaining 17% being tetraploid, hypotetraploids and hypertetraploid (Bennici *et al.*, 1976). In *Cichorum intybus* roots cultured on high sucrose haploid aneuploid and diploid cells occurred in the callus and plants regenerated were aneusomatic i.e. consisted of euploid (mostly diploid rarely tetraploid) and aneuploid (mostly hypodiploid rarely hyperdiploid) cells.

Evidence for the induction of variation in plant cells during *in vitro* culture comes indirectly through regeneration of a range of different phenotypes from calli derived from single cells (e.g. Austin and Cassells, 1983) or protoplasts (e.g. Lörz and Scowcroft, 1983). The most direct evidence for the occurrence of chromosomal variation in culture has been obtained from observations at the cell level of mitotic irregularities which are apparently induced by culture conditions (e.g. Bayliss, 1973; Ghosh and Gadgil, 1979). However it must be borne in mind that mitotic abnormalities also occur in the somatic tissues of many plants (Section *B.3.2.4.*) hence culture conditions may provide a means of perpetuating irregularities rather than being directly responsible for their induction. Irregularities observed in culture include multipolar spindles which occur frequently in the callus of *Haplopappus* (Sunderland, 1977), pea (Kallak and Yarvekgly, 1977), *Pinus coulteri* (Patel and Berlyn, 1981) and tobacco (Naylor *et al.*, 1954; Fox, 1963). This phenomenon has been reported as the main source of variation in *Daucus carota* cell cultures (Bayliss, 1977) and in highly polyploid populations may lead to a hypo- or hyperdiploid condition (cf. Sunderland, 1977; Bayliss, 1980). Cells with doubled (dumbell shaped) nuclei, binucleate cells or cells with micronuclei which may

result from anaphase bridges or sticky chromosomes (Darlington, 1942) have also been observed in callus cultures in a number of plants and have been suggested as being responsible for polyploidy or aneuploidy (Naylor *et al.*, 1954; Partanen, 1963; Cooper *et al.*, 1963; Ghosh and Gadgil, 1979; Novak, 1981; Patel and Berlyn, 1982).

Another consequence of mitotic abnormalities namely, chromosomal rearrangements, have also been observed in tissue cultures. Translocations, deletions and inversions occur quite frequently in plant cell cultures (e.g. Norstog *et al.*, 1969; Sacristan, 1971; Ashmore and Gould, 1981; Gould, 1982) and have been regarded by some as a method of somatic crossing over (Evans *et al.*, 1984). Evidence for the occurrence of these events have been elucidated from karyotype analysis. In tobacco pith callus, cells after one year of growth contained chromosomes where the ratio of the longest to the shortest was 1.5:0.36 while in the root tip it was 1:0.4 (Shimada and Tabata, 1967). In *Crepis capillaris* a number of abnormal karyotypes emerged during the first year of culture (16 percent of cells) and could be traced to random translocations and deletions (Sacristan, 1971). The more recent use of Giemsa banding in studies of chromosomes in cells and plants derived from tissue culture has allowed a more detailed karyotype analysis of structural rearrangements occuring in culture (Papěs *et al.*, 1978; Armstrong and Gould, 1981; Gould, 1982; Armstrong *et al.*, 1983).

Gould (1982) has demonstrated the total rearrangement of chromosomes in *Brachycome dichromosomatica* (2n = 4) which resulted in the production of a pseudodiploid karyotype. Chromosome counts in this instance showed no variation from the norm and Gould (1982) emphasizes that Giemsa banding techniques may be essential to determining the nature of changes occuring in some cultures. The more widespread use of this technique coupled with observations on mitotic abnormalities in amenable genotypes should give a greater insight into the changes induced in callus culture.

The review by Bayliss (1980) on chromosomal variation in callus cultures and more recent research on changes in DNA or chromosome number during the initial culture of protoplasts of tobacco (De Boucaud and Gaultier, 1981; Hayashi and Nakajima, 1984) and potato (Carlsberg *et al.*, 1984; Sree Ramulu *et al.*, 1984b) demonstrates that genetic drifts occurring in culture vary considerably between

plant species (e.g. Sunderland, 1977), explants (e.g. Novak and Vyskot, 1975; De Boucaud and Gaultier, 1981) and culture conditions (e.g. Singh and Harvey, 1972). Selection for stable chromosome populations has been reported in a number of cultures (e.g. Shimada and Tabata, 1967; Demoise and Partanen, 1969; Guo, 1972; Bayliss, 1975; Singh and Harvey, 1975; Orton, 1980). Some cultures drift towards aneuploidy (Murashige and Nakano, 1967; Singh, 1981) (see Table B.1.) and there are some rare reports of a downward drift in ploidy (Singh *et al.*, 1972; Marchetti *et al.*, 1975; Singh and Harvey, 1975). In some cultures the production of new karyotypes during culture can lead to a sudden change in the cell population if the emerging karyotype has a selective advantage e.g. 7L + 1M + 6S karyotype in *Haworthia setata* 2n = 14 (Ogihara, 1982) (see Figure B.6.). The underlying causes for these varied genetic drifts in cell cultures have yet to be conclusively defined. To some extent the range of chromosomal variation induced in culture is predetermined by the ploidy level (e.g. Sacristan, 1971) and genotype (e.g. Heinz and Mee, 1971) (see Table B.2.) of the cell or cell introduced into culture. *In vitro* conditions however may induce mitotic irregularities in normally stable genotypes and also selectively enhance or prevent division in cells of different ploidies (cf. Sunderland, 1977). Factors which have been implicated as controlling the extent of variation present in cultured cells include the disorganized nature of cell growth (Bayliss, 1980; Orton, 1984b) media composition (e.g. Torrey, 1959, 1961; Ghosh and Gadgil, 1979; Vanzulli *et al.*, 1980), period of subculture (e.g. Partanen, 1963; Smith and Street, 1974; Bayliss, 1980) and the prevailing environmental and physiological conditions imposed on cells during culture (e.g. Partanen, 1963; Smith and Street, 1974; Singh, 1975a; Singh and Harvey, 1972; Reinert *et al.*, 1977). It must be emphasized that these proposals are based on observations on a wide range of plant genotypes under different culture regimes and are a reflection of the general trends observed. They should not be taken to imply that any of the above would exclusively be responsible for the production of somaclonal variation from a given plant. The lack of critical studies which evaluate the consequences of different culture regimes on variation in cells from a single genotype is a major criticism of the present literature on tissue culture derived variation. Cultured cells cannot be considered equivalent in all species culture regimes and developmental

Table B.1. Chromosome number in stem pith tissue callus of tobacco (*Nicotiana tabacum* 2n = 4x = 48). The number in brackets denotes the mitotic figures with the indicated chromosome number (from Murashige and Nakano, 1967).

Approximate duration in culture (years)	Mitotic figures analysed (no)	Chromosome Numbers
0[a]	53	48(25), 96(28)
0[b]	44	48(4), 9(31), 112(7) aneuploids (2): 182, 184
1	15	96(4), 192(3) aneuploids (8), 54, 74, 86, 88, 92, 156, 158, 304
6	12	Aneuploids (12) 108, 122, 124, 140, 146, 148(2), 152(2), 154, 162, 174

a, b: Explants excised from stem segments at 2.5-5 cm from the apex (a) and 15.5-22.5 cm from the apex. An increase in polyploidy in the older pith is evident.

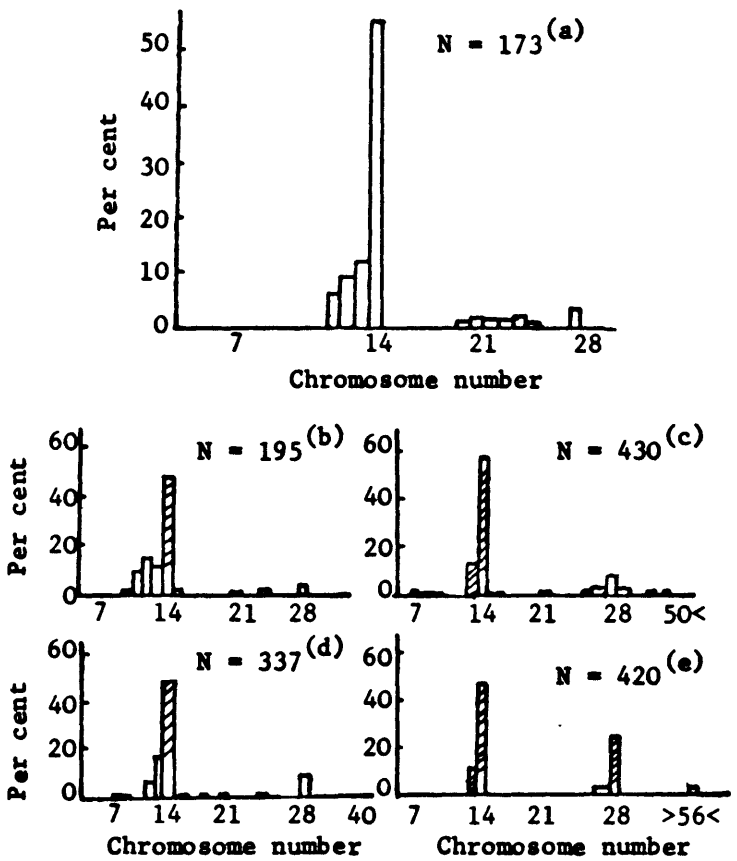


Figure B.6. Frequency of callus cells with different chromosome numbers observed in *Haworthia seta* callus over five culture generations (a) first, (b) third, (c) fourth, (d) seventh and (e) tenth culture generations. The hatched part represents the frequency of 7L + 1M + 65 karyotype and 'N' represents the number of cells observed in each culture generation (from Ogihara, 1982).

Table B.2. Chromosome numbers in tissue cultures derived from internode parenchyma of two interspecific *Saccharum* (sugar cane) hybrids and analysed after six years of suspension culture (based on data from Heinz and Mee, 1971).

Hybrid	Chromosome number		
	In the original plant (2n)	In culture	In plants regenerated from culture
H37-1933	106	-	106(8)[a]
H50-7209	108-128	71-90, 101-110, 154-160.	Chromosome mosaicism in one and the same plant. 94-120(37), 17-118(1)

a: In brackets number of regenerated plants.

fluxes. Therefore it is difficult to extract general principles from the body of descriptive literature presently available on chromosomal variation in tissue culture.

B.3.3.2. The influence of media constituents, subculture duration and level of organization on chromosomal variation in cultured cells

Hormones used in the production and subsequent maintenance of callus *in vitro* include the cytokinins kinetin and BAP and the auxins 2,4-D, IAA, IBA and NAA (Gamborg and Shyluk, 1981). Studies on these hormones both *in vivo* and *in vitro* suggest that at particular concentrations under certain environmental conditions these hormones may induce mitotic irregularities in plant cells. *In vivo* studies have been undertaken mainly on root tips of *Vicia faba* and *Allium cepa* and on *Tradescantia* stamen hairs and include those of Guttman (1956), McManus (1959), McLeod (1968), Sen (1974), Ogura (1982) and Doležěl and Novak (1984a, b). However differences between these studies in culture conditions make it difficult to make conclusive statements regarding the influence of particular hormones on mitotic irregularities. Treatments with kinetin for example were found to have no effects on the frequency of mitotic abnormalities induced in *Allium cepa* roots studied by McManus (1954), Sen (1974) and Doležěl and Novak (1984a) however Guttman (1956) observed the induction of polyploids due to lack of synchrony between mitotic division and cytokinesis after treatments with kinetin. As the range of concentrations used were similar to those used by other authors these results suggest that these discrepancies are the results of different environmental conditions and illustrate how the lack of defined conditions for such experiments has prevented a true evaluation of the influences of hormones on mitotic abnormalities *in vivo*.

While *in vivo* studies illustrate some of the influences of hormones on *in vivo* systems they do not imply that the hormones evaluated have the same effect *in vitro* on callus cells, although a number of authors have made this extrapolation (e.g. Ogura, 1982). *In vivo* studies are conducted on organized tissues such as root tips (e.g. Sen, 1974) and the influence of hormones on disorganized growth of callus *in vitro* may be significantly different. An example of the untenable extrapolations being made from *in vivo* systems to *in vitro* culture is that made by Doležěl and Novak (1984b) who do not qualify their suggestion that plant tissue culture media have no effect on the

induction of mutations in cells *in vitro*. These suggestions are based on results from studies on the frequency of somatic mutations induced by tissue culture media in *Tradescantia* stamen hairs *in vivo*. Such broad statements are likely to be misunderstood (see Scowcroft, 1984) and cannot be applied to tissue cultures not only because of the differential influence of the disorganized state but also due to the presence of specific genotype media interactions (e.g. Browers and Orton, 1982a) which have a determining influence on the nature and amount of chromosomal variation induced *in vitro*.

In vitro there are few studies which are specifically designed to evaluate the influence of hormones on the cytogenetics of cultured cells examples of these include those by Naylor *et al.* (1954), Torrey (1959, 1961), Gupta and Gadgil (1979), Vanzulli *et al.* (1980) and Browers and Orton (1982a). Many other studies which suggest a role for hormones in the induction and perpetuation of mitotic irregularities (see Bayliss, 1980) were not specifically designed to test the effects of hormones on cultured cells, hence it is difficult to validate the conclusions drawn by some authors on the basis of the results presented.

Of all the hormones used in tissue culture 2,4-D is regarded as having the most significant destabilizing influence (D'Amoto, 1977b). Its use in culture is due primarily to its growth promoting properties and its ability to induce prolific callus formation (Yeoman, 1970). While the stimulatory influence of 2,4-D on mitotic abnormalities and the division of polyploid cells has been demonstrated *in vivo* (e.g. Sen, 1974) it has been difficult to pinpoint the direct influence of this hormone *in vitro* due to the impossibility in many cases of separating the influence of hormones from the influences of the disorganized state of cell growth.

Bayliss (1980) has suggested that there is no evidence to implicate 2,4-D as the direct causal agent of mitotic aberrations leading to changes in chromosome number and that 2,4-D only affects mitosis because it stimulates disorganized growth. This suggestion is based on a review of the literature and his own studies on *Daucus carota*. Using a diploid line and a hypotetraploid habituated line which could continue undifferentiated growth in the absence of 2,4-D Bayliss (1977) found that omission of 2,4-D from the medium in which the habituated line was grown did not decrease the frequency of multipolar mitosis. Furthermore, when different 2,4-D concentrations were compared none

of the culture lines showed any correlative effect on the frequency of multipolar mitosis. Butcher *et al.* (1975) also report the absence of significant hormone influence on different isolates of *Crepis capillaris* (habituated, tumourous). Further evidence for the role of disorganized growth in the induction of mitotic abnormalities is suggested by the chromosomal heterogeneity of calli produced from a number of plants in culture in the absence of hormones (Section *B.3.3.1.*).

In contrast with the above reports however there are a number of studies where 2,4-D would appear to have a direct influence on increasing the chromosomal heterogeneity of cell populations. *Haplopappus* suspension cultures when grown on 2,4-D changed from wholly diploid to tetraploid over a period of less than six months while a corresponding culture on NAA changed less rapidly (Sunderland, 1977), but they also grew less rapidly suggesting that 2,4-D favoured growth of the tetraploid line. Shamina (1966) also working on a strain of *Haplopappus* callus likewise observed a greater rate of change with 2,4-D than NAA. In this instance, the resulting population consisted of several polyploid lines and it was concluded that 2,4-D operated by accelerating the rate of polyploidization, but no direct evidence was given in the form of increased mitotic irregularities. From these reports it would appear that the influence of 2,4-D may be dependent on cell genotype and the presence of pre-existing polyploid cells.

Few systematic studies have been made on the effects of a range of hormones or hormone concentrations on a single genotype for an analysis of their effect on chromosomal variation. One such study was undertaken by Gupta and Gadgil (1979) on *Nigella sativa*. Of the four auxins tested (2,4-D, NAA, IBA, IAA) 2,4-D induced a more rapid change producing twice as many tetraploids as the other hormones. The addition of kinetin caused a further 10 percent increase in tetraploids and also increased the number of tetraploids produced on NAA, IBA and IAA by 20 percent.

The influence of kinetin in the study of Gupta and Gadgil (1979) may be related to its effects on stimulation of mitosis in endopolyploid cells. This phenomenon has been reported in pea root (Torrey, 1959, 1961; Van't Hof and McMillan, 1961) and tobacco pith (e.g. Brossard, 1976). Torrey (1961) found that callus derived from *Pisum sativum* cv. Alaska root segments responded to different media components by exhibiting an altered distribution of 2n, 4n and 8n cells. In the

absence of additives 100% of the cells in division were diploid, with the addition of kinetin 31% of the cells were diploid, 64%, 4n and 1% 8n. The concentration of kinetin also determined the number of tetraploid cells in division, 25% at 0.1 mg/l and 90% at 1 mg/l. The influence of kinetin on the stimulation to mitosis of pre-existing endopolyploid cells was further demonstrated in a comparative study of callus production and regeneration in *Nicotiana tabacum* (polysomatic) and *Crepis capillaris* (non-polysomatic). In *Nicotiana* both the callus and shoots regenerated from it contained tetraploid cells. While in *Crepis* diploidy was maintained and increases in kinetin concentration had no effect (Brossard, 1976, 1979).

While kinetin in the above reports stimulated the division of pre-existing endopolyploid cells it is also known to induce endopolyploidy (Nagl, 1972) and studies on isolated root tips of *Allium cepa* indicate kinetin has a similar effect to that of colchicine (i.e. c-mitosis) (see Section G.) (Guttmann, 1956). In *Cymbidium* protocorms kinetin appears to specifically supress mitosis in favour of endomitosis and subsequently stimulated division in the endopolyploid cells induced (Nagl, 1972; Nagl and Rucker, 1974).

The ratio of auxin to kinetin is also believed to have significant influence on the ploidy level of cells produced from explants or in isolated calli (see D'Amato, 1978). The following examples also illustrate the differences between genotypes in response to different hormone ratios. In *Datura innoxia* a high cytokinin to auxin ratio favoured proliferation of cells with low and stable DNA content (Vanzulli *et al.*, 1980). While in *Lycopersicon* callus, an increase in the cytokinin to auxin ratio from 2.5:1 to 20:1 markedly increased cell ploidy level (Sree Ramulu *et al.*, 1976).

Reports of instability induced by other cytokinins are rare. However studies by Patel and Berlyn (1981) demonstrate the presence of a range of mitotic abnormalities in *Pinus coulteris* buds produced from callus induced on a medium containing BAP alone at 2.5 mg/l. The anaphases bridges, multipolar mitosis and nuclear extensions found in cultured buds were absent from seedling controls and would appear to confirm the role of BAP in their induction. Multipolar spindles and laggards were also observed by Ogura (1982) in isolated root tips of *Vicia faba* treated with a range of BAP concentrations. These reports have important implications in view of the widespread use of BAP in

the micropropagation of plants. While BAP's destabilizing influence may be genotype specific its use at high concentrations may induce variants in other plants and is open to further study. However it must also be recognized that treatment with BAP may simply result in the expression of pre-existing variation, as found in a chimeral *Cordyline terminalis* cv. which when pretreated with 500 mg/l BAP prior to culture produced a population 55 percent of which were phenotypic variants while pretreatments at 100 mg/l gave 100 percent true to type. This was primarily due to an increase in the number of shoots produced per explant from 7 at 100 mg/l to 35 at 500 mg/l (Maene and Debergh, 1982).

Other media constituents including adenine (Kihlmann, 1950) and the presence of nucleic acids (Venketeswaran and Speis, 1964) are also known to produce mitotic abnormalities in culture. The high nitrogen content of MS media has also been suggested as an influencing factor in the rapid rise in ploidy observed in *Haplopappus* cell cultures transferred from B5 media (Singh *et al.*, 1972).

The reported genotype dependence of hormone destabilizing influences (e.g. Browers and Orton, 1982a) demonstrates the presence of greater genome stability in some plant cells. This may be related to stricter control of the mitotic cycle and plant cells introduced into culture may maintain to a great extent the control they manifest *in vivo* (cf. Chaleff, 1981). Polyploids show a tendency towards mitotic instability *in vivo* (Section *B.3.2.4.*) and this may be intensified *in vitro* due to disorganized growth and the added destabilizing influence of hormones.

Fluctuations in ploidy level occur frequently in cell cultures (see Section *B.3.3.1.*) and subculture periods of long duration have been suggested as a causal factor in the rise or fall of cell culture populations (e.g. Mitra *et al.*, 1966; Demoise and Partanen, 1969; Bayliss, 1973; Smith and Street, 1974; Kibler and Newmann, 1980). This effect may be overcome by frequent subculture and has been successful in the maintenance of rapidly dividing diploid populations of cells (e.g. Kao *et al.*, 1970; Singh and Harvey, 1975). In *Datura innoxia* actively dividing diploid cells are present in cell clumps and during culture dead cells are sloughed off and are observed as vacuolated cells. Kibler and Neumann (1980) outline procedures for removing these cells on subculture and thereby maintained a rapidly dividing diploid cell population by preventing the build up of nucleic acids, chelating agents and other potential mutagens which Bayliss (1980)

suggests would influence the overall chromosomal composition of the culture. Moreover the maintenance of active division in diploids ensures their predominance in culture as diploid cells have a more rapid cell cycle traverse and hence a greater doubling rate which allows them to outcompete polyploids (Ashmore and Gould, 1982). The changes in karyotype and chromosomal composition which occur more rapidly in liquid as compared to solid media (e.g. Singh and Harvey, 1972) may be related not only to exposure to toxic metabolites but also to a lower level of organization.

While receiving little attention in reviews with the exception of Orton (1984b) the morphology of callus would appear to influence the chromosomal composition of a callus cell population. The organizational morphology of callus has been associated with chromosomal composition of constituent cells in pea (Libenga and Torrey, 1973), *Hordeum* (Orton, 1980) and tobacco (Zagorska *et al.*, 1974). In *Hordeum* a compact callus type A showed a relatively gradual change over a period of 16 months and chromosome number ranged between 18-26. A friable callus derived from type A proved to be unstable and produced a rapid increase in ploidy with chromosome numbers between 20-150 (Orton, 1980). In a friable callus growth is highly disorganized (Yeoman, 1970; Davidson *et al.*, 1976; Yeoman and Forsche, 1980) unlike a compact callus which may maintain a central core of actively dividing diploid cells (e.g. Krikorian *et al.*, 1982). It has been suggested that this position effect on genome stability may be linked to the fact that central core cells are by their position protected from mechanical injury during subculture and from severe disturbances such as moisture loss and changes in nutrition. These cells will continue normal growth as long as the necessary nutrient and moisture levels are available (cf. Cooper *et al.*, 1964). Specific studies aimed at assessing the influence of different callus morphologies derived from selected explant both on the chromosomal composition of the constituent cells and the regenerated plants have not yet been undertaken. Similarly the influence of 'aging' by prolonged culture on a single medium on regenerants not been commented on in the literature. This may in part be due to the fact that such treatments generally reduce or eliminate cell competence (see Smith and Street, 1974).

B.3.3.3. Possible explanations for the occurrence of genetic drifts in cultured cells

There are, as outlined, many reports in the literature of genetic instability during cell culture (see Section *B.3.3.1.*). As yet however there have been few studies which seek to find the underlying causes of this instability. It would appear that hormones play a major role in the induction or perpetuation of mitotic instability (see Section *B.3.3.2.*). The varied nature of responses however for different genotypes (e.g. Heinz and Mee, 1971; Table B.2.) and even different explants from the same plant (e.g. Murashige and Nakano, 1967; Table B.1.) would suggest that the fate of a given cell cannot readily be determined. There have been a number of suggestions put forward to explain the discrepancies between genetic drifts in cell population from different plants. Browers and Orton (1982a) for example suggest that the differences observed between populations with respect to chromosome number could be a function of genetic differences in spindle assembly and the operation of DNA replication and repair systems. The patterns of genetic drift reported in the literature (see Sunderland, 1977; Bayliss, 1980) and their own studies on *Haworthia setata* callus prompted Ogihara and Tsunewarki (1979) to suggest three modes of cell population development which are to an extent controlled by the genotype and culture conditions. Their proposals include; (1) the continual production of variants at a low level from cells of the original explant, (2) expression of a latent karyotype with selective advantage and (3) stabilization to the diploid level. Each of these systems would generate populations with different chromosomal compositions. In the case of a fluctuating variant population, expression of variation at the plant level would depend on the presence of a variant population at the time of regeneration. As yet no satisfactory hypothesis has been put forward to explain why some cultures fluctuate and others stabilize.

Mammalian tumours follow a somewhat similar pattern to callus in the development of a diversity of cell genotypes and animal biologists have not been reluctant to suggest pathways by which cells in culture generate genetic or epigenetic differences. Three pathways proposed by Nowell (1976) bear distinct resemblance to the genetic drifts found in plant cell cultures. He suggests that; (1) genetic instability may be inherent in the cell or cells introduced into culture and this mitotic irregularity is maintained in culture, (2) the continual

presence of an agent which would cause mitotic instability, (3) nutritional changes for example deficiencies of single essential amino acids which have been shown to increase the frequency of non-disjunction in cell cultures (cf. Nowell, 1976), may select for new variants on aging.

Frost and Kerbel (1983) offer an alternative explanation for the heterogeneity of cell types found in malignant tumours. Their views are based on the growing evidence that undermethylation of DNA can result in heritable alterations in gene expression (Razin and Riggs, 1980). They suggest that tumours can progress to a more heterogenous and malignant state as they become progressively more undermethylated (i.e. epigenetic effects). As gene regulation is increasingly impaired, aberrant gene expression with a random clonal distribution could occur (i.e. cells may differ in the degree of undermethylation) resulting in a heterogenous tumour and eventually overgrowth of those clones which possess an inherent growth advantage. Hence it could be the ongoing depletion of DNA methylation which is the cause of fatal neoplasma. The continued undermethylation of the genome which leads to continued deregulation of gene control they suggest could be due to continued accumulation of carcinogens or progressive impairment of methylase enzyme repair mechanisms resulting in a tumour with increasing phenotypic diversity.

While not specifically examined, it is conceivable that a similar epigenetic mechanism may be responsible for the population heterogeneity found in plant cell cultures and may explain the epigenetic effects noted (see Meins, 1983). At present there is a paucity of knowledge on the extent of methylation present in plant cells and its influence on the control of gene regulation. Hence this proposal for a role in cell diversification in callus culture is purely speculative. But parallels can be drawn between the tumour state and callus cell populations due to the rapid progression towards heterogenous populations, the production of different clones with different dominant genotypes and the reversibility of some apparent changes i.e. epigenetic effects (e.g. Meins and Binns, 1977). The exact contribution of methylation to these effects on callus cells would, on the basis of evidence available from mammalian tissue culture systems, appear to be an area worthy of further research.

B.3.4. Tissue culture and somaclonal variation: factors controlling expression and stability

B.3.4.1. Introduction

The successful application of tissue culture to the clonal propagation of plants or the production of new varieties requires a knowledge not only of the presence of somatic variation but also of factors which influence its expression in plants regenerated from tissue culture.

While high levels of chromosomal and genetic heterogeneity may exist in cell cultures (see Bayliss, 1980) and intact plants (Section *B.3.2.5.*) the subsequent expression of this variation as distinct plant phenotypes is dependent on circumstances which allow these cells to differentiate and express their totipotency (see Thorpe, 1982). Factors such as explant source (e.g. Novak and Vyskot, 1975), plant genotype (e.g. Schwartz *et al.*, 1981), regeneration procedures (Cassells, 1985), media constituents (e.g. Sree Ramulu *et al.*, 1976), subculture duration (e.g. Ahloowalia, 1983), culture environment (e.g. Cullis, 1983) and cell competence (e.g. Smith and Street, 1974) will play a significant part in the presence or absence of phenotypic variation in tissue cultured plants and their derived progeny. The above may influence the expression of pre-existing or induced genetic variation but tissue culture conditions may also result in epigenetic or physiological effects which produce phenotypes which are not stable and which breakdown in subsequent regeneration cycles. This phenomenon has led to the general ruling that identification of a variant as a somaclonal mutant requires genetic testing of regenerates by selfing and appropriate crossing for sexually propagated species. For asexually propagated species where meiotic transmission is difficult or impossible, transmission of the trait through at least two successive vegetative propagation cycles is deemed to provide reasonable surety of a true genetic base (Scowcroft, 1984).

B.3.4.2. Explant source

The distribution and presence of somatic variation in some plant tissues may vary during development and under different environmental conditions (Walbot and Cullis, 1985) and may have a determining influence on the production of phenotypic variants in regenerated plants. Examples of differences in variant production between or within organs or juvenile and mature states are limited. One such report by Van Harten *et al.* (1981) indicates that variant production

from leaf disk explants was 12.3 percent while that from petiole and rachis was 50 percent. Novak and Vyskot (1975) also found differences in variant regeneration from mature and immature stem, leaf and petiole explants of tobacco and in *B.* x *hiemalis* cv. Nixe 10 percent variation was noted in plants regenerated from petioles while 80 percent variation was recorded in a population derived from stem explants (Bigot, 1982). Differences in the number of variants produced by stem and petiole explants have also been recorded in *Pelargonium*. In petioles the level of phenotypic variation was 7 percent while in stems a level of 4 percent was recorded (Cassells and Carney, 1986). While not generally used as an explant source roots of cauliflower were cultured by Grout and Crisp (1980). These authors found a high level of phenotypic variation in the population derived from roots, including plants with altered leaf wax, multiple branching of the stem and failure to flower. In contrast, the plants they produced from the curd were all phenotypically normal.

The high incidence of variation reported in direct regeneration from explants suggests that this mode of regeneration may provide a valuable source of variant production particularly in unstable genotypes such as potato and its use has been advocated by a number of authors (Van Harten *et al.*, 1981; Cassells *et al.*, 1983). This procedure, while not widely used for the production of variants, may provide as much variation as protoplast, single cell or callus culture procedures (e.g. Cassells *et al.*, 1983).

For tissue culturists intent on clonal propagation these reports indicate that explants used for culture should be selected with caution and if possible juvenile tissues grown under non-stressed environmental conditions should be used.

B.3.4.3. Genotype

The degree of chromosomal heterogeneity present in cell cultures is significantly influenced by genotype (e.g. Browers and Orton, 1982). A similar influence is evident in the degree of variation present in regenerants from cultures of different cultivars. Oat varieties Lodi and Tippencoe after four months of culture produce variant levels of 49 percent and 12 percent respectively and after twenty months variation increased to 88 percent in Lodi and 48 percent in Tippencoe (McCoy *et al.*, 1982). Among plants derived by adventitious shoot formation from leaf explants in *B.* x *hiemalis*, Roest *et al.* (1981)

found that in one variety 43 percent of regenerates were variant (colour, size and form of leaves and flowers) whereas for another variety only 7 percent were variant. Difference in the number and type of variants produced from different genotypes in a number of other plants have also been recorded e.g. *Pelargonium* (Skirvin and Janick, 1976; Cassells and Minas, 1983), *Lycopersicon* (Sree Ramulu *et al.*, 1976), potato (see Shepard *et al.*, 1980) and wheat (Larkin *et al.*, 1984).

The evident influence of genotype on the level of tissue culture variability, which in some cases gives rise to a wide range of somaclonal variation e.g. sugarcane (Heinz and Mee, 1971) and potato (Karp *et al.*, 1982; Shepard *et al.*, 1980) while in other species no somaclonal variation is observed e.g. celery (Williams and Collins, 1976) and daylily (Krikorian and Kann, 1981) has been the subject of an ongoing debate in the literature. Bearing in mind the influence of different cultural conditions and media used it is difficult to make direct comparisons but it has been suggested by Larkin and Scowcroft (1981), Lörz (1984) and others that plants such as *Nicotiana tabacum* or *Solanum tuberosum* amphidiploid and tetraploid species, respectively, are characterized by a large genetic buffering capacity. In the latter, extensive variation of the genome is possible without disturbing the morphogenic capacity of the cultured cells. Species without this buffering capacity e.g. diploids, are possibly also affected by the genome destabilizing influences of tissue culture as manifest by changes in chromosome number and mitotic abnormalities in callus cells (see Bayliss, 1980), however in these plants there appears to be a strong bias for normal cells to proliferate and differentiate to form plants.

B.3.4.4. Epigenetic effects

The phenotypic variability observed among cultured cells (e.g. Meins and Binns, 1977) and regenerated plants (e.g. Cassells *et al.*, 1983) cannot be assumed to result only from genetic events. Physiological responses to the anomolous environment of culture and epigenetic changes also contribute to such variability (cf. Chaleff, 1981; Cassells, 1985). Epigenetic events reflect changes in gene expression which result from differential gene regulation rather than mutation. These changes are relatively stable, persist through mitosis and are expressed in daughter cells. However in contrast to altered phenotypes

having a genetic basis those resulting from epigenetic changes may be expressed in regenerated plants but will not be sexually transmissable (cf. Chaleff, 1981; Meins, 1983).

The mechanisms by which epigenetic changes are produced and controlled are not clear, we do know however, that these changes are induced by internal or external agents such as ontogenic state (e.g. Stoutemyer and Britt, 1965), hormones and environmental factors (temperature, light) (cf. Meins and Binns, 1978). Once induced they continue to be expressed in the absence of the inducing agent. Epigenetic variation has been identified at cell, tissue and plant level. Examples at the cell and tissue level include such phenomena as habituation (Meins and Binns, 1978), production of embryogenic callus (Wetherell, 1978) and differences in callus growth from juvenile and mature ivy (Stoutemyer and Britt, 1979). Cloning of individuals from internodes of tobacco produce shoots or flowers depending on location (Tran Thanh Van and Thrin, 1978). At the plant level deviant plants produced from Schwabenland red by Westerhof *et al.* (1984) reverted to the parental type after subculture indicating an epigenetic change. Variants of Aphrodite pink not normally found as sports also reverted after subculture and these authors suggest this may have been an effect of high levels of BAP in the medium which does not permanently alter the genotype but has a transient effect on the phenotype. The high frequency directed change reported for subcultured African violet regenerates (Cassells and Plunkett, 1986) may also be attributable to an epigenetic effect. While as yet not defined as epigenetic or genetic, this effect may be a consequence of changes induced by culture conditions. Stress as already outlined (Section *B.3.3.*) has been implicated as inducing rapid genomic change and may induce changes at an unstable gene locus either by amplification or gene mutation resulting in altered gene expression in regenerates.

B.3.4.5. Regeneration procedures

Micropropagation procedures play a significant role in the expression of genetic, epigenetic or non-genetic variation in tissue cultured plants (cf. Cassells, 1985). Non-genetic variation includes chimeral breakdown, physiological effects and the elimination of virus or virus-like agents (cf. Cassells, 1985) and there are a number of studies and reviews which outline the implications and consequence of this form of variation (Murashige, 1974; Reinert *et al.*, 1977; Earle

and Demarly, 1982; Larkin and Scowcroft, 1981, 1983a, b; Vaughn, 1983; Cassells, 1985).

Explant source, mode of regeneration and culture environment are the main factors controlling the expression of non-genetic variation (cf. Cassells, 1985). The presence of virus in some plants, for example *Pelargonium* causes chimeral-like variegation effects. These 'beneficial' viruses however are eliminated if these plants are propagated using meristem culture (Cassells and Minas, 1982, 1983), while petiole explant culture, by maintaining the virus, results in plants which are phenotypically similar to the parent. In contrast true chimeral *Pelargoniums* breakdown in explant culture resulting in the production of solid 'mutants' (Cassells and Minas, 1983) but are maintained in meristem culture where the integrity of the histogenic layers responsible for their chimeral nature is not disrupted.

Distinct morphological changes in leaf shape and thickness occur during development in some plants, for example *Hedera helix* (Stoutemyer and Britt, 1965) which has distinct adult and juvenile forms. These changes are termed 'phase changes' (cf. Hackett, 1983) and are regarded as semipermanent and reversible hence according to the definition of Meins (1983) may be regarded as epigenetic. In tissue culture, rejuvenation may be induced by various nutritional and environmental factors (cf. Hackett, 1983). Rapid propagation of *Vaccinium* (Lyrene, 1981) and Raspberry (Snir, 1981) lead to reversion to the juvenile form. However the mature form reappeared as the plants were grown out of doors. These phenotypic reversions while unstable can prove a problem for micropropagationists. In the case of some multicoloured foliage plants for example *Episcia*, young tissue cultured plants due to the absence of colour pattern proved difficult to sell (Bilkey and McCowan, 1979).

Adventitious bud production is the most common mode of plant regeneration from callus and complex explants (see Murashige, 1974; Hussey, 1978; Hughes, 1981). Due to the influence of bud composition on the phenotype expressed there has been much debate as to the single or multicellular origin of these buds (e.g. Broertjes *et al.*, 1968; Broertjes and Van Harten, 1978; Broertjes and Keen, 1981; Smith and Norris, 1983). The production of a high frequency of solid (non-chimeral) mutants after irradiation using the adventitious bud technique (reviewed by Broertjes and Van Harten, 1978) has led to the

view that adventitious buds are of single cell origin. Recently Broertjes and Keen (1981) restated the position regarding adventitious bud production and suggest that the apex of an adventitious bud is formed by one or a few vegetative daughter cells of one (original) cell and thus ultimately derived from a single cell, however the adventitious bud when fully developed also consists of daughter cells from cells in the zone of primary initiation. Hence, in the case of a chimera the maintenance of chimeral structure during adventitious bud formation will depend on the nature of the chimera (periclinal, mericlinal or sectorial; see Figures B.4. and B.5.). Regular division of all cell layers responsible for the chimeral structure in bud formation would ensure maintenance of the chimeral arrangement and may explain the maintenance of some chimeras propagated by the adventitious bud technique e.g. African violet (Smith and Norris, 1983). Similarly the expression of somatic mutations present in plant cells will depend on the extent to which these cells contribute to adventitious bud organization and their subsequent maintenance during shoot development, as diplontic selection could result in their elimination (cf. Broertjes and Keen, 1981). There is also the possibility that such cells may remain latent within a regenerated plant and will only become manifest under conditions which favour their expression as tissue sectors or whole plants (e.g. Sree Ramulu *et al.*, 1984a).

Indirect regeneration of plants through callus may result in the production of variants (e.g. Smith and Norris, 1983, *loc. cit.*; Cassells and Kelleher, 1984). The production of variants from callus cultures of chimeras is believed to be due to the presence of populations of cells from a number of cell layers. The participation of one or a number of cell lineages in adventitious bud organization could thereby determine the genetic architecture of the plant produced (pure variant or rearranged chimera) (see Cassells and Kelleher, 1984). Chimeral breakdown or resynthesis based on adventitious regeneration from callus has been reported in *Chrysanthemum* (Bush *et al.*, 1976; Cassells and Kelleher, 1984) and from explants where media factors have been implicated as controlling elements in the breakdown of chimeral blackberry (McPheeters and Skirvin, 1981). More recently specific attempts have been made to synthesize new chimeral arrangements by mixed callus growth (Marcotrigiano and Gouin, 1984a, b), but the frequency of chimeral regenerates to date has been extremely low.

This is surprising in view of the presence of a mixed heterogenous population of cells. But as suggested by Marcotrigiano and Gouin (1984a) the production of chimeras from calli may depend on a number of factors including; (1) the number of cells giving rise to a shoot, (2) the colony size of each genotype in the callus, (3) the stabilization of the shoot apical meristem early in development and (4) the absence of diplontic selection. This may explain why chimeras as opposed to cytochimeras (see Section G.) are rarely reported in plants derived from callus cultures. The reports of mericlinal and periclinal chimeras from *Lycopersicon* (Sree Ramulu *et al.*, 1976) and the chimeras synthesized by Marcotrigiano and Gouin (1984a) and Carlson and Chaleff (1974) being among the examples known. In contrast there are numerous observations of cytochimeras reported from callus culture (e.g. Sacristan and Melchers, 1969; Horak, 1972; Bennici, 1979; Novak, 1980; Orton, 1980; Ogihara, 1981; McCoy *et al.*, 1982; Browers and Orton, 1982b). It has been suggested that these plants arise either as regenerates from a genetically diverse population or that mitotic instability maintains chromosomal heterogeneity in plants after regeneration (Orton, 1984a). Regenerates from callus culture also demonstrate the transfer of cryptic chromosome rearrangements occurring during culture. These are manifest in the form of multicentric translocations (Orton, 1981), deletions and inversions (Ahloowalia, 1983), ring chromosomes (McCoy *et al.*, 1982), acentric and centric fragments (Novak, 1980). These chromosome rearrangements can result in loss of genetic material which may result in phenotypic variants. As well as affecting the gene on which the chromosome break occurred neighbouring genes, particularly those for which transcription is co-ordinately regulated, will also be affected (cf. Larkin and Scowcroft, 1981, 1983a).

As well as changes in chromosome number and karyotype 'Mendelian variation' (Orton, 1984a, See Section G.) may also arise in culture. Unlike gross chromosomal variation, Mendelian variation cannot at present be detected directly as we have insufficient knowledge of the fine structural organization of the genome and the primary function of genes. Several reports have appeared recently which provide genetic analysis of morphological and physiological variation in regenerated plants (reviewed by Orton, 1984a). These reports constitute the best evidence that mutations affecting functions which are manifest in

whole plants can occur in somatic tissues *in vitro*. Gross chromosomal and perhaps Mendelian mutations however impair the regenerative competency of cultured plant cells (Torrey, 1959; Mahfouz *et al.*, 1983; Mouras and Lutz, 1983). Hence, as pointed out by Orton (1984a) in some instances the variation manifested by regenerated plants misrepresents the total genetic variation in the original culture by an unknown degree. It is also true that tissue culture may simply allow the expression of mutants pre-existing in the plant (see Section *B.3.2.*). Evidence for increases in mutation rates in culture have however been conclusively determined by comparative studies using direct regenerates from the explant as contrasted with regenerates from cultured cells e.g. tobacco (Lörz and Scowcroft, 1983). In tobacco the increase in the number of variants produced with extended culture duration was attributed to the occurrence of more frequent somatic crossovers. These authors also tested the effect of growing callus under stress or altered nitrogen regimes neither of which was found to effect the frequency of mutation (Lörz and Scowcroft, 1983).

The relative phylogeny of mutations occurring in plant cell cultures have been studied in rice where an array of single recessive mutations was observed among regenerated plants; four independent unlinked mutations were present in patterns which suggested the sequence of their occurrence in the callus (Fuki, 1983). The extensive heritable genetic changes observed by De Paepe *et al.* (1981, 1983a) in plants regenerated from *N. sylvestris* after recurrent androgenic cycles led these authors to hypothesize that genetic changes which were more extensive than simple random point mutations were responsible. Molecular studies (De Paepe *et al.*, 1983b) indicate that the nuclear DNA of variants increased by an average of 10 and up to 20 percent over that of the original plant. Buoyant density separation, reassociation kinetics, and intensity of electrophoretically separated restriction fragments all point to amplification of both A-T and G-C rich highly repeated sequences and the number of inverted repeats (De Paepe *et al.*, 1983b). Similar observations of increased DNA content have been reported among androgenic doubled haploids of *Nicotiana tabacum* cv. Coker 139 (Dhillon *et al.*, 1983) and *Datura innoxia* (Sangwan-Noreel, 1983).

Just as the source of explants and genotype influence the regeneration of variants the time of callus regeneration during

subculture will influence the range of variants produced due to the presence of genetic drifts (Section *B.3.3.1.*). D'Amato *et al.* (1980) suggest that the highest level of heterogeneity in chromosomal composition of callus culture occurs in the primary callus and that the selective influences of culture conditions (media, environmental factors) may reduce the heterogeneity and result in the development of 'stable' culture populations. Studies which address this question of fluctuations in chromosomal composition during subculture are rarely reported. Results from studies by Austin and Cassells (1983) on plants regenerated from primary single cell derived callus and individual subcultures (1, 2, 3) indicate no significant changes in the level of variation produced and would appear to support the suggestion for the continual production of altered genotypes as proposed by Ogura and Tsunewaki (1979). Significant genetic drifts do however occur in other plant cultures. Diploid regenerates predominate in plants from young cultures of *Allium sativa.* However after a period of six months chimeras, tetraploids and aneuploids appeared (Novak, 1980) (see Figure B.7.). In haploid rape, plants regenerated after three years of growth were predominantly aneuploid indicating a stabilizing effect (Sacristan, 1981), while in rye-grass the first group of plants regenerated from callus were triploid but plants regenerated from later subcultures (maintained for more than 550 days) were polyploid, mixoploid and aneuploid (Ahloowalia, 1983).

In contrast to these reports a high level of stability has been reported in long term (7 years) cultures of *Lilium longiflorum* (Sheridan, 1974) although some karyotypic rearrangements have been detected (Sheridan, 1975) the plants regenerated have been predominantly diploid (Sheridan, 1975). This culture grew independently of auxin and cytokinin and this may explain the level of stability reported in this genotype.

Regeneration of plants from adventitious buds either from explants or callus, as outlined, may lead to the expression of latent variation and is not generally applicable to the clonal propagation of unstable genotypes. Plant may also arise however from explants or callus through a process of embryogenesis (Section *B.2.1.2.*). The merits of the use of somatic embryogenesis in the clonal propagation of plants has been outlined in a number of reviews (see Lawerence, 1981; Evans *et al.*, 1981; Ammirato, 1983a, b; Vasil, 1983, 1985). Its use in the

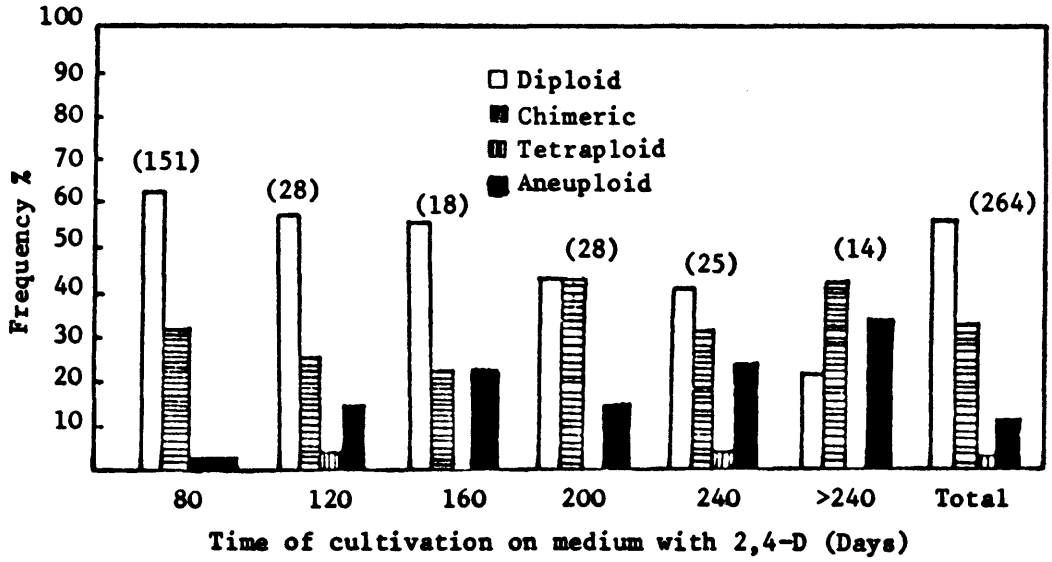


Figure B.7. Frequency of chromosome numbers in regenerants of garlic obtained from callus subcultures of different ages. The numbers in parentheses represent the number of analysed plants (from Novak, 1980).

large-scale propagation of plants however will largely depend on the maintenance of genetic stability.

It has been suggested that embryos may be derived from single cells (e.g. Cassells, 1979; Vasil and Vasil, 1982; Vasil, 1983). Indirect and direct cytological and histological evidence would suggest that embryo origin is dependent on plant genotype and mode of embryo production (direct from explants or indirectly from callus). The production of mixoploids from somatic embryos of celery (hypo-diploid and diploid) (Browers and Orton, 1982b) and Fennel (tetraploid and aneuploid) (Hunalt, 1984) indicates a multicellular origin in the case of these plant culture systems. While the apparently non-chimeral nature of plants produced from embryogenic cereal cell cultures of *Panicum maximum* Jacq. (Hanna *et al.*, 1984) and *Pennisetum americanum* (Swedlund and Vasil, 1985) would suggest a single cell origin. It must however be borne in mind that the chimeral nature of these plants may be latent and become expressed in later regeneration cycles. Thus it is difficult to unequivocally establish a single cell origin for embryos on the basis of present evidence. It is generally believed however that somatic embryos, like their zygotic counterparts, arise from single cells either directly or after the formation of a mass of proembryogenic cells (e.g. Steward *et al.*, 1964; McWilliams *et al.*, 1974; Tisserat *et al.*, 1979; Conger *et al.*, 1983; Magnusson and Bornman, 1985).

Vasil (1983) suggested that embryos derived from embryogenic cereal cell cultures were produced from diploid cells and that cells of lower or higher ploidy were embryogenically incompetent. This hypothesis was supported by reports of apparent uniformity among regenerates produced by somatic embryogenesis in *Pennisetum* spp. (Haydu and Vasil, 1981) and *Panicum maximum* (Hanna *et al.*, 1984). However, these results were based on small samples. For example in *Panicum maximum* only 20 regenerates were evaluated for cytogenetic or morphological abnormalities. Further, more detailed studies on the cytogenetics of regenerates derived from embryogenic cultures of *Pennisetum americanum* (Swedulund and Vasil, 1985) indicate that while regenerates were predominantly diploid, tetraploids also showed embryogenic competence. High levels of tetraploid cells in embryogenic culture of *Daucus* (Smith and Street, 1974) and Fennel (Hunalt, 1984) also resulted in the production of tetraploid plantlets from embryos.

In other studies the analysis of plants produced from cultures by somatic embryogenesis indicates that variation can occur in coffee (Sondahl, 1982), celery (Sharp and Evans, 1982) and sugarcane (Ahloowalia and Maretzki, 1983). These results indicate that embryogenesis does not ensure the stability in regenerates that was previously anticipated and hence the use of this technique in clonal plant propagation may be confined to plants where stability in regenerates from embryos has been conclusively demonstrated after extensive field trials.

Many authors have advocated meristem or shoot tip culture as the best option to minimize culture variation (e.g. Murashige, 1974, 1977; D'Amato, 1975; Hussey, 1978; Lawrence, 1981; Cassells, 1984). However, variants have been produced using propagation via meristem tip culture of strawberry (Schwartz *et al.*, 1981) which is one of the few studies where comparative assessment of meristem tip cultured progeny with conventionally propagated stock was undertaken. Similarly nodal cultures of chimeral plants e.g. potato may result in the production of variants if environmental conditions either *in vivo* or *in vitro* cause the rearrangement of cell layers by inducing periclinal rather than anticlinal division (Cassells *et al.*, 1986).

In view of the absence of studies on the extent of variation which may be produced from cultures using organized pre-formed meristems it is presently difficult to substantiate the claims made for the stability of these regeneration procedures as a method of clonal propagation of plants.

B.3.4.6. Latent and transient somaclonal variation

As a result of the epigenetic or non-genetic nature of some somaclonal variants (Cassells, 1985) breakdown may occur in subsequent regeneration cycles (e.g. Larkin and Scowcroft, 1983b; Sinden and Shepard, 1983; Irvine, 1984).

Phenotypic reversion has been reported in sugarcane where six somaclones deemed tolerant to eyespot toxin were found to segregate after a second tissue culture cycle. In the regenerates 40 percent showed a similar tolerance, 22 percent were more tolerant and 38 percent were susceptible (Larkin and Scowcroft, 1983b). The cause for the reversion reported is believed to be due to the effects of prolonged culture which may have caused physiological (non-genetic or epigenetic) conditioning which persisted in the leaves of

regenerates and made them appear resistant to toxin (Larkin and Scowcroft, 1983b). This study demonstrates the importance of progeny testing to determine the stability of variants derived from tissue culture. The increased tolerance of 22 percent of the population would suggest that further tissue culture cycles may uncover useful variation. This phenomenon has been referred to as character stacking (cf. Shepard *et al.*, 1980) and involves the selection of multiple characters in successive regeneration cycles while maintaining the initially selected trait and ensuring that no loss in commercial properties occurs. The overall application of this procedure to crop improvement is yet to be fully evaluated.

As well as reversion of putative variants, phenotype which initially appears stable may segregate after a further tissue culture cycle. In the potato cultivar Bintje Sree Ramulu *et al.* (1984a) report the emergence of a number of new phenotypes from the regenerates of an apparently 'normal' phenotype derived from protoplast culture. The normal phenotype and resultant off type did not show any deviation in chromosome number and suggest a qualitative rather than a quantitative change. This example of latent variation demonstrates the risks involved with regeneration from callus culture. The multicellular nature of adventitious buds and the heterogeneity of the callus being an ideal combination for the production of chimeras, cytochimeras or plants with mutant sectors or latent mutations requiring specific developmental or environmental conditions to induce expression.

Both these reports of breakdown and emergence demonstrate the need to constantly monitor the progeny of vegetatively propagated plants. Most somaclonal variants have only been processed through one or two vegetative propagation cycles (e.g. Sinden and Shepard, 1983) hence the stability of these variants is as yet not fully elucidated.

B.3.4.7. Loss of morphogenic competence on subculture

During long-term culture in many plants a gradual loss of competence has been reported and some cultures have been found to completely loose their ability to regenerate (e.g. Murashige and Nakano, 1967). It has been suggested that the causal factors which inhibit regeneration are either genetic (Torrey, 1959) or physiological (Reinert and Backs, 1968) or a combination of the two (Smith and Street, 1974).

The genetical argument was initially proposed by Torrey (1959) who suggested that the loss of rooting capacity in *Pisum sativum* cultures was related to a change in nuclear status from a mixture of diploid and tetraploid cells to an entirely polyploid cell population. Changes in chromosomal composition of calli were also found to be related to loss of morphogenic capacity in calli from single cells of tobacco (Murashige and Nakano, 1967). Competent cell lines were either diploid or tetraploid while aneuploidy was typical in morphologically repressed lines. Absence of morphogenesis in cell lines of tumourous tobacco has been linked to the presence of specific marker chromosomes for tumour virulence, only calli with low numbers of marker chromosomes were found to undergo morphogenesis (Mouras and Lutz, 1983). Changes in chromosome number however are not alone responsible for loss of morphogenic competence. Gould (1978) found complete loss of organogenic capacity in a diploid line of *Brachycome lineariloba* was not associated with karyotype change and concluded that genetic mutation may have been involved. In this culture there was a hierarchical decline in morphogenic capacity with the production of bipolar structures (embryos) being the highest and first lost, monopolar structures (roots) next and finally cytodifferentiation (i.e. trachery elements). In a later publication Gould (1984) emphasizes the importance of both spatial and temporal mitotic control and suggests that genetic changes in culture may prevent the orientation of cell divisions which are essential to the production of organized structures such as shoots or roots.

While it is apparent that some cell genotypes produced in callus may lack the capacity to regenerate there are many reports of the regeneration of variant genotypes and even aneuploid cells have been shown to produce plants (e.g. Sacristan and Melchers, 1969; Ogura, 1976; Sacristan, 1981). While the ability of variant karyotypes to regenerate may be genotype dependent it is also possible that the physiological status of cells during subculture may change resulting in the development of a new metabolic state within the callus (cf. Thorpe, 1982). This is most evident in the case of habituated tobacco calli (e.g. Meins and Binns, 1977) which produce their own supply of endogenous hormones. Similar hormonal changes have been indirectly implicated in the enhancement of regeneration from *Arabidopsis thaliana* callus after an extended subculture period of 8 weeks instead of 4

prior to regeneration (Negrutiu and Jacobs, 1978). Alterations in endogenous hormones may however influence interaction with exogenous hormones and hence the ratio previously successful for regeneration may require adjustment. Nutrient levels too have been found to change in calli and Chaturvedi *et al.* (1974) found a correlated loss of morphogenic capacity and lower levels of free amino acids, nitrogen proteins and sugar in *Citrus*.

In many cases competence in variant genotypes may be restored by providing adequate nutrients and an appropriate stimulus for active cell division and differentiation. Presently studies aimed at increasing plant regeneration from callus involve subtle alterations in media constituents (e.g. Reinert and Backs, 1968), environment (e.g. Negrutiu and Jacobs, 1978), sequential transfer to different media (e.g. Krikorian and Kann, 1981) or homogenization (Orzhinsky *et al.*, 1983). Further increases in the number of plants regenerated from calli and hence in the number of potential variants may also be achieved by manipulation of hormone levels. As already discussed (see Section *B.3.2.2.*) hormones may allow the selective division of certain cell genotypes and varying the level and ratio of hormones may increase the number of variants regenerated from a given callus. These procedures and those outlined above may allow the full exploitation of somatic variation present in callus which if from single cells or protoplasts has been both time consuming and costly to produce.

B.3.5. Tissue culture in the expression and propagation of variants induced by treatments with mutagens or colchicine

B.3.5.1. Introduction

Tissue culture as already discussed will permit the persistance of variant cells which would not normally be expressed *in vivo* and furthermore provides conditions conducive to the differentiation of plants from these variant cells. The high rate of multiplication potentially available in a tissue culture system (e.g. Hussey and Stacy, 1981) also allows the rapid cloning of any new stable genotype produced. Hence tissue culture would appear to provide a means of ensuring the production of large numbers of mutants after suitable mutagenic treatments and a method for rapidly cloning mutants for trials thus providing a useful adjuvant to conventional mutation breeding (Holdgate, 1977). These advantages are similarly applicable to polyploid research using colchicine.

Here, the application of tissue culture to conventional mutation breeding and polyploid research will be outlined and the progress and problems faced in this area of research will be discussed.

B.3.5.2. Mutation breeding

One of the main advantages of mutant induction is the ability to change one or a few characters without otherwise altering the remaining and often unique part of the genotype and this method of plant breeding has been widely used (reviewed by Broertjes and Van Harten, 1978; Gottschalk and Wolff, 1983).

Chemical mutagens such as ethyl- or methylmethanesulfonate (EMS or MMS) and various types of ionizing radiation (x-rays or gamma rays) may be used to induce mutations and have been applied *in vivo* to seeds, plants and isolated organs (see Broertjes and Van Harten, 1978; Gottschalk and Wolff, 1983) and *in vitro* to complex explants (Broertjes, 1982) cells (reviewed by Handro, 1981) and callus (e.g. Liu, 1982).

Ionizing radiation can result in disruption of DNA in plant cells. Depending on the dose rate, environmental conditions, plant tissue used and the genotype, different consequences may result from irradiation treatment; (1) some or all the disruption may be repaired (e.g. Leenhouts *et al.*, 1982) or (2) the cell may survive with irradiation damage giving rise to a mutant phenotype (e.g. Broertjes, 1969) or (3) cells may loose mitotic ability and thus the ability to differentiate (Inoue and Van Huystee, 1984).

For practical mutation breeding, acute irradiation with fairly hard x-rays or γ-rays is generally used with a dose rate of 100-1000 rad/min (and short irradiation times). For more details about radiation types, and sources, radiobiology dosimetry etc. see the 'Manual of Mutation Breeding' (IAEA, 1977). Detailed information about chemical mutagens is also available in the latter publication.

Chemical mutagens are not routinely used in *in vivo* mutation breeding programmes. They have however been widely used *in vitro* in cell cultures to induce variant cell lines and a number of mutants have been isolated at the cell level after mutagen treatment (reviewed by Handro, 1981). While single cell suspensions (monoculture) should prove ideal material for the induction and isolation of solid mutants the problems of tissue recalcitrance, cytochimera formation and epigenetic effects (see Section *B.3.4.*) must be overcome before this method can be used routinely for mutation breeding.

In general, ionization radiation is preferred as a method for inducing mutation as it is easily applicable, clean with good penetration and reproducibility and gives a high mutation frequency (cf. Broertje and Van Harten, 1978).

Vegetatively propagated plants have proved very suitable for the application of mutation breeding and it has proved particularly valuable in the breeding of sterile plants and obligate apomictics. The general high degree of heterozygosity as well as frequent polyploidy found in vegetatively propagated plants are advantages, as large variation can often be produced and in many cases mutants may be evaluated in the first generation of plants produced after irradiation. A literature review published by Broertjes and Van Harten (1978) on mutation breeding in vegetatively propagated crops contains many details on both the methods used and results from a wide range of crops and provides a source of many valuable references. This publication clearly demonstrates the progress made in mutation breeding in vegetatively propagated crops, with about 250 mutants having been developed into commercially released varieties in the last twenty or more years.

As a mutation is a one-cell-event production of chimeras and diplontic selection occurs as a result of the irradiation of multicellular apices (Broertjes and Van Harten, 1978). This is a serious drawback to mutation breeding as the recognition of mutant sectors is difficult and the recovery of mutated tissues is often not possible (Broertjes and Van Harten, 1978). These phenomena have largely been overcome however by the use of the adventitious bud technique (Broertjes *et al.*, 1968). As buds may arise from one or a small number of cells (Broertjes and Keen, 1981) high mutation frequencies and low levels of chimera formation may be obtained. The potential of this method for the production of high numbers of solid mutants have been demonstrated in several ornamental crops such as *Achimenes* (Broertjes, 1972), *Begonia* (Doorenbos and Karper, 1975), and *Streptocarpus* (Broertjes, 1969). In *Streptocarpus* irradiated half leaves of the cultivar Constant Nymph produced a total of 857 mutants, five of which were sold commercially within three years of the start of the experiment (Broertjes, 1969). In *Begonia* high levels of mutation have also been reported (Doorenbos and Karper, 1975). After x-ray irradiation doses of 2500 rads 79-80 percent of the shoots

produced from selected clones of *B.* x *hiemalis* showed one or more mutations. Controls also showed mutations but at a lower percentage frequency (18-35%) and only flower colour was affected. Irradiation induced a wide range of flower colour changes and also caused mutations in growth habit, colour and size of leaves. Almost all mutants produced were solid and two of 96 flower colour mutants one called 'Turo' and a second 'Tiara' were introduced as commercial varieties. A wide mutation spectrum has also been produced in other *B.* x *hiemalis* cultivars by Mikkelson (Mikkelson *et al.*, 1975; Mikkelson, 1976) and Brown and Harney (1974). Over ten of the mutants produced by Mikkelson (1976) were commercialized. A number of commercial mutants of *B. rex* have also been produced. By irradiating leaf cuttings of the *B. rex* cv. Winter Queen with 10 kr gamma rays Shigematsu and Matsubara (1976) produced plants with changes in leaf texture and colour pattern arrangement.

In vitro techniques have been recommended as a valuable tool to obtain non-chimeric and genetically homogenous mutant genotypes within a comparatively short time period (Broertjes, 1982) and its application has been reviewed by Roest (1976), Broertjes and Van Harten (1978) and Broertjes (1982).

In potato *in vitro* techniques were found to give a 30 percent higher mutation frequency than that found *in vivo*. Controls in the *in vitro* experiment however produced 50 percent spontaneous variation due to the inherently high somatic instability of the genotype used (Van Harten *et al.*, 1981). This result demonstrates the need to know the genetic architecture of the plant before applying expensive irradiation treatments for variant production *in vitro*. In *Chrysanthemum* all mutants produced *in vitro* were solid, which was in contrast with *in vivo* reports and a high level of mutation was induced (Broertjes *et al.*, 1976). *In vitro* techniques have also been applied to *B.* x *hiemalis* and Roest *et al.* (1981) cultured mature irradiated explants and produced plantlets after two cycles of adventitious shoot formation on axenic leaves (used to eliminate chimeras). The overall mutation frequency reported in this experiment however was low (30%) and was further complicated by the fact that one of the clones used showed a high level of spontaneous mutation. The high mortality rate of plantlets when transferred into soil (80%) however did not allow a full evaluation of the number of potential mutants produced.

UNIVERSITY COLLEGE Library CORK

These results further illustrate the need to improve tissue culture procedures to ensure the survival of high numbers of plants and will be essential to the application of *in vitro* techniques to mutation breeding in any plant.

B.3.5.3. Induction of polyploids and other variants using colchicine

While autopolyploids are regarded as contributing little to the evolution of plants in the wild (Stebbins, 1971) induced polyploids have provided a useful source of new variants for breeders of horticultural and agricultural crops (see Legros, 1964; Gottschalk, 1978; Levin, 1983). Colchicine is widely used for the induction of polyploids due to its high solubility, low toxicity and ability to specifically inhibit spindle formation (D'Amoto, 1959). In ornamental plants colchicine has been used *in vivo* to breed triploid F1 hybrids e.g. *Begonia* (Doorenbos, 1973) for re-introducing fertility e.g. *Dahlia* x *belladona* (Legros, 1964) and producing larger flowers e.g. *Streptocarpus* (Broertjes, 1974). *In vitro*, colchicine has been assessed for the production of polyploids by application either to the primary explant (e.g. Hussey and Hepher, 1976; Novak, 1983; Espino and Vazquez, 1981) to callus (e.g. Chen and Goden-Kallemeyn, 1979; Nakamura *et al.*, 1981; Orton and Steidl, 1981) or to cell suspension cultures (e.g. Heinz and Mee, 1970). Both *in vivo* and *in vitro* polyploid breeding has been hampered by the production of cytochimeras. This phenomenon may be attributed to the differential influence of colchicine on cells at different stages of the cell division cycle (Bayliss, 1976) and the treatment of multicellular apices (Broertjes, 1974). As chimeras may breakdown in subsequent regeneration cycles they create a major bottleneck to the successful application of colchicine treatment in plant breeding. This problem is well illustrated in the studies of Doorenbos and Legros (1968) on tuberous *Begonia*. These authors produced six chimeral tetraploids after colchicine treatment of 200 seedlings from a cross between *B. socotrana* x *B. dregei*. When these tetraploids were backcrossed with *B. socotrana* they produced three populations of plants with distinct variations in vigour, size and time of flowering. These plants however tended to revert to diploidy. To overcome the problem of chimeral production Broertjes (1974) has suggested the use of the adventitious bud technique (Broertjes *et al.*, 1968) a procedure which has been used successfully in the production of solid (non-chimeral)

mutants in a number of plants (reviewed by Broetjes and Van Harten, 1978). Using this technique Broertjes (1974) successfully produced 30 percent polyploidy in populations from colchicine treated *Saintpaulia* and *Streptocarpus* cuttings. Of the plants produced only 1-2 percent were chimeral. Low levels of chimeral production were also reported by Arisumi and Frazer (1968) in studies on thirteen different *Saintpaulia* cultivars. Reports on the *in vitro* application of this technique are limited to studies by Espino and Vazquez (1981) on *Saintpaulia*. These authors however report 33 percent cytochimera formation and suggest this result may be caused by the multicellular origin of buds or a permanent influence of colchicine on mitosis which caused the continued production of a range of cell ploidies in regenerates. The difference between these results and those reported by Broertjes (1974) may also be attributable to differences in time of application and concentrations of colchicine used. It must also be pointed out that Broertjes (1974) evaluated plants at maturity while Espino and Vazquez (1982) only evaluated plantlets from culture and it is possible that many of the cytochimeras recorded by these authors would have died on transplanting or become diploid due to diplontic selection (see Broertjes and Van Harten, 1978).

In callus cultures treated with colchicine, cytochimera production has been reported in a number of studies (e.g. Chen and Goeden-Kallemeyn, 1979; Nakamura *et al.*, 1981). Colchicine treatment of callus has however proved an effective means of producing a high frequency and recovery of pure tetraploids of high fertility from sterile hybrids of *Hordeum vulgare* (HV) x *H. jubatum* (HJ). Approximately 40 percent of HJ plants regenerated from colchicine treated calli appeared to be pure tetraploids (Orton and Steidl, 1980). A high percentage of tetraploids have also been induced in daylily callus treated with colchicine where 50 percent of plants initiated from treated calli were tetraploid (Chen and Goeden-Kallemeyn, 1979).

As a result of the production of polyploids from suspension cultures of sugarcane treated with colchicine Heinz and Mee (1970) have advocated the use of single cell culture for the production of polyploids. However due to the potentially destabilizing influence of the callus phase (Section *B.3.4.*) this procedure may also induce other abnormalities in these cells and hence militates against the effective use of this technique which would be particularly valuable for haploids.

In some plants low levels of polyploidy or no polyploids have been induced despite various applications of different concentrations of colchicine e.g. *Vaccinium* (Goldy and Lyrene, 1984). A number of hypothesis have been put forward to explain this phenomenon which include; lack of suitable conditions for penetration of colchicine, the absence of an actively dividing cell population or a high level of tolerance to colchicine in some genotypes (Goldy and Lyrene, 1984). A number of different approaches have been used to increase the level of polyploidy. *In vivo* the solvent Dimethylsulfoxide (DMSO) has been used as an adjuvant to colchicine treatment in a number of studies. Chemicals dissolved in DMSO are believed to have a greater penetrating capacity due to the enhanced penetration of DMSO through plant membranes (Jacobs *et al.*, 1964).

Kaul and Zutshi (1971) found DMSO carried colchicine produced 50 percent more polyploids in barley and rye and increased by 100 percent the number of polyploids produced in buckwheat. DMSO was also found to reduce the level of cytochimeras produced after treatment of garlic stem tips *in vitro* as well as increasing polyploid production by 20 percent (Novak, 1983).

Pretreatments which increase the number of actively dividing cells present prior to colchicine treatment have also been used successfully to increase polyploid production. For example in *Vaccinum* pretreatments of shoot tips in the dark at 4°C for 2 days followed by 2 days at 25°C in the light increased the number of polyploids produced (Goldy and Lyrene, 1984).

In a number of the studies cited here, distinct variation between polyploids was noted (e.g. Broertjes, 1974) and it has been suggested that this may be a result of genetic or chromosomal changes occurring in concert with duplication (Broertjes, 1974). To explain this phenomenon it is necessary to understand the action of colchicine on mitosis in plant cells. This topic has been fully reviewed by D'Amato (1959) and will only be briefly outlined here. Results from a number of studies indicate that colchicine does not always result in total spindle failure (i.e. C-mitosis, Levan, 1938) (See Figure B.8.). In plant cells chromosome movement is believed to be under the control of two groups of forces, one which is extrinsic and imposed by the mitotic spindle and a second which is intrinsic and located at the chromosome centromeres (cf. D'Amato,

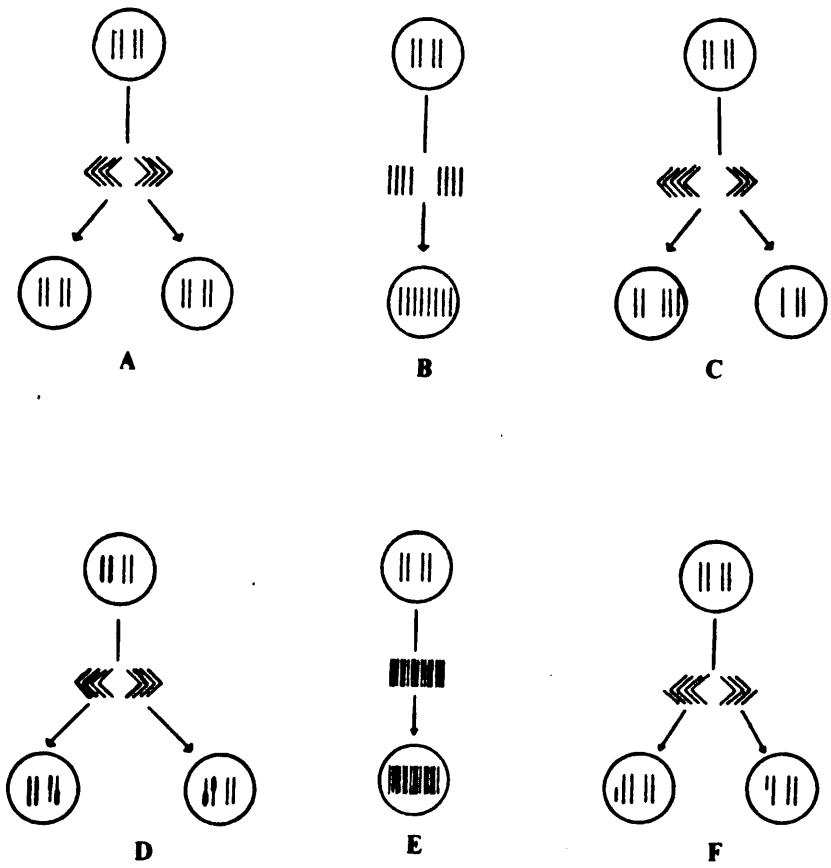


Figure B.8. Diagram illustrating the potential abnormalities in mitosis which may be induced by colchicine.

A. normal mitosis.

B. C-mitosis (total spindle failure, Levan, 1938) resulting in polyploidy.

C. Aberrant movement of chromosome in an otherwise normal bipolar mitosis. Leading to aneuploidy either (a) through loss of one or some chromosomes in the cytoplasm or (b) through inclusion of one or a few chromosomes in one of the daughter nuclei.

D. Chromosome breakage and translocation resulting in chromosome rearrangement.

E. Reiterated C-mitosis resulting in highly endopolyploid cell.

F. Chromosome breakage resulting in loss and gain of chromosome fragments.

| at anaphase indicates spindle failure

< at anaphase represents normal spindle function

1959). Hence, while inactivation of both controlling forces will result in a C-mitosis, inactivation of the extrinsic force alone will result in a disturbed C-mitosis where chromosomes are grouped in the cell. Subsequent cell membrane formation results in the production of a cell with several nuclei (micronuclei). This phenomenon has been observed in cell suspensions of sugarcane (Heinz and Mee, 1970), and *Daucus* (Bayliss, 1976) treated with colchicine. Both these authors also observed endoreduplication caused by prolonged treatment with colchicine which results in reiteration of C-mitosis i.e. replication of several C-mitotic cycles in the one cell (cf. D'Amato, 1959). Such phenomena lead to cell death or highly polyploid cells which fail to divide and differentiate or regenerate to form plants which do not survive to maturity (Heinz and Mee, 1970). Under the influence of low doses of colchicine (e.g. 100 mg/l) multipolar spindle formation (partial C-mitosis) has been observed in embryo derived callus of barley and leads to the production of aneuploid cells (Ruiz and Vazquez, 1982). Aneuploids may also arise by colchicine induced aberrant movement of one or a few chromosomes in an otherwise normal bipolar mitosis (D'Amato, 1959) but this phenomenon has not yet been recorded in tissue culture.

Colchicine has also been reported to induce chromosome breakages (Eigsti, 1940) and this phenomenon has been cited as one of the possible factors influencing the production of true breeding diploid mutants in *Sorghum* after colchicine treatment (Sanders and Franzke, 1962). A total of 67 percent of the plants produced after colchicine treatment (0.5% (w/v) in lanolin) had variant characters and 78 percent of these were found to be true breeding for complex mutations such as height and colour markings (Sanders and Franzke, 1962). Subsequent work on *Sorghum* established that;

(1) Genotype (Atkinson *et al.*, 1957) and environment (Franzke *et al.*, 1960) influence the production of complex mutants.

(2) Diploid, tetraploid and F1 diploid hybrids all give rise to complex true breeding mutants when treated with colchicine (Sanders and Franzke, 1962; Chen and Ross, 1963).

(3) There is no evidence of observable chromosomal aberrations in either the mutants of F1 hybrids or between the mutants and plants from various *Sorghum* lines including the parent (Franzke and Ross, 1952). However sporadic cytological

abnormalities have appeared in some progeny plants from non-true breeding mutants (Sanders and Franzke, 1962).

(4) Mutagenic changes may occur at many loci in different chromosomes within the one plant (Ross *et al.*, 1954).

On the basis of these observations Franzke and co-workers postulated that colchicine induced mutants arise through chromosome substitution followed by somatic reduction (Huskins, 1948) to the haploid number in some cells with subsequent redoubling (e.g. Sanders and Franzke, 1962). Substantial evidence to support this hypothesis was provided in cytogenetic studies by Simantel and Ross (1963) who used reciprocal translocations as cytological detectable chromosome markers to confirm the occurrence of chromosome substitution.

While these results have important implications for plant breeding if applicable to other genotypes this method has attracted little interest and has been tried only on a few other genotypes including flax (Dirks *et al.*, 1956), barley (Gilbert and Patterson, 1965) and sunflower (Downes and Marshall, 1983). At present, due to the emphasis placed on the production of polyploids from *in vitro* culture the production of other variants after colchicine treatment has not yet been reported.

B.3.6. Generation and expression of somatic variation: general conclusions

This review demonstrates the diversity of approaches which may be used to generate and express somatic variation *in vitro* from culture explants (see summary Figures B.9. and B.10.). Of the systems outlined here, callus culture is presently the most widely used in the production of variants. Callus culture procedures in many cases however, have not been adequately developed to fully exploit pre-existing (Section *B.3.2.*) or induced (Section *B.3.3.*) somatic variation and extensive research is required on selected genotypes to define the relative impact of media constituents and culture conditions on the production of somaclonal variation.

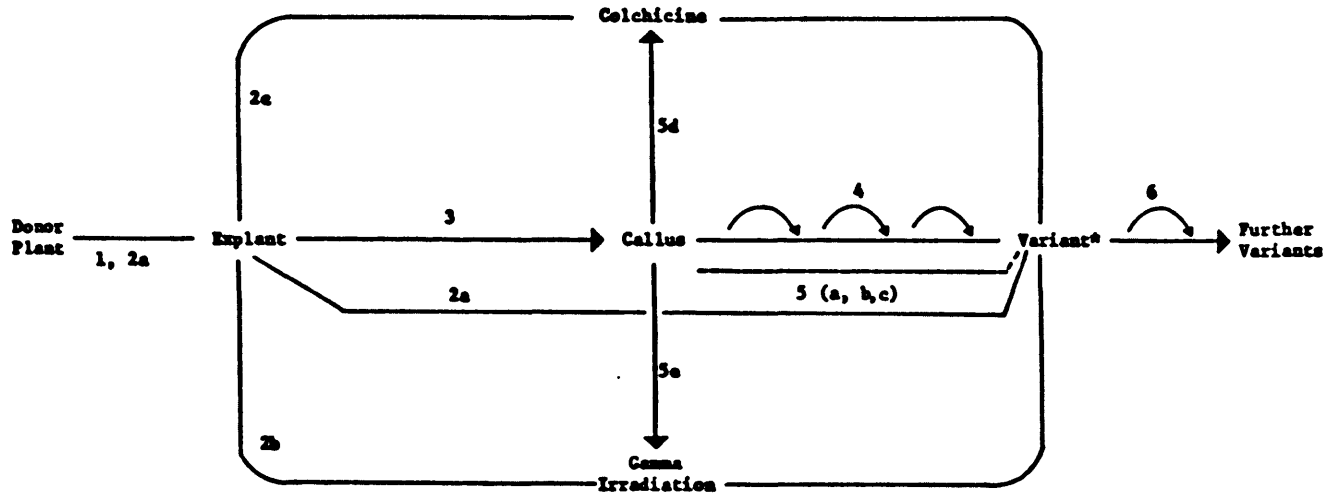
Colchicine treatment and mutagenesis as outlined have not been widely used in *in vitro* studies and research in this area should provide worthwhile results particularly with the use of explants derived from axenic plantlets (see Section *B.2.4.*).

The wide range of somatic variation potentially available in plant tissues (Section *B.3.2.*) would also suggest that direct

culture from explants on media with appropriate nutrient and hormone supplementation may allow the production of an array of somaclonal variants.

For tissue culturalists intent on clonal propagation of plants, results from the literature reviewed here indicate how important it is to know the genetic architecture of the plant prior to its introduction into culture. With a knowledge of plant genetic architecture and the relative stability of the genotype when exposed to stress, appropriate procedures which may ensure genetic stability *in vitro* can be applied. In some plants however it may not be possible to maintain trueness to type and achieve high multiplication rates, particularly in chimeras and unstable genotypes, hence in these plants a certain level of variation should be anticipated.

Figure B.9. Generation and expression of variation from explants and cultured cells *in vitro*



* development dependent on totipotency of variant genotype

1. Pre-existing variation, dependent on ontogeny and environmental conditions under which the plant is grown.
2. Generation of variation at the explant level by inducing mitotic abnormalities or genome reorganization of cells prior to organogenesis by (a) environmental stress *in vivo* or *in vitro*, (b) irradiation treatment, (c) colchicine treatment.
3. Callus cell heterogeneity dependent on explant and media supplementation.
4. Subculture of callus, nature of variants produced depends on subculture duration, hormones used and original genotype of callus.
5. Generation of further variation in callus cells *in situ* by (a) aging, (b) osmotic stress, (c) nutrient or hormone supplementation, (d) treatment with colchicine, (e) gamma irradiation.
6. Utilization of somatic variation potentially inherent in variants or putative normal plants by reculturing to produce further variants.

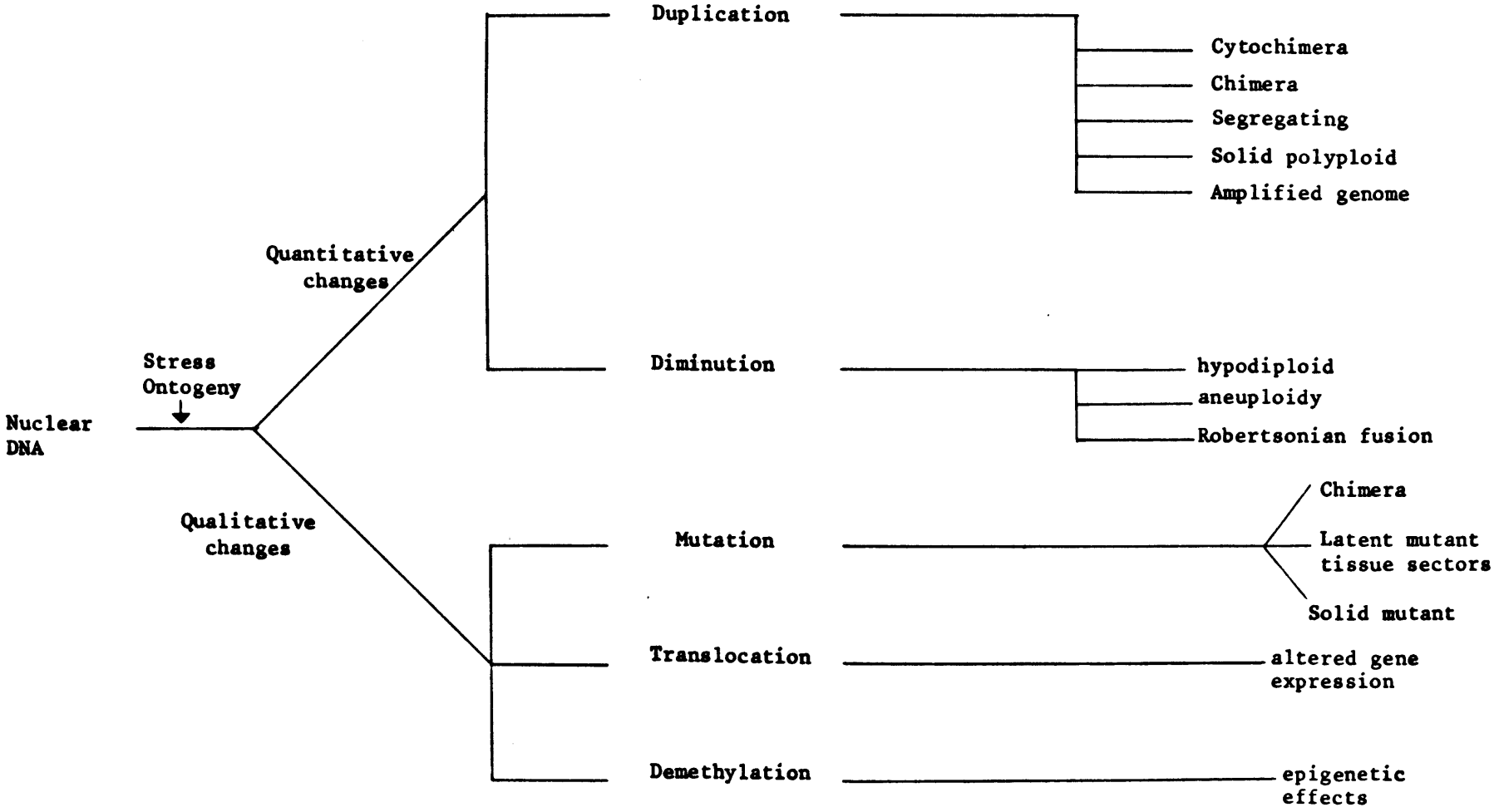


Figure B.10. Qualitative and quantitative changes which may be induced in plants as a result of stress or ontogeny which may contribute to the production of somaclonal variants in tissue culture.

SECTION C. GENERAL MATERIAL AND METHOD

C.1. Stock plants: source and growth conditions

The *Begonia rex* Putz. cultivar Lucille Closon and *Begonia* x *hiemalis* cultivar Violeta plants used in this study were purchased as adult plants from a commercial nursery (Leeside Nurseries, Killinardrish, Co. Cork). Stock plants of *Begonia bowerii* Ziesenhenne var. Nigramara Ziesenhenne were established from leaf petiole cuttings taken from a plant purchased in Nottingham Garden Centre, Nottingham, UK. Tubers of *Begonia* x *tuberhybrida* 'Crispa margenata' were purchased from Atkins Ltd., Popes Quay, Cork, Ireland. Other *Begonia rex* Putz. cultivars used in this study were grown from seed (Suttons Seeds Ltd., Torquay, UK) or established from leaf cuttings.

All plants were grown in a glasshouse where daylight was extended to 16 hours with supplementary lighting from high pressure sodium lamps (HPS/H 10 solarcolour 400 W, Simplex, Cambridge, UK) or 60 watt light bulbs (Solus, Ireland).

The minimum night temperature was 15°C. Plants were grown in soilless compost which consisted of Irish Peat Moss (Bord na Mona, Dublin, Ireland) and sand in the ratio peat:sand, 3:1 by volume, supplemented with Bio P Fertilizer (Pan Brittanica Industries, Herts., UK). All plants were treated during the growing period with Bio P Fertilizer at manufacturer's recommended rates. Plants were sprayed every ten days with Nimrod (active ingredient Triforin; Imperial Chemicals Industries PCL., Surrey, UK) to prevent establishment of powdery mildew.

C.2. Tissue culutre methods

C.2.1. Explant culture

Sterilization procedures for explants were modified to suit individual tissues as follows. Petioles were surface sterilized in 80 percent (v/v) aqueous ethanol for 30 seconds followed by 10 minutes in 10 percent (v/v) aqueous Domestos (active ingredients: 10.5% (w/v) sodium hypochlorite; Lever Bros., Liverpool, UK). Petioles were then rinsed twice in sterile distilled water. The ethanol treatment was omitted for leaves and these were treated directly in 5% (v/v) Domestos for 10 minutes and then rinsed three times in sterile distilled water.

After sterilization, explants of the required size were cut and placed on culture media. In preliminary experiments responses on a factorial combination of hormones (see Section D.) were examined. Standardized media for shoot and callus induction once established (Table C.1.) were routinely used.

All hormones were added prior to autoclaving at 105 kPa, 120°C for 15 minutes. Culture media was solidified with agar at a concentration of 6 g/l (No. A-7002, Sigma, Dorset, UK) or 5.8 g/l for leaf explants.

Explants were cultured in 60 ml screwtop glass jars (Richardson's Leicester, UK) containing 15 mls of culture medium. Culture vessels were placed in a growthroom (25°C, 5Wm^{-2}, 16 hour photoperiod) with light provided by 'white' 65/80 Liteguard fluorescent tubes (Osram Ltd., UK).

C.2.2. Axenic plantlets: culture procedures and storage

Axenic leaf, root and petiole explants used in the course of this work were taken from axenic plantlets of approximately 2 cm in height. Leaves of approximately 1 cm in length were wounded at random with a scalpel before explanting lower surface downward, onto culture media. Roots and petioles were cut into 1 cm segments. Leaves, roots and petioles were cultured on appropriate media in 60 ml culture jars under standard growthroom conditions (see Section *C.2.1.*).

Axenic shoots which were not immediately required for culture were transferred in their original culture vessels to a cold cabinet (Eurofrigo, STA. Via Casteff, Romani, Italy) at 10°C, 24 hour photoperiod 2 Wm^{-2} with light provided by daylight 58 W fluorescent tubes (Atlas Ltd., London, UK).

C.2.3. Development and rooting of adventitious shoots

After 4-5 weeks on SIM or APM media (Table C.1.) explants with buds were subdivided and transferred to shoot development media (SDM, Table C.1.).

C.2.4. Establishment of plantlets

Plantlets greater than or equal to 2 cm in height were planted 2 cm apart in propagators at 20°C containing soilless compost (see Section *C.1.*). Propagators were placed in shaded locations in the greenhouse to prevent scorching of plantlets. After 8 to 10 weeks

propagator lids were removed for 5 days to acclimatize plantlets to lower humidity prior to transfer to 7.5 cm diameter pots.

C.3. Callus culture

C.3.1. Induction and proliferation of callus

Initial callus induction and proliferation studies were conducted on both mature and axenic leaf and petiole explants on a wide range of media. The established callus medium (CM, Table C.1.) for axenic leaves was routinely used for the production of callus. Leaves of approximately 1 cm in length were removed from axenic plantlets and cultured on CM (Table C.1.) in 60 ml screwtop jars. After 4-6 weeks of culture under standard growthroom conditions, callus was removed from the explant and subcultured onto fresh CM medium (Table C.1.) in petri dishes (8.5 cm diameter) (Sterilin, Middlesex, UK) and cultured in the dark at 22°C. Where necessary callus was subcultured onto fresh CM medium (Table C.1.) at 4-5 week intervals.

C.3.2. Regeneration of plants

Clumps of approximately 25 mg of callus were transferred to S1M or SM3 (Table C.1.) in 60 ml screwtop jars and cultured under controlled growthroom conditions. After 12 weeks regenerated shoots were transferred to SDM (Table C.1.) for a further 4 weeks of culture before being planted out and grown on as outlined in Section *C.2.3.*

C.4. In vitro gamma irradiation

C.4.1. Preparation of material

One centimeter leaves were excised from axenic plantlets of *B. rex* and wounded at random before transfer to petri dishes (8.5 cm diameter) containing a thin layer (10 mls) of APM (Table C.1.). A total of 27 plates were set up with 10 leaves per plate.

C.4.2. Radiation treatment

Using a Teletherapy machine (Toshiba, Japan) with a Co^{60} source, nine petri dishes containing axenic leaf explants of *B. rex* were aligned in a field size 60 x 60 cm and irradiated at 2,000 rads. The distance from source to material was 140 cm. Using a Farmer Substandard dose rate meter (Baldwin Instruments, Dartford, Kent, UK) ninety five percent uniformity of dose was confirmed in the field in which the dishes were aligned.

C.4.3. Culture of irradiated explants

Leaves were left overnight in petri dishes under standard growth-room conditions and the next day transferred onto fresh APM (Table C.1.) in 60 ml culture jars and cultured under standard growthroom conditions. After 5-6 weeks of culture responding explants were transferred to SDM (Table C.1.) for plantlet development. When fully developed plantlets greater than 2 cm were transferred to peat compost in heated propagators. A control population of plantlets derived from unirradiated axenic explants was also planted out. All plantlets were transferred to 7.5 cm pots after 2-3 months. Plantlets were evaluated after 5 months of growth and obvious variants and controls were potted on into 12 cm pots where they were grown to maturity.

C.5. Cytology

*C.5.1. Fixing hydrolysis and staining**

For cytological analysis tissues were fixed without pretreatment in Carnoy's solution (ethanol acetic acid 3:1 v:v) for 4 hours on a rotary shaker (MKV, Orbital Shaker, Lh Engineering, UK) at 180 rpm. The tissue was then macerated in 2:1 (acetic acid:INHCL) for 10-15 minutes, rinsed once in 95% ethanol and twice in chilled distilled water before storing in distilled water overnight at 4°C. The next day specimens were prepared by the standard squash method and the coverslip removed by freezing (Conger and Fairchild, 1953). Slides were dried on a warming plate at 50°C and could be stored for staining as required.

In preparation for staining, slides were soaked in Sorenson's phosphate buffer (Gurr buffer tablets, pH 6.8 BDH Ltd., Poole, UK) for 30 minutes, hydrolysed in INHCL for 6-8 minutes and rinsed for 1 hour in running water. Slides were then stained in 2% (v/v) Giemsa stain (2 mls Giemsa stain (see Sharma and Sharma, 1981) in 100 ml Sorenson's phosphate buffer pH 6.8) for 5-8 minutes. After staining slides were rinsed in buffer, air dried and mounted in DPX mountant (BDH Ltd., Poole, UK). For permanent preparations, slides after rinsing were dehydrated through a four step ethanol series of 50%, 70% 95%, absolute (5 min. for each step) before mounting in DPX.

*J.C. Arends, personal communication

C.5.2. Chromosome analysis

All observations were made with 1,000 x Nikon planopoachromate phase contrast or bright field optics using an oil immersion objective and dry condensor (Nippon, Kogaki, KK, Japan).

Counts of chromosomes were determined from Giemsa stained heterochromatin blocks according to the method used by Banks and Evans (1976). In counting chromosomes only those cells which met the following criteria were recorded; (1) an intact cell membrane, (2) the cells were not superimposed and (3) individual chromosomes were clearly visible.

C.6. Light microscopy

C.6.1. Hand sectioning

This procedure was used in evaluating changes in starch and pigment distribution and origin of meristematic centers in callus and pith during plantlet regeneration. Sections were cut with a sharp double sided razor blade, mounted in distilled water or iodine solution (see Section *C.6.2.*) and viewed under 100X or 400X magnification.

C.6.2. Estimation of chloroplast number in guard cells

This procedure was used on the basis that chloroplast number in guard cells has been positively correlated with increase in chromosome number in *Petunia* (Santos and Handro, 1983).

Using a cork borer discs of 1 cm in diameter were taken from mature leaves at 12.00 pm and fixed in Carnoy's solution (ethanol 3:acetic acid; v:v) on a rotary shaker (180 rpm) overnight. Discs were rinsed in 75% ethanol and twice in distilled water before staining with iodine solution (1 g iodine, 2 g potassium iodide in 100 ml distilled water) for 5 minutes. Discs were then rinsed and mounted in distilled water.

Chloroplasts were counted in a random selection of 25 guard cells. Observations were made at magnification 400X.

C.6.3. Staining with Fluorescein diacetate

Unfixed razor cut sections of callus were stained in the dark for 5 minutes on a slide with 0.01% (v/v) Fluorescein diacetate (Fluorescein diacetate (No. F-7250; Sigma, Dorset, UK) dissolved in acetone at a concentration of 5 mg/ml, diluted to a final concentration of 0.01%

by the addition of 0.05 m phosphate buffer at pH 5.8). Stained sections were viewed by fluorescence microscopy using an ultra violet light for excitation.

C.7. Evaluation of leaf colour variant

Colours observed on variant phenotype leaves were described using the Methuen Handbook of Colour (Kornerup and Wanscher, 1967).

Colours have been given here under two headings, their colour names and a Munsell reference. A Munsell reference has three terms: *Hue* - position across the spectrum; *Value* - position on a lightness darkness scale: and *Chroma* - position on a neutral - colourfulness scale.

Hue: The symbols for Hue are numbers placed in front of the initials R, RP, P, PB, B, BG, G which stand for red; purple; blue and green with their combinations. Each of the hues is indicated with intervals based on a unit of 0.5.

Value: The value symbol is the second number provided. The Munsell value is based on a gradation of 0.1. Value is the Munsell term for tone.

Chroma: Figures for Chroma are placed after value symbols i.e. third term provided and go from 0 for neutrals, these are greys, whites and blacks - to 16 or more for strong lines. Chroma is graded in steps as fine as 0.1. Chroma is the Munsell term for intensity.

By estimating Hue, Value and Chroma colours produced on variants were given a colour rating which could be used for comparison and future reference.

Notation: Colour name (Hue, Value, Chroma).

Table C.1. Components of culture media used in the present study.

Components	Culture Media Codes							
	SIM	SIM2	SIM3	APM	SDM	EM	CM	CM2
Murashige and Skoog (1962) basal nutrients medium without sucrose, IAA or kinetin (Flow Laboratory Ltd., Ayrshire, Scotland) g/l	4.7	4.7	4.7	4.7	4.7	2.3	4.7	4.7
2,4-D mg/l	-	-	-	-	-	-	0.75	1
NAA mg/l	0.01	0.1	0.01	1	-	1	-	-
BAP mg/l	0.1	0.01	1	0.01	-	1	0.001	-
Kinetin mg/l	-	-	1	-	-	-	-	1
Sucrose g/l	30	30	30	30	30	30	30	30
Agar g/l	6	6	6	5.8	5.8	5.8	5.8	6
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

SECTION D. EXPERIMENTAL WORK I

D.1. Micropropagation of Begonia

D.1.1. Introduction

More recent developments in tissue culture research indicate that tissue culture may not only provide a method for the micropropagation of plants but may also prove a useful means of both mother stock storage (Section *B.2.3.*) and the production and multiplication of new varieties (Section *B.3.*).

At present the absence of effective micropropagation procedures for most *Begonia* means that such developments cannot be used for improving the propagation and breeding of new varieties of *Begonia*. For this reason the present study has been undertaken to assess the development and comprehensive assessment of continuous and discontinuous micropropagation procedures (see Section G.) for the production of large numbers of vigourous true to type plants from *B. rex*. This plant is known for its natural propensity to produce plantlets and would appear not to require tissue culture for propagation and this fact is not denied. It is also true however that regeneration in *B. rex* is subject to seasonal variation (Bigot, 1971) and stock plants are susceptible to disease. Hence tissue culture provides a means of year round propagation and storage and could be cost effective for crop production. Furthermore, in the case of sterile cultivars, such as the one studied here, tissue culture may provide a useful means of both producing and propagating new genotypes (Section *B.3.*).

To establish procedures for the discontinuous and continuous micropropagation of *B. rex* studies were undertaken on the optimization of media for plantlet regeneration from both mature and axenic explants. Studies on optimization of explant source were also undertaken in view of the influence of the epigenotype on responses *in vitro* (Section *B.2.2.6.*). In conjunction with these experiments a number of other commercially important *Begonia* species hybrids were assessed for their regenerative potential *in vitro* and media for the production of plantlets from mature and axenic explants were established.

D.2. Responses of explants from a number of Begonia species hybrids to exogenous auxin and cytokinin

D.2.1. Introduction

The tissue culture of a number of *Begonia* has been achieved using various combinations of media and hormones and a selection of different explants and there are reports of differences in morphogenic capacity

between species (Ringe and Nitsch, 1968; Bigot, 1971) and cultivars (Welander, 1977). The recalcitrance or low morphogenic capacity demonstrated by some begonias in these studies may be a result of the use of an inappropriate explant source and/or exogenous hormones and/or suboptimal growth conditions either *in vivo* or *in vitro* which would reduce or inhibit morphogenesis (Section *B.2.2.*).

In the present study the morphogenic responses of *Begonia* species hybrids *B. rex* Putz., *B.* x *tuberhybrida*, *B.* x *hiemalis* and *B. bowerii* were assessed on a basal Murashige and Skoog (1962) nutrient medium supplemented with a factorial combination of the hormones NAA and BAP. The prime objective of this study was to establish media for the production of plantlets from mature and immature axenic tissues of *B. rex* (Plate D.1.). This study also allowed a comparison of the morphogenic responses of the *Begonia* species hybrids studied on a wide range of hormonal combinations. The influence of explant source on regenerative capacity was also studied, where possible, by an assessment of organogenesis from mature leaves and petioles and from axenic leaves derived from plantlets produced in culture. These latter organs having been induced and grown *in vitro* under controlled environmental conditions should have similar physiological and ontological states and hence the variation in morphogenic response which is caused by these factors (Section *B.2.2.*) is greatly reduced. The range of media and explants tested also allowed the establishment of media for plantlet regeneration for each of the begonias tested.

D.2.2. Material and method

D.2.2.1. Begonia rex Putz. cv. Lucille Closon

Leaves of approximately 9 cm in length and their petioles were removed from stock plants. After the appropriate sterilization and preparation of explants (Section *C.2.1.*) these tissues were cultured on media containing Murashige and Skoog (1962) nutrients, 3% (w/v) sucrose and 0.6% (w/v) agar with the concentration range 0, 0.01, 0.1, 1.0, 10 mg/l of the hormones NAA and BAP in a 5 x 5 factorial with pH adjusted to 5.8.

A total of 10 leaf and petiole explants per treatment were cultured under standard growthroom conditions. Explant responses were evaluated after 3 months of culture.



Plate D.1. *Begonia rex* 'Lucille Closon'

D.2.2.2. Begonia x hiemalis

Three centimetre leaves and their petioles excised from stock plants were used as explants. For this plant half strength Murashige and Skoog (1962) nutrients were used, as explants failed to respond on full strength MS. Also, leaf explants were cut into 1 cm diameter discs using a cork borer instead of into squares as in *B. rex*. With the exception of these modifications, sterilization and culture procedures were similar to those outlined in Section *D.2.2.1.*).

D.2.2.3. Begonia x tuberhybrida 'Crispa Margenata'

For this plant leaves of approximately 9 cm and their petioles were used as an explant source and culture procedures were the same as those outlined in Section *D.2.2.2.*

D.2.2.4. Begonia bowerii

Leaves of 6 cm in length and their petioles were removed from stock plants. Sterilization and culture procedures were the same as those outlined in Section *D.2.2.1.*

D.2.2.5. In vitro derived explants

For each of the begonias tested, leaves of approximately 1 cm in length were removed from axenic plantlets under aseptic conditions. These were wounded at random using a scalpel and placed with their lower surface downwards on the factorial combination of media outlined in Section *D.2.2.1.* Ten explants per treatment were cultured in 60 ml culture jars under standard growthroom conditions. Explant response for all cultures were evaluated after 3 months of culture. In *B. rex* both leaf and petiole explants were evaluated.

D.2.2.6. Development of plantlets

Bud producing cultures were subdivided and transferred to a shoot development medium (SDM Table C.1.). The development of plantlets was assessed after 2 months of culture under controlled growthroom conditions.

D.2.3. Results

Of the named begonias cultured *B.* x *tuberhybrida* proved to be the least responsive and mature leaf and petiole explants failed to undergo morphogenesis on all but two of the hormone combinations assessed (Table D.1.). Responses were observed on NAA at 10 mg/l and a combination of NAA and BAP of 1 mg/l. On the latter medium explants produced a large number of buds near the wound edge and scattered at random over the surface. These buds developed into plantlets once

Table D.1. Factorial response of *in vivo* derived petiole explants of four *Begonia* species hybrids.

NAA mg/l	BAP mg/l 0	0.01	0.1	1	10	
0	*p*	*p*	S	b	-	*B. rex*
	-	-	-	-	-	*B.* x *hiemalis*
	-	b	S	B	-	*B. bowerii*
	-	-	-	-	-	*B.* x *tuberhybrida*
0.01	*p*	*p*	B	B	-	*B. rex*
	-	-	-	*b*	-	*B.* x *hiemalis*
	R	S	P	B	-	*B. bowerii*
	-	-	-	-	-	*B.* x *tuberhybrida*
0.1	P	P*	s	B	-	*B. rex*
	-	-	*p*	-	-	*B.* x *hiemalis*
	R	S	P*	b	-	*B. bowerii*
	-	-	-	-	-	*B.* x *tuberhybrida*
1	*p*	R	-	-	-	*B. rex*
	-	-	p*	B	-	*B.* x *hiemalis*
	R	R	R	R	-	*B. bowerii*
	-	-	-	B*	-	*B.* x *tuberhybrida*
10	c	-	-	-	-	*B. rex*
	-	-	-	-	-	*B.* x *hiemalis*
	-	-	-	-	-	*B. bowerii*
	c	-	-	-	-	*B.* x *tuberhybrida*

*optimum medium for the production of large numbers of plantlets

Intensity of morphogenic response is represented by the typescript e.g. P = intensive total explant coverage, p = medium scattered over explant and *p* = sparse localized production. Morphogenic responses are represented by the letters p for plantlets, s for shoots, b for buds, r for roots and c for callus.

transferred to SDM. On 10 mg/l NAA a small amount of friable callus was produced at the wound edges of the explant. The response of axenic explants of this plant could not be assessed due to the presence of latent bacterial contamination.

Mature tissues from the other begonias studies proved more responsive (Table D.1.) and these begonias demonstrated distinct differences in their morphogenic responses on varying levels of exogenous hormones. *B. rex* responded both in the absence and presence of one or both hormones, while *B. bowerii* petiole explants demonstrated an absolute requirement for at least one exogenously supplied hormone for morphogenesis. In *B.* x *hiemalis*, petiole explants only underwent morphogenesis in the presence of relatively high levels of BAP (0.1, 1 mg/l) in combination with 0.01 and 0.1 mg/l NAA. However leaf explants responded in the absence of BAP at high levels of NAA (1 and 10 mg/l). For leaf explants, increases in BAP concentrations within the NAA 1 mg/l range also increased the number of buds induced and optimal plantlet induction was observed on a combination of BAP at 0.1 mg/l and NAA at 1 mg/l. Both leaf and petiole explants produced large numbers of buds on NAA and BAP at 1 mg/l and these readily developed into shoots when transferred to SDM.

B. bowerii responded to a wide range of media (Table D.1.) and optimal plantlet induction was observed on a combination of 0.1 mg/l NAA and BAP. Attempts to culture mature leaf explants of this hybrid were unsuccessful due to the presence of latent fungal infection.

In *B. rex*, mature leaf and petiole explants both gave similar morphogenic responses. Plantlets were produced on high levels of NAA (0.1 and 1 mg/l) both in the presence and absence of 0.01 mg/l BAP. This response was in contrast to that obtained for the other begonias studied where rooting predominated on this medium (Table D.1.). In *B. rex* however, rooting was followed by the production of plantlets. On increasing concentrations of BAP, leaf and petiole explants of *B. rex* showed a reduced polarity in organogenesis and bud induction was intensified with a concomitant limitation in development. Buds produced on a combination of NAA 0.01 mg/l and BAP 0.1 mg/l readily developed into plantlets when transferred to SDM.

Where studied, morphogenesis on axenic tissues was rapid and plantlets were fully developed in 2 months as compared with 3 months for mature tissues.

Table D.2. Factorial response of *B. rex* immature (axenic) and mature leaves.

NAA mg/l	BAP mg/l	0	0.01	0.1	1	10	
	0	-	-	-	B	-	Mature
		p	p	P	B	-	Axenic
	0.01	-	*p*	*p*	B	-	Mature
		p	*p*	P	B	B	Axenic
	0.1	P	*p*	*p*	B	-	Mature
		P	P	P	B	B	Axenic
	1	P	*p*	*p*	-	-	Mature
		P	P	P	B	*b*	Axenic
	10	-	C	-	-	-	Mature
		C	*c*	-	-	-	Axenic

Intensity of morphogenic response is represented by the typescript e.g. P = intensive total explant coverage, p = medium scattered over explant and *p* = sparse localized production. Morphogenic responses are represented by the letters p for plantlets, s for shoots, b for buds, r for roots and c for callus.

Table D.3. Factorial response of *B. rex* axenic leaf and petiole explants.

		BAP mg/1					
		0	0.01	0.1	1	10	
NAA mg/1	0	*p*	p	P	B	-	Leaf
		b	b	*p*	B	B	Petiole
	0.01	*p*	*p*	P	B	B	Leaf
		p	*p*	*p*	B	B	Petiole
	0.1	P	P	P	B	B	Leaf
		p	*p*	*p*	B	B	Petiole
	1	P	P	P	B	*b*	Leaf
		p	*p*	R	*b*	*b*	Petiole
	10	C	*c*	-	-	-	Leaf
		c	*c*	-	-	-	Petiole

Intensity of morphogenic response is represented by the typescript e.g. P = intensive total explant coverage, p = medium scattered over explant and *p* = sparse localized production. Morphogenic responses are represented by the letters p for plantlets, s for shoots, b for buds, r for roots and c for callus.

Table D.4. Factorial response of axenic leaf explants of three *Begonia* species hybrids.

NAA mg/l	BAP mg/l	0	0.01	0.1	1	10	
		p	p	P	B	-	*B. rex*
	0	*p*	*p*	p	b	B	*B.* x *hiemalis*
		-	-	-	P	-	*B. bowerii*
		p	*p*	P	B	B	*B. rex*
	0.01	R	R	s	b	B	*B.* x *hiemalis*
		r	P	P	b	-	*B. bowerii*
		P	P	p	B	*b*	*B. rex*
	0.1	R	R	s	s	B	*B.* x *hiemalis*
		R	R	P	P	B	*B. bowerii*
		P	P	p	B	*b*	*B. rex*
	1	P	p	p	S	B	*B.* x *hiemalis*
		b	b	*p*	B	B	*B. bowerii*
		C	*c*	-	-	-	*B. rex*
	10	c	C	c	S	S	*B.* x *hiemalis*
		c	c	C	c	-	*B. bowerii*

Intensity of morphogenic response is represented by the typescript e.g. P = intensive total explant coverage, p = medium scattered over explant and *p* = sparse localized production. Morphogenic responses are represented by the letters p for plantlets, s for shoots, b for buds, r for roots and c for callus.

In *B. rex*, axenic leaf explants responded on a wider range of media (Table D.2.) compared to mature leaf explants. On media where both explants showed competence the intensity of response of axenic explants appeared greater but the nature of morphogenic response did not change. Leaf explants when compared with petiole explants were found to give a more intensive response (see Table D.3.).

An increase in the number of hormone combinations on which axenic explants responded also occurred in the other 2 hybrids tested but like *B. rex* there were no major differences in the nature of morphogenic responses (Table D.4.).

Unlike mature tissues, axenic explants responded more frequently on high levels of NAA and callus was produced. This callus however was not prolific and rapidly organized to produce roots.

D.2.4. Discussion

These studies demonstrate the differences which exist between different *Begonia* hybrids in their responses to exogenous hormones and also illustrate how explant age and source can influence a tissue's response *in vitro*. The necessity to test a wide range of media to develop a medium which can be used in the micropropagation of a given plant is also evident.

The more pronounced differences in responses of mature explants and the narrower range of media responses, particularly in *B.* x *tuberhybrida*, may be the result of epigenotype influences. Flowering has been found to influence explant response in a number of plants e.g. *Lilium* (Nimi and Ohozawa, 1979) and the fact that *B.* x *hiemalis* and *B.* x *tuberhybrida* were both flowering when this experiment was performed may have influenced the results of the present study. This suggestion is substantiated by the increased morphogenic capacity of axenic leaf explants of *B.* x *hiemalis*. Both of these plants also have cauline habits as compared to the acauline habits of *B. rex* and *B. bowerii* and hence changes in endogenous hormone distribution throughout these plants may be more pronounced. Similar differences have been reported in morphogenic responses in cauline and acauline Gesneriads (Plunkett, 1984).

The bud inducing capacity of *B. rex* and *B. bowerii* on a wide range of media may be related to an inherent high propensity for plantlet formation. In both these plants the organs cultured are used in conventional propagation while in *B.* x *tuberhybrida* leaves and petioles are not normally used for propagation.

The differences observed here between the responses of axenic and mature tissues illustrates clearly the greater morphogenic capacity present in axenic explants. This increased responsiveness may in part be due to the aseptic nature of the tissue and growth under controlled environmental conditions, features which eliminate potential loss due to sterilization and reduce the influence of the epigenotype on explant response.

In *B. rex* a number of media were evident which could be used to produce plantlets. The number produced and the procedures involved vary but the overall applications of these procedures cannot be assessed until mature plants have been produced and evaluated. While one medium may produce large numbers of plantlets, plantlet survival and growth habit may not be as favourable as that from procedures where small numbers of plantlets are produced.

D.3. Optimization of explant source for the micropropagation of B. rex

D.3.1. Introduction

In considering a routine source of explant material for micropropagation it is essential that variability between explants is not excessive and that a high percentage of explants respond in culture. As the epigenotype plays a major role in the response of explants *in vitro* (Section *B.2.2.5.*) it is important to establish a source of explant which will ensure reproducible results at all times of the year.

On factorial media both petiole and leaf explants of *B. rex* demonstrated a high morphogenic capacity. However the number of responding explants on a given medium differed. Petioles were 100 percent responsive while leaves were between 30 to 50 percent responsive when evaluated. For this reason mature petioles were used in preference to leaves in the following studies. The position of explantation and age may influence explant response *in vitro* (Section *B.2.2.5.*) and the influence of these parameters on the responses of mature petiole explants of *B. rex* were examined in the studies which follow.

Studies of responses of axenic leaf and petiole explants of *B. rex* (Figure D.4.) indicate that plantlet production is more vigourous on leaf explants. This is possibly due to the smaller size of petiole explants. Hence leaf explants were used in micropropagation studies.

As explant size has been cited on many occasions as an influencing factor in morphogenic responses (Section *B.2.2.5.*) the response of axenic leaves of different sizes was assessed to determine an optimum size for use in micropropagation.

D.3.2. Effect of petiole ontogeny on responses in B. rex in vitro

D.3.2.1. Material and method

Petioles from leaves of 5, 9 and 11 cm were removed from stock plants, sterilized and prepared for culture as outlined in Section *C.2.1.* A total of 20 petiole explants were cultured on SIM2 (Table C.1.) under standard growthroom conditions for 3 months prior to evaluation.

D.3.2.2. Results

As indicated in Table D.5. petioles showed no significant difference in the number of shoots produced for any of the leaf sizes assessed. All shoots produced were scattered at random over the explant and there was no evident polarity in morphogenesis. Shoots from each treatment were also of similar vigour and morphology. Due to the high mean number of shoots produced from explants of leaves of 9 cm in length this leaf size was used as a source of petiole explants in future experiments.

D.3.3. Explant zonation influence on morphogenesis in B. rex petiole explants

D.3.3.1. Material and method

Fifteen petioles were removed from leaves 9 cm in length and divided into 3 zones; upper (near the leaf), middle and basal (near the rhizome). Each zone was separately sterilized (Section *C.2.1.*) and explanted as 1 cm petiole halves.

D.3.3.2. Results

The percentage response of explants and the number of shoots formed per explant gradually decreased from the basal to the upper zone of the petiole (Table D.6.) with a significant difference between shoot numbers induced at basal and upper zones. This difference may be attributable to both explant size and position. Petiole diameter decreased in the last 3 cms before the leaf and hence explants from this zone (upper) were smaller in total dimension than those from the basal zone. Plants produced from the upper zone were also less

Table D.5. Effect of petiole ontogeny on petiole explant response of *B. rex* in vitro.

Leaf size cm	Explant[a] response	Number of shoots produced (mean±SE)	Intensity of growth	Level of significant difference
5	100	22±4.5	++	NS*
9	100	34±3	++	NS
11	100	26±2	++	NS

* Non significant ($P > 0.05$) according to student t test

a Result from 20 replicas

++ Plantlets produced over total surface and well developed

Table D.6. Effect of position of petiole explantation on petiole explant response *in vitro*.

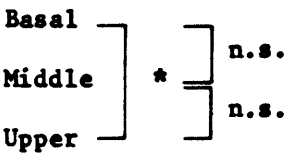
Zone	Explant Response %	Number of shoots produced (mean±SE)	Intensity of shoot production
Upper	82	13±3	+
Middle	86	16±2.5	++
Basal	100	22±2	++

Analysis of variance table

Item	55	N	Variance	VR	P
Total	8042	80			
Zone	864	2	432	4.69	0.05
Error	7178	78	92		

LSD (p = 0.05) = 8

Using LSDs the following differences were found:



* P = 0.05
n.s. = non significant

++ - plantlets produced over total surface and well developed.

\+ - plantlets scattered over the surface and not well developed

Table D.7. Axenic leaf size influence on regeneration *in vitro* of *B. rex*.

Leaf length (mm)	Responding explants* %	Explants producing shoots %
5	70	50
10	100	100
20	50	100

* From a total of 20 replicas

vigourous and developed more slowly than those from other zones. For this reason only the basal 5 cm of petioles was used in subsequent experiments.

D.3.4. Axenic leaf size influence on regeneration.

D.3.4.1. Material and method

Twenty leaves of approximately 0.5, 1, and 2 cm were removed aseptically from axenic plantlets in culture and wounded at random before being transferred to APM (Table C.1.). Explant response was assessed after 2 months of culture under standard growthroom conditions.

D.3.4.2. Results

Of the three leaf sizes tested those of 10 mm in length gave the highest percentage response and shoots developed more rapidly than those produced from explants of 20 and 5 mm in length. (see TABLE 3▼) Only 50 percent of explants from 5 mm leaves produced shoots the remaining 30 percent underwent extensive rooting. Leaves of 20 mm tended to senesce and only 50 percent of these explants produced shoots. On the basis of these results explants of 10 mm in length were used in subsequent experiments using axenic leaves.

D.3.5. Discussion

The results presented here demonstrate the need to optimize explant source to reduce variability between explants and ensure a high percentage of responding explants.

In *B. rex* leaf age did not appear to influence the number of plantlets produced from petiole explants. This is in contrast to studies on other plants where explant age has been shown to play a significant role in the number of shoots induced e.g. *Nicotiana tabacum* (Long, 1982). In plants such as *Nicotiana* differences in morphogenic competence may be attributed to variance in endogenous hormone levels, reduced cell competence due to senescence or distance from the growing point, (see Letham *et al.*, 1978). In *B. rex* the lack of a true stem and hence the absence of major spatial differences between leaves may explain the absence of significant differences in morphogenic responses between leaves of different ages. Similar responses have been noted in *Saintpaulia* (Plunkett, 1984).

Petioles showed a distinct gradient in the production and growth of adventitious shoots relative to their position of explantation.

This gradient in response along an organ has also been demonstrated in other begonias in culture (e.g. Khoder *et al.*, 1981) and is believed to be the result of endogenous hormone gradients.

The differences in responses observed between axenic leaves of different sizes would suggest different levels of endogenous hormones within these leaves. The intensive rooting which is induced by the medium used, may have depleted endogenous resources in small explants while cells in larger explants retained the competence to produce shoots once roots had been formed. The cause of senescence in leaves of 20 mm is not clear. It is possible that these larger leaves may normally senesce in culture due to their inability to undergo further growth in the confines of the culture vessel.

D.4. Comparative analysis of B. rex plants propagated from leaf cuttings and from tissue cultures of mature petioles and axenic leaves

D.4.1. Introduction

Where reported, the evaluation of plants produced from tissue culture of *Begonia* has been limited to a subjective analysis of trueness-to-type based on maintenance of flower colour and leaf shape (Section *B.2.1.6.*). No comprehensive study has yet been undertaken on the assessment of the general morphological characters and the level of interpopulation variation present in tissue culture populations as compared with control populations of vegetatively propagated plants. These studies are important if tissue culture is to be used as a means of plant propagation.

In the present study the influence of tissue culture propagation on plant habit, vigour and interplant variation in *B. rex* has been evaluated using a quantitative assessment of 10 plant characters from control and tissue culture populations, the latter being derived from axenic leaves and mature petioles.

Somatic variation *in vitro* has in some cases been ascribed to the concentration of hormones used or explant source (Section *B.3.4.*). In *B. rex* variation in chromosome number has been reported in somatic cells (e.g. Sharma and Bhattacharyya, 1957) and some cultivars are known to produce a wide range of variants from leaf cuttings (Bigot, 1971). Hence explant source or the concentration of hormones used may significantly influence the stability of the plants produced. In

the present study the different explant sources and the use of media which contain high and low concentrations of cytokinin should allow an assessment of the influence of these factors on the production of true to type plants from tissue culture of *B. rex* 'Lucille Closon'.

D.4.2. Material and method

D.4.2.1. Leaf cuttings

Leaves approximately 9 cm in length were removed from mature stock plants. Three discs of 2 cm in diameter and including a portion of the main vein were cut, using a cork borer, from the base of 36 leaves. After immersion in 10 percent aqueous captan solution (v/v) discs were placed 1 to 2 cm apart in heated propagators (20°C) containing peat:sand (1:1).

After 6 to 8 weeks shoots appeared at the soil surface. After 19 weeks single adventitious plantlets were removed and transferred to uncovered seed trays containing peat compost (Section *C.2.3.*). After a further 8 weeks of growth plants were transferred to 7.5 cm diameter pots. Finally, after a total of 39 weeks plants were transferred to 12 cm diameter pots containing peat compost (Section *C.2.3.*) where they remained for a further 13 weeks before evaluation.

D.4.2.2. Petiole explants

The basal 5 cm of petioles taken from 9 cm leaves were surface sterilized and prepared for culture as outlined in Section *C.2.1.* before transfer onto a medium which induced large numbers of buds (SIM, Table C.1.) and a medium which induced small numbers of well developed plantlets (SIM2, Table C.1.). A total of 20 segments per treatment were set up. After 4 weeks of culture in standard growth-room conditions petioles on SIM were cut into 4 and transferred to SDM (Table C.1.) for plantlet development. After 8 to 10 weeks, plantlets on SIM2 media were fully developed and cultures were transferred to cold storage at (10°C) to await the development of plantlets on SDM. After a total of 12 weeks in culture plantlet number per explant was recorded for the responding explants on each of the media tested and a total of 50 plantlets greater than or equal to 2 cm in height were taken from each media source and planted in peat compost in heated propagators at 20°C. After 2 months plantlets were transferred to 7.5 cm pots. Further transfers and growth period prior to evaluation were as outlined for cuttings.

D.4.2.3. Axenic leaf explants

Twenty, 1 cm leaves were aseptically removed from axenic plantlets. These were wounded at random and placed with their lower epidermis in contact with APM (Table C.1.). After 1 month of culture under controlled growthroom conditions responding explants were transferred to SDM (Table C.1.) for further plantlet development. Plantlets were fully developed after a total of 10 to 12 weeks of culture and some were stored at 10°C for 2 weeks before planting out. Prior to planting the number of plantlets produced per explant was counted and 50 plantlets greater than or equal to 2 cm in height were selected for planting. Procedures for planting, establishment and growth to maturity were as outlined for petiole explants (see Section *D.4.2.2.*).

D.4.2.4. Growth analysis

At maturity vegetative characteristics of all populations were evaluated by measuring the following, plant diameter, plant height, leaf number, leaf area (leaf area measuring machine Crump Scientific Products, Essex, UK), leaf length, leaf width, petiole length, number of rhizomes, rhizome weight and root weight. Variation in leaf shape was given by the leaf asymmetry index $\frac{LW - RW}{W}$ where LW = left width RW = right width and W = leaf width (Ogura, 1978).

D.4.3. Results

There were significant differences in the number of plantlets produced from each of the propagation procedures assessed, axenic leaf explants producing the highest number of shoots (see Table D.8.). The number of shoots produced per explant however influenced overall shoot development. The most vigourous shoots were produced on SIM2 media. These shoots had large leaves and a well developed root system, while those from the remaining tissue culture procedures where large numbers of shoots were produced had small leaves and many had no roots. Leaves of shoots initiated from the latter procedures were green in colour (seedling foliage) and plantlets retained this colour up to 8 weeks after planting out, after which the typical adult leaf colour emerged. The plantlets produced on SIM3 media and from cuttings had adult foliage.

As can be seen from Table D.9. population means for each of the 10 characters assessed were very similar, with the exception of rhizome weight. When statistically assessed however no significant differences

Table D.8. Shoot numbers produced for different propagation procedures in *B. rex*.

Explant Source	Mean shoot number (mean±SE)
Leaf disc cutting	1
Petioles SIM	57±10
Petioles SIM2	23±9
Axenic leaf	92±35

Analysis of variance table

Item	SS	N	Variance	VR	P
Total	71626	50			
Treatment	22208	3	7403	7	0.01
Error	49418	47	1051		

LSD (P = 0.01) = 31

Using LSD values the following differences were found:

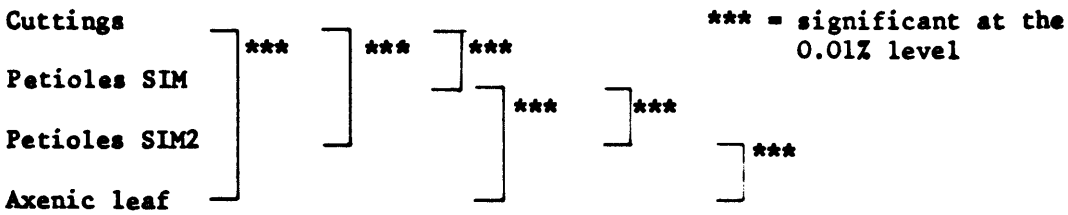


Table D.9. Vegetative characteristics of plants produced from cuttings and from tissue cultures of mature petiole explants and axenic leaf explants. Figures in parenthesis represent percentage.

Characteristic[a]	Tissue source			
	Leaf cutting	Mature petiole explant SIM	Mature petiole explant SIM2	Axenic leaf explant
No. of plants surviving (%)	24(70)	32(64)	58(29)	32(70)
Plant height cm (mean±SE)	14±3	14±3	14±3	13±2
Plant diameter cm (mean±SE)	25±5	25±5	18±6	23±4
No. of leaves/ plant (mean±SE)	24±9	26±9	22±7	25±8
Leaf area mean/ plant cm^2 (mean±SE)	29±6	29±8	29±7	24±9
Total leaf area/ plant cm^2 (mean±SE)	646±265	731±305	563±177	611±200
Petiole length cm (mean±SE)	5±1	6±1	5±1	5±2
Rhizome number (mean±SE)	5±2	5±2	4±1	4±1
Rhizome weight gms (mean±SE)	8±4	8±4	6±2	6±3
Leaf asymmetry index (mean±SE)	0.18±0.02	0.17±0.02	0.17±0.02	0.18±0.02

a - all differences between populations in vegetative characteristics were non-significant ($P > 0.05$).

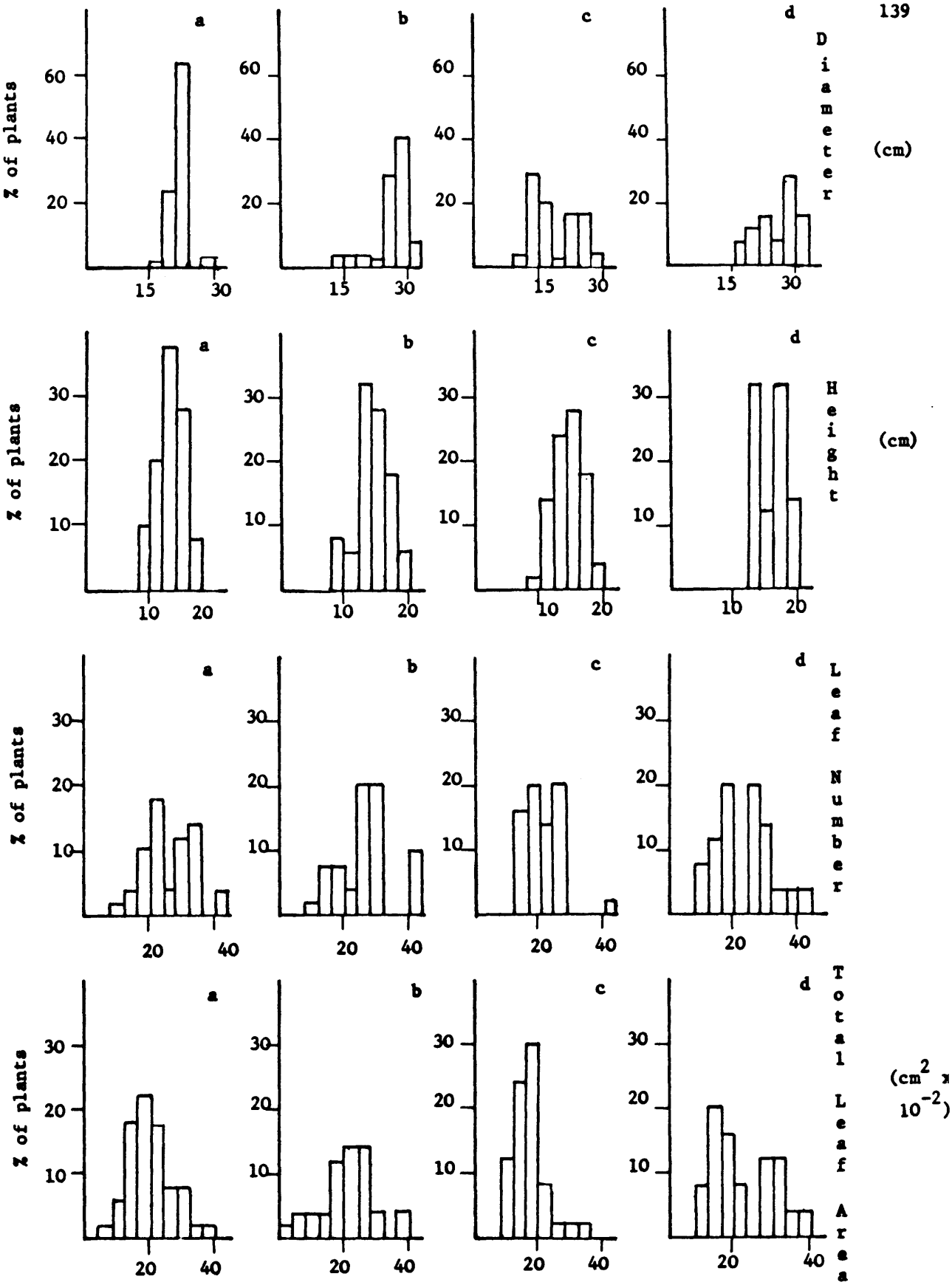


Figure D.1. Frequency plots for plant populations derived from (a) axenic leaf explants, (b) petiole explants cultured on SIM, (c) petiole explants cultured on SIM2 and (d) leaf cuttings.



Plate D.2. Variant produced from axenic explant culture of *Begonia rex*.

were found between populations for any of the characters assessed. A subjective assessment of plant habit of each population also indicated no major alterations in habit and interpopulation variation for all 10 characters was similar.

Frequency plots for four characters (Figure D.1.) give a clear illustration of the interpopulation variation which exists. Of the plant characters plotted, plant diameter in the population derived from axenic leaf explants would appear most uniform (Figure D.1(a) and plants derived from petioles on SIM2 also showed more uniform total leaf area.

These results indicate that the tissue culture procedures used here do not have any detrimental effect on habit, vigour or intra-population variation. Furthermore all of the plants produced, with the exception of one silver variant produced from axenic leaf culture (Plate D.2.) were true to type.

D.4.4. Discussion

The large number of plantlets produced from explants in tissue culture of *B. rex* and the absence of significant phenotypic changes in mature plants demonstrate the potential application of tissue culture to the propagation of *B. rex*. While many of the plantlets produced in the present study were too small to be planted out further subdivision cycles on SDM may ensure full development of these plantlets.

The fact that only one variant was produced also indicates that the adventitious bud method of propagation used here does not significantly influence the phenotype of the plants produced.

Tissue culture progeny of some plants have been shown to produce more uniform crops, a feature which has been of commercial advantage in particular in Banana (Hwang *et al.*, 1984) and *Stevia* (Tamura *et al.*, 1984). Tissue culture progeny of *B. rex* however do not show any significant improvements in any of the characters assessed. Axenic explant culture however does appear to be suitable for the development of an aseptic and relatively uniform source of explants. The large number of plants produced from a single explant (100) and the potential for continuous subculture also suggests that this procedure may provide a rapid means of introducing new genotypes. On the basis of present results the number of plants which could be produced in 9 months has been outlined in Figure D.2.

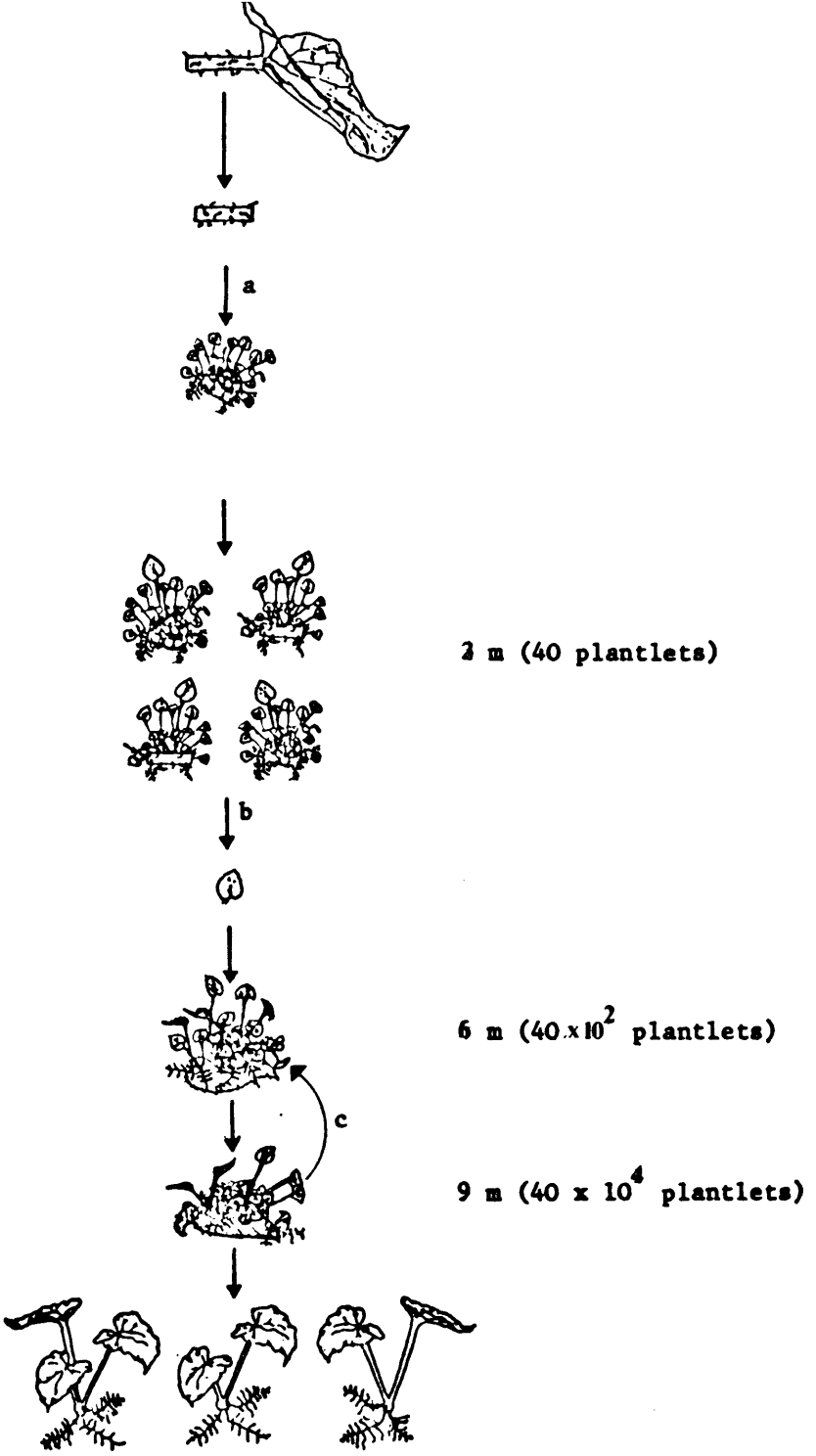


Figure D.2. Schematic diagram of potential multiplication of *B. rex* plants using axenic leaf explants derived from a single petiole explant. (a) Culture of petiole explant; (b) culture of axenic leaf explants derived from plantlets produced on the petiole explant; (c) subculture of axenic leaf explants derived from plantlets produced on axenic leaves. (m = months after culture initiation).

D.5. Evaluation of the application of continuous subculture to the propagation of B. rex

D.5.1. Introduction

The development of a continuous subculture system using axenic leaves for the propagation and maintenance of *B. rex* appears feasible in view of the demonstrated success of axenic explant culture (Section D.4.). From the limited number of studies available on continuous explant subculture (Section *B.2.4.1.*) there are reports of decreases in multiplication rate and vigour (Bigot, 1981) and the development of genetic/epigenetic changes which resulted in the production of off-type (Cassells and Plunkett, 1986). As these irregularities would prevent the application of subculture procedures to stock maintenance or multiplication of new genotypes, the establishment of subculture procedures for a given plant must involve a detailed assessment of the number of plantlets produced and the number of responding explants in each subculture. Most importantly this procedure must result in the production of true to type plantlets of acceptable habit and vigour.

The following study, which is aimed at the establishment of a continuous subculture procedure for *B. rex* involves a detailed evaluation of the regenerative capacity, survival, vigour and trueness to type of *B. rex* during subculture. In view of the changes observed in endogenous hormone levels reported during callus subculture (e.g. Meins and Binns, 1977) this study will also undertake an indirect assessment of possible changes which may occur in endogenous hormone levels during complex explant subculture. This study has been achieved using the assessment of responses of subcultured *B. rex* leaf explants to exogenous hormones.

D.5.2. Material and method

D.5.2.1. An analysis of the productivity and survival of axenic leaf explants in vitro

Axenic plantlet cultures were initially established from mature petiole explants on SIM (Table C.1.). Initial studies on leaf size demonstrated that leaves of approximately 1 cm were the most responsive in culture (Section *D.3.3.*). Hence this leaf size was routinely used during subculture.

Procedures adopted for each subculture included; (1) counting of shoots produced from a random sample of 10 cultures, (2) culture of 80 leaf explants and (3) storage of 5 cultures at 10°C (as a precaution) for use in the event of accidental contamination.

For each subculture leaves of approximately 1 cm in length were aseptically removed from axenic plantlets and wounded at random, using a scalpel, before being placed with their lower epidermis in contact with the plantlet induction medium (APM) (Table C.1.). After 4 weeks of culture under standard growthroom conditions the number of responding explants was noted before transfer to SDM (Table C.1.) for further plantlet development. After a total of 12 weeks, cultures were either counted, transferred to the cold (10°C) or used as a source of axenic leaf material for further subculture.

D.5.2.2. Comparative analysis of progeny from leaves of axenic plantlets and leaves of multiply recycled axenic plantlets

Twenty leaves of approximately 1 cm in length derived from both the sixth subculture generation and from axenic plantlets produced on mature petiole explants were wounded at random and transferred to APM (Table C.1.) in 60 ml screwtop jars. After 4 weeks of culture under standard growthroom conditions responding explants were transferred to SDM (Table C.1.) for plantlet development. When fully developed, after a total of 12 weeks of culture, plantlet numbers were counted and graded into size classes which indicated the number of plantlets greater than 2 cm, between 1 to 2 cm and less than 1 cm in height. From each source a total of 90 plantlets approximately 2 cm in height were planted out in peat compost in heated propagators at 20°C. After 8 weeks established plantlets were transferred to 7.5 cm pots and finally after a further 12 weeks of growth plants were transferred to 12 cm pots where they remained for 12 weeks prior to evaluation. Chromosome counts were undertaken as outlined in Section *C.5.*

D.5.2.3. Indirect assessment for changes in endogenous hormone levels during subculture

Axenic leaves of approximately 1 cm in length derived from both the eighth subculture generation and from axenic plantlets produced on mature petiole explants were wounded at random and separately cultured on media containing the hormones NAA and BAP in a 5 x 5 combination of 0, 0.01, 0.1, 1, 10 mg/l with 5 replicas for each treatment. Explants were cultured for 2 months under standard growthroom conditions before evaluation.

D.5.3. Results

Counts of shoots and the number of responding explants from successive subculture demonstrates that there is no reduction in explant regenerative capacity (Table D.10.). The timescale for regeneration also remained constant at 12 to 14 weeks. While the number of responding explants in subcultures did not vary, there were however always some explants on which shoot development was slow. Hence it was important to culture adequate numbers of explants to prevent delays in the establishment of subsequent subculture cycles.

Shoot numbers produced per explant in each subculture cycle fluctuated (Table D.10.) and while there were differences in the shoot numbers produced in some culture passages as compared with other cycles (e.g. S5) this difference was not maintained. In most culture passages a high level of variation was found between shoot numbers produced by different explants. This variation appears to have decreased in the more recent culture passages i.e. 11 and 12 where the number of shoots produced also appears to have stabilized (see Table D.10.).

Assessment of shoot size classes of explants from S1 and S8 indicate that there was no significant change in the size range of shoots produced from subcultured explants (Table D.11.) (Plate D.3.) and factorial studies on S1 and S8 also indicate no difference in the response of subcultured explants to exogenous hormones. The maintenance of plant vigour and normal phenotypic characters in mature plants regenerated from subculture is evident from results of the quantitative assessment of progeny from S1 and S6 (Table D.12.). While there was an apparent lower mean value in the S6 generation for many of the characters assessed this was not significant ($P > 0.05$).

Frequency plots (Figure D.3.) indicate that in the S6 plant population there are more plants of a uniform height and the median value is higher than that of the S1 population. A larger population of plants of similar leaf area were also recorded in the S6 population. Other characters plotted show the extent of interpopulation variation which is present in both S1 and S6.

Chromosome counts from S1 and S8 demonstrate the aneusomatic nature of this *B. rex* plant (see Table D.13.) for both S1 and S8 plant populations the most frequent chromosome numbers recorded were 22, 24, 26 and hence subculture would not appear to have influenced the relative range of chromosome numbers present.

Table D.10. Adventitious shoot regeneration in recycled axenic leaf culture.

Culture generation	Explant response[a] (%)	Adventitious shoot number[b]/ leaf explant (mean±SE)
1	100	80±19
2	90	114±49
3	95	130±40
4	90	118±35
5	100	169±30
6	100	138±33
7	100	123±40
8	92	111±64
9	100	142±30
10	100	94±31
11	84	76±16
12	92	75±15

a - from a total of 80 replicas

b - 10 replicas counted



Plate D.3. Comparison of plantlets produced from the first (S1) and eighth (S8) subculture of young axenic leaves.

Table D.11. Size classes of shoots produced in the first (A1) and eighth (A8) subculture cycle.

	Culture origin	
Shoot size class*	A1	A8
Shoots > 2 cm	51±22	47±31
Shoots 1-2 cm	65±51	48±33
Shoots < 1 cm	22±9	20±12
Total shoots	116±56	111±64

* Differences between means were non significant ($P > 0.05$)

Table D.12. Vegetative characteristics of progeny plants from singly and multiply recycled axenic leaves.

Characteristic[a]	Plant population	
	Axenic leaf 1st generation	Axenic leaf 6th generation
No. of plants surviving (%)	27(34)	19(23)
Plant height cm (mean±SE)	11±3	9±2
Plant diameter cm (mean±SE)	17±4	15±4
No. of leaves (mean±SE)	10±5	8±4
Leaf area mean/plant cm^2 (mean±SE)	24±9	20±7
Petiole length cm (mean±SE)	4±1	13±1
No. of rhizomes (mean±SE)	1±1	1±1
Weight of rhizomes grms (mean±SE)	2±1	1±1
Total leaf area/plant cm^2 (mean±SE)	239±132	161±93

a - All differences between populations in vegetative characteristics were non-significant ($P > 0.05$).

Figure D.3. Frequency histograms of vegetative characteristics of progeny populations from the first (S1) and sixth (S6) subculture cycle of axenic leaf explants.

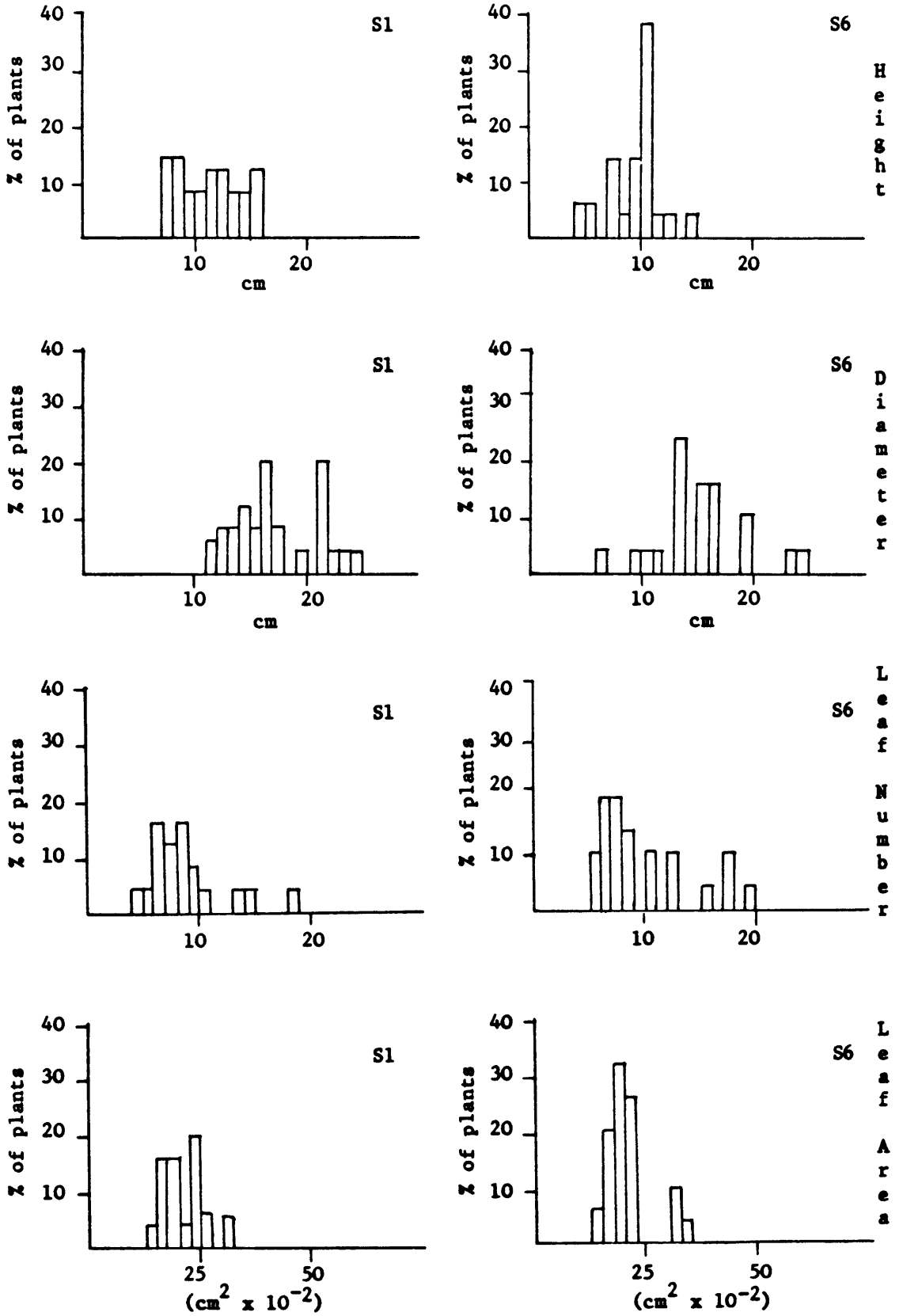


Table D.13. Chromosome number frequency in plantlets regenerated from the first (S1) and 8th (S8) subculture of axenic leaf explants. Frequency in 100 counts using a random sample of 10 cells from 10 plants per population.

Chromosome number	S8	S1
18	1	2
20	2	10
21	2	3
22	19	18
23	1	3
24	39	27
25	3	3
26	26	24
27	1	2
28	6	8

D.5.4. Discussion

The present study indicates that subculture of axenic leaf tissue over a period of one year has no significant influence on the vigour or stability of *B. rex* plants produced from tissue cultures. Furthermore, multiplication rate and explant response is maintained, a feature which has allowed the development of standardized procedures. These results demonstrate that a continuous subculture system may be used for the maintenance and propagation of *B. rex* cv. Lucille Closon for at least one year and give supportive evidence to the proposals of Lê and Collet (1981) for the development of an axenic explant subculture system which allows *in vitro* maintenance and multiplication of plant stock.

While there were no differences in the sizes range of shoots produced from S1 and S8, the difference observed in shoot numbers produced between explants for successive subculture deserves comment. These variations in explant responses demonstrate that despite controlled environmental conditions and apparently similar ontological states individual leaf explants produced different numbers of shoots. Also, while not quantified, there were differences in the rate of shoot development between explants. These results demonstrate that synchrony and productivity have not been fully controlled by the use of axenic explant culture. Differences in explant response may be linked to differences in endogenous hormone levels between explants either as a result of the prevailing cultural conditions, plantlet ontogeny or due to differences in the quantity of tissue cultured. The use of standardized leaf discs would reduce the latter influencing factor. Preliminary studies using discs however indicate that the survival rate of explants is reduced due to excessive wounding. Furthermore, this procedure increases the risk of contamination and is more time consuming than randomly wounding intact leaves.

While plants produced from subculture after one year were phenotypically normal this does not imply that this stability will be maintained in future subcultures. The continued assessment of plants at intervals will be required to ensure that stability and vigour are maintained in subculture especially as changes observed in *B. rex*, like those reported for African violet (Cassells and Plunkett, 1984), only become apparent when plants have been grown in the greenhouse for at least three months (see Section E.).

The aneusomaty observed in the *B. rex* cultivar studied here has previously been reported in *Begonia* (Section *B.1.2.*). There have been numerous proposals to explain the cause of aneusomaty (see Section *B.1.2.*), in the present study no direct evidence for the underlying mechanism causing aneusomaty in this *B. rex* cultivar was observed.

D.6. Assessment of the regenerative responses of a number of B. rex cultivars in vivo and in vitro.

D.6.1. Introduction

In micropropagation the establishment of single defined media which would allow the propagation of a number of different genotypes is desirable as it would make this procedure more cost effective. Presently many tissue culturists when assessing genotype responses utilize only one medium and explants derived from mature tissues (see Section *B.2.2.2.*). The cause of recalcitrance or low morphogenic response in some genotypes may therefore be related to epigenotype effects or the use of suboptimal media. As epigenotype influences are reduced in explants derived from axenic plantlets the culture of these explants on a broad range of media may allow the establishment of common regenerative media for a number of different genotypes.

In the present study, procedures developed for the micropropagation of *B. rex* cv. Lucille Closon using both mature and axenic explants will be assessed for their application to the propagation of other *B. rex* cultivars. Studies have also been undertaken on the development of broad spectrum media using axenic leaf explant culture on a range of hormone combinations. To demonstrate the influence of *in vitro* procedures and conditions on morphogenic capacity of individual cultivars and to assess the extent of genotype as compared with epigenotype influences on media responses, the following study also evaluates *in vivo* and *in vitro* responses of both mature and axenic tissues.

D.6.2. Material and method

D.6.2.1. Cuttings

The *B. rex* Putz. plants used in this study include 'Silver Corkscrew', 'Inimitable', 'Jubilium Weinstephan', 'Lucille Closon', 'Regia' and 'Bodnant'. The names 'Bodnant' and 'Jubilium Weinstepahn' were ascribed to seed progeny which were identified as having similar leaf colours to these cultivars. The remaining four cultivars

were part of a *Begonia* collection in the Botany Department, University College, Cork.

For *in vivo* propagation, 10 discs of 2 cm in diameter and including a portion of the main vein were punched, using a cork borer, from each of the cultivars under study. These discs were immersed in Captan 10% (v/v) for 5 minutes before being placed 2 cm apart in a cutting compost (peat:sand, 1:1) in a heated propagator (20°C). Leaf cutting responses were evaluated after 8 weeks of growth.

D.6.2.2. Petiole explants

Petioles from leaves approximately 9 cm in length were removed from each of the six cultivars under study and prepared for culture as previously outlined (Section *C.2.1.*). A total of 30 replicas per cv. were cultured on SIM (Table C.1.) in 60 ml screwtop jars. After 4 to 6 weeks of culture under standard growthroom conditions, explants were scored for site and intensity of bud induction. Responding explants were subdivided and transferred to SDM (Table C.1.) for plantlet development. After a further 2 months of culture plantlet number per explant was evaluated for 10 of the replicas and the remainder were used as source material for axenic leaf factorial studies.

D.6.2.3. Axenic leaf culture

For each of the cultivars under study, leaves of 1 to 1.5 cm in length were removed from axenic plants, wounded at random and placed on the appropriate medium. For studies of axenic explant response on a single defined medium explants were cultured for one month on APM (Table C.1.) and then transferred to SDM (Table C.1.) for further shoot development. Shoot numbers were counted after a further 2 months of culture. For factorial studies, explants were placed on media containing NAA and BAP in a 5 x 5 combination of the concentrations 0, 0.01, 0.1, 1 and 10 mg/l. Explant response was subjectively assessed after 2 months of culture and quantified by measurement of fresh weight.

D.6.3. Results

Based on budding intensity and percentage response, the cultivar Jubilium Weinstephan proved the least responsive *in vivo*. Only 20 percent of the cuttings responded, producing shoots which were low in vigour, all other cultivars were 70 to 100 percent responsive and all produced vigourously growing plantlets (Table D.14.).

Table D.14. Evaluation of responses of six *B. rex* cultivars *in vivo* and *in vitro* (using mature leaves and immature axenic leaves). Results are the mean of 10 replicas taken after 12 weeks of growth.

	Response of Cuttings *in vivo*			Response of Petioles *in vitro*			Response of axenic leaves *in vitro*		
Cultivar	Budding Intensity	Shoot numbers (mean/ disc)	Response %	Budding Intensity	Shoot numbers mean ±SE	Response %	Budding Intensity	Shoot numbers	Response %
Regia	+++	1	100	+++	33±4	100	++	128±28	90
Bodnant	+++	1	100	+++	50±10	100	+	20±10	100
Silver Corkscrew	+++	1	70	++	20±12	100	-	0	100
Inimitable	+++	1	100	+++	23±7	100	++	32±14	90
Jubilium Weinstephan	+	1	20	+	12±5	100	+++	30±9	90
Lucille Closon	+++	1	90	+++	39±8	100	+++	80±13	100

+++ = large numbers of buds produced ++ = scattered bud production medium vigour + = few buds produced low vigour

- = no buds produced



Plate D.4. Morphogenic responses of six cultivars of *B. rex* on APM (see Table C.1.).

BR26 'Regia'
BR27 'Silver Corkscrew'
BR28 'Inimitable'
BR 5 'Jubilium Weinstephan'
BR30 'Lucille Closon'
BR22 'Bodnant'

In cultures of petiole explants *in vitro* the response of the cultivar Jubilium Weinstephan increased to 100 percent but shoots were few in number and grew slowly. Other cvs. showed differences in the number and intensity of bud induction (Table D.14.) and plantlets grew at different rates when transferred to SDM media.

In axenic leaf explant culture 'Jubilium Weinstephan' (BR5) showed an increase in the number and intensity of plantlet produced (Plate D.4.). In other cultivars while explants were 100 percent responsive, there were differences in the nature and intensity of morphogenesis. The cultivar Silver Corkscrew (BR27) produced only roots and the response of the cultivars Bondant (BR22) and Inimitable (BR28) were evidently suboptimal (Plate D.4.). When axenic leaf explants were cultured on factorial however, the range of hormone concentrations assessed did allow the establishment of media on which these cultivars gave an optimal response. On this factorial range of media all cultivars showed distinctive differences in morphogenic response. The overall morphogenic response of each cultivar is outlined briefly here (see also Table D.15.; Plate D.5. a-f).

'Bodnant' (BR22). No morphogenesis occurred in the absence of hormone. Bud production predominated on all media with 1 mg/l or greater BAP concentrations. On lower BAP concentrations plantlets were produced while on high concentrations of NAA at low BAP concentrations roots were produced.

'Silver Corkscrew' (BR27). This cultivar also failed to respond in the absence of hormones. In contrast to B22 however the presence of auxin had a greater influence with rooting predominating on all media with low cytokinin while in combinations of high auxin and high cytokinin no morphogenesis occurred.

'Inimitable' (BR28). Unlike the above cultivars plantlets production occurred in this cultivar on media without hormones. The predominant response on all media with low auxin concentrations was for plantlet production. Budding occurred on high cytokinin while on high auxin and low cytokinin roots or callus were produced. The influence of auxin however was eliminated on media with high cytokinin where budding occurred.

Plate D.5. Morphogenic responses of six *B. rex* cultivars on factorial combinations of the hormones NAA and BAP. a = 'Regia', b = 'Bodnant', c = 'Jubilium Weinstephan', d = 'Lucille Closon', e = 'Silver Corkscrew', f = 'Inimitable'.


BAP mg/l
0 0·01 0·1 1 10
NAA mg/l
0 0·01 0·1 1 10
a
b
c
d
e
f

BAP mg/l →

NAA mg/l ↓

NAA mg/l	1	0.01	0.1	1	10
0	-	-	-	B	*b*
0.01	*p*	*p*	p	b	B
0.1	*p*	P	P	B	B
1	R	R	R	B	B
10	R	R	R	B	B

BR22

NAA mg/l	1	0.01	0.1	1	10
0	-	-	-	S	B
0.01	-	*r*	*s*	S	B
0.1	R	r	p	S	B
1	R	R	B	S	*b*
10	R	S R	R S	-	-

BR27

NAA mg/l	0	0.01	0.1	1	10
0	p	P	P	S	B
0.01	P	P	B	B	B
0.1	P	P	P	S	B
1	C	r C	P	S	B
10	C	R C	S C R	B C	B C

BR28

BAP mg/l →

NAA mg/l ↓

NAA mg/l	0	0.01	0.1	1	10
0	*p*	*p*	*p* *b*	*s*	B
0.01	p	*b*	p	S	B
0.1	P	p	p	S	B
1	*p* R	*b* R	P	B	B
10	*r* C	R C	R C	B	B

BR26

NAA mg/l	0	0.01	0.1	1	10
0	*p*	*p*	S	S	B
0.01	*p*	*p*	B	B	*b*
0.1	P	P	p	B	B
1	R	R	P	B	B
10	C	R	R	*r*	*b*

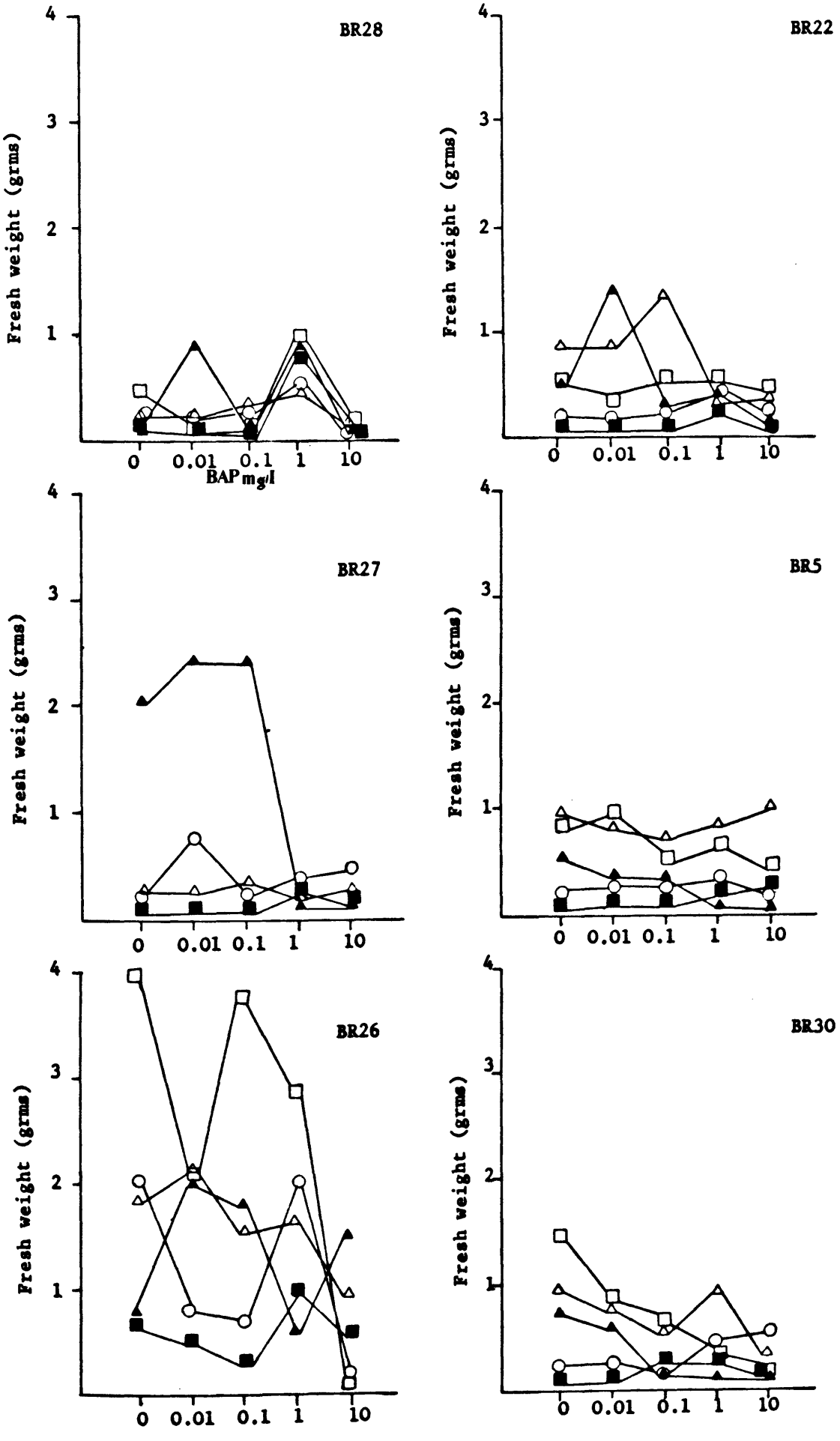
BR5

NAA mg/l	0	0.01	0.1	1	10
0	*p*	*p*	B	B	*b*
0.01	p	P	B	B	B
0.1	P	*p*	B	B	B
1	P	P	s	B	*b*
10	R	R	-	*c*	-

BR30

Table D.15. Morphogenic responses of axenic leaves of six *B. rex* cultivars on combinations of the auxin NAA and cytokinin BAP. Legend as in Table D.1.

Figure D.4. Fresh weight plots of morphogenic responses from six *B. rex* cultivars an combinations of auxin and cytokinin. (■ = 0, 0 = 0.01, Δ = 0.1, □ = 1, ▲ = 10 mg/1 NAA).


BR28
BR22
BR27
BR5
BR26
BR30
Fresh weight (grms)
BAP mg/l
0
0.01
0.1
1
10
1
2
3
4

'Regia' (BR26). This cultivar responded on all the media tested and proved the most responsive. Morphogenesis occurred over the total surface of the explant and was generally intensive. The predominant response was bud or shoot morphogenesis and auxin only antagonized shoot production when present at very high concentrations (10 mg/l).

'Jubilium Weinstephan' (BR5). Morphogenesis occurred on all media tested but was not as intensive as that produced on BR26 (see Table D.15.) and high auxin inhibited budding even at 1 mg/l BAP. In this cultivar as in other cultivars budding was prolific at high concentrations of BAP.

'Lucille Closon' (BR30). Morphogenesis occurred on all but two of the media tested. In common with BR26 only very high levels of auxin antagonized bud or shoot production resulting in the production of roots or callus. All other media produced buds, shoots or plantlets at varying intensities (see Table D.15.).

As indicated in Table D.15. none of the media tested provided a single hormone combination on which plantlets were induced from all cultivars. Overlap in response occurred however at high concentrations of BAP where all cultivars produced buds at varying intensities over the leaf explant surface.

Fresh weight data (Figure D.4.) illustrates the quantitative differences which exist between the responses of individual cultivars on the media tested. These data incorporate the overall morphogenic responses of all replicas while the results illustrated in Plate D.5. a-f are based on the best response produced on a given medium, hence any apparent discrepancy between fresh weight data and these illustrations are due to differences between replicas. Fresh weight results indicate the high morphogenic competence of 'Regia' (BR26) while other cultivars show a fluctuating response and no obvious similarities appear to occur in fresh weight gain on the media tested.

D.6.4. Discussion

The results of the present study indicate that individual *B. rex* cultivars show distinctive morphogenic responses both *in vivo* and *in vitro*. This confirms the observations made by Bigot (1971) on different *B. rex* cultivars (see Section *B.1.1.1.*).

The manifest low morphogenic responses of some cultivars *in vitro* when mature explants were used may, as suggested by Welander (1977), be the result of epigenotype influences or the use of suboptimal media. The former influence was reduced by the use of axenic explants which may explain the increased morphogenic response of some cultivars e.g. Jubilium Weinstephan. The reduced response of some cultivars which had previously appeared highly responsive using mature explants would suggest that each genotype has a specific response to exogenous hormones. This suggestion was confirmed with results from a study of genotype responses on a factorial combination of media where a wide range of responses were noted for individual cultivars. Similar studies of different *Lycopersicon* cultivars on a range of media, but using mature explants, by Kurtz and Lineberger (1983) also demonstrate differences between cultivars both in the nature and intensity of morphogenesis.

The present study was also undertaken to assess the potential for the development of broad spectrum media for propagation of *B. rex* cultivars. Results indicate that overlap in media for plantlet formation only occurs in one or two cultivars however, the production of intensive budding in all cultivars on high BAP containing media does indicate a similarity in response and further studies on a narrower range of media coupled with studies on the subsequent development of these buds using SDM may reveal a medium which may be used for the propagation of a number of *B. rex* cultivars in tissue culture.

D.7. In vitro storage of Begonia

D.7.1. Introduction

The maintenance of plants *in vitro* by minimal growth storage at low temperature has been successfully applied to a number of plants (Section *B.2.3.1.*) thereby eliminating the cost of greenhouse maintenance of mother stock and the risk of disease.

While stocks of *B. rex* may be maintained by continuous subculture (Section *D.5.*) it would also be useful to maintain plants for prolonged periods in cold storage, a procedure which has the added advantage of reducing the risk of contamination and genome instability.

In most studies tissue cultures are stored as whole plantlets or shoot tips (Section *B.2.3.1.*). However in the case of complex explant

culture it may also be possible to slow down growth at the bud development stage by transfer to low temperatures. Once low temperature treatment does not affect the vigour and growth characteristics of the plantlets produced, this procedure could be effective in maintaining axenic plantlets which may be used as explants for the initiation of fresh stock.

For the storage of whole plantlets the presence of a cryoprotectant such as glycerol in the medium may protect plantlets from the detrimental influence of low temperature treatment (Bajaj and Reinert, 1977).

The present study is a preliminary examination of the survival of *Begonia* plantlets at low temperatures in the presence and absence of glycerol. This study also examines the maintenance of budding cultures by delayed growth at low temperatures, the subsequent establishment of cultures from stored axenic plantlets for the production of new stock and the survival of plants produced when compared with controls.

D.7.2. Material and method

D.7.2.1. Plantlet development on media in the presence and absence of glycerol

Petioles were prepared for culture as outlined in Section *C.2.1.* After 4 weeks of culture on SIM (Table C.1.) under standard growthroom conditions budding explants were transferred to SDM (Table C.1.) containing 0, 1, and 2 percent (v/v) glycerol. Once plantlets had fully developed, after a further 2 months of culture, all cultures were transferred to a cold cabinet at 10°C. Plantlet survival was evaluated after 2 and 4 months in culture.

D.7.2.2. Storage of budding petiole explants of B. rex by delayed growth at low temperatures

Petioles were prepared for culture as outlined in Section *C.2.1.* and a total of 20 explants were cultured on SIM (Table C.1.) for 4 weeks under standard growthroom conditions. After subdivision and transfer to SDM (Table C.1.), cultures were placed in a cold cabinet at 10°C where they remained for 6 months. By this time plantlets had fully developed in the majority of cultures. Axenic leaves were removed from surviving cultures and 40 leaves were wounded at random before transfer to APM (Table C.1.). A control population of 40

axenic leaves from non-stored plantlets were also cultured on APM. After 4 weeks of culture responding leaves were transferred to SDM (Table C.1.) for further plant development. After a further 8 weeks of culture 50 plantlets approximately 2 cm in height were selected from each population and planted out in peat compost in heated propagators (20°C). The number of surviving plants, plant height and number of leaves was recorded for each population 12 weeks after transfer.

D.7.2.3. Storage by delayed growth of buds on leaf explants of B. x *hiemalis*

Leaves were prepared for culture as outlined in Section *D.2.* A total of 20 explants were cultured on EM (Table C.1.) under standard growthroom conditions. After 4 weeks of culture budding leaves were subdivided and transferred to SDM (Table C.1.) and placed in a cold cabinet at 10°C where they remained for 6 months. Twenty axenic leaves were removed from the surviving cultures and wounded at random before transfer to EM (Table C.1.). In this experiment controls were leaf explants taken from plants which had been maintained for 6 months in the glasshouse. These explants were prepared for culture as outlined in Section *D.2.* and 20 replica discs were transferred to EM (Table C.1.). All cultures were cultured under standard growthroom conditions for 8 weeks before evaluation.

D.7.3. Results

D.7.3.1. Plantlet storage using glycerol treatment

After 2 months of low temperature treatment 30 percent of cultures from both control and glycerol treated plantlets had senesced. After 4 months both populations had senesced and hence it would appear that the glycerol concentrations used here did not enhance the survival of *B. rex* at low temperatures (10°C).

D.7.3.2. Plantlet storage by delayed growth in B. rex

Low temperature treatment delayed plantlet development by 2 months. Of the cultures transferred to low temperatures 80 percent developed to produce plantlets. Thus an adequate number of cultures were available to provide the axenic leaves required to reinitiate plantlets. On transfer to APM explant response of stored plantlet leaves was similar to that of controls (70%) and the plantlets produced were equally as vigourous. Results of plant survival,

Table D.16. Effect of low temperature storage for 6 months on axenic leaf explant response and plantlet survival.

Leaf explant source	Responding[a] explants %	Plantlet[b] survival %	Leaf number (mean±SE)	Plant height (cm) (mean±SE)
Cold stored	95	70	7±3	5±2
Control	95	79	6±2	5±2

a - total of 40 replicas

b - total of 50 plantlets

height and leaf number (Table D.16.) indicate that plantlets derived from axenic leaves of stored plantlets have similar survival capacities and growth habits as controls.

D.7.3.3. Storage by delayed growth of buds on leaf explants of B. x hiemalis

Of the 40 explants transferred to low temperature 40 percent survived and developed to produce plantlets. When axenic leaves were cultured after 6 months of storage they gave 100 percent response. In contrast explants from plants maintained for the same period of time in the glasshouse only produced buds on 50 percent of explants.

D.7.4. Discussion

It must be emphasized that the results reported here are from a preliminary study on the potential for cold storage in *Begonia* and have been designed to provide a guideline for future work.

The failure of glycerol to enhance survival of plantlets at low temperatures may be due either to a requirement in *B. rex* for higher concentrations or different combinations of cryoprotectants.

The results from studies of storage of *B. rex* and *B. x hiemalis* by delayed growth suggest that this procedure with further modifications may be used for the maintenance of stock from one growth season to another. However long term storage of unique genotypes would require more elaborate preservation treatments (see Section *B.2.3.*).

SECTION E. EXPERIMENTAL WORK II

E.1. The use of tissue culture in the production of variants from B. rex

E.1.1. Introduction

The extensive range of habits, leaf colours and forms presently found in *B. rex* plants has been achieved as a result of hybridization and the production of spontaneous and induced mutations (Thompson and Thompson, 1981). The *B. rex* cultivar Lucille Closon which is the subject of the present study is sterile and would appear, on the basis of results from previous studies, (see Section D.) to have a low rate of spontaneous mutation. Hence improvements in the habit of this plant or the production of colour or pattern variants may only be achieved with procedures which will induce somatic variation which could subsequently be expressed via vegetative propagation.

Conventional methods used for the production of somatic variation include, chemical or physical mutagenesis and colchicine treatment (Section *B.3.5.*). These treatments can also be applied *in vitro* and may result in the production of a high frequency of variants due to the large number of plants which may be produced from treated explants (Section *D.4.*).

A number of studies also indicate that some tissue culture procedures for plant regeneration result in the production of variants (Section *B.3.4.5.*). Regeneration from callus culture which has been shown to destabilize a number of plant genomes has resulted in the production of an array of mutants (see Orton, 1984a) and variants have also been produced by regeneration from pre-existing somatic variant cells which may be present in plant tissue (Hermsen *et al.*, 1981). Furthermore, variants produced from any of the above procedures are not necessarily stable and further tissue culture cycles may cause reversion to the parental genotype or further variation which may result in the production of a range of plant phenotypes (cf. Larkin and Scowcroft, 1983b).

When variants are produced under *in vivo* conditions their propagation for trialing can take a number of years thus delaying the release of new commercial varieties. With tissue culture however potentially valuable variants can be rapidly propagated, thereby reducing the timescale required before release of valuable commercial varieties (Holdgate, 1977).

The following studies have been undertaken to examine the potential for the production of variants from *B. rex in vitro* using gamma

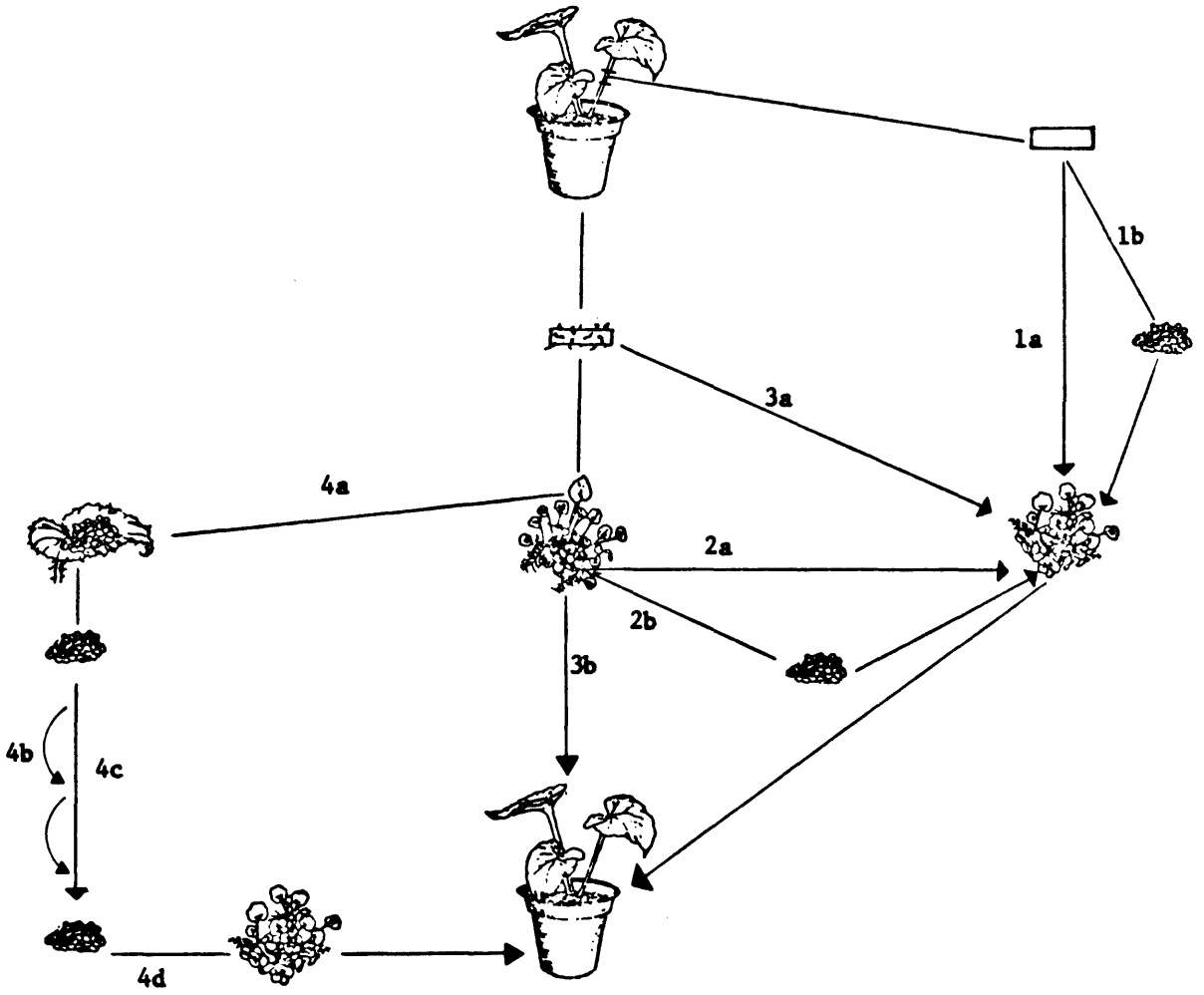


Figure E.1. Schematic diagram of methods evaluated in the present study for the production of variants in *B. rex* 'Lucille Closon'.
(i) Expression of pre-existing variation in pith (1) and root (2), by direct (a) and indirect (b) adventitious regeneration.
(ii) Induction of somatic variation by treatment of petiole explants with colchicine (3a) and axenic leaves with C_o^{60} irradiation (3b).
(iii) Callus culture. Induction of somatic variation in cultured cells by treatment with colchicine (4a), by subculture (4b), by aging (4c) and by regeneration on media supplemented with hormones and/or casein hydrolysate and adenine (4d).

irradiation, callus culture and colchicine treatments. The presence and expression of somatic variation in tissues of *B. rex* will also be evaluated. In the event of variant production tissue culture systems which have been developed here for the rapid propagation of *B. rex* (see Figure D.2.) are available to bulk up and assess the stability of these variants. The methods investigated here for the production of variants using tissue culture have been outlined in Figure E.1.

E.2. Mutation breeding of B. rex using in vitro culture of axenic leaves

E.2.1. Introduction

Gamma irradiation *in vivo* of *B. rex* leaf cuttings has been successful in the production of a number of commercially valuable mutants (cf. Broertjes and Van Harten, 1978) and x-ray irradiation of *B.* x *hiemalis* has resulted in the production of a wide spectrum and high frequency of mutants (Doorenbos and Karper, 1975). However the low productivity of leaf cuttings (ca. 1-2 plants per cutting) means that many potential valuable mutants may be lost. One of the major advantages therefore of *in vitro* mutagenesis is the increased potential for the production of mutants due to the increase in the number of plants which may be regenerated from irradiated tissue.

Many *in vitro* mutagenesis studies use *in vivo* derived explants (reviewed by Broertjes and Van Harten, 1978). As previously outlined (Section *B.2.2.1.*) the health and physiological state of the donor plant will influence explant response *in vitro*, hence the use of *in vivo* derived explants has resulted in some reports of systemic contamination and senescence which reduces the number of responding explants and subsequently the number of plants produced (e.g. Plunkett, 1984). The use of *in vitro* derived explants potentially reduces these losses and thereby eliminates the need for the large numbers of replicas which may have to be set up to allow for contamination and senescence. Furthermore, responses from axenic explants are generally more rapid than those from *in vivo* derived explants (Section *D.2.*) and thus reduce the timescale required for production of plants for assessment.

The present study on *B. rex* is aimed at utilizing an axenic leaf explant culture procedure both to express the mutations induced by gamma irradiation treatment and to assess the stability, and rapidly propagate any new variants which may be of commercial significance.

E.2.2. Material and method

Preparation of explants, irradiation treatments and subsequent culture and establishment of plants were as outlined in Section *C.4.*

Variants induced were cultured using axenic leaf explant culture on APM (Table C.1.) or petiole explant culture on SIM (Table C.1.) and plantlets produced were subsequently grown on for visual assessment as outlined in Section *C.2.4.*

Chromosomal analysis was undertaken as outlined in Section *C.5.* and chloroplast counts in guard cells as outlined in Section *C.6.2.*

E.2.3. Results

As indicated in Table E.1. the overall level of contamination recorded for explants was low (37%) with higher levels of contamination occurring in irradiated explants due to the more frequent transfer steps. A high level of senescence was also recorded in irradiated explants and the growth rate of 13 explants was also reduced.

While plantlets were developing in culture, only one phenotypic variant was observed. This variant had lighter colour markings and smaller leaves when compared with other plantlets (see Plate E.1.). Axenic leaves of this variant were cultured on APM to produce a sizable population of plants for assessment.

When selecting plantlets for planting out no phenotypic variants were observed. On transfer to 7.5 cm pots however a number of variants were evident. These included plants with changes in leaf colour, pattern and shape and plants with abnormal habits or reduced vigour (see Plates E.2. and E.3.). Many of the latter variants did not survive to maturity.

Changes in plant phenotypes were more pronounced when plants had matured. Putative chimeras were observed only at this stage after the foliage had been pruned. This was necessary as many of the leaves had become scorched due to exposure to excessive levels of sunlight.

As indicated in Table E.1. a total of 83 phenotypic variants were produced, this was equivalent to 6 percent of the total population of plants produced from irradiated explants. A wide range of phenotypic characters were affected and the spectrum of phenotypic changes induced have been outlined in Table E.2. Changes in one or more character occurred in 37 percent of the variants produced and 3 plants showed changes in 6 characters which was the maximum number of changes



Plate E.1. Axenic plantlets produced on axenic leaves of a variant observed in culture after gamma irradiation (V2 = variant, C = control).



Plate E.2. Plants with reduced vigour produced after gamma irradiation (C = control).



Plate E.3. Plants with abnormal leaf shape, altered colour and pattern from gamma irradiation treatment (C = control).

UNIVERSITY COLLEGE Library CORK

Table E.1. Gamma irradiation treatments: culture data, percentage variation and mutation spectrum induced.

	C^{60} irradiated (%)	Control (%)
Total explants	270	40
Total contaminants	50(19)	7(18)
Total senesced	90(33)	0
Total shooting	117(43)	33(75)
Total plants planted	1777	330
Total plants assessed	1366	300
Total variants	83(6)	0
Number of vegetative characters showing variation	11	0

Table E.2. Spectrum of phenotypic variants produced in *B. rex* after gamma irradiation.

Nature of change	Number of variants	Number of variants % of total
Plant habit		
Dwarf	5	6
Spindly	3	4
Planar	12	14
Leaf size		
Smaller	13	15
Leaf colour		
Ruby/magenta	3	4
Grey/green	4	5
Greyish rose/dark green	21	25
Dark ruby/deep magenta	4	4
Greyish rose	2	2
Leaf pattern		
Polka dot	9	11
Blotched	2	2
Reduced margin flecking	4	4
Solid margin	3	3
Striated	2	2
Leaf sheen		
Dull	1	1
Shiny	3	4
Leaf margin		
Serrated	2	2
Fluted	8	11
Ruffled	2	2
Indented	3	4
Leaf shape		
Obicular	2	2
Lanceolate	2	2
Distorted	5	6
Lamina texture		
Leathery	2	2
Papery	1	1
Puckered	3	4
Blistered	2	2

Table E.2. continued

Nature of change	Number of variants	Number of variants % of total
Petioles		
Fusion	1	1
Reduced diameter	2	2
Reduced length	17	20
Rhizome sheath		
Green	1	1

Colour code for colour variants. Munsell notation

Greyish Rose (7.5 RP, 7.2, 3.7)[a]

Ruby (9.5 RP, 3.3, 8.9)

Magenta (5.5P, 3.4, 1.2)

Greyish Ruby (8 RP, 4.3, 9.1)

Dark Green (7 G, 3.1, 4.3)

Grey (1 RR, 5.7, 1.6)

Deep Magenta (3 RP, 3.7, 11.5)

a - (Hue, Value, Croma)

induced in any one plant. There were certain characteristic combinations of changes, the most common syndrome being reduced petiole length and a planar habit.

The most frequently recorded phenotypic change occurred in leaf colour, 36 of the variants produced being colour variants. The majority of these variants had normal plant habits (see Plate E.5.). Colour changes only occurred in the leaves and petioles were never affected. In one plant green rather than red rhizome scales were observed. Eighteen plants (21%) showed variation in the pattern of pigmentation on the leaf surface (see Plate E.4. for examples), 12 of these plants also had colour changes. The pattern changes observed appear to be associated with changes in the number and/or distribution of cells forming the two main pigments produced in the leaf.

Gross alterations in growth habit were also observed in some plants. Twelve plants were found to have planar habits, all had small petioles which gave the plant a flattened appearance. Reduction in petiole length was also generally associated with a reduction in leaf size and plant vigour (Plate E.2.). In one plant however leaf size was not affected and this plant was abnormal in appearance with normal leaves or small petioles. Another abnormal plant was typified by extensive leaf puckering which gave the leaf a diseased appearance (see variant 22359 Plate E.6.). Uneven leaf growth and 'blistering' also caused abnormal leaf shapes (see variants 22579, 2161 and 22735 Plate E.6.). Other changes in leaf form included an increased serration of the leaf margin which occurred in two plants and gave the leaves a ruffled appearance (see Plate E.4.). Changes in leaf texture also influenced leaf shape and leaves with thickened leaf lamina were also slightly fluted in appearance (see variant 2910 Plate E.6.). In a number of plants with smaller leaves, leaf shape was also affected some producing obicular rather than obivate leaves (see Plate E.4.).

Putative chimeras produced both normal and variant leaves, on a single individual a wide range of leaf types were found and these have been illustrated in Plate E.7a along with leaves of similar developmental stage from a control (Plate E.7b). Three variant phenotypic characters were observed in the leaves of these plants including; fluted leaf form, polka dot pattern and colour change,



Plate E.4. Examples of leaf pattern and colour variation induced after gamma irradiation treatment. Control leaf, in top right hand corner.



Plate E.5. Plant habits of some leaf colour variants produced after gamma irradiation treatments. Control is in the bottom right hand corner.

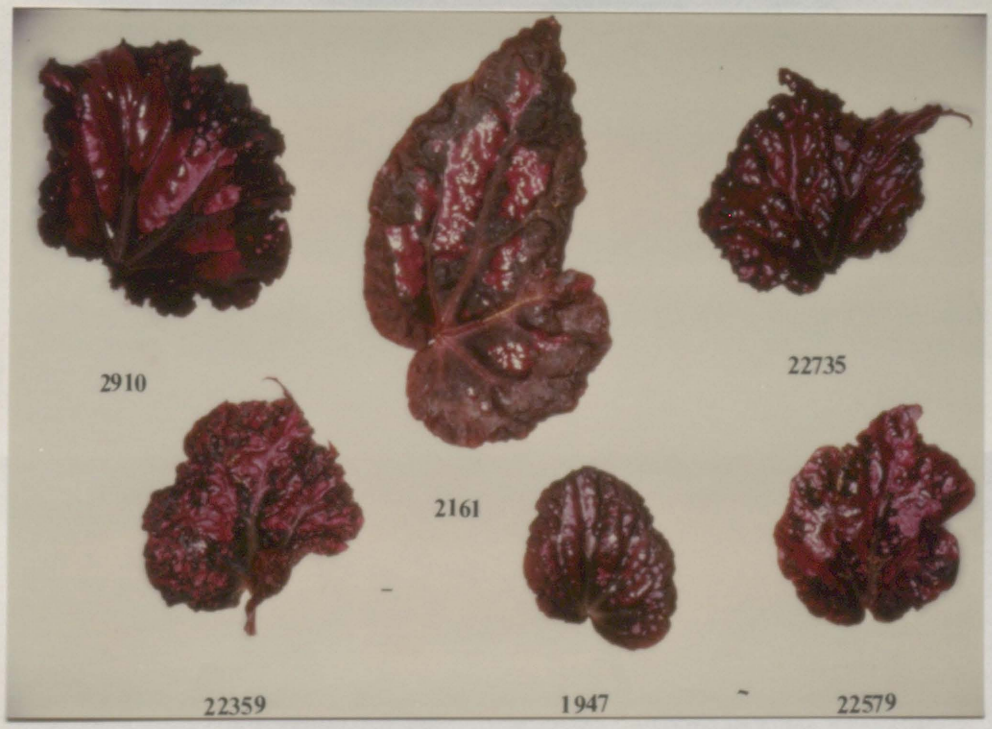


Plate E.6. Abnormal leaf types produced on plants from gamma irradiation treatments.



PlateE7a. Range of leaf types produced by a putative chimera.



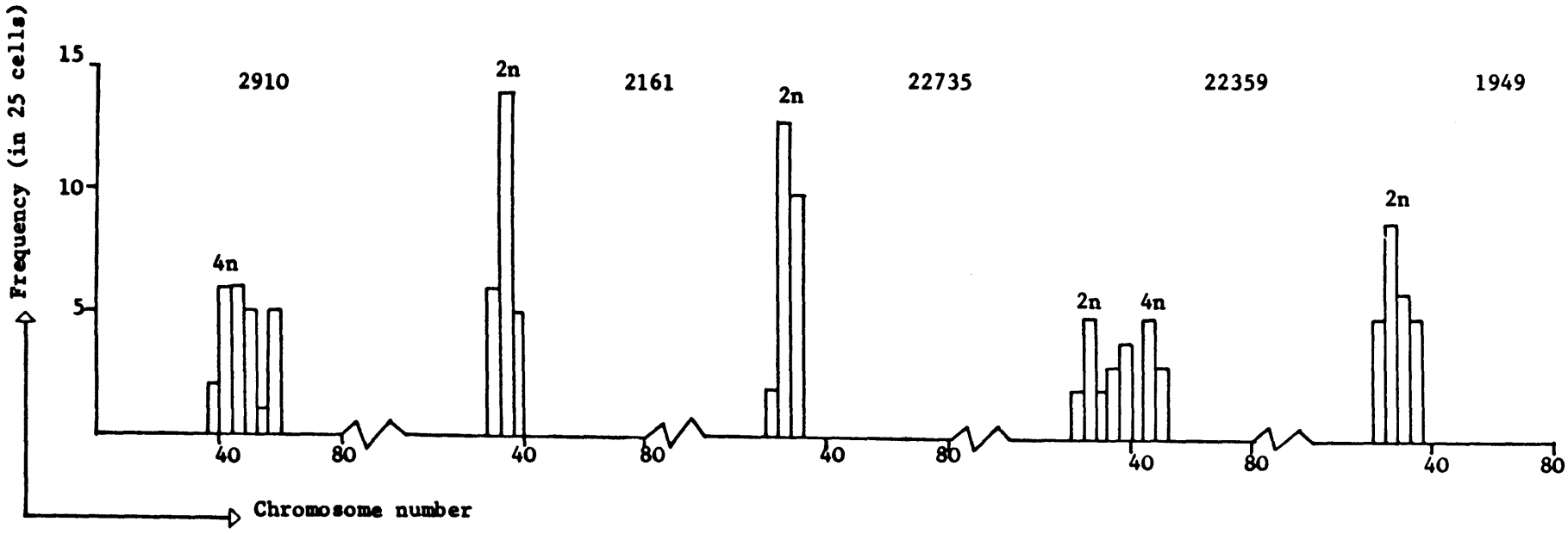
Plate E.7b. Range of leaf types produced by a control.

these were found in various combinations, for example some leaves with a normal pattern and colour were fluted.

The variant which occurred at the highest frequency was a silver colour leaf variant (see Plate D.1.) which made up 25 percent of the total variants produced. A similar colour variant was also produced in a previous experiment using unirradiated axenic explants (Section *D.4.*). Irradiation treatment however does not appear to have increased the frequency of its occurrence.

Chromosome counts from variants with colour or pattern changes indicate no major change in chromosome number although in some plants a low number of cells were observed with asynchronous division (Plate E.8.). Counts from some of the more abnormal variants outlined in Figure E.2. demonstrate distinctive changes in chromosome number in some plants. The leaf puckering which caused a diseased appearance in one variant (22359; see Plate E.6.) was found to be directly linked to the presence of diploid and polyploid cells (mixoploid). The reduction in leaf size and overall vigour of variant 1949 may be related to the wider range of diploid chromosome numbers found in this plant when compared with variants 22579 or 22735 which showed no major chromosome changes. The abnormal leaf shape, altered colour and blistered appearance of variant 2161 was associated with the mixoploid state of this plant. Variant 2910 which had a thickened leaf lamina, small petioles and reduced vigour was found to be polyploid. This polyploid was also characterized by an increase in chloroplast number in the guard cells. Control plants have a mean of 18 chloroplasts per guard cell while the polyploid mean was 26 (see Plate E.9a, b). A second plant with an apparent polyploid habit was found to contain low levels of diploid cells. In this plant asynchronous division of the chromosome complement, manifest as premature chromosome condensation, was observed in a number of cells (Plate E.8.). This phenomenon was also evident in putative chimeral plants which were otherwise found to have a normal chromosome number.

A number of variants with ploidy changes and the putative chimera have been cultured and their progeny will be assessed at a later date. Preliminary counts on plantlets derived from the unstable polyploid (3370) indicate that this variant produces both polyploid and mixoploid plants (see Figure E.3.). Further studies to produce plants from subculture cycles and from callus (see Figure E.4.) have been undertaken



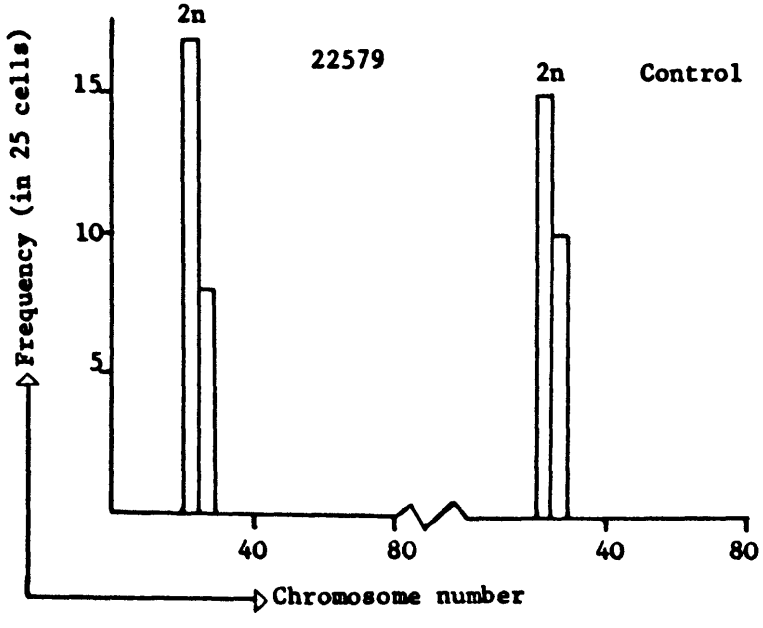
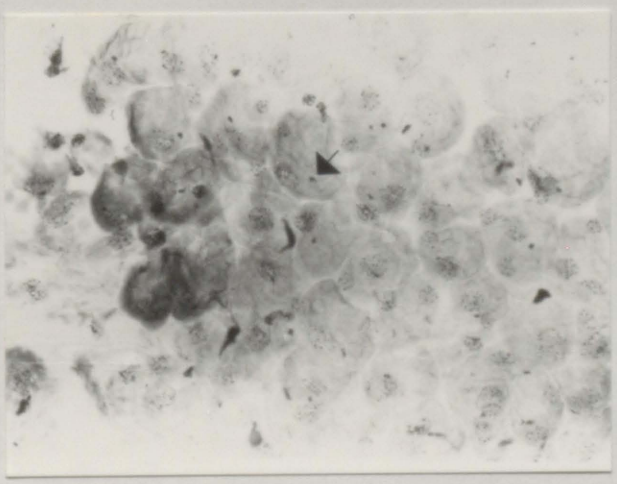
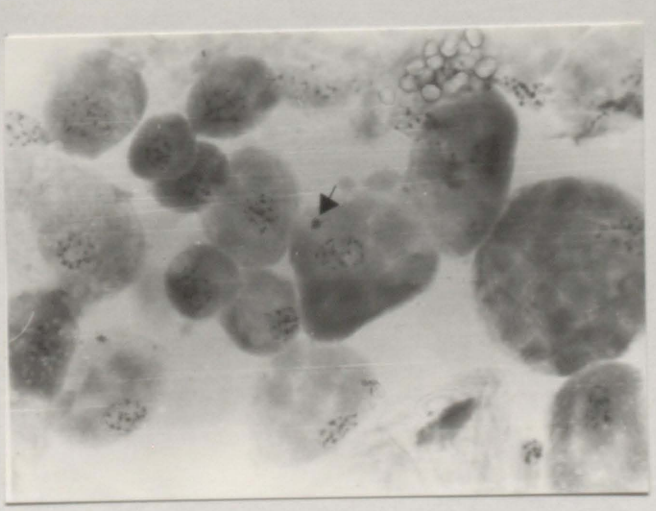


Figure E.2. Frequency plots of chromosome numbers in plants with abnormal leaf morphologies produced after gamma irradiation treatment (see Plate E.6.).

Plate E.8. Asynchronous division in cells from an induced polyploid (arrow marks condensed chromosomes). Enlargement a x 1,000, b x 2,500.





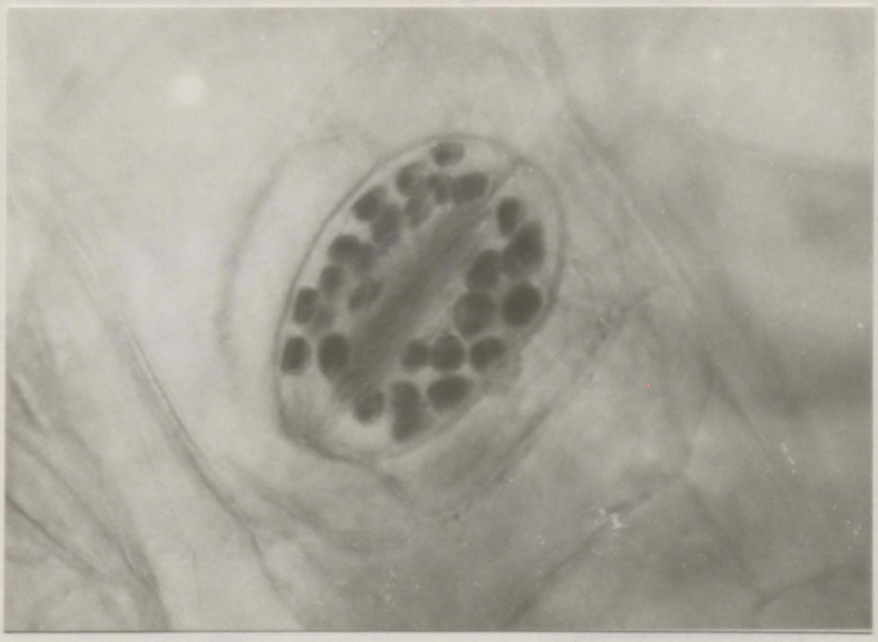


Plate 9a. Chloroplasts in guard cells of a polyploid. Enlargement x 1,520.

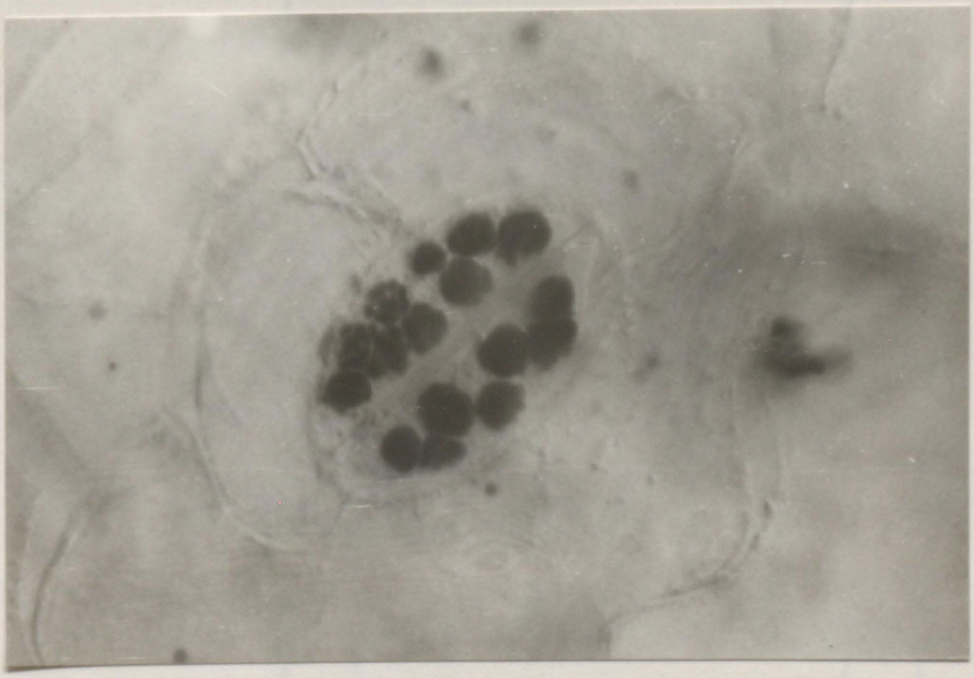


Plate 9b. Chloroplasts in guard cells of a diploid. Enlargement x 1,520.

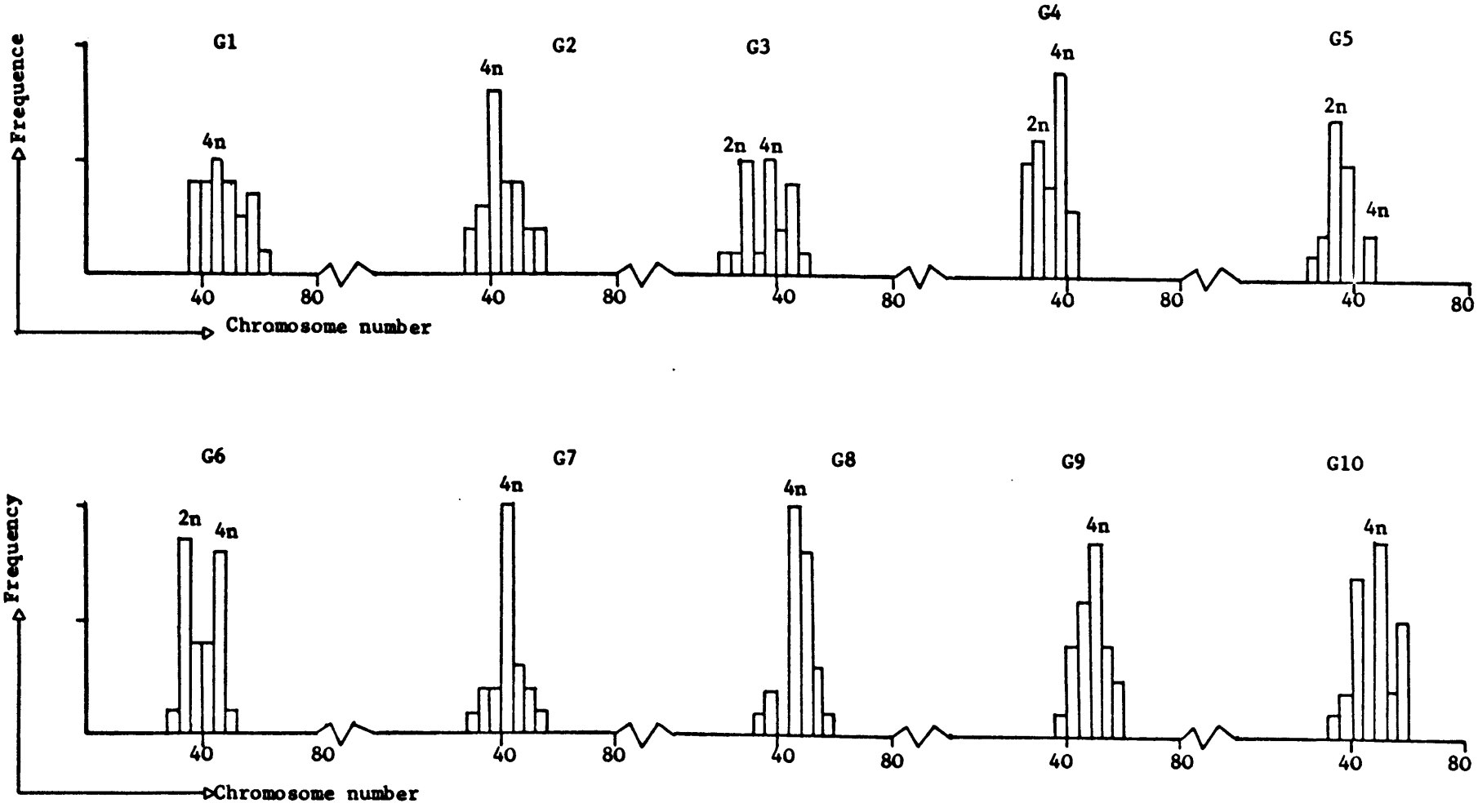


Figure E.3. Frequency histograms of chromosome counts for progeny of the unstable polyploid variant (3340) induced after gamma irradiation (frequency in 25 cells).

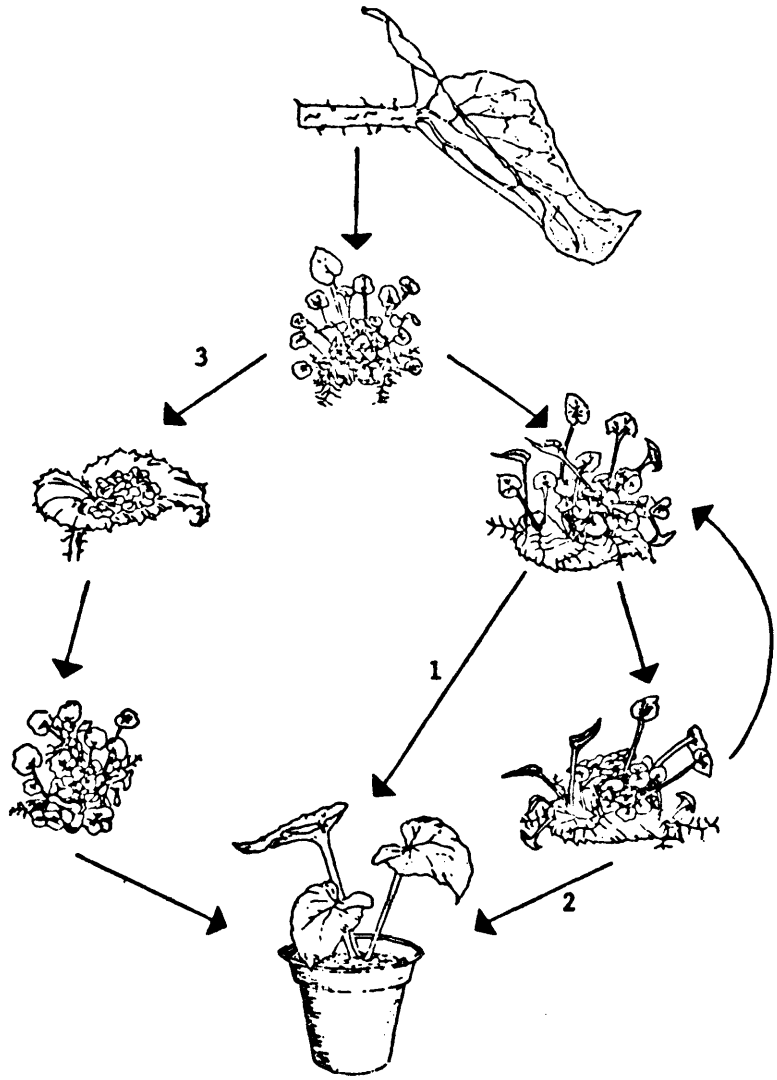


Figure E.4. Schematic diagram of micropropagation pathways for the expression of somatic variation which may be present in unstable polyploids induced after gamma irradiation.

1. Discontinuous culture from singly subcultured axenic explants.
2. Continuous culture of axenic leaf explants.
3. Callus culture.

to establish the degree of instability present in this plant and the potential for the production of variants from callus.

Other gamma irradiation induced variants introduced into culture include the small apparently green leaf colour variant (see Plate E.1.) observed early in culture. A large population of plantlets were successfully produced from the original two axenic leaves after a number of subculture cycles. When planted out all but one of 50 plants failed to establish. The surviving plant had a dwarf habit and closely compacted small leaves. Low survival rate however makes this variant unsuitable for commercial production. The silver leaf colour variant (Plate D.1.) which occurred at a high frequency was also introduced into culture and has maintained stability after one subculture passage and through callus indicating this variant can be readily propagated *in vitro*. A further 5 leaf colour variants have been cultured and are presently being trialled in a commercial nursery in Germany.

E.2.4. Discussion

Axenic leaf explant culture has been successfully used here in the production and propagation of variants from *B. rex*. The procedures and results outlined illustrate the efficiency of the system which gives a high percentage of responding explants and a large population of plants for assessment.

In the present experiment phenotypic variants were, with one exception, only evident after 8 weeks of growth under greenhouse conditions. This emphasizes the necessity for planting out *B. rex* tissue cultured plantlets for the assessment of variants. A similar requirement has also been noted for other plants produced from tissue culture e.g. potato (Cassells *et al.*, 1983, 1986).

The mutation frequency reported here (6%) for *B. rex* is considerably less than that reported for *B.* x *hiemalis* (40%) (Doorenbos and Karper, 1975) and *Streptocarpus* (Broertjes, 1969). A low frequency of mutation has also been reported in diploid *Achimenes* treated with x-rays and Broertjes (1972) suggests that this may be due to the low level of heterozygosity present, as evident from the absence of spontaneous mutants in controls. A similar explanation may apply to the *B. rex* cultivar studied here.

The wide spectrum of mutations induced here has not previously been reported in other gamma irradiation treatments of *B. rex*. Previous mutants induced include colour pattern and texture changes (Shigematsu and Matsubara, 1976).

Chromosome counts have not previously been provided for mutants produced from irradiation treatment of *Begonia*. In the present study chromosome counts on leaf colour and pattern variants indicate no major change in chromosome number suggesting these changes may be a result of gene mutation, chromosome rearrangements or deletion. Single gene changes have also been suggested as the source of flower colour variants produced by vegetative propagation of unstable chimeral *B.* x *hiemalis* cultivars (Arends, 1970). Here, only plants with major leaf morphological changes showed a distinct change in chromosome number. The extensive leaf puckering which gave variant 22359 a diseased appearance was found to be associated with the mixoploid state of the leaf. This may explain the puckering observed in the leaf as cells of different ploidies may have different rates of growth. Similar observations have been made by Dermen (1965) on colchicine induced mixoploid pear and apple leaves. Another plant with a thickened leaf lamina, short petioles and a dwarf habit was found to be polyploid and these vegetative characteristics have been previously ascribed to induced polyploids in other plants (Stebbins, 1971).

The exact origin of the mixoploid and polyploid plants produced here may only be speculated on. The origin of the polyploid and mixoploid plants may lie in the induction of mutation in a gene or genes controlling mitosis (cf. Orton, 1984b) resulting in the production of a polyploid cell or cells which give rise totally (solid polyploid) or in part (mixoploid) to the cellular composition of an adventitious bud. It is also possible that mixoploids or unstable polyploids may have arisen by mutations in a mitotic gene which caused a permanent effect or spindle function in one or a number of cells. The presence of these cells in a bud apex would result in the continuous production of a mixoploid population of cells (see Vaarma, 1949; Ogura, 1978).

A plant with an apparent polyploid morphology after chromosome analysis was found to contain a small population of diploid cells. While the presence of these cells may be attributed to either of the above origins, the presence of diploid cells in induced polyploids has also been ascribed to genetic instability and the occurrence of mitotic and nuclear abnormalities (cf. Fukumato, 1962). Some cells of this plant were found to show asynchronous division of their constituent genomes with differential contraction of chromosomes in

different metaphase plates. Other cells showed the presence of condensed chromatin in one half of the cell. This premature chromosome condensation may act as a mechanism for directional chromosome elimination, as suggested for some hybrids (e.g. Finch and Bennett, 1983).

Adventitious shoots produced from tissue cultures of this predominantly polyploid plant showed distinctive thickening of their leaf lamina. Chromosome counts of the progeny indicate that some of the shoots are solid polyploids, which suggests they arose from a zone of polyploid cells, while others are mixoploids possibly arising from zones of polyploid and diploid cells. It is possible that these progeny may develop to produce a range of different phenotypes and plants are presently being grown on for further study.

In the present experiment nearly all the variants produced were solid (non-chimeral). Putative chimeras being observed as 12 percent of the total mutant population or 0.7 percent of the total population. This suggests that each adventitious bud originates from one or a small number of cells either mutated or non-mutated. Where cells are mutated in many cases surrounding cells fail to divide (cf. Broertjes, 1976), hence allowing the development of a solid mutant. Where mutated and non-mutated cells survive and are incorporated into the structure of an adventitious bud, non-mutated cells may outcompete mutated cells (diplontic selection cf. Broertjes and Keen, 1981) (or vice versa), or mutated cells may be retained within the plant as random mutated tissue sectors or may be incorporated into the apex in a chimeral organization (sectorial, mericlinal or periclinal, cf. Broertjes and Van Harten, 1978) (See Figure B.4.). The expression of mutated cells may occur at a specific stage in development or after events which specifically induce the division and differentiation of mutated cells (see Section *B.3.4.1.*). The above events may underly the production of leaf sports in some of the plants which were observed only on maturity or after leaf pruning due to scorching. These individual plants produced a number of different leaf sports, some of which occurred in combination on the same plant. These leaf sports were characterized by changes in colour, pattern or leaf form. The origin of mutated cells in these plants cannot be fully resolved until mature plants derived from both leaf sports and normal leaves are assessed.

E.3. The effect of colchicine treatment on plantlet regeneration from tissue culture of B. rex by the adventitious bud technique

E.3.1. Introduction

A number of studies demonstrate that chromosome changes other than chromosome reduplication may be induced by colchicine treatment (see Section *B.3.5.3.*). Furthermore, the propagation of plants produced after colchicine treatment can result in the production of phenotypic variants as a consequence of the segregation of unstable genotypes (e.g. Sanders and Franzke, 1962). Hence colchicine treatment may cause extensive variation in the genotype and may be used not only as a method for the induction of polyploids but may, depending on the genotype, result in the production of a range of plant phenotypes.

The uptake and effect of colchicine on plant cells is influenced by a number of factors including genotype, concentration and the method and duration of application (Section *B.3.5.3.*). Thus, in establishing a procedure for the treatment of explants with colchicine to induce variation, it is essential to assess a range of colchicine concentrations and evaluate methods which may enhance uptake and effect.

The success of any procedure is dependent on the presence of a population of actively dividing cells and hence a suitable temperature for active division is essential. The addition of growth hormones may increase the number of cells affected. Also the addition of an adjuvant such as DMSO, which enhances the uptake of colchicine (Section *B.3.5.3.*), may increase polyploid production and decrease the number of chimeras produced (e.g. Novak, 1983).

The mode and duration of pretreatment with colchicine can play a significant role in the production of polyploids (e.g. Goldy and Lyrene, 1984). Liquid pretreatment with colchicine has been advocated as a superior method for polyploid induction as it ensures immersion of all cells and facilitates the treatment of large numbers of explants.

The present study is a preliminary assessment of the influence of liquid pretreatment of *B. rex* petiole explants with a range of colchicine concentrations, in the presence or absence of DMSO, on the production of polyploids and other variants in *B. rex*.

E.3.2. Material and method

Petioles were surface sterilized and prepared for culture as previously outlined (Section *C.2.1.*).

Petiole sections were cultured in liquid media containing Murashige and Skoog (1962) basal nutrients 30 g/l sucrose, 1 mg/l BAP and colchicine in the concentrations 0, 300, 500, 1000, 1800 mg/l both in the presenceand absence of 4% (v/v) DMSO in 120 ml screwtop jars with a total of 10 petiole segments per treatment. Cultures were placed on an orbital shaker at a speed of 180 rpm under standard growth room conditions. After 48 hours explants were removed from colchicine, rinsed twice in sterile water and cultured on SDM (Table C.1.) in petri dishes under standard growthroom conditions. Responses of petioles were assessed after a month of culture and responding explants were cut into 4 and transferred to SDM, in 60 ml jars for further plantlet development. After a further 2 months of culture when plantlets were fully developed plantlets greater than or equal to 2 cm in height were planted out. Plantlets were established and grown on to maturity as outlined in Section *C.2.4.*

Leaves from plantlets showing obvious polyploid characteristics, such as a thickened leaf lamina, if observed in culture were cultured on APM (Table C.1.) and the plantlets produced were grown on for assessment. *In vivo*, developing plantlets showing obvious polyploid characteristics were assessed for chromosome number using the procedure outlined in Section *C.5.* Chloroplast counts were also undertaken using the procedure outlined in Section *C.6.*

E.3.3. Results

Contamination in most treatments was high due to the use of liquid pretreatment (Table E.3.). With 5 explants per jar, contamination if present, affected a large number of explants. High concentrations of colchicine (1,000-1,800 mg/l) when combined with DMSO reduced the induction and development of shoots. Treatments with 1,800 mg/l were particularly affected and plantlet production in these treatments was either inhibited or the plantlets produced were not vigourous.

While in culture, polyploid plantlets were only observed in treatments with 500 mg/l colchicine. A leaf of one of these plantlets when cultured produced both normal and polyploid shoots which suggests the presence of both polyploid and diploid cells in this leaf. Other leaves which were cultured failed to produce shoots. Since planting out a total of 28 plantlets with polyploid morphologies (see Plate E.10a) have been observed from all treatments. The highest percentage of polyploids occurred in the 1,800 mg/l colchicine treatment (total

Table E.3. Colchicine and DMSO treatment of petiole explants of *B. rex*.

Colchicine treatment mg/l	Total number of responding explants	Intensity[a] of response	Total plantlets planted	Total* putative polyploids (%)
300	4	+++	60	7(11)
500	5	+++	71	5(7)
1,000	3	+++	50	2(4)
1,800	3	+++	50	12(24)
500 + DMSO	5	+++	50	2(4)
1,000 + DMSO	7	++	50	0
1,800 + DMSO	4	+	50	0
Controls	5	+++	50	0

* - observations after 2 months of growth in the glasshouse.

a - +++ = total explant coverage with buds
++ = buds scattered over explant surface
+ = 10-20 buds produced of low vigour



Plate E.10a. Comparison of leaves from a diploid (a) and a polyploid (b) plantlet.

Table E.4. Chromosome counts in plantlets without polyploid morphologies induced on colchicine treated petiole explants.

Plant Number	300		500		1000		1,800		500+DMSO		1,000+DMSO		1,800+DMSO	
	2n	non-2n[a]	2n	non-2n	2n	non-2n	2n	non-2n	2n	non-2n	2n	non-2n	2n	non-2n
1	21	4	25	-	22	3	25	-	25	-	25	-	24	1
2	25	-	19	6	25	-	24	1	25	-	25	-	25	-
3	20	5	24	1	17	8*	24	1	24	1	25	-	23	2
4	25	-	24	1	24	1	25	-	24	1	25	-	25	-
5	25	-	21	4	24	1*	25	-	25	-	24	1	25	-
6	25	-	17	8	25	-	-	25	24	1	24	1	25	-
7	25	-	21	4	24	1*	25	-	25	-	25	-	25	-
8	25	-	24	1	25	-	25	-	25	-	25	-	25	-
9	25	-	25	-	24	1	25	-	25	-	25	-	25	-
10	24	1	25	-	25	-	25	-	25	-	25	-	25	-
Total	240	10	229	21	235	15	223	27	246	4	248	2	247	3
% non 2n	4		8		6		10		2		0.8		1	

a - haploid or polyploid cells

* - indicates haploid cells

of 12 plantlets). A number of these plantlets once established were assessed for chloroplast number and chromosome counts (see Appendix).

An assessment of chromosome numbers in a random sample of 10 plantlets from each treatment prior to planting (Table E.4.) demonstrates the presence of a mixed population of diploid and polyploid or haploid cells in some plantlets. Polyploid cells occurred most frequently in plantlets from treatment with 500 mg/l colchicine while a high frequency of haploid cells were found in treatments with 1,000 mg/l colchicine. In all treatments genetic instability manifest as condensation of part of the chromosome complement was evident in a varying percentage of cells. There was no set trend in the occurrence of this form of instability, although DMSO treatment appeared to increase its occurrence in treatments with 500 and 1,000 mg/l colchicine. The effect of DMSO on the level of polyploidy and variants induced will be fully assessed when plantlets are grown to maturity. For chromosome and chloroplast counts of putative polyploid progeny from these colchicine treatments see variants 5315, 5304, 5265 and 5290 in Appendix Figures I.1. and I.2.

E.3.4. Discussion

In *B. rex* high concentrations of colchicine (up to 1,800 mg/l) applied for 48 hours do not significantly influence explant survival or plantlet induction. This is in contrast with other ornamentals e.g. *Saintpaulia* which is highly sensitive to colchicine (Broertjes, 1974).

From the results recorded to date increased in the colchicine concentration used for pretreatment do influence the number of polyploids produced and demonstrate the need to test a range of colchicine concentrations when assessing for optimal levels to induce polyploidy. While DMSO did not have any apparent affect it may however have caused colchicine to induce other effects e.g. chromosomal rearrangement (Eigsti, 1940). The influence of these changes on the phenotype of *B. rex* are not evident at the plantlet stage and may become manifest at a later stage of growth.

To date the percentage of polyploids induced is low. This may be due to a number of factors. Colchicine may not have had an effect on the total population of dividing cells present during treatment either due to lack of penetration, rapid recovery, resistance to colchicine influence or differences in cell division rates (see Section *B.3.5.3.*). It is also possible that plantlets may have arisen from cells dividing

after transfer to control media once the treatment was carried out. The low number of polyploids produced may also be a consequence of the multicellular nature of adventitious buds. As previously discussed (Section *B.3.4.5.*) adventitious buds are composed of cells derived from one or a small number of cells generally originating in the epidermis (cf. Broertjes and Keen, 1981) hence only when a sector of polyploid cells are produced and when all cells contributing to the bud apex are polyploid will a solid polyploid develop. On the basis of present results this appears to occur very rarely in the treatments applied here.

Due to the multicellular nature of bud apices a small population of polyploid cells if present may not show phenotypic expression until a later stage of growth and hence the possibility of their presence must be borne in mind when interpreting results of progeny assessment at maturity. It is also possible that these cells may remain latent and may only be expressed when procedures for propagation are applied which allow their participation in the development of adventitious buds.

The genetic instability observed in some plants as chromosome condensation has also been observed in chimeras induced by gamma irradiation (Section *E.2.3.*) and in the tissue culture progeny of gamma irradiation induced polyploids (Section *E.2.3.*). The influence of this instability on the phenotype of *B. rex* plantlets produced after colchicine treatment remains to be evaluated. Similar genetic instability has been recorded in colchicine treated *Rubus* plants and their progeny (Vaarma, 1949). Cytological studies on these plants also demonstrated the presence of diploid, haploid and polyploid cells within individual tissues and similar mixoploid populations have been recorded in colchicine treated *B. rex* plants (Table E.4., see also Figure I.1.).

Reduction in chromosome number may be based on the presence of a number of different mitotic abnormalities (see Figure B.1.), a number of these have been recorded in the colchicine treated plants observed in the present experiment suggesting such events may underlie the differences in chromosome number observed between individual cells of *B. rex* progeny arising from explants treated with colchicine. Mixoploids however may, as already suggested (Section *E.2.4.*) also be the result of the multicellular nature of adventitious buds production in *B. rex*.

Increases in the numbers of polyploids induced in *B. rex* using the adventitious bud technique may possibly be achieved by pretreating explants for longer periods with colchicine, thereby exposing a greater number of dividing cells to the influence of colchicine and increasing the likelihood of the production of polyploid sectors. Despite the low number of polyploids induced in the present experiment however, it must also be remembered that polyploids may not be the only variants induced by colchicine treatment and the presence of other variant phenotypes may be demonstrated in future progeny assessment.

E.4. Callus culture of Begonia rex

E.4.1. Introduction

The production of plants from tissue culture using adventitious regeneration from callus culture has in many cases resulted in the production of a wide range and high frequency of variant phenotypes (see Section *B.3.*). For this reason callus culture has been advocated as providing a useful adjuvant to conventional plant breeding (see Larkin and Scowcroft, 1981, 1983a).

Callus culture may be used in a number of ways to produce variants. Regeneration from primary callus has been shown to produce variants from many plants, particularly those with a complex genetic architecture or unstable genotype (e.g. D'Amoto *et al.*, 1980). In both these and other plants subcultured calli if competent may produce a wider range and higher frequency of variants (e.g. McCoy *et al.*, 1982). While not yet reported aging of callus cells by extended maintenance on a single medium without subculture may also increase the number of variants produced. This proposal is based on the reported increase in spontaneous mutations produced in plants derived from plant cells aged *in vivo* (D'Amoto and Hoffman Ostenhoff, 1957).

In *Begonia* the potential for the production of variants from callus culture has not yet been assessed. While callus has been produced and plants regenerated (Section *B.2.1.2.*) none of the plants were grown to maturity for assessment. Also the effect of callus culture on the genetic stability of cells in *Begonia* and the chromosome number present in regenerated plants is not known.

The present study of callus culture of *B. rex* firstly establishes a system for the rapid production, subculture and regeneration of callus. Preliminary studies on the influence of subculture and aging on the

production of variants have also been undertaken. To establish the presence and source of chromosomal variation in callus (i.e. pre-existing or induced) cytological studies on the explant derived callus, subcultured and aged calli were evaluated. Studies on chromosome numbers in regenerated plants from these calli were also performed to assess to what extent chromosomal variation, or mitotic irregularities, if any, were present in regenerated plants.

E.5. Induction, proliferation and regeneration of callus from B. rex

E.5.1. Introduction

Callus production in many plants has been successfully induced using high concentrations of auxin (cf. Thorpe, 1982). In *B. rex*, Bigot (1971) produced callus from pith and petiole explants using 3 mg/l 2,4-D. 2,4-D has also been used to induce callus in other begonias (e.g. Margara and Piollat, 1982).

In the factorial studies reported in Section *D.2.1.* a small amount of callus was produced on explants cultured on high concentrations of NAA. This callus when subcultured on the induction medium failed to proliferate and underwent rapid organization to produce roots.

The present study, by assessing the effect of different concentrations of 2,4-D on callus production and proliferation from mature and axenic leaf and petiole explants, aims to increase the quantity of callus produced and to standardize an explant source and medium for callus production, proliferation and regeneration. To determine the origin of plantlet production and the nature of change occurring prior to callus regeneration histological and histochemical studies were also undertaken on regenerating calli.

E.5.2. Material and method

E.5.2.1. Callus induction

Mature and axenic leaf and petiole explants were prepared for culture as outlined in Section *C.2.* Ten replicas of each explant were placed aseptically on media containing Murashige and Skoog (1962) basal nutrients, 30 g/l sucrose, 6 g/l agar, and combinations of the hormones 2,4-D and BAP (see Table E.5.) in 60 ml screwtop jars. After 2 months of culture in the light under standard growthroom conditions explants were assessed for callus production.

E.5.2.2. Callus proliferation

Callus produced on explants was aseptically excised and plated onto media in petri dishes containing the same concentrations of hormones responsible for its induction. After a month of culture in the dark at 22°C the degree of proliferation of callus was assessed.

E.5.2.3. Regeneration of callus

Subcultured callus clumps of approximately 25 mg were transferred to SIM (Table C.1.) for regeneration in the light. After 12 weeks of culture induced buds were transferred to SDM for development of plantlets. The number of plantlets produced per callus was evaluated after a further 4 weeks of culture. Events occurring during callus regeneration were observed in sections prepared as outlined in Section *C.6.1.* Staining procedures for starch and cell viability were as outlined in Sections *C.6.1.* and *C.6.3.* respectively.

E.5.3. Results

Explants from mature tissue failed to respond on many of the media tested, and on media where they did produce callus, the number of responding explants and the amount of callus produced was very low (Table E.5.). The callus produced on mature leaf explants was limited to zones adjacent to the main vein and was highly organized into compact cream coloured nodules. Callus on petiole explants was scattered at random over the surface as discrete nodules which grew slowly. Callus from both mature leaf and petiole explants when subcultured proliferated very slowly on all of the media tested and none of the calli regenerated when transferred to SIM.

In contrast to mature explants, callus production on axenic explants was rapid and in general replicas gave 100 percent response. Callus when produced on axenic leaves was generally produced over the total surface of the leaf and organized into compact nodules of creamy yellow callus. When subcultured, particularly on high levels of 2,4-D callus proliferated rapidly but maintained the nodular form of growth (Plate E.10.).

Callus produced on axenic petioles was similar in appearance to that produced on axenic leaves but did not grow as rapidly and it was difficult to separate callus from the explant. The small amount of callus produced on these explants furthermore limited its proliferation on subculture.

Table E.5. Responses of mature and immature leaf (L) and petiole (P) explants of *B. rex* to combinations of the hormones 2,4-D and BAP.

Hormone concentration mg/l		Cultures with callus %				Intensity of callus production				Proliferation intensity			
		Immature		Mature		Immature		Mature		Immature		Mature	
2,4-D	BAP	L	P	L	P	L	P	L	P	L	P	L	P
0.1	0.01	100	100	25	20	+	±	+	±	+	±	±	±
0.1	0.05	100	100	0	20	+	+	0	±	+	±	0	±
0.1	0.1	0	0	0	20	0	0	0	±	0	0	0	±
0.5	0.1	60	40	50	40	++	++	±	±	+	±	±	+
0.5	0.05	100	80	50	40	++	+	±	±	++	+	±	+
0.5	0.1	50	50	50	40	+	±	±	±	+	±	±	+
0.75	0.01	100	100	0	0	+++	++	0	0	+++	+	0	0
0.75	0.05	70	60	0	0	++	+	0	0	++	+	0	0
0.75	0.1	80	80	0	0	+	±	0	0	+	+	0	0

0 = no response
± = sparse
+ = scattered clumps
++ = medium growth
+++ = intensive growth high quantity

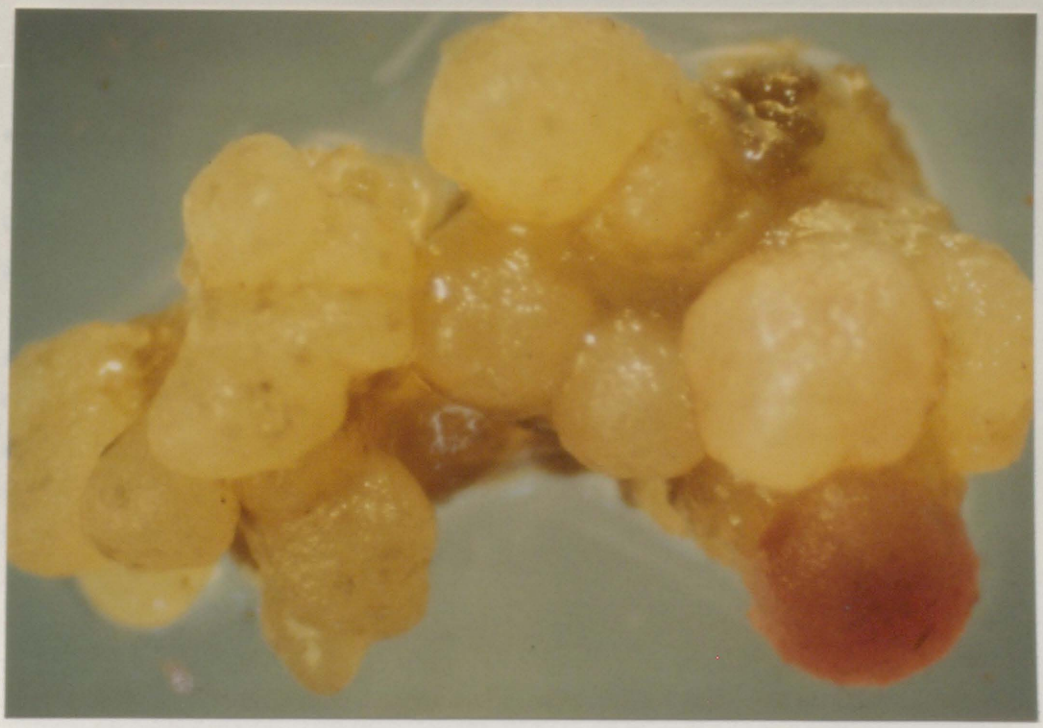


Plate E.10. Callus nodule of *B. rex*.



Plate E.11. Regeneration sequence for callus of *B. rex*.

Due to the rapid production and proliferation of callus from leaf explants this explant was used for further studies on callus culture. Of the media tested a combination of 2,4-D at 0.75 mg/l and BAP at 0.001 mg/l (Table C.1., CM) was selected as a standard medium for the induction and proliferation of callus.

Regeneration in *B. rex* callus was found to follow a particular sequence of morphogenic responses which are illustrated in Plate E.11. On transfer to regeneration medium calli accumulated a red pigmentation after one week of culture. After 4 weeks the originally smooth surfaced nodules became pitted and root like structures emerged. Nodules also began to enlarge and after a total of 8 weeks of culture chlorophyll accumulation was evident. Shoot primordia emerged after 10-12 weeks of culture and these rapidly developed into plantlets once transferred to SDM. The mean number of plantlets produced per callus was 100. All the plantlets produced were morphologically normal in appearance and many had their own root system.

The histological and histochemical events observed during the 12 weeks prior to regeneration have been outlined in Table E.6. The nodule of the callus may be divided into 3 distinct zones, this includes a central core of meristematic cells, an outer layer of parenchymatous cells and an extreme outer layer of highly irregularly shaped cells. As outlined in Table E.8. changes occurred in the distribution and concentration of starch, anthocyanin and chloroplasts during callus regeneration. The viability of cells also changed particularly in the first 2 weeks on regeneration media where rapid death occurred in all cells except those in the central core. Anthocyanin accumulated gradually in the first 2 weeks and was limited to a distinct layer of cells surrounding the central core. These cells grew out in a layered arrangement with the development of the root and only a few anthocyanin synthesizing cells were retained within the callus. Chloroplasts too, showed a distinct accumulation with a rapid build up within the central core prior to meristem formation. The accumulation of starch within the central core was very pronounced with a very high level of starch being produced in weeks 9 and 11, starch levels declined rapidly once shoots were produced. The presence of trachery elements was evident after 2 weeks of culture and were also observed for a further 4 weeks. Due to the accumulation of starch over the last 6 weeks it was not possible to observe either meristematic zones or trachery elements in the central core of cells.

Table E.6. Histological and histochemical observations on changes occurring during callus differentiation in *B. rex*. Observations on hand sections of callus on regeneration media taken at 1 to 12 weeks.

Week	Chloroplasts		Anthocyanin		Starch		Viable Cells	Trachery Elements	Meristematic Zones
	Presence	Location	Presence	Location	Presence	Location			
1	±	OC	±	OC	±	OC	AC	-	-
2	±	OC	±	OC	+	OC	AC	+	-
3	+	AC	++	OC	++	OC	CC	+	+
4	+	AC	+	RC/OC	++	OC	CC	+	+
5	+	AC	+	RC/OC	++	OC	CC	+	±
6	+	AC	±	RC/OC	++	CC	CC	*	*
7	++	AC	±	RC/OC	++	AC	CC	*	*
8	++	AC	±	OC	++	AC	CC	*	*
9	++	CC	±	OC	+++	CC	CC	*	*
10	++	CC	±	OC	+++	CC	CC	*	*
11	++	CC	±	OC	+++	CC	CC	*	*
12	+++	CC	±	OC	+	CC	CC	*	*

CC = central core
AC = All cells except outer layer
OC = outer layer of cells
RC = Root cells

- = absent
± = sparse
+ = scattered presence

++ = medium accumulation
+++ = intensive accumulation

* = observations not possible due to intensity of starch grain accumulation

Sections taken after shoot production indicate that all the shoots produced originated in the central core of cells.

E.5.4. Discussion

The more intensive production of callus from axenic leaf explants as compared to mature explants, may, as previously discussed (Section *D.2.1.*), be linked to limitations in the ability of cells in mature explants to respond to exogenous hormones. Similar restrictions in callus production from mature explants as compared with immature explants on a range of media have also been noted in other plants e.g. Pea (Rubluo *et al.*, 1984).

The nodular growth form of the *B. rex* callus contrasts markedly with the friable and disorganized growth normally associated with calli (Aitchenson *et al.*, 1980). As indicated by histological studies, this growth form may be related to the presence of a central core of dividing cells rather than a scattered population of meristematic cells in outer zones as found in other calli e.g. *Helianthus* (Yeoman, 1970) where the inner core of cells is dead.

In studies on changes occurring during differentiation of these calli, all the major changes were found to occur primarily in central core cells. These studies suggest that differentiation in *B. rex* callus is associated with the gradual accumulation of anthocyanin, starch and chlorophyll. The changes which occur in starch accumulation prior to shoot production and the rapid decline once shoots are initiated have also been recorded in tobacco callus during regeneration (reviewed by Thorpe, 1982) and is believed to be associated with a requirement for a high level of starch for organogenesis. Complex leaf explants of *B. rex* have also been shown to have a requirement for starch to undergo organogenesis (Chlyah, 1972).

The accumulation of anthocyanin in callus after transfer to the regeneration medium may be associated with cell differentiation. Anthocyanin accumulation has also been reported to occur with cell differentiation in carrot (Ozeki and Komamine, 1981). The gradual greening which occurred in callus prior to shoot organogenesis indicates chlorophyll accumulation and is of common occurrence in regenerating calli (e.g. Thorpe, 1974).

The production of high numbers of shoots by calli is indicative of a high level of competence in callus cells of *B. rex* and may be related to the presence of an active meristematic population of cells as described above.

E.6. Assessment of the morphogenic potential and phenotype of plants produced from primary and subcultured calli of B. rex

E.6.1. Introduction

Primary calli, in particular those produced by nuclear fragmentation have been postulated as providing a greater potential source of chromosomal variation than calli derived from subculture cycles (D'Amoto *et al.*, 1980). Evidence from cytological studies on the chromosomal composition of a number of plant calli however suggest that during subculture the chromosomal composition of callus cells can fluctuate undergoing periods of stabilization and diversification (see Section *B.3.3.*). At the plant level there are many examples of increases in the range of variants produced from callus subcultures (e.g. Novak, 1980). But there are also examples of studies where 1) the number and range of variants produced is similar at different subculture stages (e.g. Cassells *et al.*, 1983) or 2) no variants are produced even after long periods of subculture (e.g. Sheridan, 1974). These differences have been attributed to a number of factors including culture conditions, media and plant genotype (Section *B.3.3.*). Due to this range of influencing factors the effect of subculture on the phenotype of plants regenerated from a given plant callus cannot be predetermined. For this reason the present study has assessed regenerants from both singly and multiply recycled *B. rex* callus to evaluate the influence of callus subculture on the production of variants in *B. rex*.

During subculture many plant calli undergo a reduction or loss of morphogenic potential (see Section *B.3.4.7.*). Here the morphogenic potential of *B. rex* calli has also been assessed by a quantitative evaluation of the number of shoots produced per callus.

E.6.2. Material and method

Callus induced on axenic leaves on CM (Table C.1.) was subcultured at 4 week intervals in the dark for a period of 6 months. At appropriate times callus clumps of approximately 25 mg from the first, fourth, fifth and sixth subculture were inoculated onto regeneration medium (SIM Table C.1.) in 60 ml screwtop jars and cultured under standard growthroom conditions for 12 weeks before transfer to SDM (Table C.1.). After a further 4 weeks of culture the number of plantlets produced per callus were counted. Plantlets greater than or equal to 2 cm in

height were planted out in potting compost (Section *C.2.3.*) in heated propagators in the glasshouse. A control population of plantlets derived from axenic leaves produced as outlined in Section *C.2.2.* was also planted out. Further transfers to allow plant growth to maturity were as outlined in Section *C.2.4.*

E.6.3. Results

There was no noticeable difference in callus growth rate during subculture and calli retained their nodular organization. When transferred onto regeneration media the sequence of events leading to shoot production was similar for each subculture generation assessed (see Plate E.11.). However, as indicated in Table E.7. both the number of responding calli and the number of shoots produced per callus decreased in successive subcultures. In contrast to the 75 percent response observed for primary calli and the production of an average of 100 plantlets per callus only 23 percent of calli from the sixth subculture responded producing on average 11 plantlets per callus. Many of these subcultured calli produced roots but failed to produce shoots and instead accumulated large amounts of phenolics which caused rapid senescence. The low number of shoots produced from subcultured calli meant that only a small population of plants were available for assessment.

On planting out none of the plantlets produced from any of the calli populations showed any significant phenotypic variation. The percentage plant survival was highest in populations from the fifth and sixth subculture, 60 and 70 percent respectively as compared with 40 percent for the first, this may be related to the low number of shoots induced in these subcultures which enhanced shoot development.

At maturity, because of the large population available, the plants produced from the first subcultured callus were quantitatively assessed for vegetative characteristics and compared with controls (see Table E.8.). Mean values suggest differences between populations in total leaf area per plant, however the difference was not significant ($P > 0.05$). Frequency plots (Figure E.5.) for both populations also demonstrate that interpopulation variation is similar for all characters with the exception of plant diameter and leaf area. Median value in controls is higher than that in callus populations and there are more plants of a similar diameter and area in this population than in the callus population.

Table E.7. Results of callus subculture and plant regeneration in *B. rex*. Figures in brackets represent percentages.

	Callus Source			
	Sub. 1	Sub. 4	Sub. 5	Sub. 6
Total calli cultured	10	8	20	22
Contaminants	2	1	1	2
Total shoot producing calli	6(75)	3(43)	3(15)	5(23)
Total plantlets/callus (mean)	109	56	23	11
Total plantlets planted	142	41	41	24
Total survival (%)	60(42)	17(41)	25(60)	17(70)
Total variants	1	0	0	0

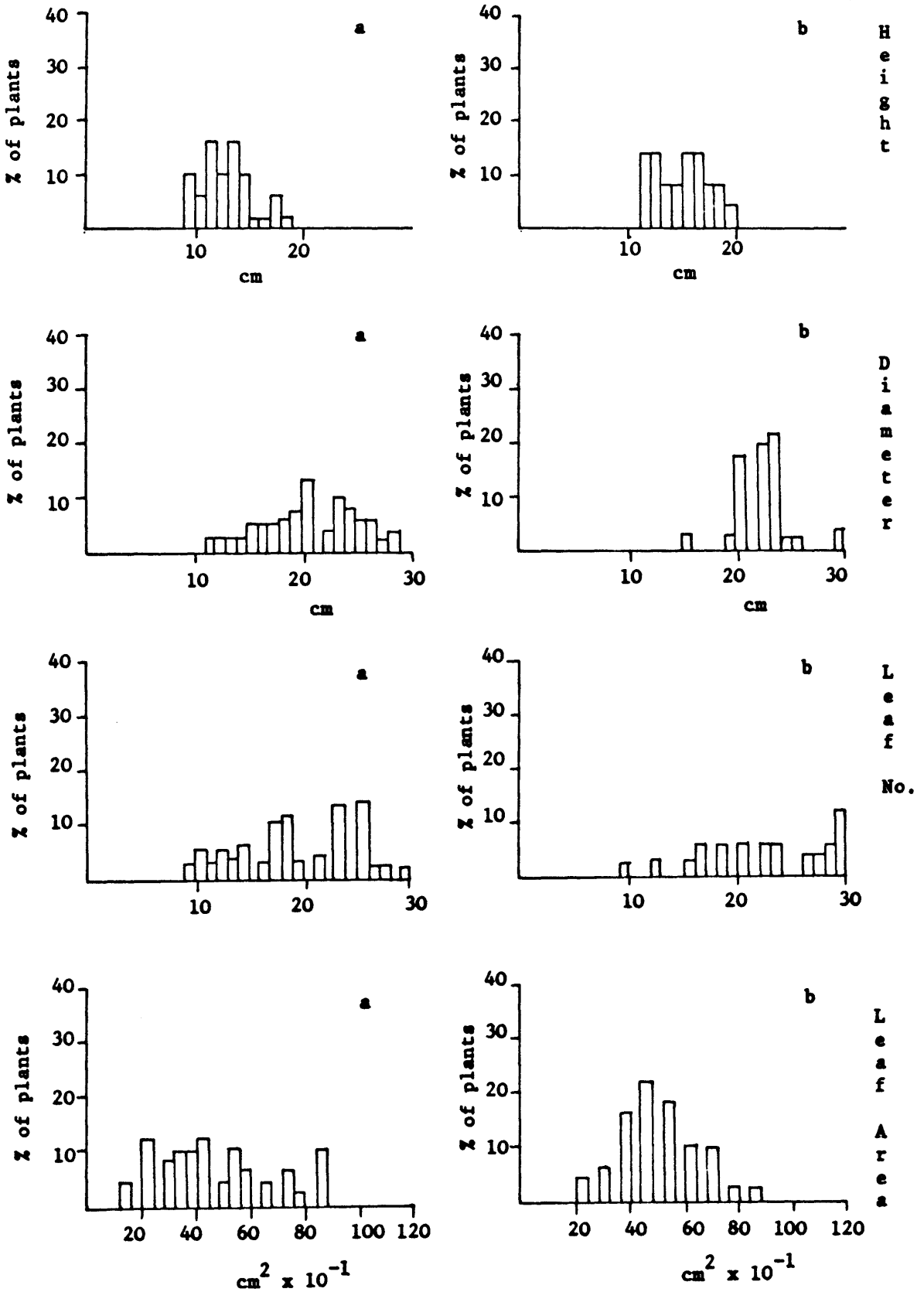


Figure E.5. Frequency plots of four vegetative characteristics of populations derived from singly subcultured callus (a) and controls (axenic leaves) (b).

Table E.8. Vegetative characteristics of plants produced from singly subcultured callus and axenic leaves (controls).

Character	Population	
	Controls	Callus
Plant height (cm)	14±3	14±4
Plant diameter (cm)	25±5	22±5
Leaf number	24±9	17±7
Total leaf area/plant cm^2	646±265	540±304
Mean leaf area/plant cm^2	24±9	29±9
Petiole length (cm)	5±1	5±1
Rhizome number	5±2	2±1
Rhizome weight (grms)	8±4	5±4

All but one plant produced from callus culture were phenotypically similar to the control. The variant produced was a colour variant with ruby centre and magenta margin (Plate E.5.C.V.).

Observations on the habit, leaf number, vigour, leaf morphology and colour characteristics of each of the remaining subculture populations compared with controls indicate that these plants showed no change in phenotype.

E.6.4. Discussion

The reduction in plantlet production reported here for subcultured callus of *B. rex* has also been reported for subcultured calli of other plants (Section *B.3.4.7.*). The direct cause for this loss of competence is unknown but it has been suggested that genetic or physiological factors may be involved (Smith and Street, 1974). In some plants increases in plantlet regeneration in subcultured calli showing reduced morphogenic capacity have been achieved by environmental manipulations or changing the hormone ratios (Section *B.3.4.7.*) and similar procedures may increase the number of shoots produced from subcultured calli of *B. rex*. To fully determine the degree of variation or stability present in regenerates of *B. rex* callus a large population of plants will need to be assessed hence studies on hormone requirements to increase the number of regenerates from *B. rex* subcultured callus have been undertaken.

While plantlet production in later subcultures was low, plantlet numbers per callus from the first subculture were comparable with those produced from axenic leaf explants (Section *D.4.* c. 100/explant) and it would appear that this callus has maintained the high regenerative capacity found in the axenic leaves from which it was derived. This high level of competence and the absence of any significant deviation in vegetative characters or interpopulation variation in callus plant population would suggest that callus culture may provide a useful means of propagating and storing *B. rex* plants (see Section *B.2.1.2.*). Improvements in plantlet survival and the problem of reduction in adventitious shoot production on subculture would have to be overcome however before this method could be used for large scale propagation.

In the present study plantlets produced from subcultured calli of the fourth, fifth and sixth generations showed no significant phenotypic changes from control populations. Chromosome studies give

further confirmation of the stability of plants produced from callus (Section *E.8.*). While the population assessed was small, these results do suggest that subculture of *B. rex* callus may not result in the production of variants. Reports of stability in other plants regenerated from subcultured callus are rare (e.g. Sheridan, 1974) and there has been speculation regarding the validity of these reports (Bayliss, 1980). It has been suggested that calli which show high levels of stability are not truly dedifferentiated and maintain an actively dividing population of meristematic diploid cells from which plantlets are produced (Halperin, 1973). This would also appear to be the case in *B. rex* as cytological studies on calli indicate the presence of a central core of meristematic diploid cells in all calli assessed (see Section *E.8.*). Hence selective regeneration of plants from these cells may account for the stability manifest in regenerants.

E.7. Callus aging and plantlet regeneration from aged callus in B. rex

E.7.1. Introduction

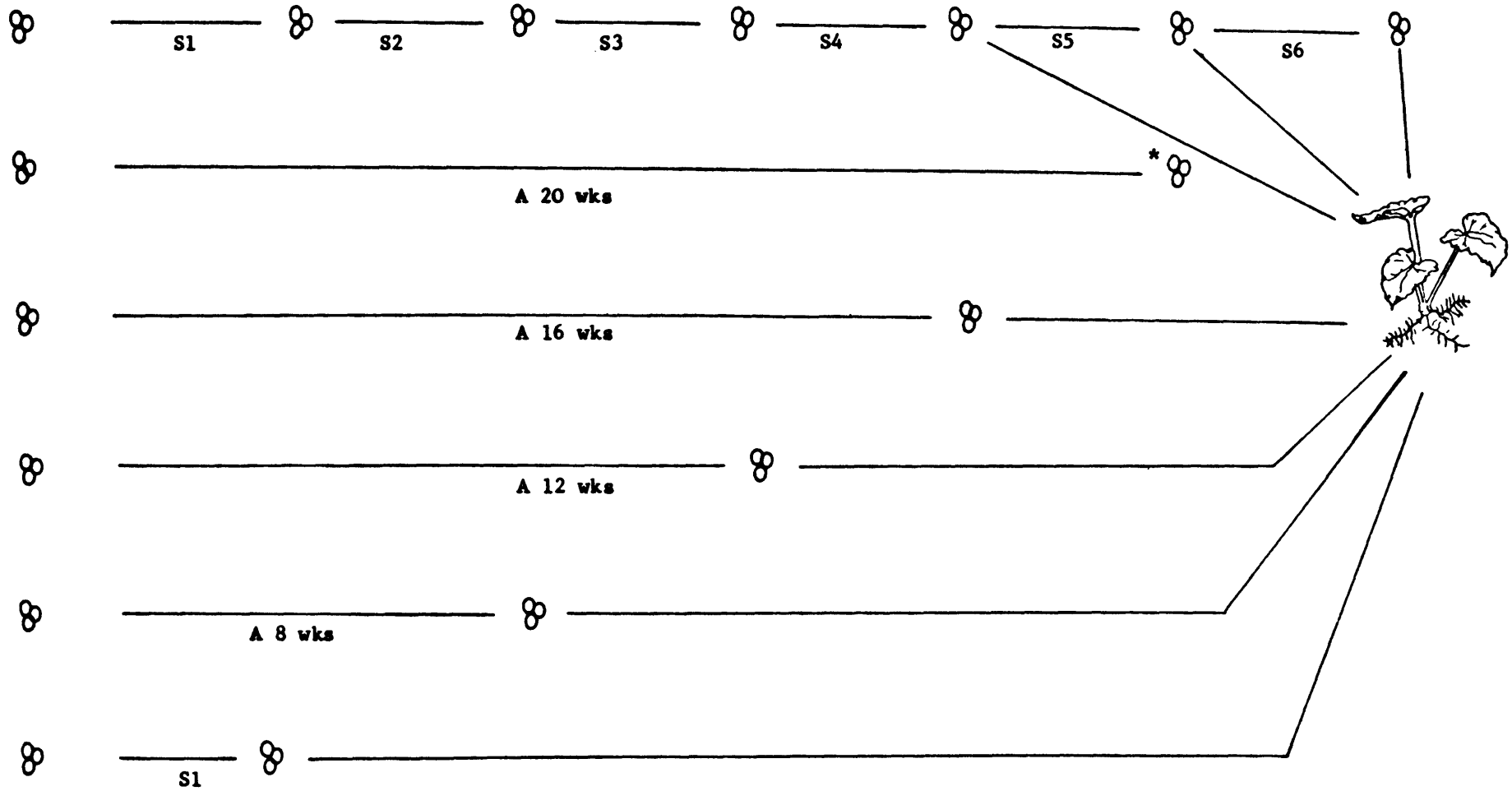
Increases in the production of spontaneous mutations and changes in nuclear DNA in plants *in vivo* have been reported to occur as a result of cell aging or stress (Section *B.3.2.*). These effects have been attributed either to the accumulation of automutagens within aged plant tissue, to a reduction in DNA repair mechanisms or to rapid genome reorganization (Section *B.3.2.*). Similar effects may occur in the cells of plant calli aged and stressed by maintenance for extended subculture passages on one medium. However, the influence of this form of aging and stress on the phenotype of plantlets regenerated from callus culture has not yet been evaluated.

While callus subculture failed to produce any appreciable variation in regenerated plants of *B. rex* (Section *E.6.*) it is possible that if cells of *B. rex* callus maintain their competence extended periods of aging may result in the production of variants. The present study is an assessment of the effect of different periods of aging as illustrated in Figure E.6. on regenerative capacity and variant production from *B. rex* callus.

E.7.2. Material and method

Callus was produced and proliferated as outlined in Section *C.3.* Clumps of approximately 25 mg of aged callus were removed at 8, 12, 16 and 20 weeks after transfer of callus to proliferation medium and

Figure E.6. Schematic diagram of callus subculture and aging in *B. rex* prior to plant regeneration (S = subculture, A = aged). *No regeneration.



placed on regeneration medium SIM (Table C.1.) or SIM3 (Table C.1.) in 60 ml screwtop jars. Calli were cultured in the light under standard growthroom conditions. After 12 weeks bud producing calli were recorded and transferred to SDM (Table C.1.). Plantlets had fully developed after a further 4 weeks of culture and after counting the total number of plantlets produced all plantlets greater than or equal to 2 cm in height were planted out into potting compost in heated propagators ($20^{o}C$). A control population of 50 plantlets produced from petiole explants, regenerated as outlined in Section *C.2.* was also planted out. Further transfers and evaluation were undertaken as outlined in Section *D.4.*

E.7.3. Results

Calli accumulated large amounts of phenolics on aging and nodules became pitted and rough in appearance. When transferred to regeneration media these calli produced shoots directly instead of undergoing a rooting step (see Section *E.5.*).

The percentage of responding calli did not, as anticipated, decrease gradually with aging. While aging reduced the percentage of responding calli as compared with non-aged calli (see Table E.9.) the level remained constant for the 8, 12 and 16 week aging treatments but decreased suddenly to zero after 20 weeks of aging. An evaluation of cell viability in the latter calli using FDA (see Section *C.6.*) indicated that all cells in these calli were dead.

Large numbers of plantlets were produced from some aged calli (e.g. 144 plantlets/callus for 12 weeks aging) indicating that aging did not decrease the regenerative capacity of calli. The majority of plantlets produced from aged calli showed no obvious phenotypic changes. A small population of shoots with a thickened leaf lamina however were produced from one callus clump which had been aged for 16 weeks. Chromosome counts on these plantlets indicate that they were either mixoploid or polyploid (see Table E.12a.).

Of the calli which produced plantlets, calli aged for 8 weeks gave the lowest shoot number, this may be a result of the use of SIM medium for regeneration from this callus, other calli were regenerated on SIM3. Mature plants produced from this callus have not shown any significant phenotypic variation, they are however less vigourous than controls. Plantlets produced from calli aged for 12 and 16 weeks are presently been grown on for assessment.

Table E.9. Results of callus aging and plant regeneration in *B. rex*.

	Callus Source			
	Aged 8 weeks	Aged 12 weeks	Aged 16 weeks	Aged 20 weeks
Total calli cultured	10	5	12	10
Contaminants	4	3	4	2
Total shoot producing calli (%)	3(50)	2(40)	5(41)	0
Total plantlets mean per callus	21	144	100	0
Total plantlets planted	16	130	150	0
Total survival	10	*	*	0
Total variants[a]	0	0	3†	0

a - Noted to date

* - In progress

† - putative polyploids

E.7.4. Discussion

The production of large numbers of shoots from aged calli despite their moribund appearance suggests that a population of highly competent cells is maintained in these calli. This suggestion has been confirmed in studies on callus cytology (Section *E.8.*) where it was shown that a central core of meristematic cells was present in all calli with the exception of those aged for 20 weeks. It is possibly as a result of the absence of meristematic cells that these latter calli failed to produce shoots.

Direct shoot production in aged calli and the absence of rootlike structures which are produced as a stage in regeneration of non-aged calli may be the result of changes occurring in endogenous hormones during aging (e.g. Meins and Binns, 1977) or the use of higher levels of cytokinin in the regeneration medium.

Increases in cytokinin may also be responsible for the high number of shoots produced and the greater response of aged calli when compared with subcultured calli. This suggestion is supported by the fact that calli aged for 8 weeks and regenerated on the same medium as subcultured calli also showed a low number of shoots per callus.

The production of plants with polyploid phenotypes from one clump of aged callus would suggest that aging influences the phenotype of plants produced from callus in *B. rex*. The presence of obvious variants in only one callus may be a result of unique changes which occurred within this particular callus. Inter-callus differences in variant production have previously been noted in other plants (e.g. Austin and Cassells, 1983) and studies at the cell level on *Haworthiana setata* indicate that calli can vary significantly with respect to the relative heterogeneity of their cell populations (Ogihara and Tsunewaki, 1979). It has been suggested that during culture new genotypes may emerge and the relative presence of these cells within a callus will depend on their adaptability to prevailing culture conditions (Ogihara, 1982; see Figure B.6.). Similar phenomenon may have occurred in *B. rex* callus.

The underlying cause for the production of the variants observed may only be speculated on. In view of the presence of postulated mutagenic agents which would have accumulated in calli as a result of cell death (Bayliss, 1980) it is possible that disruptions in the mitotic cycle may have been induced. The depletion of nutrients in

the culture medium and hence in callus cells may also have interfered with the functioning of the mitotic cell cycle (Roest, 1977). These events may also have caused genotypic changes other than polyploidy. The detection of other genotypic effects however will only be evident when plantlets have been grown on in the greenhouse.

E.8. Cytogenetic analysis of callus and regenerated plants in B. rex

E.8.1. Introduction

There have been many studies which focused either on the cytology of plants regenerated from callus culture or on the cytogenetics of cultured cells (Section *B.3.3.*). While these studies provide an evaluation of the degree of variation present in culture or the presence of chromosome variation in regenerated plants they do not allow an assessment of the source of this variation or the degree of attenuation or increase in variation which may occur during plant regeneration. Such an assessment requires a comprehensive study which encompasses the cytology of the explant introduced into culture, the callus produced and the plants regenerated from this callus. Results from these studies help to increase our understanding of the factors which control the presence or absence of variation in cultured cells and regenerated plants knowledge which, as already discussed (Section *B.3.2.1.*) is important to the successful use of tissue culture in both micropropagation and the production of variants.

While the presence of chromosomal variation has been evaluated in more than 50 different culture systems (Bayliss, 1980) there are however very few studies which identify the mitotic abnormalities which underlie the production of this variation (e.g. Sunderland, 1977). These studies are important, as they demonstrate the nature of mitotic and nuclear abnormalities which may be induced in cells from different plants, under different cultural regimes and help to identify conditions which would limit or intensify the degree of instability induced and hence the extent of chromosomal variation present in callus cell populations. Such studies furthermore may allow an assessment of the level of competence in some calli and help to determine whether attempts to regenerate from these calli by the manipulation of environmental conditions or application of hormones would be worthwhile.

It has already been established that leaves of *B. rex* contain cells with a range of chromosome numbers (see Table D.13.). The effect of disorganized growth induced by 2,4-D and the influence of sub-culture and aging on genome stability in these cells has been evaluated here in an assessment of the chromosome number and mitotic abnormalities present in callus cells in culture. Chromosome numbers in regenerated plants have also been assessed to establish whether any latent chromosomal variation or mitotic instability exists in phenotypically normal plants and to evaluate the nature of change induced in putative polyploids produced from aged callus.

E.8.2. Material and method

Fixing maceration and staining of all tissues were undertaken as outlined in Section *C.5.*

For all calli 10 slides were prepared from separate callus clumps. For analysis of regenerated plants single, emerging leaves were removed either from culture or from mature plants. Leaves from a total of 10 randomly sampled plants were evaluated for each treatment and for controls. In both leaves and calli a total of 25 counts were taken from 25 separate fields in each of the 10 prepared slides. Criteria for assessment of cells suitable for counting were as outlined in Section *C.5.* Assessment of the presence of mitotic abnormalities and cell types was based on percentage presence in a scan of 10 fields on 10 slides.

E.8.3. Results

As indicated in Table E.10. and Figure E.7. chromosome numbers in all calli when compared with those of leaves indicates *de novo* production of aneuploid, hypodiploid and haploid cells. These respective cell populations being most prevalent in subcultured and aged calli. All calli with the exception of those aged for 20 weeks maintained a high percentage of diploid cells (never lower than 58 percent) hence a diploid population of cells was always present from which diploid plants could be regenerated.

The presence of different chromosome numbers was notably linked to cell type. In all four distinctive cell populations were observed in calli. These included meristematic cells, parenchymatous cells, elongated cells and degenerating cells, all of which were present at varying levels in each of the calli assessed (see Table E.11.).

Table E.10. Chromosome numbers in callus and axenic leaf explants of *B. rex*. Figures in brackets represent percentages.

Tissue source	Chromosome number frequency[b] 8-11	12-17	18-32	>32	Aneuploids[a]
Leaf			250(100)		
Primary callus	1(0.4)	54(22)	192(76)	3(0.8)	12(5)
Subculture 1	-	28(11)	222(89)	-	13(5)
Subculture 5	1(0.4)	105(42)	144(58)	-	20(8)
Aged 8 weeks	-	25(10)	224(90)	2(0.8)	10(4)
Aged 12 weeks	1(0.4)	11(4)	238(95)	-	-
Aged 16 weeks	10(4)	69(28)	171(68)	-	28(11)

a - all aneuploids were from the 11-17 chromosome number range

b - from a total of 250 cells

- indicates, not observed

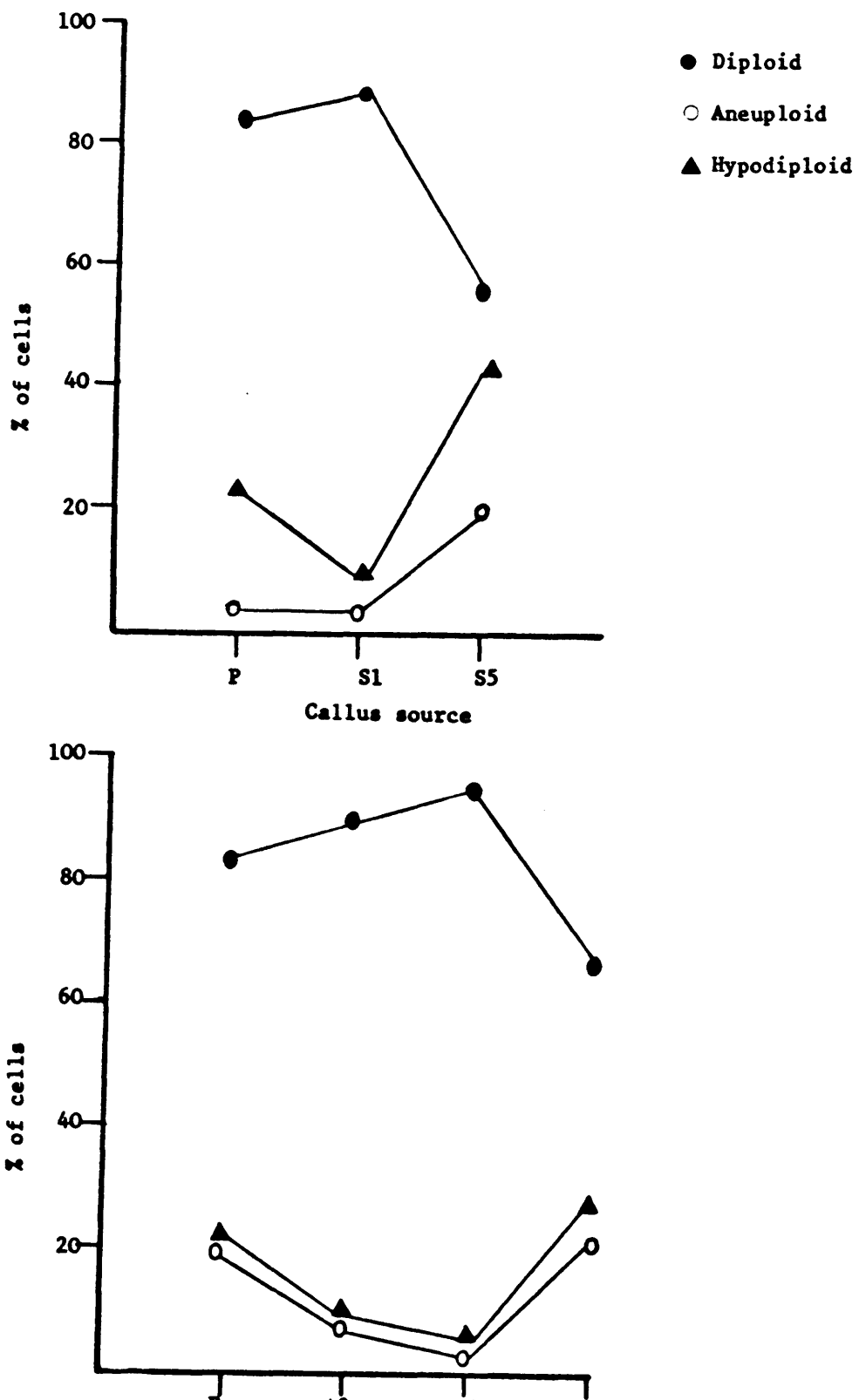
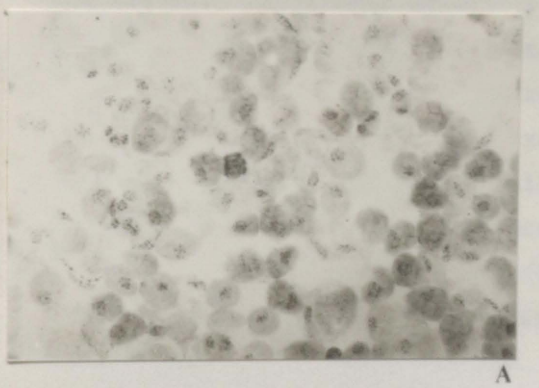


Figure E.7. Comparison of changes occurring in chromosome numbers in callus cell populations from primary (P), singly subcultured (S1) and fifth subculture (S5) calli and callus aged for 8 weeks (A8), 12 weeks (A12) and 16 weeks (A16). (Counts taken from total of 250 cells per callus)

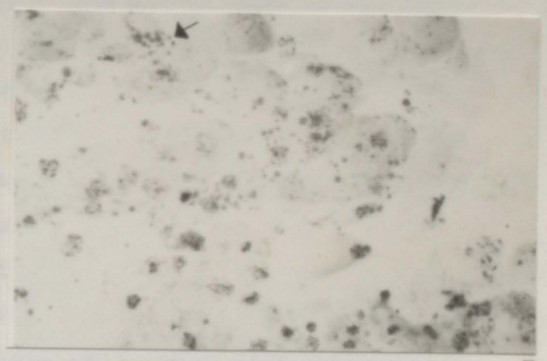
Table E.11. Mitotic abnormalities and cell types observed in *B. rex* callus.

	Mitotic abnormalities*			Cell types*		
Callus source	C-mitosis	Bilobed and micronuclei	Degenerated	Elongated	Parenchymatous	Meristematic
Primary	-	9	20	-	21	50
Subculture 1	2	15	19	2	45	30
Subculture 5	5	2	40	14	67	19
Aged 8 weeks	-	-	43	-	27	30
Aged 12 weeks	-	4	39	-	60	53
Aged 16 weeks	24	5	57	-	60	53
Aged 20 weeks	-	-	100	-	-	-

* Based on percentage presence in 100 randomly sampled cells from 10 callus nodules from each source.
- Indicates, not observed.



A



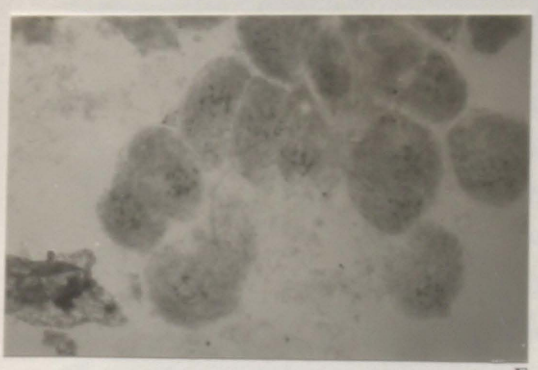
B



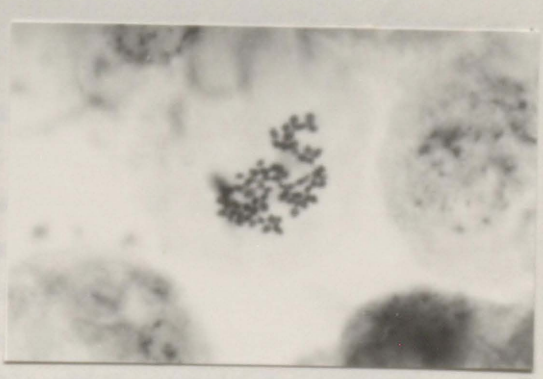
C



D



E



F

Plate E.11. Cell types found in callus of *B. rex* and a metaphase plate. A = meristematic cells. B = micronuclei. C = elongated cells. D = degenerating cells. E = parenchymatous cells. F = metaphase plate. Enlargement A-D x 400, E,F x 1,000.

Meristematic cells were generally small containing high levels of starch and with a diploid chromosome number. These cells were tightly packed and localized in the central core of the callus nodule and occurred in all calli with the exception of those aged for 20 weeks (see Plate E.11a). Elongated cells were devoid of starch and generally contained clumped chromosomal material with an apparently actively dividing hypodiploid complement (Plate E.11c). These cells appeared to be associated with meristematic cells and were most noticeable in callus from the fifth subculture. These cells were also present at low levels in primary calli but were not observed in aged calli. All calli contained parenchymatous cells (see Plate E.11e) which were devoid of starch and predominantly diploid although some hypodiploid and aneuploid cells were also observed. These cells appeared to occur randomly scattered in the outer core of the callus. Many of these cells became highly irregular in shape and gradually degenerated. Degenerating cells were characterized by large nuclei, micronuclei, densely packed chromosomal material or chromosomal material scattered at random throughout the cell (Plate E.11d). These cells were generally very large and found in the outer core of the callus nodule. The percentage of degenerating cells present in calli increased with aging and in calli aged for 20 weeks all cells had degenerated (Table E.11.). It is possible that each of these cell types is indicative of a gradual progression towards cell death in a population of callus cells.

The occurrence of mitotic abnormalities in calli was evident from the presence of multinucleate cells and cells with micronuclei and figures resembling C-mitosis. These abnormalities were limited to certain cell types hence their presence was correlated with the relative percentage presence of individual cell types within a callus. Micronuclei were found both in degenerating cells and in isolated zones of small apparently meristematic cells which contained no starch (Plate E.11b). Other abnormalities were limited to parenchymatous, elongated or degenerating cells.

Assessment of chromosome numbers in plants regenerated from the aforementioned calli (see Figure E.8., Table E.12.) indicate that attenuation of variation occurs during regeneration and changes in chromosome number and mitotic instability present in calli are only expressed in plants regenerated from callus aged for 16 weeks. Both

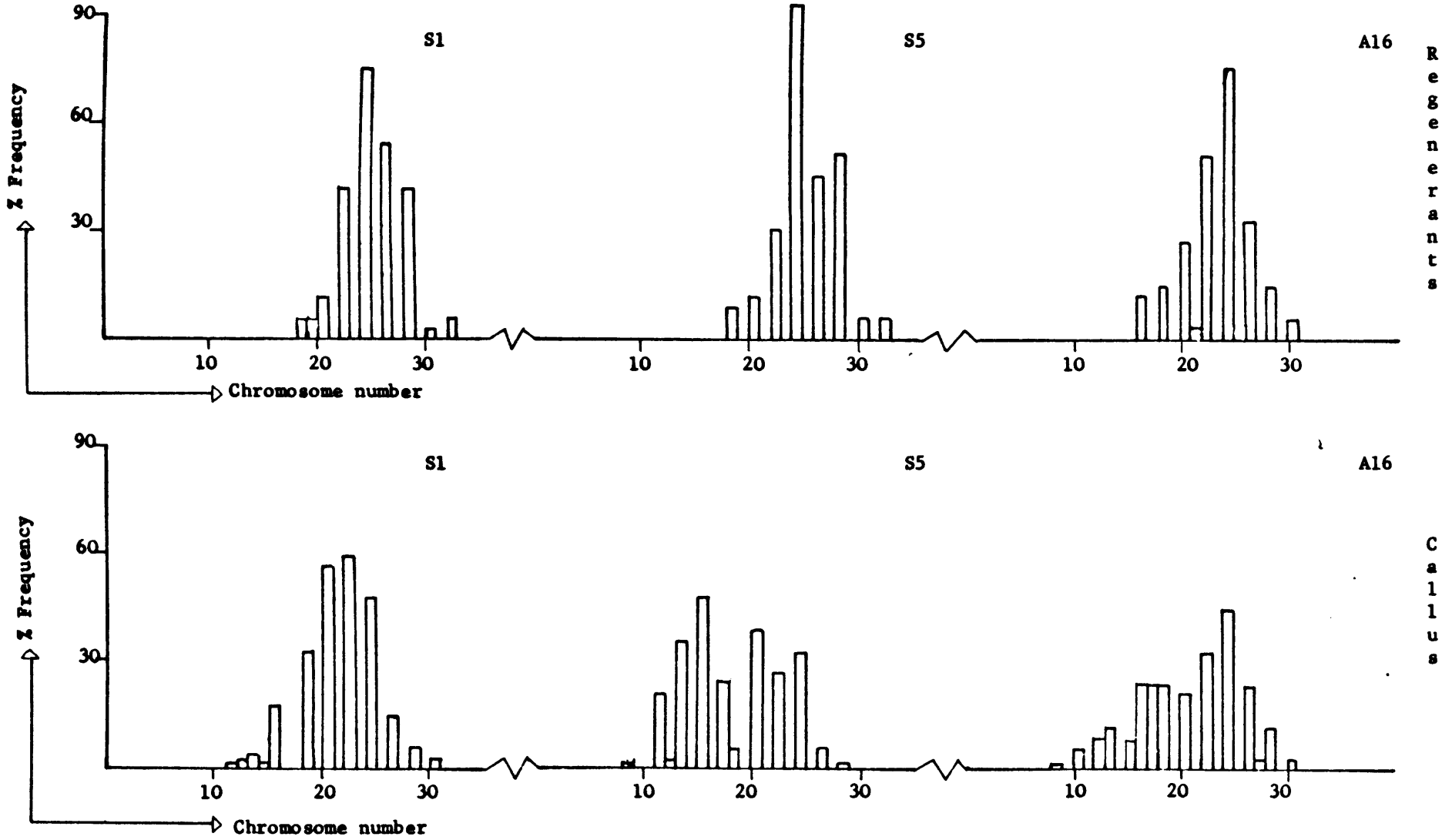


Figure E.8. Frequency plots of chromosome numbers in callus cultures and regenerants of single subcultured (S1), fifth subculture (S5) callus and callus aged 16 weeks (A16).

Table E.12. Chromosome counts in plants from callus culture of *B. rex*.

Sample number	Subculture 1		Subculture 5		Aged 8 weeks		Aged 12 weeks		Aged 16 weeks	
	2n	non-2n[a]	2n	non-2n	2n	non-2n	2n	non-2n	2n	non-2n
1	25	-	25	-	25	-	25	-	25	-
2	25	-	25	-	25	-	25	-	25	-
3	25	-	25	-	25	-	25	-	25	-
4	25	-	25	-	25	-	25	-	25	-
5	25	-	25	-	25	-	25	-	23	2
6	25	-	25	-	25	-	25	-	24	1
7	25	-	25	-	25	-	25	-	19	6
8	25	-	25	-	25	-	25	-	23	2
9	25	-	25	-	25	-	25	-	20	5
10	25	-	25	-	25	-	25	-	25	-
Total	250	0	250	0	250	0	250	0	234	16*
% non-2n	0		0		0		0		6	

* haploids, hypodiploids

a - includes hyper or hypodiploids

Table E.12a. Chromosome number in aged calli (16 weeks) putative polyploid progeny.

	Chromosome number frequency in a count of 25 cells									
Plant number	34	38	40	42	44	46	48	50	52	58
6175	3	4	1	4	5		3	2	3	
6225		3	2	3	3	3	3	1	3	4
6318	1	2	1	2	4	5	3		2	5
6321	2	1	4	3	3	5		4		3

putative polyploids and phenotypically normal plants regenerated from this callus were evaluated. In non-variant phenotypes asynchrony in mitosis occurred in a number of cells. Some of these plantlets also contained cells with chromosome numbers between 12 to 16.

Four putative polyploids evaluated from aged calli were found to have chromosome numbers ranging from 34 to 58 and hence were aneusomatic but with a polyploid rather than a diploid range of chromosome numbers (see Table E.12a). In all these plants mitotic abnormalities were observed, these included asynchronous division of constituent genomes, separate plate metaphases and laggards at anaphase.

E.8.4. Discussion

Cells in callus cultures derived from axenic leaves of *B. rex* showed distinct changes in chromosome numbers and cell morphology. These changes were associated with the presence of mitotic abnormalities. Alterations in the chromosome complement of all callus populations studied was predominantly towards a reduction in chromosome number resulting in the presence of populations of haploid, hypodiploid and aneuploid cells which increased with time in culture. This is in contrast to changes found in many other plant calli where callus cells become polyploid (Section *B.3.5.*). Calli with a high incidence of cells with reduced chromosome number have however been recorded in *Phaseolus coccineus* (Bennici *et al.*, 1976). Haploid cells in these calli are believed to be the result of segregation of a diploid nucleus into two homologous chromosome groups followed by mitosis i.e. true somatic haploidization (Huskins, 1948). A similar phenomenon may be responsible for haploidization in *B. rex* calli and may explain the presence of micronuclei in some cells. Haploidization may however occur by other mechanisms. In general, cells with a reduced chromosome complement were recorded in elongated cells and parenchymatous like cells, many of which displayed extensive chromosome aggregation which is possibly due to differential chromosome condensation due to restitution cycles (cf. Nagl, 1978). The loss of the affected portion of chromosomes on cell division resulting in haploidization or hypodiploidy.

Aneuploidy in callus cells has generally been associated with the presence of lagging chromosomes at anaphase (e.g. Bayliss, 1973). Very few anaphase plates were however observed in the *B. rex* callus studied here and none of those recorded had laggards. Aneuploidy may

also occur as a result of amitosis followed by mitosis and the presence of micronuclei which may be a consequence of amitosis would suggest that these may be the source of aneuploid cells. Aneuploids may also arise from spindle abnormalities (Bennici *et al.*, 1976) and the aggregation of chromosomes in many callus cells suggests that this may also be a source of aneuploidy in *B. rex* callus.

The chromosome complement of subcultured callus does not differ greatly from that of aged calli where regeneration capacity is high and both calli contain similar levels of actively dividing diploid cells. Hence the low regenerative capacity recorded in subcultured calli (Section *E.7.*) may be due to physiological changes which may be overcome by changes in hormone concentrations.

Chromosome counts indicate that plants regenerated from all calli, with the exception of a small population of plants derived from callus aged for 10 weeks, were diploid. Chromosome number alone however does not imply that callus culture did not affect the genotype as gene mutations or chromosomal rearrangements may also have been induced (Section *B.3.*). The size of *B. rex* chromosomes however (see Plate E.11f) does not allow a study of karyotypic changes. Furthermore, chromosome abnormalities which could be observed in meiosis cannot be studies in this *B. rex* cultivar because of its sterility. Hence the presence of these changes may only be detected in the present study if they are expressed in primary regenerates and have a significant effect on the phenotype such as the changes induced by gamma irradiation (Section *E.2.*). As yet no such variants have been observed in plants regenerated from *B. rex* callus.

Despite the absence of polyploid population of cells in calli aged for 16 weeks a number of polyploids were produced from one of these calli. To date, polyploids have not been recorded in any of the other calli aged for 16 weeks. The selective production of polyploids in one callus may be related to intercallus differences in chromosomal composition (cf. Ogihara and Tsunewaki, 1979) and the production of a polyploid population of cells in one callus which were maintained in the absence of the selective influences which are present in subcultured calli. The exact origin of these polyploid cells can only be speculated on. They may have resulted from restitution cycles, endocycles or been induced as a result of mitotic error caused by mutation in a gene or gene controlling mitoses (cf. Orton, 1984b). Mutations may also

have been induced by the byproducts of degenerating cells e.g. nucleic acids which are believed to have a mutagenic effect (cf. Bayliss, 1980). Also, abnormalities in the mitotic cycle may have been caused by limitation in nutrients or other factors essential to the regular functioning of the cell cycle (see Section *B.3.3.*).

E.9. The influence of supplementing regenerative media with casein hydrolysate, adenine or high concentrations of cytokinin on variant production from B. rex callus

E.9.1. Introduction

The results of studies on *B. rex* and on other plants which incorporate cytological analysis of both calli and regenerated plants indicate that in the majority, attenuation of variation occurs during regeneration (Section *E.8.*, see Orton, 1984a). Hence much of the variation present in calli is not expressed at the plant level and it is evident that the potential of this system as a method for the regeneration of variants may not be fully utilized.

Studies on calli during subculture indicate that media constituents can both induce further genome modification or enhance the division of existing variant cell lines (Section *B.3.3.*). The influence of media constituents in regeneration media on the production of variants however has not been investigated.

The present study is an assessment of the influence of the supplementation of regeneration media with high concentrations of BAP and kinetin, adenine and high nitrogen in the form of casein hydrolysate on the production of variants from *B. rex* callus.

E.9.2. Material and method

E.9.2.1. Media supplementation with kinetin and BAP

Callus was produced and proliferated as outlined in Section *C.3.* Clumps of approximately 25 mg of primary subcultured callus was transferred to media containing different concentrations of the hormones kinetin and BAP in combination with 0.01 mg/l NAA. The concentrations of cytokinins used were 1, 2.5, 5 mg/l. After 12 weeks calli were evaluated for their responses on individual media and responding calli were transferred to SDM for further shoot development. When fully developed after a further 4 weeks of culture the number of plantlets produced was counted. Shoots greater than or equal to 2 cm in height were planted out and grown to maturity as

outlined in Section *C.2.4.* Chromosome counts from plantlets were undertaken as outlined in Section *C.5.*

E.9.2.2. Media supplementation with casein hydrolysate and adenine

Calli were produced and proliferated as outlined in Section *C.3.* Clumps of approximately 25 mg of callus were transferred to SDM3 (Table C.1.) supplemented with either 1000 mg/l casein hydrolysate or 40 mg/l adenine sulphate or a combination of these two nutrients at the above concentrations. After 12 weeks of culture under controlled growthroom conditions calli were evaluated for their responses on individual media and responding calli were transferred to SDM (Table C.1.) for further development of plantlets. Plantlets greater than or equal to 2 cm were planted out, established and grown to maturity as outlined in Section *C.2.4.*

E.9.3. Results

The degree of morphogenesis induced in calli on the media assessed was found to vary with concentration (see Table E.13.). At low concentrations (1-2.5 mg/l) kinetin failed to induce shoots and similarly at the highest concentration of BAP (5 mg/l) no shoots were produced. On media where calli produced shoots, 60 percent response was generally recorded. Plantlets developed from these calli were vigourous with the exception of those induced on high concentrations of BAP (2.5 mg/l). The induction of multiple shoots on this medium meant that many plantlets were too small for planting out.

None of the shoots produced from any of these calli showed deviation from type at the plantlet stage. Of the treatments applied, plantlets induced on 5 mg/l of kinetin and 2.5 mg/l BAP were grown on for assessment. All plants which survived to maturity (20-40 percent) were true to type.

Chromosome analysis of a random sample of 10 plants from each of the populations did not detect any significant change in chromosome number or the behaviour of chromosomes in mitosis.

E.9.3.1. Adenine and casein hydrolysate treatments

While not quantitatively assessed the presence of casein and adenine supplements in the medium appeared to reduce the number of shoots induced hence only a small population of shoots were available for planting out. None of the shoots produced any evident deviation from type and to date established plants have shown no sign of variation.

Table E.13. Results of supplementation of callus regeneration media with cytokinins, casein hydrolysate and adenine sulphate.

Treatment mg/l	Number of shoots producing calli	Number of shoots produced per callus (mean)	Number of plantlets planted	Number of variants observed
Kinetin 1	0	0	0	0
BAP 1	3	52	0	0
Kinetin 2.5	0	0	0	0
BAP 2.5	3	150	50	0
Kinetin 5	3	55	41	0
BAP 5	0	0	0	0
Casein hydrolysate (CH) (1,000 mg/l)	3	*	54	0
Adenine sulphate (Ad) (40 mg/l)	2	*	10	0
CH + Ad†	3	*	20	0

* Not quantified

† Concentrations as above

E.9.4. Discussion

In the present study BAP was found to induce more shoots than kinetin in *B. rex* callus. The superiority of BAP as a cytokinin for shoot induction in *Begonia* has also been noted by Reuther and Bandari (1981).

The low survival rate recorded here for plants from treatment with 2.5 mg/l BAP may be a result of the high numbers of shoots induced and competitive effects in the culture vessel which may have reduced plant vigour.

The apparent reduction in shoot numbers in calli cultured on supplements of adenine and casein hydrolysate may be due to the intolerance of *B. rex* cells for high concentrations of these organics (see Section *B.2.*) as both are already present in MS media.

The absence of polyploidy and any gross morphological changes in young plantlets from all treatments would indicate that these supplementations of the medium have not induced major morphological variants. These however are the results of a preliminary study and data from a larger population of plants which assesses the phenotypic characteristics at all stages of growth would provide a greater insight into the potential for variant induction using nutrient and hormonal supplementation. Such a study will require the treatment of a large number of calli and the use of subdivision procedures to ensure the development of the many shoots induced. Furthermore conditions for establishment and growth of plants will have to be carefully monitored to ensure a high survival rate of plantlets.

E.10. Callus induction on colchicine and its influence on regenerated plants of B. rex

E.10.1. Introduction

The mitotic abnormalities induced in cells of *Begonia rex* in callus culture result in somatic reduction rather than polyploidization as found in calli of many other plants (Section *B.3.3.*). The production of polyploids in callus culture is desirable, as these cells, due to their inherent 'buffering capacity' (e.g. Broertjes, 1976) are more likely to undergo and survive extensive genetic and chromosome variation when compared to diploid cells under similar cultural circumstances. Hence plants regenerated from altered polyploid cells may bear desirable genotypic characters which may be expressed in the primary or secondary regenerates.

As previously outlined (Section *B.3.5.3.*) treatment of cells with colchicine can have a number of different effects other than direct duplication of the genome. Hence by inducing callus on colchicine containing media it is possible that a number of different chromosomal changes may be induced (in cells), these may include polyploidization or karyotype rearrangement. While there are reports on the cytology of calli induced on colchicine containing media e.g. Barley embryos (Ruiz and Vazquez, 1982b) there are no reports in the available literature on the effects on the phenotype of regenerates from callus produced on media containing colchicine. The following study of callus induction from axenic leaves on colchicine containing media and subsequent regeneration of plants evaluates the influence of colchicine on the production of variants from *B. rex* callus.

E.10.2. Material and method

Axenic leaf explants of 1 cm in length were aseptically removed from axenic plantlets, wounded at random and placed on CM (Table C.1.) supplemented with 0, 1,000 and 2,000 mg/l colchicine in 60 ml culture jars. Twenty replicas of each treatment were cultured in the growth-room under standard conditions for 6 weeks before evaluation. Planting and establishment of plantlets was as outlined in Section *C.2.4.* Chromosome counts were undertaken on a random population of plantlets produced in culture using the procedure outlined in Section *C.5.* and developing plantlets with obvious polyploid morphology were assessed both for chromosome number and guard cell chloroplast counts. The latter were undertaken as outlined in Section *C.6.2.*

E.10.3. Results

No callus was produced on media containing 2,000 mg/l colchicine. On 1,000 mg/l colchicine only 60 percent of explants produced callus and the intensity of callus production was less than that of controls, and only small clumps were available for transfer to regeneration media. Of these only 50 percent produced shoots. On emergence some of these shoots were distinctly abnormal in appearance with thickened petioles and leaves. These plantlets were also stunted in growth and were not suitable for planting. Chromosome counts in these shoots indicate they were polyploid and there was a high level of mitotic instability manifest as asynchronous division of part of the full chromosome complement. Later studies on fully developed plantlets

which did not show any signs of polyploid morphology indicated that these plants also contained cells with asynchronous division of the chromosome complement which may possibly be a carry over effect of colchicine treatment. None of these plants however contained polyploid cells. Of the 310 plantlets planted out, 6 putative polyploids have been observed. Assessments at a later stage may however detect other phenotypic changes which have been induced by colchicine. For counts from polyploid plantlets produced from callus treated with colchicine see variants 5555, 5565, 5564 illustrated in the Appendix Section I Figure I.1.

E.10.4. Discussion

The absence of callus production on media containing 2,000 mg/l concentrations of colchicine and the small amount of callus produced on 1,000 mg/l may be a result of the combined destabilizing influences of both colchicine and 2,4-D. Callus production requires regular division of a meristematic population of cells (cf. Yeoman, 1970) hence the inhibition of regular division in meristematic cells by the continued presence of colchicine may have limited the amount of callus produced. It is also true that polyploid cells grow less rapidly than diploids (see Torrey, 1969) hence the presence of a polyploid population of cells in the callus would also reduce the growth rate.

The small number of polyploids induced and the genetic instability manifest in the cells of regenerated plants would suggest that colchicine did have some influence on the cell population in callus culture which was transferred to regenerated plants. Colchicine treatment may also have caused chromosomal rearrangements or deletions which have not yet been expressed as phenotypic changes but which may become evident in mature plants.

E.11. Studies on the presence of somatic cell variation in pith and root explants of B. rex and expression in in vitro culture

E.11.1. Introduction

It is clear from both cytological studies and the production of bud sports (Section *B.3.2.*) that plant tissues provide a valuable source of genetic variation which, if expressed as regenerated plants may produce useful new plant genotypes. In tissue culture however direct regeneration from explants as a source of variants has not been widely acknowledged or used.

In the present study on *B. rex*, plant regeneration either directly through bud formation on explants or indirectly via callus (primary or subcultured) has not resulted in the production of a wide array of variants (see Sections *D.1.* and *E.5.*). This suggests that cells in the epidermis and underlying tissue, from which these plants were originally derived do not provide a source of somatic variation in this *B. rex* cultivar. Other tissues however, as demonstrated in other plants (Section *B.3.2.*), may contain endopolyploid cells or other forms of somatic variation. Hence, direct regeneration or regeneration from primary calli of these tissues may result in the production of variants.

In *B. rex* the presence of polysomaty or a chimeral structure has not yet been reported. Tissues most likely to contain endopolyploid cells include the root and pith (Nagl, 1978). Studies by Bigot (1971) on regeneration in pith and pith derived callus of *B. rex* demonstrated that these tissues would not produce buds on media used for bud production in intact petioles. However hormone manipulation may overcome the recalcitrance of these tissues. No attempts have yet been made to regenerate plants from roots or root derived callus of *B. rex*.

To assess for the presence of endopolyploid cells in root and pith of *B. rex* and their derived callus kinetin was included in culture media, as studies by Torrey (1961) indicate that kinetin will specifically induce the division of endopolyploid cells (see Section *B.3.3.2.*). A preliminary study of media on which roots and pith may be induced to regenerate has also been undertaken.

E.11.2. Material and method

E.11.2.1. Explants

As systemic infection would pose a problem in culture of roots grown *in vivo*, axenic roots were used in the present experiment. The whole root consisting of main root and laterals was used.

Pith explants consisted of the petiole devoid of the epidermis and primary mesophyll layer.

E.11.2.2. Pre-culture of explants for cell division prior to chromosome analysis

Root explants derived from axenic plantlets and petioles sterilized and prepared as outlined in Section *C.2.1.* were cultured in petri dishes on SDM3 (Table C.1.). After 2 weeks of culture under standard

growthroom conditions tissues were fixed, macerated and stained as outlined in Section *C.5.*

E.11.2.3. Callus culture

Pith explants were prepared from petioles sterilized and prepared for culture as outlined in Section *C.2.1.* Prior to transfer to regeneration media their epidermis and underlying mesophyll layer was removed using a fine forceps. Both pith and axenic roots derived from axenic plantlets were cultured on CM2 (Table C.1.). After culture under standard growthroom conditions for 6 weeks 10 callus nodules were removed.from each of these explant sources and prepared for chromosome counts as outlined in Section *C.5.* A total of 25 counts were taken from 25 separate fields of view in each of 10 slides.

Calli from both explant sources were transferred to SIM3 (Table C.1.) for regeneration. Bud producing callus was transferred to SDM (Table C.1.) for further development.

E.11.3. Results

E.11.3.1. Cytological studies of root and pith explants and calli

While putative endopolyploid nuclei were observed in pith sections these did not appear to divide on the medium used as no polyploid cells were recorded in chromosome counts from pith tissues. In root explants, however a small population of polyploid cells were found on one slide. The small size of the cells and their chromosomes however made it impossible to take chromosome counts from these cells. Chromosome numbers in other cells of the root were all diploid.

The range of karyological abnormalities found in other calli (Table E.11.) were also observed in callus induced from pith and root explants. The frequency of diploid, hypodiploid and haploid cells in these calli were also similar to those derived from leaf explants (see Figure E.9.). Hence explant source would not appear to influence the degree of chromosome variation present in *B. rex* callus.

E.11.3.2. Pith culture

On the factorial media tested none of the pith explants produced shoots. However a number of green budlike primordia were observed at the base of some roots and a green callus like growth was produced at the cut ends of the explants (Table E.14.). Histological observations indicated the presence of extensive cell division and the organization

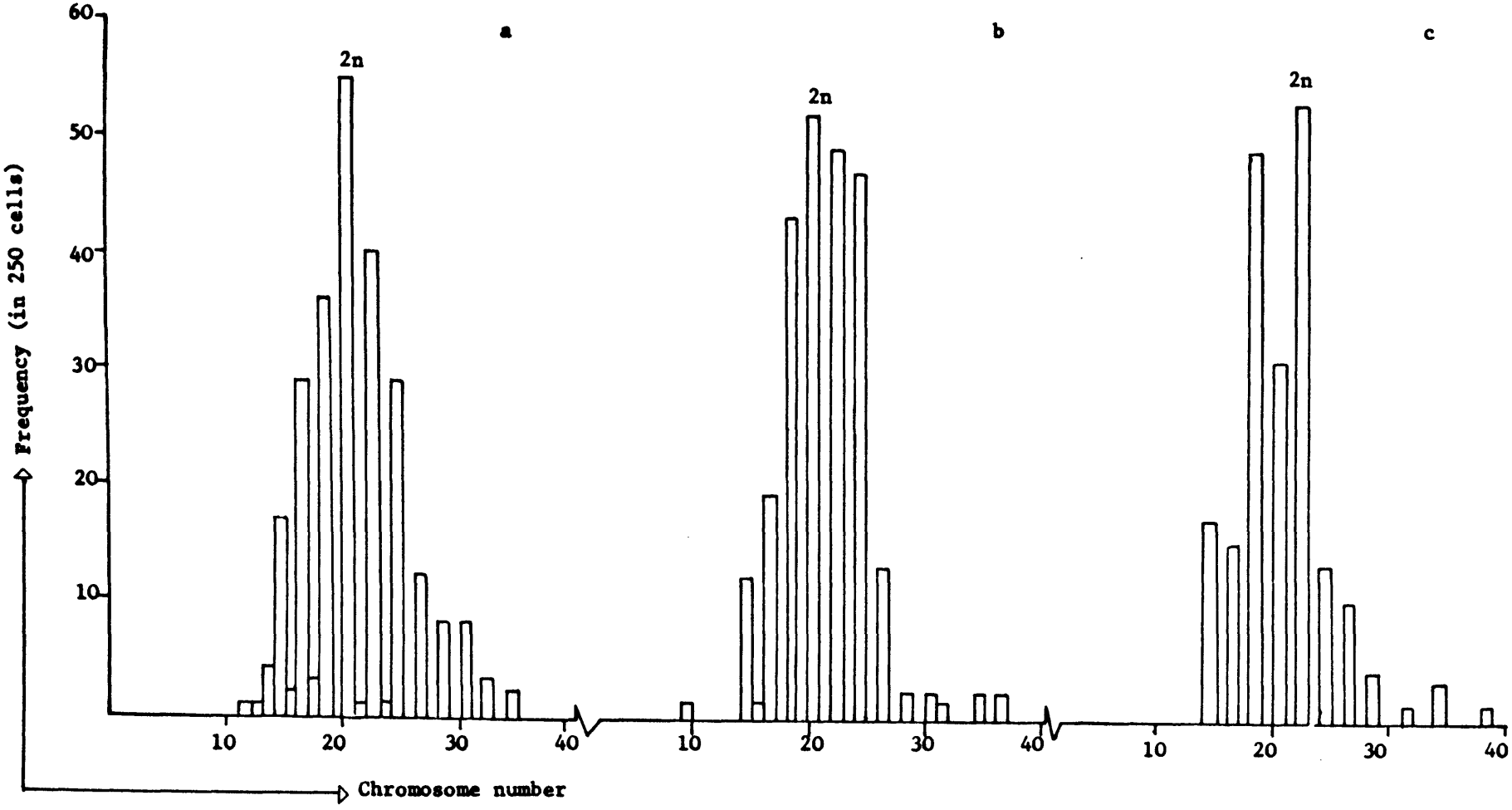


Figure E.9. Chromosome number in calli cell populations derived from leaf (a), pith (b) and root (c) explants of *B. rex*.

Table E.14. Responses of pith explants on combinations of kinetin and NAA.

Hormone Concentration mg/l		Responding explants (%)	Nature of response
Kinetin	NAA		
0.1	0.01	2(40)	Scattered root production with bud primordia at root base. Distinct swelling at cut basal ends.
1.0	0.01	2(40)	Swelling and emergence of green callus at cut ends.
2	0.01	1(20)	*Nodular green callus produced at cut basal ends.
0.1	0.1	2(40)	As above (see *)
1.0	0.1	2(40)	As above (see *)
2.0	0.1	1(20)	As above (see *)

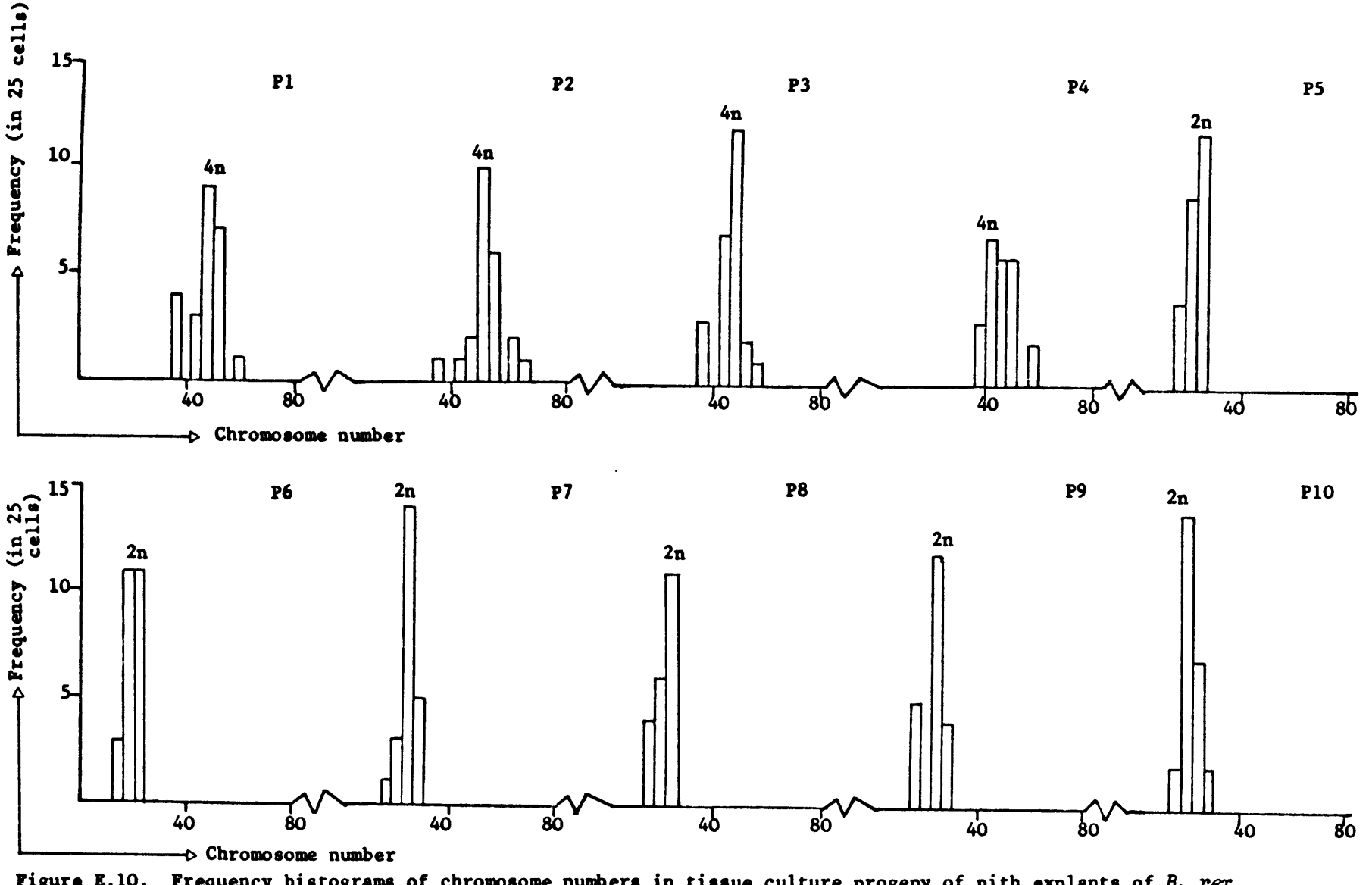


Figure E.10. Frequency histograms of chromosome numbers in tissue culture progeny of pith explants of *B. rex*

of bud meristems accompanied by the accumulation of starch. As the meristems appeared quiescent, attempts were made to induce development by transfer to SIM3. After 4 weeks of culture shoot primordia emerged from the cut ends of the explant. On transfer to SDM these began development to produce shoots. Results from chromosome counts indicate that on one pith explant 30 percent of the shoots produced were polyploid (see Figure E.10.). While this was a small sample it does indicate that polyploid shoots can be produced from pith tissues. Total culture time for shoot induction and development was 6 months which is 3 months longer than that required to induce shoots from petioles with an epidermis.

Due to the prolonged regeneration period and frequent subcultures only a small number of explants have survived. Approximately 30 plantlets derived from these cultures have been planted out and these will be assessed for phenotypic characteristics at a later date. Calli from petiole explants failed to regenerate when transferred to SIM3.

E.11.3.3. Root culture

While some growth was observed in roots particularly on high concentrations of BAP no direct bud regeneration occurred on any of the media tested. Callus produced from roots on CM2 media however has been induced to shoot and the induced shoots have been transferred to SDM for further development.

E.11.4. Discussion

The regeneration of polyploid plantlets from pith and the presence of polyploid cells in root callus suggests that *B. rex* tissues contain both diploid and polyploid cells and hence may provide a useful source of pre-existing variation which may be used in the production of variants from tissue culture. This is in contrast to regenerates from petiole or leaf explants where regenerates were diploid (Section *D.5.*). Another major difference observed between these two systems was the timescale required for regeneration. In pith the extra 3 months required for bud induction and development may relate to the need for extensive division (dedifferentiation) and reprogramming of cells before bud differentiation can occur. A similar phenomenon occurs in many plants which undergo a callus phase prior to bud regeneration (see Thorpe, 1982). In epidermal cells of

leaves of *B. rex* programming for bud formation occurs within the first 48 hours after excision or treatment with BAP (Bigot, 1971). The rapidity of this response may be related to the position of arrest in the cell cycle of quiescent epidermal cells. The control of cell cycle resting state i.e. G_1 (2C) or G_2 (4C) on the rate of cell differentiation has been discussed by a number of authors (e.g. D'Amato, 1977a; Dodds, 1981) and it is suggested that the rate of entry of a given cell into a differentiation pathway may be dependent on its position of arrest.

The absence of bud regeneration from roots on the combination of hormones assessed may be due to the lack of appropriate stimuli for cell differentiation and the application of different hormones or hormone concentrations may result in regeneration from these tissues. the recalcitrance of roots on media which readily stimulate organogenesis in leaf and petiole explants (Section *D.2.*) may be attributed to the extent of determination of cells in root tissues (Wareing, 1978) or to the presence of different levels of endogenous hormones. Shoots have been induced on callus derived from roots indicating that root callus cells are competent and the ploidy level and morphological characteristics of the plantlets produced will be assessed at a later date.

The absence of significant differences between the chromosome numbers of cells in leaf derived calli and those from pith suggests that endopolyploid cells which are present in the pith are not involved in callus formation. Callus production from pith tissue was evident after 8 weeks of growth but was selectively induced as scattered nodules on the surface of the explant. Hence unlike other plants where extensive callusing occurs over the surface and particularly at the basal ends of the explant and which is in many cases derived from a number of cell layers (see Yeoman and Forsche, 1980) the callus produced here from pith of *B. rex* was only derived from the storage parenchyma. A change in the callus inducing medium or a more prolonged growth period which would allow the participation of cells from other cell layers e.g. vascular cambium (see Ruiz and Vazquez, 1982a) may allow the participation of endopolyploid cells in callus production. Similar requirements may apply to the production of callus from roots where although a small population of polyploid cells were observed, the majority of cells

of the callus had a similar cytology to those found in calli of leaf origin.

As a preliminary study these experiments demonstrate that it is possible to produce polyploid plants from *B. rex* pith explants and more variants may be observed in the population which has been planted out. To fully exploit this source of variation, further investigations on the media constituents are required both to reduce the timescale for plant regeneration and to increase the number of variants produced.

SECTION F. GENERAL DISCUSSION

F.1. General discussion

One of the primary objectives of the present study was to develop micropropagation procedures which maintained genome stability and allowed the rapid and continuous propagation of *B. rex* 'Lucille Closon'. Results from the studies reported here demonstrate that procedures for the production of plants in tissue culture from *B. rex* can be developed using mature leaf petioles (discontinuous culture) or young axenic leaves either singly or multiply recycled (continuous culture). These procedures allow the production of large numbers of plants which self root thereby eliminating a rooting step and increasing cost effectiveness. When evaluated mature plants produced from these cultures showed similar interpopulation variation in vegetative characteristics to those found in a control population derived from leaf disc cuttings. Furthermore, all tissue culture progeny, with the exception of one anthocyanin variant, were true to type. The only similar comparative assessment available on tissue culture progeny of *Begonia* was reported more recently by Samyn *et al.* (1984) on *B.* x *tuberhybrida*. In this plant tissue culture progeny were also true to type but were more vigorous than controls as a result of virus eradication. This study and those on other plants e.g. *Saintpaulia* (Cassells and Plunkett, 1984) and Banana (Hwang *et al.*, 1984) demonstrate that comparative growth measurements and assessment of multiplication rates are the most appropriate means of evaluating the cost effectiveness and reliability of a tissue culture procedure thus giving important guidelines to horticulturists intending to use tissue culture as a means of plant propagation.

Like *B. rex* there are other examples of plants where progeny from discontinuous culture proved stable e.g. *Stevia* (Tamura *et al.*, 1984). But the limited number of studies available on progeny from continuous culture indicate that this procedure can result in genome instability (e.g. Westerhof *et al.*, 1984; Cassells and Carney, 1986) (see also Section *B.2.4.*). Hence continuous culture is not suitable for the micropropagation of true to type progeny from these plants. This instability was not induced in the *B. rex* cultivar studied here and progeny produced after a year of subculture were all true to type. Furthermore, shoot vigour and a high multiplication rate was also maintained. This is in contrast to reports of reduced multiplication rates in *B.* x *hiemalis* (Bigot, 1981a) and reduced shoot vigour in

Actinidia cinensis (Standari, 1983) and suggests that continuous culture may be used for rapid plant multiplication in *B. rex*.

The maintenance of genome stability and multiplication rates in *B. rex* during subculture is encouraging as it indicates the potential for the development of a mother stock independent tissue culture system as suggested by Lê and Collet, (1981). This could provide a source of relatively uniform aseptic explants for the establishment of an efficient micropropagation procedure simply by the successful regeneration of plantlets from a single mature explant.

The extent to which *B. rex* can be subcultured without any detrimental effect on the phenotype remains to be evaluated and will require further progeny assessment. The application of continuous culture to other *Begonia* genotypes or other plants for the development of stock independent tissue culture procedures will require a similar detailed study as somatic variation or other influencing factors may cause a reduction in multiplication rate, shoot size or genetic/epigenetic instability (see Section *B.2.4.*).

Here, a maintenance programme for the storage of stocks of axenic plantlets was successfully developed by cold storage of developing plantlets *in vitro* (see Section *D.7.*). This confirms the suggestion made by Henshaw (1982) that minimal growth storage would provide an effective means of storing plants from one season to the next. The procedure developed here when used in conjunction with a continuous culture procedure can readily be used to bulk up stocks for micropropagation. Thus eliminating the need for glasshouse maintenance of mother stock plants, a tradition which as pointed out by Holdgate (1977), is a major cost and risk factor in vegetative propagation.

The increased morphogenic responses of explants from a number of *B. rex* cultivars and *Begonia* species hybrids when axenic leaf explants were used (see Sections *D.1.* and *D.6.*) indicates a further advantage of using continuous culture as a means of plant propagation. In *B. rex* cultivars the increased morphogenic responses of axenic explants when compared with mature explants cultured on defined media (see Section *D.6.*) confirms the suggestion made by Welander (1977) that differences between genotypes in tissue culture responses (see Section *B.2.2.2.*) may in part be attributable to the growth conditions and age of the donor plant used. With the reduced epigenetic/ontological influence, culture of axenic explants allows a more accurate

assessment of the potential for the development of a single medium for the propagation of a range of *B. rex* cultivars. The successful development of this procedure would greatly reduce the cost of micropropagation of *B. rex* genotypes. Here, the morphogenic responses of axenic leaf explants from a number of *B. rex* cultivars assessed on a factorial combination of hormones indicates that genotype influences on morphogenic responses *in vitro* are very pronounced (see Section *D.6.*). These differences may be a result of differences between cultivars in endogenous levels, metabolism or uptake of hormones as suggested for *Petunia* (Skvirsky *et al.*, 1984). While similarities or differences in morphogenic responses in other plants have been traced to a genetic base (see Section *B.2.*) this is not possible in *B. rex* due to infertility and the difficulty in tracing the origins of the cultivar used (see Section *B.1.1.2.*). Despite the extensive differences in morphogenic responses between cultivars (see Section *D.6.*) and the absence of common plantlet regeneration media, overlap in morphogenic response did occur on high cytokinin and with further more detailed assessment a single medium may be developed on which these cultivars could be propagated. This result suggests that in other plants similar studies using axenic explants should be used to assess the potential for the development of media on which a range of genotypes may be propagated. If successful these procedures could increase the cost effectiveness of micropropagation.

Due to the sterility of the *B. rex* cultivar under study tissue culture provided a potentially useful means for the development of new variants (see Section *B.3.*). The procedures adopted for the production of variants (see Figure E.1.) also allowed an assessment of the relative stability of the *B. rex* genome when subjected to various chemical and environmental stresses. The fact that suitable procedures had been developed for storage and rapid multiplication of plants from single explants also meant that any new variant produced could be rapidly propagated for trials and cultures could also be stored to guard against loss in the field. Furthermore, the high morphogenic capacity of the *B. rex* cultivar used both in continuous and discontinuous culture, allowed the production of large numbers of plants for evaluation, a feature which is essential to the evaluation of the relative merits of different methods for destabilizing plant genomes *in vitro*.

Due to the natural propensity of *B. rex* 'Lucille Closon' for plantlet formation both from complex explants and from callus a number of different procedures were assessed for their influence on genome stability and the production of variants. Also a contrast was made between the effectiveness of conventional methods for producing variants e.g. gamma irradiation and colchicine treatment as compared with somaclonal variants arising via regeneration from callus or explants.

The variants induced after irradiation treatments included leaf colour and pattern variants, plants with abnormal leaf morphologies and reduced vigour and putative chimeras (see Section *E.1.*). Variants from other treatments, for the present, are limited to putative polyploids but other variants may appear when these plants have matured.

Changes in leaf colour and pattern found in variants of *B. rex* may be a consequence of a qualitative change in the genome involving gene mutation or chromosome rearrangement. Colour changes in *B.* x *hiemalis* have previously been ascribed to qualitative rather than quantitative changes (Arends, 1970).

Leaf variegation in plants has been attributed to beneficial virus infection, chimerism, pattern genes or air blisters resulting from a detachment of the epidermal cells from the palisade layer beneath causing a difference in the light scattering of the leaf (cf. Vaughn, 1983). In *B. rex* pattern changes are a natural consequence of development (see Plate E.7b), however the distinct change in pattern induced in leaves after irradiation (see Plate E.4.) suggests a mutation in pattern genes or rearrangement in chromosomes carrying these genes which results in a new pattern of expression such as that found in maize after induced breakage fusion bridge cycles (McClintock, 1951).

The genetic change underlying the development of leaf sports (see Plate E.7a) which were produced on a number of plants from irradiation treatments, cannot be conclusively determined as yet. Plants produced from petiole explants of these leaves may however give some insight into the origin and stability of this form of variation. Similar putative chimeras have been reported after irradiation treatment of *B.* x *hiemalis* by Mikkelson (1976), this author however did not propose any reason for their occurrence. In

UNIVERSITY COLLEGE Library CORK

B. rex the production of leaf sports suggests a change in the genetic architecture (see Section G.) of the plant. This change was possibly induced by irradiation treatment but remained latent until suitable developmental or environmental conditions allowed the expression of variant somatic cells. The nature of the new genetic architecture induced by irradiation treatment may only be speculated on.

Mutation treatments have been shown to induce chimeras in plants (see Section *B.3.* and Figure B.4.). The pattern of leaf types produced and the fact that mutated and normal leaves co-exist on the same plant suggests that these plants may be sectorial chimeras (see Figure B.5.) and hence high temperature and pruning, both of which have been found to stimulate rearrangement of cell layers in chimeras (Dowricks, 1953; Broertjes, 1976), may have stimulated periclinal and anticlinal division leading to expression of variant cell layers. The diverse range of leaf phenotypes observed (see Plate E.7a) would thus result from the production of leaves from different sites on the rhizome and the displacement of mutated layers so that the newly formed adventitious buds have mutated cells in positions where they contribute to the nature of leaf pattern, form or colour. The leaf sports produced, however, could equally arise if the plant is composed of a mosaic of mutated tissue sectors arising as a result of mitotic instability (see Dyer, 1976). Leaves arising from the mutated sectors would bear the solid variant trait while combinations of the mutated sectors and normal tissue may be responsible for the rearrangement that occurs in apparently normal leaves i.e. fluted phenotypes (see Section *E.1.*).

Sports may also arise from rearrangement of the genome such as that induced by stress (McClintock, 1978) or loss of chromosomes as found in *Chrysanthemum* sports (Sampson *et al.*, 1958). In the putative chimeras studies here the presence of condensed heterochromatin during metaphase in a number of cells of the young leaves assessed may be indicative of an ensuing loss of chromosomes in the mature leaf. If genes on the remaining chromosomes are mutated or rearranged this loss of DNA may result in the expression of recessive traits.

Cytogenetic confirmation of genetic changes occurring in variants as a result of irradiation treatments were limited to a study of chromosome number (based on counts of heterochromatin blocks) and their behaviour in mitosis. Karyotype changes, however, could not be characterized due to chromosome size (see Section *B.1.2.1.*). Furthermore the sterility of the plants prevented meiotic analysis. This limitation

applies to all cytogenetic studies conducted on this plant and has limited the comprehensive analysis of changes occurring in the genome as a result of different culture treatments. The evaluation of changes in chromosome number is hampered further by the aneusomatic nature of the plant which only allows the detection of gross chromosomal changes such as hyper- or hypodiploids.

Aneusomaty has previously been observed in *B. rex* (Sharma and Bhattacharyya, 1961) and a number of potential causes have been suggested (see Section *B.1.2.1.*). Here, the underlying cause of aneusomaty was not studied in detail but the use of heterochromatin blocks as a measure of chromosome number and the potential presence of putative B-chromosomes which may vary within individual tissues (Jones, 1975) may have caused discrepancies in chromosome counts.

Chromosome analysis of plants produced from irradiation treatments did however allow the characterization of putative polyploids and plants with mitotic abnormalities. Putative polyploids were found to show varying degrees of mixoploidy and hence were unstable. The low level manifest in one variant (see Section E.) suggests that this may be a result of diminution occurring as a result of genetic instability due either to the polyploid state or the mutation of genes controlling mitosis (see Section *B.3.*). Preliminary studies on tissue culture progeny of this plant demonstrate the production of both mixoploid and solid polyploids and it is possible that further diversity may arise in subsequent regeneration cycles or in regeneration from callus (see Figure E.4.). These procedures may result in a return to the diploid state due to somatic reduction (Huskins, 1948) as found in other induced polyploids (Gottschalk, 1978). The genotype produced, will not necessarily be that of the parent as changes other than direct duplication may have occurred as a result of irradiation and latent variation repressed in the polyploid state may be expressed.

The second conventional method assessed for the production of variants involved colchicine treatments which were applied to explants prior to direct or indirect adventitious regeneration and resulted in the production of a number of putative polyploids which on cytogenetic analysis were found to be cytochimeras (see Section G.). These plants also exhibited a high level of mitotic irregularities which may be responsible for their cytochimeral state. This genetic architecture may also be a result of the multicellular nature of adventitious buds

(Broertjes and Keen, 1981) or the persistence of colchicine induced mitotic abnormalities (see Espino and Vazquez, 1981). To date a low level of variation has been induced using colchicine. Due to the wide array of mitotic abnormalities which may be induced by colchicine (see Section *B.3.5.*) more variants may emerge at a later date when other presently normal phenotypes mature.

In assessing non-conventional means for destabilizing plant genomes and producing variants in *B. rex* 'Lucille Closon', procedures were developed for callus production, subculture and regeneration. On cytogenetic analysis primary and subcultured calli were shown to produce an array of cell karyotypic variants *in vitro* with a distinct genetic drift towards somatic reduction. Attenuation of variation however occurred during regeneration due to selective regeneration from diploid cells (see Section *E.8.*). The maintenance of an actively dividing diploid population of cells in *B. rex* callus may be related to their origins from axenic leaf explants. These cells as suggested by Halperin (1973) may not be completely de-differentiated and may maintain the epigenetic machinery characteristic of their explant origin. As already outlined regenerants from axenic leaves were all true to type (see Section D.).

While the population of plants produced from callus cultures was small, all regenerants showed no deviation in phenotype when compared with a control population derived from axenic leaves suggesting *B. rex* plants are stable when regenerated from subcultured callus. This putative maintenance of stability is important in view of the limited number of genotypes which have proved stable in callus cultures (see Section *B.3.4.*). It must be remembered however, that these results are only from an evaluation of the first generation of progeny and further vegetative regeneration cycles may result in the emergence of latent variation such as that found in phenotypically 'normal' potato (see Section *B.3.4.*).

The absence of any significant difference in vegetative characteristics of the first callus subculture progeny with those of controls derived from axenic leaves (see Section E.) suggests that callus culture may provide a means of propagating and storing *B. rex* 'Lucille Closon' (see Section *B.2.*). This will only be possible if large populations of callus progeny prove stable and stability is also retained in subsequent vegetative regeneration cycles. Callus

culture could furthermore be used in the development of procedures for the production of embryos, such as those found in *B. semperflorens* (Sehgal, 1975). Thus allowing the mechanization of *B. rex* micropropagation (Lawrence, 1981) and increasing cost effectiveness (see Section *B.2.*). The realization of a mechanized micropropagation procedure for the propagation of *B. rex* from somatic embryos however will only be achieved when a combination of efficient, synchronized embryogenesis with a procedure for encapsulation and planting of somatic embryos has been developed. More recent research (see Tomes, 1984) and studies by Ammirato (1983a, b) and Vasil (1985 *loc. cit.*) would suggest that such developments will be attainable in the near future.

Cytogenetic analysis of both axenic leaves and their derived calli, indicate that the process of disorganized growth and the presence of 2,4-D, does upset the normal functioning of the mitotic cell cycle in *B. rex* resulting in a diversity of cell genotypes. Due to an array of factors which may control genome destabilization in callus culture (see Section *B.3.3.*) the origin of genome instability in *B. rex* callus cannot be conclusively defined. Unlike calli of most other plants where cellular cytogenetics has been studied (see Section *B.3.2.*) the genetic drift in *B. rex* calli is towards somatic reduction. The major changes observed in callus cells include restitution nuclei, cell degeneration, hypopolyploidization and the formation of multimucleate cells. The latter may be a result of breakdown in cell wall formation (see Mahlberg *et al.*, 1975) (i.e. absence of cytokinesis) or the production and subsequent amitosis of endopolyploid nuclei (see Section *B.3.2.*). A similar array of mitotic abnormalities have been reported in a number of other studies on callus cells (see Section *B.3.3.*). In these studies however no attempt was made to link the mitotic abnormalities observed with the cell type or the zone in the callus in which the cells arose. In the present study distinct correlations were observed between cell type and mitotic abnormalities and a zonation of cell types was observed, radiating from small meristematic diploid cells in the central core to 'giant' degenerating cells in the outer core (see Plate E.11.). This gradient in cell types could be related to the presence of an endogenous hormone and nutrient gradient (Cooper *et al.*, 1964) and the build up of waste metabolites such as nucleic acids which may contribute to a

breakdown in cell cycle control resulting in endocycles, restitution cycles or mitotic abnormalities (Bayliss, 1980). The degeneration of cells in the outer core of the callus may as suggested by Gahan (1981) also be a consequence of an environmentally induced switch in groups of genes into a pattern of activity which may result in restitution or endocycles (see Section *B.3.2*) or a moribund mitosis as described by Darlington (1942) which results in multinucleation and cell death. The reason for maintenance of a central zone of diploid cells in *B. rex* calli is unclear but may be a consequence of their position and the nature of callus organization (see Orton, 1984b).

As well as subculturing calli, calli were also aged for different periods of time (see Figure E.8.) and in this way cells were subjected to a range of stresses as imposed by limitations in nutrients, a reduction in water potential and a build up of metabolic waste products. Such stresses have been implicated in genome reorganization (see Walbot and Cullis, 1985) and would normally reduce cell competence (Smith and Street, 1974). Despite these conditions, a high level of cell competence was maintained in *B. rex* callus resulting in the production of large numbers of plantlets (ca. 140 per callus), the majority of which were normal in phenotype. While no major difference was observed in the cytology of aged calli when compared with sub-cultured calli (see Figure E. 4.) a number of polyploids were produced from one callus. This suggests that stress did cause some form of genome instability. Polyploid production may be linked to the emergence of a karyotype with a selective advantage induced by the conditions present in the callus as found by Ogihara (1982) (see Figure B.6.). The origin of the karyotypic change induced may be in the induction of mutations by automutagens present in the aged callus as found in aged seed (D'Amoto and Hoffman Ostenhof, 1957) or due to nutrient starvation which may lead to mitotic abnormalities (Rost, 1977), genome reorganization or mutation (Curtis, 1976; Nowell, 1976; Walbot and Cullis, 1985). Other mutations which may have arisen in calli as a result of aging will only be fully evaluated when the phenotypes of mature plants derived from these calli are assessed.

As previously discussed, attentuation of variation occurs during regeneration from callus and this has been observed in many other plant calli (see Orton, 1984a). Few attempts however have been made to reduce this attenuation and exploit the variation present in

callus through selective enhancement of differentiation in variant cells by incorporating nutrients or hormone supplements in regenerative media. In the present study attempts to produce variants by allowing the expression of pre-existing variation in the callus or inducing variation *de novo* by providing appropriate media supplementation have not as yet resulted in the production of variants. This approach, however, does merit further study due to the evident requirement of some cells for specific nutrient and hormonal stimuli for growth as demonstrated by Torrey (1961).

While somatic variation, as discussed above, may be generated in callus cultures complex explant culture also provides a means of expressing pre-existing variation in plant tissues by inducing the differentiation of variant cells. Studies here on *B. rex* indicate that variants may be produced from pith tissue (see Section *E.11.*) and give further supportive evidence to the suggestions by Van Harten *et al.* (1981) and Cassells *et al.* (1983, 1986) that explants may be as useful as isolated cells or protoplasts for the generation of variants in tissue culture.

In contrasting the present study with other reports on the destabilization of plant genomes using callus culture, explant culture or colchicine and mutagenic treatments (Section *B.3.*) it is evident that the genome under study is highly stable. Many plants which show a high level of instability *in vitro* are either chimeral or polyploid (see Cassells, 1985). A low level of heterozygosity has been suggested as a cause for genome stability in some plants (Broertjes, 1976) and may apply to the *B. rex* cultivar studied here. Stability may also be related to the regeneration procedures used, which concentrated mainly on the use of young axenic tissues as explants. Cells in these tissues may be less liable to undergo mitotic abnormalities and may have more rapid mechanisms for DNA repair than cells from mature tissues (see Osborne *et al.*, 1984). It is also possible that stability may be related to the inherent propensity of the genome for plantlet production and an overall mechanism such as DNA methylation (see Section G.) which ensures fidelity of replication and prevents breakdown of regular mitotic division once cells are provided with adequate nutrients and a stimulus for growth. In callus cells demethylation may result in epigenetic effects which lead to increased cellular heterogeneity as suggested for mammalian tumours (Frost and Kerbel,

1983, see Section *B.3.3.3.*). Similarly irradiation treatments may result in demethylation allowing the expression of latent genes. This change may not be permanent (epigenetic) hence further vegetative cycles may cause breakdown of these variants.

Here, the presence and expression of pre-existing variation in *B. rex* and the manifest attenuation of variation in regenerates from callus cultures prompts a discussion on the relative merits of attempting regeneration from *in vivo* or *in vitro* sources of somatic variation (see Section *B.3.*). Somatic variation *in vivo* has the advantage of existing within the differentiated tissue and consequently may be expressed if explants are provided with appropriate stimuli. In contrast genetic variation in cultured cells, which can arise at a more rapid rate, does so outside any normal developmental context and hence valuable mutations may be accompanied by those which eliminate developmental competence (e.g. Gould, 1978). Also, as indicated in studies by Van Harten *et al.* (1981), the range of spontaneous variation produced from some genotypes in complex explant culture may be as great as that produced after irradiation treatment. Hence in some cases, particularly unstable genotypes and chimeras (see Section *B.3.*), explants may provide a major source of variants. Other genotypes may not have the inherent instability or genetic architecture which would provide an array of somaclonal variants when introduced into culture. In these plants the generation of genetic heterozygosity by the polyploidization of the genome (see Broertjes, 1976), as demonstrated here, may prove an effective method of generating genetic diversity.

Results of the present study and other reports on variation *in vivo* and *in vitro* (see Section *B.3.*) indicate that mitosis plays a major role in generating genetic diversity. The culture of tissues *in vitro* appears to speed up the process of induced variation by environmental and selective pressures and furthermore allows the maintenance of cell genotypes which would normally degenerate.

Our understanding of events occurring *in vitro*, which will be essential to the use of tissue culture for the dual purpose of inducing variation and/or cloning, depends on the development of a greater awareness of the effect of stress on genome stability *in vitro*. Furthermore more attention should be focused on the genetic architecture of the plant introduced into culture (see Section *B.3.*) and the development of suitable regeneration procedures for limiting variant production from unstable and chimeral plants.

F.2. Proposals for future work

While the micropropagation procedures developed here for *B. rex* produced large numbers of plantlets (see Section *D.2.*) many of these were too small for planting out. To fully avail of the high rate of multiplication available, particularly in continuous culture (see Figure D.1.) procedures which ensure the development of these shoots should be assessed. Liquid culture as used by Takayama and Misawa (1981) for shoot development in *B.* x *hiemalis* may prove a useful means of producing large numbers of well developed shoots. In developing such a system the problem of vitrification and desiccation on transplanting (see Section *B.2.*) should be prevented by the use of appropriate culture vessels and acclimatization procedures.

An increase in the efficiency of the micropropagation procedures developed here will also require the development of conditions for plant acclimatization which are optimal at all times of the year. Here, low survival rates and reduced plant vigour in some experiments was due to a seasonal effect. *B. rex* like many other tissue culture plants (see Section *B.2.1.5.*) require an extensive acclimatization period. Elimination of this step by appropriate pretreatments *in vitro* or the application of antitranspirants (see George and Sherrington, 1984) deserves further study as it would increase the cost effectiveness of micropropagation.

The differences between morphogenic responses of *B. rex* genotypes in studies on axenic explants may be related to intrinsic differences in endogenous hormone levels. If on the establishment of suitable procedures for the assessment of endogenous hormone a correlation can be established between differences in morphogenic responses and endogenous hormone levels it may be possible to pretreat explants with exogenous hormones prior to explantation to decrease relative differences in endogenous hormones between genotypes.

Latent systemic bacterial infection, as observed in axenic explants of *B.* x *tuberhybrida*, may hamper the development of continuous culture systems in other plants. Hence suitable precautions should be undertaken to eliminate latent infection. For *B.* x *tuberhybrida* the procedures developed by Reuther and Bandari (1981) or Hakkaart and Versluijs (1983b) may prove successful.

Here, a range of tissue culture procedures have been assessed for their effect on genome stability in *B. rex*. There remain many other

tissue culture methods which could be studied for their effects on the production of variants and genome stability in *B. rex*, these include;

1. Single cell or protoplast culture which may give a wider range of variants due to the removal of organizational influences of the explant. Protoplasts could also be used to produce new variants if the bottlenecks which have to date limited the success of somatic hybridization and the integration of foreign DNA into plant cell genomes can be overcome.
2. Production of less organized callus which may result in a wider diversity of cell genotypes within calli as found in *Hordeum* (Orton, 1982).
3. Cell suspension culture which may increase the rate of genetic drift.
4. Media supplementation with hormones, organics or sucrose to induce variation during (a) callus induction, (b) proliferation and subculture, (c) regeneration.
5. Media supplementation to enhance the production of variants from somatic tissues of complex explants.
6. Culture stress at the callus or explant level to induce genome reorganization.

In the present study the low level of variation induced in *B. rex* after the application of treatments shown to induce wide range of variants in other genotypes may be related to a low level of heterozygosity. This would appear to confirm the suggestion made by a number of authors (e.g. Chaleff, 1981; Browers and Orton, 1982a) that the genotype may control the level of genetic diversity which can be induced in tissue culture. To establish the extent of genotype influence on the generation of genetic diversity *in vitro* a comparative analysis of a diploid and induced polyploid of *B. rex* could be undertaken. This study would also provide results which would allow an evaluation of the suggestion that the polyploid state enhances the level of variation which may be induced in tissue culture (see Section *B.3.3.*). These results would also allow an assessment of the relative merits of polyploidizing genomes which show a low level of heterozygosity to increase the range of variants induced. Bearing in mind the instability of many induced polyploids (Gottschalk, 1978) and the fact that some polyploidizing treatments may cause mutations in the genes

controlling mitosis (e.g. Ogura, 1978) it will be important to determine the genetic architecture of these plants and their potential for producing stable variants. To maintain stability in induced variants, suitable regeneration procedures will have to be devised. If embryos can be developed from single cells of *B. rex* and the plants produced are true to type then this method may prove an effective means of maintaining genome stability (see Vasil, 1985).

The initial aim of the present study was to assess the potential use of tissue culture for micropropagation and the production of variants in *B. rex* with a view to its future application to other begonias. The procedures and results outlined here suggest that tissue culture methods for producing variants are useful in the breeding of sterile plants such as *B. rex*. The wider application of tissue culture procedures of continuous culture for stock independent micropropagation and for the production of variants, to other begonias should provide a fruitful area of research and deserves further study.

SECTION G. GLOSSARY

Aneusomaty - used to describe somatic cells which contain chromosome numbers which are not multiples of the haploid number. Usually there are more chromosomes than the diploid but hypodiploid cells are also found. Variability in many cases is due to heterochromatic chromosomes (→ B-chromosomes).

Asynchronous division - caused by timing imbalance in division of chromosomes. Appears most often as differential contraction of chromosomes in metaphase plates.

B-chromosomes - unstable supernumary or accessory chromosomes.

Continuous culture - refers to the culture of complex explants derived from plantlets grown *in vitro*. The culture is continuous as it is maintained by continuous cycles of plantlet induction from explants derived from preceding regeneration cycles e.g. S2 derived from axenic leaf explants of S1.

Cytochimera - a chimera in which different tissue sectors or tissues differ in chromosome number.

Cytotype - used to describe progeny which show changes in chromosome number which may arise from vegetative propagation of aneusomatic plants. Any variety of a species whose chromosome complement differs qualitatively (in chromosome number) or qualitatively (in chromosome structure) from the standard complement of the species.

DNA methylation - cytosine moiety of DNA methylated by DNA methyl-transferase. Thereby limiting the expression of certain genes.

Discontinuous culture - refers to culture of complex explants derived from *in vivo* grown donor plants.

Endocycles - defines DNA replication cycles within the nuclear envelope and without spindle formation. May be total or differential (underreplication or amplification).

Epigenotype - the biochemical and physiological state of a plant under certain environmental and developmental constraints.

Genetic architecture - the total somatic karyotpyes which exists in plant tissues. Includes diploid cells and all forms of somatic variant karyotypes.

Mendelian variation - does not result from gross chromosomal change, is heritable and segregates in crosses with individuals exhibiting a disparate phenotype (Orton, 1984a).

Mixoploid - plant with somatic cells of different ploidies (i.e. 2n, 4n, 8n etc) unlike polysomatic plants the polyploid cells are not confined to specific tissues of the plant.

Mutant - genetic stability and sexual transmission of a mutated trait.

C-mitosis - Occurs when as a result of spindle failure chromosomes do not move to the poles at anaphase. Leads to polyploidy.

Polysomatic - describes plants which undergo endocycles as a normal course in differentiation producing endopolyploid cells.

Robertsonian translocations - represent chromosome structural and karyotype changes due to centric fusion or fusion.

Somaclonal variants - describes variants arising from cells or tissues cultured *in vitro*. In this way this term covers both pre-existing and induced variation derived from tissue cultures.

Stress - indicates some form of disequilibrium where the buffering capacity of plants fails and the survival of the organism is dependent on the induction of a mechanism which generates rapid genomic change (see Walbot and Cullis, 1985).

Variant - novel phenotypes which are insufficiently characterized to justify their designation as novel genotypes.

SECTION H. BIBLIOGRAPHY

Ahloowalia, B.S. (1983). Spectrum of variation in somaclones of triploid ryegrass. Crop. Sci. 23: 1141-1147.

Ahloowalia, B.S. and Maretski, A. (1983). Plant regeneration via somatic embryogenesis in sugarcane. Plant Cell Rep. 2: 21-25.

Aitken, J., Horgan, K.J. and Thorpe, T.A. (1981). Influence of explant selection on the shoot forming capacity of juvenile tissues of *Pinus radiata*. Can. J. Forest. 11(1): 112-117.

Alleweldt, G. and Radler, F. (1961). Interrelationship between photoperiodic behaviour of grapes and growth of plant tissue cultures. Plant Physiol. 36: 376-379.

Ammirato, P.V. (1983a). The regulation of somatic embryo development in plant cell cultures: Suspension culture techniques and hormone requirements. Bio Technology 1: 68-74.

Ammirato, P.V. (1983b). Embryogenesis. In: Handbook of Plant Cell Cultures. Vol. 1. (Eds. D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada). MacMillan, New York, pp. 82-112.

Anderson, W.C. and Meagher, G.W. (1978). Cost of micropropagating plants through tissue culture using lilies as an example. North West Res. Exp. Unit Mimeo.

Appelgren, M. (1985). Effect of supplementary light to mother plants on adventitious shoot formation in flower peduncle segments of *Begonia* x *hiemalis* Fotsch *in vitro*. Scientia Hortic. 25: 77-83.

Appelgren, M. and Heide, O.M. (1972). Regeneration in *Streptocarpus* leaf discs and its regulation by temperature and growth substances. Physiol. Plant. 24: 417-423.

Arends, J.C. (1970). Somatic chromosome numbers in 'Elatior'-begonias. Meded. Landbouwhogeschool Wageningen 70-20: 1-18.

Arisumi, T. and Frazier, L.C. (1968). Cytological and morphological evidence for the single-cell origin of vegetatively propagated shoots in 13 cultivars of *Saintpaulia* treated with colchicine. Proc. Amer. Soc. Hort. Sci. 93: 679-685.

Armstrong, K.C., Nakamura, C. and Kelleher, W.A. (1983). Karyotype instability in tissue culture of regenerates of *Triticale* (x *Triticosecale* Wiltmack) cv. Welsh from 6-month-old callus cultures. Z. Pflanzenzüchtg. 91: 233-245.

Arora, Y.K., Nakao, S. and Nakajima, T. (1970). Perpetuation of *Begonia rex* by aseptic cultures with micro-leaf cuttings under various conditions of auxin and cytokinin. Jap. Jour. Breeding 20(5): 275-281.

Ashmore, S.E. and Gould, A.R. (1982). Karyotype evolution in a tumour derived plant tissue culture analysed by Giemsa C-banding. Protoplasma 106: 297-308.

Atkinson, G.F., Franzke, C.J. and Ross, J.G. (1957). Differential reaction of two varieties of *Sorghum* to colchicine treatment. J. Hered. 48: 259-264.

Bajaj, Y.P.S. (1977). Initiation of shoots and callus from potato-tuber sprouts and axillary buds frozen at -196°C. Crop. Improv. 4: 48-53.

Bajaj, Y.P.S. and Reinerts, J. (1977). Cryobiology of plant cell cultures and establishment of gene banks. In: Plant Cell Tissue and Organ Culture (Eds. J. Reinert and Y.P.S. Bajaj). Springer Verlag, New York, pp. 757-778.

Bajaj, Y.P.S., Ram, H.K., Labana, K.S. and Singh, H. (1981). Regeneration of genetically variable plants from the anther derived callus of *Arachis hypogaea* and *Arachis vidlosa*. Plant Sci. Lett. 23: 35-40.

Banks, M.S. and Evans, P.K. (1976). The use of heterochromatin spot counts to determine ploidy levels in root tips and callus cultures of *Nicotiana otophora* and its hybrids. Plant Sci. Lett. 7: 417-427.

Barlass, M. and Skene, K.G.M. (1982). *In vitro* plantlet formation from *Citrus* species and hybrids. Scientia Hortic. 77: 333-341.

Barlow, P.W. (1976). Towards an understanding of meristem behaviour. J. Theor. Biol. 57: 433-451.

Barnhill Jones, J. (1979). Commercial use of tissue culture for the production of disease free plants. In: Plant Cell and Tissue Culture (Eds. W.R. Sharp, P.O. Larsen, G.F. Paddock and V. Raghaven). Ohio State Univ. Press, Columbus, pp. 441-451.

Baroncelli, S., Buiatti, M. and Bennici, A. (1974). Genetics of growth and differentiation *in vitro* of *Brassica oleracea* var. botrytis. III. Genetic correlations and ontogenetic unity. Z. Pflanzenzüchtg. 72: 257-282.

Barton, K.A. and Brill, W.J. (1983). Prospects in plant genetic engineering. Science 219: 671-676.

Bastiaens, L., Maene, L., Harbaui, Y., Van Suiniere, C., Vande Casteele, K.L. and Debergh, P.C. (1983). The influence of antibacterial products on plant tissue cultures. Med. Fac. Landbouww. Rijsuniv. Gent. 48: 13-24.

Bauer, E. (1930). Einführung in die experimentelle vererbungshehre. Berlin. (Cited from Neilson-Jones, 1969).

Bayliss, M.W. (1973). Origin of chromosome number variation in cultured plant cells. Nature 246: 529-530.

Bayliss, M.W. (1976). Variation of cell cycle duration within suspension cultures of *Daucus carota* and its consequence for the induction of ploidy changes with colchicina. Protoplasma 38: 279-285.

Bayliss, M.W. (1977). The effect of 2,4-D on growth and mitosis in suspension cultures of *Dacus carota*. Plant Sci. Lett. 8: 99-

Bayliss, M.W. (1980). Chromosomal variation in plant tissues in culture. Int. Rev. Cytol. Suppl. 11A: 113-144.

Beadle, G.W. (1931). A gene in maize for supernumary cell division following meiosis. Cornell Univ. Expt. St. Mem. 135: 1-12.

Beadle, G.W. (1933). A gene for sticky chromosomes in maize. Z. Ind. Abstramm. Verebungel 63(3): 195-217.

Bedson, F. (1954). Successful *Begonia* culture. Collidridge Ltd., New York.

Bennett, M.D. (1973). Nuclear characters in plants. Brookhaven Symp. Biol. 25: 344-366.

Bennici, A. (1979). Cytological chimeras in plants regenerated from *Lilium longiflorum* tissue grown *in vitro*. Z. Pflanzenzüchtg. 82: 349-353.

Bennici, A., Cionnini, P.G. and D'Amoto, F. (1976). Callus formation from the suspensor of *Phaseolus coccineus* in hormone-free medium: a cytological and DNA cytophotometric study. Protoplasma 89: 251-261.

Bennici, A. and D'Amoto, F. (1978). *In vitro* regeneration of *Durum* wheat plants. I. Chromosome number of regenerated plantlets. Z. Pflanzenzüchtg. 81: 305-311.

Berghoef, J. and Bruinsma, J. (1979). Flower development of *Begonia franconis* Liebm. IV. Adventitious flower bud formation on excised inflorescence pedicels *in vitro*. Z. Pflanzenphysiol. 94: 407-416.

Bigot, C. (1971). Analyse de quelque facteurs du bourgeonnement sur des explant foliaires, en particular chez un clone de *Begonia rex*. Thèse de Doctorat d'Etat. CNRS N° AO 6333 Fac. Sci. Clermont Ferrand, 274 pp.

Bigot, C. (1975). Multiplication végétative de *Gloxina* hybrida a partir d'organes cultives *in vitro*. Ann. Amelior. Plantes 25(3): 337-351.

Bigot, C. (1981a). Multiplication végétative *in vitro* de *Begonia* x *hiemalis* ("Rieger" et "Schwabenland"). I. Methodologie. Agronomie 1(6): 433-440.

Bigot, C. (1981b). Multiplication végétative *in vitro* *Begonia* x *hiemalis* ("Rieger" et "Schwabenland"). II. Conformité des plantes elevées en serre. Agronomie 1(6): 441-447.

Bigot, C. (1982). *In vitro* propagation of *Begonia* x *hiemalis* Fotsch: plant conformity. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara), Maruzen, Tokyo, pp. 685-696.

Bilkey, C. and Cocking, E.C. (1981). Increased plant vigour by *in vitro* propagation of *Saintpaulia ionantha* Wendl. from sub-epidermal tissue. HortScience 16(5): 643-644.

Bilkey, P.C. and McCowan, B.H. (1979). *In vitro* culture and propagation of *Episcipa* species. J. Am. Soc. Hortic. Sci. 104: 109.

Boxus, P. (1974). Production of strawberry plants by *in vitro* micropropagation. J. Hort. Sci. 49(3): 209.

Brainerd, K. and Fuchigami, L. (1981). Leaf anatomy and stomatal functioning in the acclimatization of tissue cultured apples and plums. New Horizon HRI Washington DC.

Brainerd, K. and Fuchigami, L.H. (1983). Tips for transplanting tissue cultured plants. J. Environ. Hortic. 1: 23-25.

Brink, R.A., Styles, E.D. and Artel, J.D. (1968). Paramutation: directed genetic change. Science 159: 161-170.

Britton, D.M. and Hull, J.W. (1956). Mitotic instability in blackberry seedlings. Jour. Hered. 47: 205-210.

Broertjes, C. (1969). Mutation breeding in *Streptocarpus*. Euphytica 18: 333-339.

Broertjes, C. (1974). Mutation breeding of *Achimenes*. Euphytica 21: 48-62.

Broertjes, C. (1974). Production of polyploids by the adventitious bud technique. In: Polyploidy and Induced Mutations in Plant Breeding. IAEA, Vienna, pp. 29-34.

Broertjes, C. (1976). Mutation breeding in vegetatively propagated crops. Acta. Hortic. 63: 187-202.

Broertjes, C. (1982). Significance of *in vitro* adventitious bud techniques for mutation breeding of vegetatively propagated crops. In: Induced Mutations in Vegetatively Propagated Plants II. IAEA, Vienna, 1982, pp. 1-10.

Broertjes, C. and Van Harten, A.M. (1978). Application of Mutation Breeding Methods in the Improvement of Vegetatively Propagated Plants. An Interpretative Literature Review. Elsevier, Amsterdam.

Broertjes, C. and Keen, A. (1980). Adventitious shoots: do they develop from one cell? Euphytica 29: 73-87.

Broertjes, C., Haccius, B. and Weidlich, S. (1968). Adventitious bud formation on isolated leaves and its significance for mutation breeding. Euphytica 17: 321-344.

Brossard, D. (1974). Etude cytophotometric des variations des content en DNA nucléaire en cours de la differentiation de la moelle de Tabac (*Nicotiana tabacum*) cultivée *in vitro*. C.R. Acad. Sc. Paris 278 Ser. D: 2517-2520.

Brossard, D. (1976). The influence of kinetin on formation and ploidy level of buds arising from *Nicotiana tabacum* pith tissue grown *in vitro*. Z. Pflanzenphysiol. 78: 323-333.

Brossard, D. (1978). Microspectrophotometric and ultrastructural analysis of a case of cell differentiation without endopolyploidy: the pith of *Crepis capillaris*. Protoplasma 93: 369-380.

Brossard, D. (1979). Neoformation of vegetative and reproductive buds from foliar discs of *Crepis capillaris* L. Wallr. cultured *in vitro*. Z. Pflanzenphysiol. 93(1): 67-81.

Browers, M.A. and Orton, T.J. (1982a). A factorial study of chromosomal variability in callus cultures of celery (*Apium graveolens*). Plant Sci. Lett. 26: 65-73.

Browers, M.A. and Orton, T.J. (1982b). Transmission of gross chromosomal variability from suspension cultures into regenerated celery plants. Jour. Hered. 73: 159-162.

Brown, J.L. and Harney, P.M. (1974). Induction of non-chimeral mutants in *Begonia*. HortScience 9(3): 276. Abstract.

Brown, D.W.C., Leung, D.W.M. and Thorpe, T.A. (1979). Osmotic requirement of shoot formation in tobacco callus. Physiol. Plant. 46: 36-41.

Bush, B.S.R., Earle, E. and Langhans, R.W. (1976). Plantlets from petal segments, petal epidermis and shoot tips of the periclinal chimera *Chrysanthemum morifolium* 'Indianapolis'. Amer. Jour. Bot. 63(6): 729-737.

Buiatti, M., Baroncelli, S., Bennici, A., Pagliai, M. and Tesi, R. (1974). Genetics of differentiation *in vitro* in *Brassica oleraceae* var. botrytis. II. An *in vitro* and *in vivo* analysis of diallel cross. Z. Pflanzenzüchtg. 72: 269-274.

Butcher, D.N., Sogeke, A.K. and Tommerup, I.C. (1975). Factors influencing changes in ploidy and nuclear DNA levels in cells from normal, crown-gall and habituated cultures of *Helianthus annus* L. Protoplasma 86: 295-308.

Butterfass, T. (1965). Der Endopolyploidiegrad als neur Aspekt der Ploidiezuchtung Zuchter 35: 293-296 (cited from Nagl, 1978).

Butterfass, T. (1966). Neue Aspekte der Polyploidieforschung und Züchtung. Mitt. Max. Plank. Ges. 1: 47-58 (cited from Nagl, 1978).

Buxton, B.R. (1939). Begonias. (ed. E.I. Farrington). Adams Press, Massachusetts (cited from Thompson and Thompson, 1981).

Caffaro, L., Dameri, R.M., Profumo, P. and Bennici, A. (1982). Callus induction and plantlet regeneration in *Cichorium intybus* L. I. A cytological study. Protoplasma 111: 107-112.

Cambell, C.T. and Tomes, D.T. (1983). Establishment and multiplication of red clover plants by *in vitro* shoot tip culture. Plant Cell Tissue Organ Culture 3: 49-57.

Carlsberg, I., Glimelius, K. and Eriksson, T. (1984). Nuclear DNA content during the initiation of callus formation from isolated protoplasts of *Solanum tuberosum* L. Plant Sci. Lett. 35: 225-230.

Carlson, P.S. and Chaleff, R.S. (1974). Heterogenous associations of cells formed *in vitro*. In: Genetic Manipulation with Plant Materials (Ed. L. Ledoux). Plenum Press, New York, pp. 245-261.

Carmona, M.J. and Gutiérrez, C. (1981). DNA replication during differentiation of cortical cells in *Allium* roots. Plant Cell Reports 1: 23-25.

Cassells, A.C. (1979). The effect of 2,3,5-triiodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol. Plant. 46: 159-164.

Cassells, A.C. (1984). Micropropagation in Horticulture and in Conservation. Moorea 3: 11-21.

Cassells, A.C. (1985). Genetic, epigenetic and non-genetic variation in tissue culture derived plants. In: In vitro Techniques. Propagation and Long-term Storage (Ed. A. Schafer-Menher). Martinus Nijhoff, (in press).

Cassells, A.C. and Carney, B.F. (1985). Adventitious regeneration in *Pelargonium* x *domesticum* 'Bailey'. Acta Hortic. (submitted).

Cassells, A.C. and Plunkett, A. (1984). Production and growth analysis of plants from leaf cuttings and from tissue cultures of discs from mature leaves and young leaves of African violet (*Saintpaulia ionantha* Wendl.). Scientia Hortic. 23: 361-369.

Cassells, A.C. and Plunkett, A. (1986). Habit differences in African violet produced from leaf cuttings and *in vitro* from leaf discs and recycled axenic leaves. In: Plant Tissue Culture and its Agricultural Applications (Eds. P.G. Alderson and L.A. Withers). Butterworth, London, (in press).

Cassells, A.C., Minas, G. and Long, R.D. (1980). Culture of *Pelargonium* hybrids from meristems and explants: chimeral and beneficially infected varieties. In: Tissue Culture Methods for Plant Pathologists (Eds. D.S. Ingram and J.P. Helgeson). Blackwell, Oxford, pp. 125-130.

Cassells, A.C., Long, R.D. and Mousdale, D.A. (1982). Endogenous IAA and morphogenesis in tobacco petiole cultures. Physiol. Plant. 56: 507-512.

Cassells, A.C., Goetz, E.M. and Austin, S. (1983). Phenotypic variation in plants produced from lateral buds, stem explants and single-cell derived callus of potato. Potato Res. 26: 367-372.

Cassells, A.C., Farrell, G. and Goetz, E.M. (1986). Variation in the tissue culture progeny of the chimeral potato (*Solanum tuberosum* L.) variety Golden Wonder. In: Somaclonal Variation and Plant Improvement (Ed. J. Semel). Martinus Nijhoff, (in press).

Chaleff, R.S. (1981). Genetics of Higher Plants. Applications of Cell Culture. Cambridge University Press.

Chaleff, R.S. (1983). Isolation of agronomically useful mutants from plant cell cultures. Science 219: 676-682.

Chaturvedi, H.C. and Mitra, G.C. (1975). A shift in morphogenic pattern in *Citrus* callus tissue during prolonged culture. Ann. Bot. 39: 683-687.

Chaturvedi, H.C., Chowdhur, A.R. and Mitra, G.C. (1974). Morphogenesis in stem callus tissues of *Citrus grandis* in long-term cultures - biochemical analysis. Curr. Sci. 43(5): 139-142.

Chen, C.H. and Ross, J.G. (1963). Colchicine induced somatic chromosome reduction in *Sorghum*. Crop Sci. 3: 355-358.

Chylah, A. (1972). Neoformation caulinaire et radiculaire chez les fragments de jeuille de *Begonia rex* Putz: Action de divers facteurs regulateurs trophiques et d'environment. Biologia Plantarum 14(3): 204-212.

Chlyah, A., and Tran Thanh Van, M. (1975). Differential reactivity in epidermal cells of *Begonia rex* excised and grown *in vitro*. Physiol. Plant 35: 16-20.

Chylah, A. and Tran Thanh Van, M. (1984). Histological changes in epidermal and subepidermal cell layers of *Begonia rex* induced to form de novo unicellular hairs, buds and roots. Bot. Gaz. 145: 55-59.

Cionini, P.G., Bennici, A. and D'Amoto, F. (1978). Nuclear cytology of callus induction and development *in vitro*. I. Callus from *Vicia faba* cotyledons. Protoplasma 96: 101-112.

Clutter, M., Brady, T., Walbot, V. and Sussex, T. (1974). Macromolecular synthesis during plant embryogeny cellular rates of RNA synthesis in diploid andpolyploid cells in bean embryos. J. Cell Biol. 63: 1097-1102.

Cohl, H. and Moser, B.C. (1976). Effect of photoperiodic manipulation on seasonal variation in bud and shoot regeneration in Rieger Begonia leaf cuttings. HortScience 11(4): 376-377.

Conger, B.V. (1981). Cloning Agricultural Plants via *in vitro* Techniques. CRC Press Boco Raton, Florida.

Conger, A.D. and Fairchild, L.M. (1953). A quick freeze method for making smear slides permanent. Stain Technol. 28: 281-283.

Chen, C. and Goden-Kallemeyn, Y.C. (1979). *In vitro* induction of tetraploid plants from colchicine treated diploid daylily callus. Euphitica 28: 705-709.

Conger, B.V., Hilenski, L.L., Lowe, K.W. and Carabia, J.V. (1982). Influence of different auxins at varying concentrations on callus induction and growth from embryos and leaf tip explants in Graminaceae. Env. Expt. Bot. 22: 39-48.

Conger, B.V., Hanning, G.E., Gray, D.J. and McDaniel, J.K. (1983). Direct embryogenesis from mesophyll cells of Orchardgrass. Science 221: 850-851.

Conner, A.J. and Thomas, M.P. (1981). Re-establishment of plantlets from tissue culture: A review. Proc. Inter. Plant. Proc. Soc. 31: 343-357.

Constantin, M.J. (1981). Chromosome instability in cell and tissue cultures and regenerated plants. Env. Expt. Bot. 2(3/4): 359-368.

Cooke, R.C. (1977). Tissue culture propagation of African violet. HortScience 12(6): 549.

Cooke, R.C. (1979). Homogenization as an aid in tissue culture propagation of *Platycerium* and *Davallia*. HortScience 14: 21-22.

Cooper, L.S., Cooper, D.C., Hildebrandt, A.C. and Riker, A.J. (1964). Chromosome numbers in single cell clones of tobacco tissues. Amer. J. Bot. 51(3): 284-290.

Cullis, C.A. (1983). Environmentally induced DNA changes in plants. CRC Review in Plant Science 1: 117-131.

Curtis, H.J. (1966). Biological Mechanisms of Aging. Springfield: C.C. Thomas Publisher.

Curtis, H.J. (1971). Genetic factors in aging. Adv. Genet. 16: 305-324.

Dale, J.E. (1976). Cell division in leaves. In: Cell Division in Higher Plants. (Ed. M.M. Yeoman). Academic Press, London, pp. 315-347.

D'Amato, F. (1952). Polyploidy in the differentiation and function of tissues and cells in plants. A critical examination of the literature. Caryologia 4: 311-358.

D'Amato, F. (1959). C-mitosis and experimental polyploidy in plants. Genetica Agraria 11: 117-126.

D'Amato, F. (1975). The problem of genetic stability in plant tissue and cell culture. In: Crop Genetic Resources for Today and Tomorrow (Eds. O.H. Frankel and E.G. Hawkes). Cambridge University Press, pp. 333-348.

D'Amato, F. (1977a). Nuclear Cytology in Relation to Development. Cambridge University Press.

D'Amato, F. (1977b). Cytogenetics of differentiation in tissue and cell cultures. In: Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture. (Eds. J. Reinert and Y.P.S. Bajaj). Springer-Verlag, Berlin, pp. 343-357.

D'Amato, F. (1978). Chromosome number variation in cultured cells and regenerated plants. In: Frontiers of Plant Tissue Culture (Ed. T.A. Thorpe). Intern. Ass. Plant Tissue Culture, Calgary, pp. 287-295.

D'Amato, F. and Hoffman Ostenhof, O. (1956). Metabolism and spontaneous mutations in plants. Adv. Genet. 8: 1-28.

D'Amato, F., Bennici, A., Cionini, P.G., Baroncelli, S. and Lupi, M.C. (1980). Nuclear fragmentation followed by mitosis as a mechanism for wide chromosome number variation in tissue cultures: Its implications for plant regeneration. In: Plant Cell Cultures, Results and Perspectives (Eds. F. Sala, B. Parisi, R. Cella and D. Ciferri). Elsevier, North Holland, Amsterdam, pp. 62-72.

Darlington, L.D. (1942). Chromosome chemistry and gene action. Nature 149: 66-69.

Darlington, L.D. (1973). Chromosome botany and the origin of cultivated plants. Third revised edition. Allen and Unwin, London.

Davidson, A.W., Aitchison, P.A. and Yeoman, M.M. (1976). Disorganized systems. In: Cell Division in Higher Plants (Ed. M.M. Yeoman). Academic Press, London, pp. 407-435.

Debergh, P.C. (1983). Effect of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59: 270-276.

Debergh, P.C. and Maene, L.J. (1981). A scheme for commercial propagation of ornamental plants by tissue culture. Scientia Hortic. 14: 335-345.

Debergh, P., Harbaui, Y. and Lemeur, R. (1981). Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53: 181-187.

De Boucaud, M.T. and Gaultier, J.M. (1983). Evidence of a different nuclear population in chimerae of regenerated plants from tobacco aneuploid tissue cultures. Cytophotometric and statistical analysis. Z. Pflanzenzüchtg. 110(2): 97-105.

De Fossard, R.A. (1976). Tissue culture for plant micropropagators. University of New England Printery, Armidale, Australia.

De-Langhe, E. and De Bruijne, E. (1976). Continuous propagation of tomato plants by means of callus cultures. Scientia Hortic. 4: 221-227.

Demoise, C.F. and Partanen, C.R. (1969). Effects of subculturing and the physical condition of the medium on the nuclear behaviour of a plant tissue culture. Amer. J. Bot. 56(2): 147-152.

De Paepe, R., Bleton, D. and Gnangbe, F. (1981). Basis and extent of genetic variability among doubled haploid plants obtained by pollen culture in *Nicotiana sylvestris*. Theor. Appl. Genet. 59: 177-184.

De Paepe, R., Prat, D. and Knight, J. (1983a). Effects of consecutive androgenesis on morphology and fertility in *Nicotiana sylvestris*. Can. Jour. Bot. 61(7): 2038-2046.

De Paepe, R., Prat, D. and Hugnet, T. (1983b). Heritable nuclear DNA changes in doubled haploid plants obtained by pollen culture of *Nicotiana sylvestris*. Plant Sci. Lett. 28: 11-28.

Dermen, H. (1965). Colchiploidy and histological imbalance in triploid apple and pear. Amer. J. Bot. 42(4): 353-359.

Dermen, H. and Bain, F. (1944). A general cytohistological study of colchicine polyploidy in Cranberry. Am. J. Bot. 31(8): 451-463.

Depta, H., Eisel, K.H. and Hertel, R. (1983). Specific inhibitors of auxin transport action on tissue segments and *in vitro* binding to membranes from maize coleoptiles. Plant Sci. Lett. 31(2-3): 181-192.

de Wilde, J.J. and Arends, J.C. (1980). *Begonia* section *Squamibegonia* Warb. A taxonomic revision. Publication 19 Landbouwhogeschool Wageningen, The Netherlands.

Dhillon, S.S., Wernsman, E.A. and Miksche, J.P. (1983). Evaluation of nuclear DNA content and heterochromatin changes in anther-derived dihaploids of tobacco (*Nicotiana tabacum*) cv. Coker 139. Can. J. Genet. Cytol. 25: 169-173.

Dirks, V.A., Ross, J.G. and Harpstead, D.D. (1956). Colchicine induced true-breeding chimeral sectors in flax. Jour. Hered. 47: 229-233.

Dix, P.J. (1977). Chilling resistance is not transmitted sexually in plants regenerated from *Nicotiana tabacum* and *Capsicum annuum*. Plant Sci. Lett. 5: 231-237.

Dodds, J. (1981). The role of the cell cycle and cell division in xylem differentiation. In: Xylem Cell Development (Ed. J.R. Barnett). Castlehouse Publications Ltd., UK, pp. 153-167.

Doerfler, W. (1983). DNA methylation and gene activity. Ann. Rev. Biochem. 52: 93-124.

Doležel, J. and Novák, F.J. (1984a). Cytogenetic effects of plant tissue culture medium with certain growth substances on *Allium sativum* L. meristem root tip cells. Biologia Plantarum (Praha) 26(4): 293-298.

Doležěl, J. and Novak, F.J. (1984b). Effect of plant tissue culture media on the frequency of somatic mutations in *Tradescantia* stamen hairs. Z. Pflanzenphysiol. 114(1): 51-58.

Donnelly, D.A. and Vidaver, W.E. (1984). Leaf anatomy of red raspberry transferred from culture to soil. J. Amer. Soc. Hort. Sci. 109(2): 172-176.

Doorenbos, J. (1973). Breeding 'Elatior' begonias (*B.* x *hiemalis* Fotsch). Acta Hortic. 31: 127-132.

Doorenbos, J. and Karper, J.J. (1975). X-ray induced mutations in *Begonia* x *hiemalis*. Euphytica 24: 13-19.

Doorenbos, J. and Legros, R.A.H. (1968). Breeding Gloire de Lorraine Begonias. Meded. Landbouwhogeschool Wageningen, 68-19: 1-14.

Dover, G.A. and Flavell, R.B. (1982). Genome Evolution. Academic Press, London, New York.

Dover, G., Brown, S., Coen, E., Dallas, J., Strachen, T. and Trick, M. (1982). The dynamics of genome evolution and species differentiation. In: Genome Evolution (Eds. G.A. Dover and R.B. Flavell). Academic Press, London, pp. 343-372.

Dowerick, G.J. (1953). The chromosomes of *Chrysanthemum*. II. Garden varieties. Heredity 7: 59-72.

Downes, R.W. and Marshall, D.R. (1983). Colchicine induced variants in sunflower. Euphytica 32: 757-766.

Duncan, R.E. (1945). Production of variable aneuploid numbers of chromosomes within the root tips of *Paphiopedilum wardii*. Amer. J. Bot. 32: 506-509.

Durante, M., Geri, C., Grisvard, J., Guille, E., Partenti, R. and Buiatti, M. (1983). Variation in DNA complexity in *Nicotiana glauca* tissue cultures. I. Pith tissue dedifferentiation *in vitro*. Protoplasma 114: 114-118.

Durrant, A. (1981). Unstable genotypes. Phil. Trans. R. Soc. London 292: 467-474.

Dyer, A.F. (1976). Modification and errors of mitotic cell divisions in relation to differentiation. In: Cell Division in Higher Plants (Ed. M.M. Yeoman). Academic Press, London, pp. 199-253.

Earle, E.D. and Langhans, R.W. (1974). Propagation of *Chrysanthemum in vitro*. II. Production, growth and flowering of plantlets from tissue cultures. J. Am. Soc. Hortic. Sci. 99: 352-358.

Earle, E.D. and Langhans, R.W. (1975). Carnation propagation from shoot tips cultured in liquid medium. HortScience 10(b): 608-610.

Earle, E.D. and Demarly, Y. (eds) (1982). Variability in plants regenerated from tissue culture. Praeger, New York.

Economou, A.S. and Reid, P.E. (1984). *In vitro* shoot proliferation of Minnesota deciduous *Azaleas*. HortScience 19(1): 60-61.

Eigsti, O.J. (1940). Effect of colchicine upon the nuclear and cytoplasmic phases of cell division in the pollen tube. Genetics 25: 11b Abstract.

Espino, F.J. and Vazquez, A.M. (1981). Chromosome numbers of *Saintpaulia ionantha* plantlets regenerated from leaves cultured *in vitro* with caffeine and colchicine. Euphytica 30: 847-853.

Evans, I.H. (1976). Polyploidization in the rat liver: the role of binucleate cells. Cytobios 16: 115-124.

Evans, D.A. and Sharp, W.R. (1983). Single gene mutations in tomato plants regenerated from tissue culture. Science 221: 949-951.

Evans, D.A., Sharp, W.R. and Flick, C.E. (1981). Growth and behaviour in cell cultures: Embryogenesis and organogenesis. In: Plant Tissue Culture: Methods and Applications in Agriculture (Ed. T.A. Thorpe). Academic Press, New York, pp. 45-113.

Evans, D.A., Sharp, W.R. and Medina-Filho, H.P. (1984). Somaclonal and gametoclonal variation. Amer. Jour. Bot. 71(6): 759-774.

Fedoroff, N.V. (1983). Controlling elements in maize. In: Mobile Genetic Elements (Ed. J.A. Shapiro). Academic Press, New York, pp. 1-63.

Feng, K.A. and Linck, A.J. (1970). Effect of N-1 naphthylphthalamic acid on the growth and bud formation of tobacco callus grown *in vitro*. Plant Cell Physiol. 11: 589-598.

Finch, R.A. and Bennet, M.D. (1983). The mechanism of somatic chromosome elimination in *Hordeum*. In: Kew Chromosome Conference II (Eds. P.E. Brandham and M.D. Bennett). Allen and Unwin Ltd., London, pp. 147-154.

Fonnesbech, M. (1974a). Temperature effects on shoot and root development from *Begonia* x *cheimantha* petiole segments grown *in vitro*. Physiol. Plant. 32: 282-286.

Fonnesbech, M. (1974b). The influence of NAA, BAP and temperature on shoot and root formation from *Begonia* x *cheimantha* petiole segments grown *in vitro*. Physiol. Plant. 32: 49-54.

Fotsch, K.A. (1933). Die Begonien. Stuttgart 130-134 (cited from Arends, 1970).

Fox, E. (1963). Growth factor requirements and chromosome number in tobacco tissue culture. Physiol. Plant. 16: 793-803.

Franzke, C.J. and Ross, J.G. (1952). Colchicine induced variants in *Sorghum*. Jour. Hered. 43: 107-115.

Franzke, C.J., Sanders, M.E. and Ross, J.F. (1960). Influence of light from an infrared bulb on the mutagenic effect of colchicine on *Sorghum*. Nature 188: 242-243.

Freeling, M. (1984). Plant transposable elements and insertion sequences. Ann. Rev. Plant Physiol. 35: 277-298.

Frost, P. and Kerbel, R.S. (1983). On a possible epigenetic mechanism(s) of tumour cell heterogeneity. Cancer Metastasis Reviews 2: 375-378.

Fuki, K. (1983). Sequential occurrence of mutations in a growing rice callus. Theor. Appl. Genet. 65: 225-230.

Fukumoto, K. (1962). Nuclear instability and chromosomal mosaicism in high polyploids of *Solanum* species and hybrids. Japan J. Botany 18(1): 19-53.

Gahan, P.B. (1977). Increased levels of euploidy as strategy against rapid aging in diploid mammalian systems: An hypothesis. Exp. Geront. 12: 133-136.

Gahan, P.B. (1981). Cell senescence and death in plants. In: Cell Death in Biology and Pathology (Eds. I. Bowen and R. Lockshin). Chapman and Hall, London, pp. 145-169.

Galston, A.W. and Davies, P.J. (1969). Hormonal regulation in higher plants. Science 163: 1288-1297.

Gamborg, O.L., Murashige, T., Thorpe, T.A. and Vasil, I.K. (1976). Plant tissue culture media. *In vitro* 12: 473-478.

Gamborg, O.L. and Shyluk, J.P. (1981). Nutrition, media and characteristics of plant cell and tissue cultures. In: Plant Tissue Culture Methods and Application in Agriculture (Ed. T.A. Thorpe). Academic Press, London, pp. 21-45.

Geitler, L. (1939). Die entstehung der polyploiden somakern der heteropteren durch chromosomenteilung ohne kernteilung. Chromosoma 1: 1-22 (cited from D'Amoto, 1977).

George, E.F. and Sherrington, P.P. (1984). Plant Propagation by Tissue Culture. Eastern Press, Reading, Berks.

Ghosh, A. and Gadgil, V.N. (1979). Shift in ploidy level of callus tissues: A function of growth substances. Ind. J. Expt. Biol. 17: 562-564.

Gilbert, S.K. and Patterson, F.L. (1965). Colchicine induced mutants in 'Decatur' barley. Crop Sci. 5: 44-47.

Goldsmith, M.H.M. (1968). The transport of auxin. Ann. Rev. Plant Physiol. 19: 347-360.

Goldy, R.G. and Lyrene, P.M. (1984). *In vitro* colchicine treatment of 4x blueberries *Vaccinium* sp. J. Amer. Soc. Hort. Sci. 109(3): 336-338.

Gautheret, R.J. (1983). Plant tissue culture: A history. Bot. Mag. Tokyo 96: 393-410.

Gottschalk, W. and Wolff, G. (1983). Induced Mutation in Plant Breeding. Springer-Verlag, Berlin.

Gottschalk, W. (1978). Open problems in polyploid research. The Nucleus 21(2): 99-112.

Gould, A.R. (1978). Diverse pathways of morphogenesis in tissue cultures of the composite *Brachycome lineariloba* (2n = 4). Protoplasma 97: 125-135.

Gould, A.R. (1984). Control of the cell cycle in cultured plant cells. Critical Reviews in Plant Science 1(4): 315-344.

Green, C.E. (1977). Prospects fro crop improvement in the field of cell culture. HortScience 12(2): 131-134.

Green, C.E. and Phillips, R.C. (1975). Plant regeneration from tissue cultures of maize. Crop Sci. 15(3): 417-421.

Griesbach, R.J. (1983). The use of indoleactylamine acids in the *in vitro* propagation of *Phalaenopsis* orchid. Scientia Hortic. 19: 363-366.

Grout, B.W. and Aston, M. (1977). Transplanting of cauliflower plants regenerated from meristem culture. 1: Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hortic. Res. 17: 1-7.

Grout, B.W. and Crisp, E.T. (1980). The origin and nature of shoots propagated from cauliflower roots. J. Hort. Sci. 55: 65-70.

Gunkel, J.E., Sharp, W.R., Williams, B.W., West, W.C. and Drinkwater, W.O. (1972). Root and shoot initiation in sweet potato explants as related to polarity and nutrient media variations. Bot. Gaz. 133(3): 254-262.

Guo, C.L. (1972). Effect of chemical and physical factors on the chromosome number in *Nicotiana* anther callus cultures. *In Vitro* 7(6): 381-386.

Gustafson, J.P. (ed.) (1984). Gene Manipulation in Plant Improvement. Plenum, New York.

Guttman, R. (1956). Effect of kinetin on cell division with special reference to initiation and duration of mitosis. Chromosoma 8: 341-350.

Haberlandt, G. (1902). Kulturersuche mit isolierten Pflanzenzellen. Sitz. Akad. Wiss. Wien. 111: 69-92 (cited from Gautheret, 1983).

Hackett, W.P. (1983). Phase change and intra-clonal variability. HortScience 18(6): 12-16.

Hagemann, J. (1979). Tuberous Begonias. Origins and Development. J. Cramer (Vaduz).

Hagemann, R. and Snoad, B. (1971). Paramutations in the 'Sulf' locus of *Lycopersicon esculentum*. Heredity 27: 409-418.

Hakkaart, F.A. and Versluijs, J.M.A. (1983a). Some factors affecting glassiness in carnation meristem tip cultures. Neth. J. Plant Path. 89: 47-53.

Hakkaart, F.A. and Versluijs, J.M.A. (1983b). Control of leaf curl and xanthomonas Begoniae in 'Elatior' begonias by meristem culture and an isolation test. Acta Hortic. 131: 299-301.

Halperin, W. (1973). The use of cultured tissue in studying developmental problems. Can. J. Bot. 51(10): 1801-1806.

Hammerschlag, F.A. (1978). Influence of light intensity and date of explantation on growth of geranium callus. HortScience 13(2): 53-154.

Handro, W. (1981). Mutagenesis and *in vitro* selection. In: Plant Tissue Culture, Methods and Applications in Agriculture (ed. T.A. Thorpe). Academic Press, New York, pp. 155-180.

Hanna, W.W., Lu, C. and Vasil, I.K. (1984). Uniformity of plants regenerated from somatic embryos of *Panicum maximum* Jacq. (Guinea grass). Theor. Appl. Genet. 67: 155-159.

Harney, D.M. and Knap, A. (1979). A technique for the *in vitro* propagation of African violets using petioles. Can. J. Plant Sci. 59: 263-266.

Harris, M. (1982). Induction of thimidine kinase activity in enzyme deficient Chinese hamster cells. Cell 19: 483-492.

Harris, R.E. and Mason, E.B. (1983). Machines for *in vitro* propagation of plants in liquid media. Can. J. Plant 6(1): 311-316.

Harrison, B.J. and Fincham, J.R.S. (1964). Instability in the PAL locus in *Antirrhinum majus*. I. Effect of environment on frequencies of somatic and germinal mutation. Heredity 19: 237-258.

Hartman, H.T. and Kester, D.E. (1975). Plant Propagation. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

Hartsema, A. (1926). Anatomische und experimentelle untersuchungen über der anftreten von neunbildungen an Blattern von *Begonia rex*. Rev. Trav. Bot. Neerl. 23: 305-361 (cited from Bigot, 1971).

Hasegawa, P.W., Murashige, T. and Takatori, F.H. (1973). Propagation of asparagus through shoot apex culture. II. Light and temperature requirements, transplantability of plants and cytological characteristics. J. Am. Soc. Hortic. Sci. 98: 143-148.

Hayashi, M. and Nakajima, T. (1984). Genetic stability in regenerated plants derived from tobacco mesophyll through three step culture. Japan J. Breed. 34(4): 405-415.

Haydu, Z. and Vasil, I.K. (1981). Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* Schum. Theor. Appl. Genet. 59: 269-273.

Heide, O.M. (1964). Effect of light and temperature on the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 17: 789-804.

Heide, O.M. (1965a). Photoperiodic effects on the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 18: 185-190.

Heide, O.M. (1965b). Interaction of temperature, auxins and kinins in the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 18: 891-920.

Heide, O.M. (1972). The role of cytokinins in regeneration processes. In: Hormonal Regulation of Plant Growth and Development (Eds. H. Kadeweg and Y. Vardar). Verlag Chemie, Weinheim, pp. 207-219.

Heinz, D.J. and Mee, W.P. (1970). Colchicine induced polyploids from cell suspension cultures of sugarcane. Crop Sci. 10: 696-699.

Heinz, D.J. and Mee, G.W.P. (1971). Morphological, cytogenetic and enzymatic variation in *Saccharum* species hybrid clones derived from callus tissue. Amer. Jour. of Bot. 58(3): 257-262.

Henshaw, G.G. (1982). Tissue culture and germplasm storage. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 789-792.

Hermsen, J.G., Ramanna, M.S., Roest, S. and Bokelmann, G.S. (1981). Chromosome doubling through adventitious shoot formation on *in vitro* cultivated leaf explants from diploid interspecific potato hybrids. Euphytica 30: 239-246.

Hewett, E.W. and Wareing, P.F. (1973). Cytokinins in *Populus* x *robusta*. Qualitative changes during development. Physiol. Plant. 29: 386-389.

Hildebrandt, A.C. (1971). Induced differentiation of *Gladiolus* plants from stem tip and callus cultures. *In vitro* 6(5): 399.

Hilding, A. and Welander, T. (1976). Effects of some factors on propagation of *Begonia* x *hiemalis in vitro*. Swed. Jour. Agric. Res. 6: 191-199.

Holdgate, D.P. (1977). Propagation of ornamentals by tissue culture. In: Plant Cell Tissue and Organ Culture (Eds. J. Reinert and J.P.S. Bajaj). Springer-Verlag, Heidelberg, New York, pp. 18-43.

Holliday, R. and Pugh, J.E. (1975). DNA modification mechanisms and gene activity during development. Science 187: 226-232.

Holmes, J., Dubus, F. and Bourdon, J.L. (1982). Cold storage of plant tissues. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 801-802.

Horak, J. (1972). Ploidy chimeras in plants regenerated from the tissue culture of *Brassica oleracea* L. Biologia Plantarum (Praha) 14(6): 423-426.

Hosoki, T. and Asahiri, T. (1981). *In vitro* propagation of *Bromeliads* in liquid culture. HortScience 15(5): 603-604.

Hughes, K.W. (1981). Ornamental species. In: Cloning Agricultural Plants via *in vitro* Techniques (Ed. B.V. Conger). CRC Press Ind., Boca Raton, Florida, pp. 5-50.

Hunalt, G. (1984). *In vitro* culture of fennel tissues (*Foeniculum vulgare* Miller) from cell suspension to mature plant. Scientia Hortic. 22: 55-65.

Huskins, C.L. (1948). Chromosome multiplication and reduction in somatic tissues. Nature 161: 80-83.

Hussey, G. (1978). The application of tissue culture to the vegetative propagation of plants. Sci. Prog. Oxf. 65: 185-208.

Hussey, G. and Hepher, A. (1978). Clonal propagation of sugar beet plants and the formation of polyploids by tissue culture. Ann. Bot. 42: 477-479.

Hussey, G. and Stacey, N.J. (1981). *In vitro* propagation of Potato (*Solanum tuberosum* L.). Ann. Bot. 48: 787-796.

Huxter, T.J., Thorpe, T.A. and Reid, D.M. (1980). Shoot initiation in light grown and dark grown tobacco callus. The role of ethylene. Physiol. Plant. 53(3): 319-326.

Hwang, S.C., Chen, C.L., Liu, J.C. and Lin, H.C. (1984). Cultivation of Banana using plantlets from meristem cultures. HortScience 19(2): 231-233.

IAEA (1977). Manual of Mutation Breeding. Technical Reports Series No. 119, IAEA, Vienna 2nd ed., 288 pp.

Ingram, D.S. and Helgeson, J.P. (1980). Tissue Culture Methods for Plant Pathologists. Blackwell Scientific, London.

Inoue, M. and Van Huystee, R.B. (1984). Age dependent effects of gamma-exposure on form growth and peroxidase activity of cultured peanut cells. Env. Expt. Bot. 24: 53-62.

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

Irvine, J.E., Fitch, M. and Moore, P.H. (1983). The induction of callus in sugarcane cultures by selected chemicals. Plant Cell Tissue Organ Culture 2: 141-149.

Izhar, S. and Power, J.P. (1977). Genetical studies with Petunia leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8: 375-383.

Jackson, R.C. and Newmark, P. (1960). Effect of supernumary chromosomes on production of pigment in *Haplopappus gracilis*. Science 132: 1316-1317.

Jacob, S.W., Bischel, M.D., Eberle, G.A. and Herschler, R.J. (1964). The influence of dimethyl sulfoxide on transport of insulin across a biological membrane. Fed. Proc. 23(2 Pt.): 410 Abstract.

Jarret, R.J., Hasegawa, P.M. and Bressan, R.A. (1981). Gibberellic acid regulation of adventitious shoot formation from tuber discs of potato. *In Vitro* 17: 825-830.

John, B. and Lewis, K.R. (1968). The Chromosome Complement. Protoplasmatologia VIA.

Johnson, R., Crisp, C. and Strehler, B. (1972). Selective loss of ribosomal RNA genes during the aging of post-mitotic tissues. Mech. Age. Dev. 1: 183-198.

Jones, R.N. (1975). B-chromosome systems in flowering plants and animal species. Int. Rev. Cytol. 1-100.

Jones, K. (1978). Aspects of chromosome evolution in higher plants. Adv. in Bot. Res. b: 120-194.

Kado, C.I., Kleinhofs, A. (1980). Genetic modification of plant cells through uptake of foreign DNA. Int. Rev. Cytol. Suppl. 11B: 47-80.

Kallak, H. and Yarvekylg, C. (1977). Nuclear behaviour in callus cells: Morphology and division. Biol. Plant. 19(1): 48-52.

Kao, K.N., Miller, R.A., Gamborg, O.L. and Harvey, B.L. (1970). Variation in chromosome number and structure in plant cells grown in suspension cultures. Can. J. Genet. Cytol. 12: 297-301.

Karp, A., Nelson, R.S., Thomas, E. and Bright, S.W.J. (1982). Chromosome variation in protoplast derived potato plants. Theor. Appl. Genet. 63: 265-272.

Kato, Y. (1955). Polyploid mitoses, extranuclear bodies and mitotic aberrations in seedlings of *Lilium maximowiczii* Regel. Cytologia 20: 1-10.

Kato, Y. (1974). Bud formation on excised *Helionopsis* leaf fragments. Effect of leaf age and the mid rib. Plant Cell Physiol. 15: 363-372.

Kato, Y. (1978a). Induction of adventitious buds on undetached leaves, excised leaves and leaf fragments of *Helionopsis orientalis*. Physiol. Plant. 42: 39-44.

Kato, Y. (1978b). The involvement of photosynthesis in inducing bud formation on excised leaf segments of *Helionopsis orientalis* (*Liliaceae*). Plant Cell Physiol. 19(5): 791-799.

Kaul, B.L. and Zutshi, U. (1971). Dimethyl sulphoxide as an adjuvant of colchicine in the production of polyploids in crop plants. Indian J. Exp. Biol. 9: 522-523.

Keevers, C., Coumans, M., Coumans-Gilles, M.F. and Gasper, Th. (1984). Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. Physiol. Plant. 61: 69-74.

Kessler, B. (1973). Hormonal and environmental modulation of gene expression in plant development. In: The Biochemistry of Gene Expression in Higher Organisms (Eds. J.K. Pollak and J.W. Lee). Australia and New Zealand Book Co., Sydney, pp. 333-356.

Kessler, B. and Reches, S. (1977). Structural and functional changes of chromosomal DNA during aging and phase changes in plants. Chromosomes Today 6: 237-246.

Khoder, M., Villemur, P. and Jonard, R. (1981). La multiplication végétative de l'espèce florale *Begonia elatior* (cultivar Rieger) a partir de differentes organes cultivees *in vitro*. CR. Acad. Sc. Paris, t. 293 Série 111: 403-408.

Kibler, R. and Neumann, K.H. (1980). On cytogenetic stability of cultured tissues and cell suspensions of haploid and diploid origin. In: Plant Cell Culture, Results and Perspectives (Eds. K. Sala, B. Parisi, R. Cella and D. Ciferi). Elsevier, Amsterdam, pp. 59-67.

Kihlmann, B. (1950). Induction of structural chromosome changes with adenine. Hereditas 36: 103-105.

Kihlmann, B. (1967). Action of Chemicals on Dividing Cells. Prentice Hall, New York.

Kornerup, A. and Wauscher, J.H. (1967). Methuen Handbook of Colour. Methuen Co. Ltd., London.

Krikorian, A.D. and Kann, R.P. (1981). Plantlet production from morphologically competent cell suspensions of Daylily. Ann. Bot. 47: 679-686.

Krikorian, A.D., O'Connor, S.A., Fitter, M.S. and Kann, R.P. (1982). Karyotype analysis of daylily plants clonally derived from a long term totipotent suspension culture. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 429-430.

Kurtz, G.M. and Lineberger, D. (1983). Genotypic differences in morphogenic capacity of cultured leaf explants of tomato. J. Amer. Soc. Hort. Sci. 108(5): 710-714.

Kunakh, V.A. (1972). Variability of the chromosome number in the ontogenesis of higher plants. Tsitologiya. Genetika 12(2): 160-173.

Lane, W.D. (1982). *In vitro* culture of fruit trees. In: Applications of Plant Cell and Tissue Culture to Industry (Eds. D.T. Tomes, B.E. Ellis, P.M. Harney, K.J. Kasha and R.L. Peterson). University of Guelph, Guelph, pp. 163-186.

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

Larkin, P.J. and Scowcroft, W.R. (1983a). Somaclonal variation and crop improvement. In: Genetic Engineering of Plants (Eds. T. Kosugi, C.P. Meredith, and A. Hollaender). Plenum, New York, pp. 289-314.

Larkin, P.J. and Scowcroft, W.R. (1983b). Somaclonal variation and eyespot toxin tolerance in sugarcane. Plant Cell Tissue Organ Culture 2: 111-121.

Larkin, P.J., Ryan, S.A., Brettell, R.I.S. and Scowcroft, W.R. (1984). Heritable somaclonal variation in wheat. Theor. Appl. Genet. 67: 443-455.

Lawrence, R.H. (1981). *In vitro* plant cloning systems. Env. and Expt. Bot. 21: 289-300.

Lê, C.L. and Collet, G.F. (1981). Advantages and limitation of *in vitro* propagation of *Saintpaulia ionantha*. Revue Suisse de Viticulture, d'Arboriculture et d'Horticulture 13: 265-270.

Lee, T.T. and Skoog, F. (1965). Effects of substituted phenols on bud formation and growth of tobacco cultures. Physiol. Plant. 18: 386-402.

Leenhouts, H.P., Broertjes, C., Sijsma, M.J. and Chadwick, K.H. (1982). Radiation stimulated repair in *Saintpaulia*: its cellular basis and effect on mutation frequency. Env. and Expt. Bot. 22(3): 301-306.

Legros, R.A.H. (1964). Improvement of flowering plants by polyploidy and hybridization. Publication 262 Laboratorium voor Tuinbouw-plantenteelt der Landbouwhogeschool Wageningen, The Netherlands.

Legros, R.A.H. and Doorenbos, J. (1969). Chromosome numbers in *Begonia*. Neth. Jour. Agric. Sci. 17: 189-202.

Legros, R.A.H. and Doorenbos, J. (1971). Chromosome number in *Begonia* 2. Neth. Jour. Agric. Sci. 19: 176-183.

Legros, R.A.H. and Doorenbos, J. (1973). Chromosome numbers in *Begonia* 3. Neth. J. Agric. Sci. 21: 167-170.

Legros, R.A.H. and Haegemann, J.F.V. (1971). Chromosome numbers of hybrid tuberous begonias. Euphytica 20: 1-13.

Leshen, B., Lilien-Kipmis, H. and Steinitz, B. (1982). The effect of light and of explant orientation on the regeneration of subsequent growth of bulblets on *Lilium longiflorium* Thumb bulbscales sections cultured *in vitro*. Scientia Hortic. 17: 129-136.

Letham, D.S., Goodwin, P.B. and Higgins, T.J.V. (eds.) (1978). Phytohormones and Related Compounds a Comprehensive Treatise Vol. I and II. Elsevier/North-Holland Biomedical Press.

Letouze, R. and Beauches, G. (1969). Effect of monochromatic illumination on rhizogenesis in Jerusalem artichoke. C.R. Ac. Sci. D. 269: 1528.

Levan, A. (1938). The effect of colchicine on root mitoses in *Allium*. Hereditas 24: 471-486 (cited from D'Amoto, 1959).

Levin, D.A. (1983). Polyploidy and novelty in flowering plants. The American Naturalist 122(1): 1-25.

Libbenga, K.R. and Torrey, J.G. (1973). Hormone induced endoreduplication prior to mitosis in cultured pea root cortical cells. Am. J. Bot. 60: 293-299.

Liu, M. (1982). Effect of gamma irradiation on growth of callus and its differentiated plantlets in sugarcane. Nucl. Sci. J. 19(4): 208-215.

Liu, M.C. and Chen, W.C. (1979). Tissue and cell culture as aids to sugarcane breeding. 1. Creation of genetic variation through callus culture. Euphytica 25: 393-403.

Long, R.D. (1982). Tissue culture and *in vitro* antiviral chemotherapy of tobacco and potato. PhD Thesis, London.

Lörz, H. (1984). Variability in tissue culture derived plants. In: Genetic Manipulation: Impact on Man and Society (Eds. W. Arber, K. Illmensee, W.J. Peacock and P. Starlinger). Garden City Press Ltd., Hertfordshire, pp. 103-114.

Lörz, H. and Scowcroft, W.R. (1983). Variability among plants and their progeny regenerated from protoplasts of Su/su heterozygotes of *Nicotiana tabacum*. Theor. Appl. Genet. 66: 67-75.

Lundergan, L.A. and Janicke, J. (1979). Low temperature storage of *in vitro* apple shoots. HortScience 14(4): 514.

Lupi, M.C., Bennici, A., Baroncelli, S., Gennai, D. and D'Amoto, F. (1981). *In vitro* regeneration in Durum wheat plants. II. Diplontic selection in aneusomatic plants. Z. Pflanzenzüchtg. 87: 167-171.

Lurquin, P.F. and Kleinhofs, A. (1983). Engineering in Eukaryotes. Plenum, New York.

Lyrene, P.M. (1979). Micropropagation of rabbiteye blueberries. HortScience 15(1): 80-81.

Lyrene, P.M. (1981). Juvenility and production of fast-rooting cuttings from blueberry shoot cultures. J. Amer. Soc. Hort. 106: 396-398.

MacLeod, R.D. (1968). Changes in the mitotic cycle in lateral root meristem of *Vicia faba* following kinetin treatment. Chromosoma 24: 177-187.

Maene, L.J. and Debergh, P.C. (1982). Stimulation of axillary shoot development of *Cordyline terminalis* 'Celestine Queen' by foliar sprays of 6-benzylamino purine. HortScience 17(3): 344-345.

Magnusson, I. and Bornman, C.H. (1985). Anatomical observations on somatic embryogenesis from scutellar tissues of immature embryos from *Triticum aestivum*. Physiol. Plant 63: 137-145.

Mahlberg, P.G., Turner, F.R., Walkinshaw, C., Venketeswaren, S. and Mehrotra, B. (1975). Observations on incomplete cytokinesis in callus cells. Bot. Gaz. 136(2): 189-195.

Mahfouz, M.N., de Boucaud, M.T. and Gaultier, J.M. (1983). Caryological analysis of single cell clones of tobacco. Relationship between the ploidy and the intensity of the caulogenesis. Z. Pflanzenphysiol. 109: 251-257.

Maliga, P. (1984). Isolation and characterization of mutants in plant cell cultures. Ann. Rev. Plant. Physiol. 35: 519-543.

Marcotrigiano, M. and Gouin, F.R. (1984a). Experimental synthesized plant chimeras. I. *In vitro* recovery of *Nicotiana tabacum* L. chimeras from mixed callus cultures. Ann. Bot. 54: 503-511.

Marcotrigiano, M. and Gouin, F.R. (1984b). Experimentally synthesized plant chimeras. 2. A comparison of *in vitro* and *in vivo* techniques for the production of interspecific *Nicotiana* chimeras. Ann. Bot. 54: 513-521.

Margara, J. and Piollat, J.T. (1982). Organogenèse normale on atypique en culture *in vitro* à partir du pétale de *Begonia* x *elatior*. C.R. Acad. Sc. Paris, t. 294, Série 111: 127-132.

Margara, J. and Piollat, M.T. (1983). Novelle observation sur l'organogenése *in vitro* à partir de petales de *Begonia* x *elatior*. C.R. Acad. Sc. Paris, t. 297, Série 111: 161-164.

Margara, J. and Piollat, M.T. (1984). L'aptitude a l'organogenèse des petales de *Begonia* x *elatior* néoformés *in vitro*. C.R. Acad. Sc. Paris, 5. 298, Série iii: 583-586.

Martini, G. and Nuti Ronchi, V. (1974). Microdensitometric and autoradiographic analysis of cell proliferation in primary culture of *Nicotiana glauca* pith tissue. Cell Diff. 3: 239-247.

Matsuura, H. and Okuno, S. (1943). Cytogenetical studies on *Begonia* (Preliminary Survey). Cytologia 13: 1-18.

Matthysee, A.G. and Torrey, J.G. (1967). Nutritional requirements for polyploid mitosis in cultured pea root segments. Physiol. Plant. 20: 661-672.

McClintock, B. (1951). Chromosome organization and genic expression. Cold Spr. Harb. Quant. Biol. 16: 13-47.

McClintock, B. (1978). Mechanisms that rapidly reorganize the genome. In: Stadler Genetic Symposium (Ed. G.P. Redei). Vol. 10: 25-47.

McCowan, (1980). The use of microculture (tissue culture) in the production and improvement of nursery crops. New Horizons, HRI, Washington DC.

McCoy, T.J. and Bingham, E.T. (1977). Regeneration of diploid *Alfalfa* plants from cells grown in suspension culture. Plant Sci. Lett. 10(1): 59-66.

McCoy, T.J., Phillips, R.L. and Rines, H.W. (1982). Cytogenetic analysis of plants regenerated from oats (*Avena sativa*) tissue cultures; high frequency of partial chromosome loss. Can. Jour. Genet. Cytol. 24: 37-50.

McManus, M.A. (1959). Certain mitotic effects of kinetin, gibberellic acid, maleic hydrazide and indoleacetic acid in onion roots. Proc. Iowa Acad. Sci. 56: 74-80.

McPheeters, K. and Skirvin, R.M. (1983). Histogenic layer manipulation in chimeral 'Thornless Evergreen'. 1. Trailing blackberry. Euphytica 32: 351-360.

McWilliams, A.A., Smith, S.M. and Street, H.E. (1974). The origin and development of embryoids in suspension cultures of carrot (*Dacus carota*). Ann. Bot. 38: 243-250.

Meins, F. (1983). Heritable variation in plant cell culture. Ann. Rev. Plant Physiol. 34: 327-346.

Meins, F. and Binns, A. (1977). Epigenetic variation in cultured somatic cells: Evidence for gradual changes in the requirement for factors promoting cell division. Proc. Nat. Acad. Sci. USA 74(7): 2928-2932.

Meins, F. and Binns, A.N. (1978). Epigenetic clonal variation in the requirement of plant cells for cytokinins. In: The Clonal Basis of Development (Eds. S. Subtelny and I.M. Sussex). Academic Press, New York, pp. 185-201.

Mele, E., Messegner, J. and Campbrubo, P. (1982). Effect of ethylene on carnation explants grown in sealed vessels. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 69-70.

Menzel, Y.M. and Brown, M.S. (1952). Polygenomic hybrids in *Gossypium*. II. Mosaic formation and somatic reduction. Am. J. Bot. 39: 59-69.

Meredith, C.P. (1984). Selecting better crops from cultured cells. In: Gene Manipulation in Plant Improvement (Ed. J.P. Gustafson). Plenum, New York, pp. 503-528.

Middelton, J. and Gahan, P.B. (1979). A quantitative cytochemical study of acid phosphatases in rat liver parenchymal cells of different ploidy levels. Histochem. Journ. 11: 649-659.

Mikkelson, J.C. (1976). Enlarging the scope of flower crop improvement mutation breeding. Acta. Hortic. 63: 197-202.

Mikkelson, J.C., Ryan, J. and Constantin, M.J. (1975). Mutation breeding of Rieger's Elatior begonias. Amer. Hort. 54: 18-21.

Mikkelson, E.P. and Sink, K.C. (1978). Histology of adventitious shoot and root formation on leaf-petiole cuttings of *Begonia* x *hiemalis* Fotsch. 'Aphrodite Peach'. Scientia Hortic. 8: 179-192.

Mitra, J., Mapes, M.O. and Steward, F.C. (1966). Growth and organized development of cultured cells. IV. The behaviour of the nucleus. Amer. J. Bot. 47(5): 357-368.

Morel, G.M. (1964). Tissue culture. A new means of clonal propagation of orchids. Bull. Am. Orchid Soc., pp. 472-478.

Mouras, A. and Lutz, A. (1983). Plant tumour reversal associated with the loss of marker chromosomes in tobacco cells. Theor. Appl. Genet. 65: 283-288.

Mullins, R.H. and Schlegel, D.E. (1976). Cold storage maintenance of strawberry meristem plantlets. HortScience 11: 100-101.

Mullins, M.G., Nair, Y. and Bampert, P. (1979). Rejuvenation *in vitro* induction of juvenile characters in an adult clone of *Vitis vinifera* L. Ann. Bot. 44: 623-627.

Murashige, T. (1974). Plant propagation through tissue culture. Ann. Rev. Plant. Physiol. 25: 135-166.

Murashige, T. (1977). Manipulation of organ initiation in plant tissue cultures. Bot. Bull. Academia Sinica 18: 1-24.

Murashige, T. and Nakano, R. (1966). Tissue culture as a potential tool in obtaining polyploid plants. Jour. Hered. 57: 114-118.

Murashige, T. and Nakano, R. (1967). Chromosome complement as a determinant of the morphogenic potential of tobacco cells. Amer. Jour. of Bot. 54(8): 963-970.

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

Murashige, T., Serpa, M. and Jones, B. (1975). Clonal multiplication of *Gerbera* through tissue culture. HortScience 9(3): 175-180.

Nabors, M.W. (1976). Using spontaneously occurring and induced mutations to obtain agriculturally useful plants. BioScience 26(12): 761-768.

Nagl, W. (1972). Evidence of DNA amplification in the orchid *Cymbidium in vitro*. Cytobios. 5: 145-154.

Nagl, W. (1976). Nuclear Organization. Ann. Rev. Plant. Physiol. 27: 39-69.

Nagl, W. (1978). Endopolyploidy and Polyteny in Differentiation and Evolution Towards an Understanding of Quantitative and Qualitative Variation in Nuclear DNA in Ontogeny and Phylogeny. North Holland, Amsterdam.

Nagl, W. (1979). Differential DNA replication in plants: A critical review. Z. Pflanzenphysiol. 95: 283-314.

Nagl, W. (1983). Nuclear organization - physical aspects of gene regulation. In: Kew Chromosome Conference (Eds. P.E. Brandham and M.D. Bennett). George Allen and Unwin Press, London, pp. 55-63.

Nagl, W. and Rucker, W. (1974). Shift of DNA replication from diploid to polyploid cells in cytokinin controlled differentiation. Cytobios. 10: 137-144.

Nakamura, C., Keller, W.A. and Fedak, G. (1981). *In vitro* propagation and chromosome doubling of *Triticum crassum* x *Hordeum vulgare* intergeneric hybrid. Theor. Appl. Genet. 60: 89-96.

Navashin, M. (1933). Aller der Samen als Ursache von Chromosomen-mutationen. Planta 20: 233-243 (cited from D'Amoto, 1977).

Naylor, J., Sander, G. and Skoog, F. (1954). Mitosis and cell enlargement without cell division in excised tobacco pith tissue. Physiol. Plant. 7: 25-29.

Negrutiu, I. and Jacobs, M. (1978). Factors which enhance *in vitro* morphogenesis of *Arabidopsis thaliana*. Z. Pflanzenphysiol. 90: 423-430.

Negrutiu, I., Jacobs, M. and Cattoir-Reynaerts, A. (1984a). Progress in cellular engineering of plants: biochemical and genetic assessment of selectable markers from cultured cells. Plant. Mol. Biol. 3: 289-302.

Negrutiu, I., Jacobs, M. and Caboche, M. (1984b). Advances in somatic cell genetics of higher plants - the protoplast approach in basic studies on mutagenesis and isolation of biochemical mutants. Theor. Appl. Genet. 67: 289-304.

Neilson-Jones, W. (1969). Plant Chimeras, 2nd edition. Methuen and Co. Ltd., London.

Nešický, M., Novák, F.J., Provarči, A. and Doleželová, M. (1983). Genetic analysis of callus growth of maize (*Zea mays* L.) *in vitro*. Z. Pflanzenzüchtg. 91: 322-328.

Nimi, Y. and Ohozawa, T. (1979). *In vitro* bulblet formation from leaf segments of Lilies especially *Lilium rubellum* Baker. Scientia Hortic. 11: 379-389.

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

Norstog, K., Wall, W.E. and Howland, G.P. (1969). Cytological characteristics of ten-year old rye-grass endosperm tissue cultures. Bot. Gaz. 130(2): 83-86.

Novak, F.J. (1980). Phenotype and cytological status of plants regenerated from callus cultures of *Allium sativum* L. Z. Pflanzenzüchtg. 84: 250-260.

Novak, F.J. (1981). Chromosomal characteristics of long-term callus cultures of *Allium sativum* L. Cytologia 46: 371-379.

Novak, F.J. (1983). Production of garlic (*Allium sativum* L.) tetraploids in shoot-tip *in vitro* culture. Z. Pflanzenzüchtg. 91: 329-333.

Novak, F.J. and Petru, E. (1981). Tissue culture propagation of *Lilium* hybrids. Scientia. Hortic. 14: 191-199.

Novak, F.J. and Vyskot, B. (1975). Karyology of callus cultures derived from *Nicotiana tabacum* L. haploids and ploidy of regenerates. Z. Pflanzenzüchtg. 75: 62-70.

Nowell, P.C. (1976). The clonal evolution of tumour cell populations. Science 194: 23-28.

Nuti Ronchi, V., Bennici, A. and Martini, G. (1973). Nuclear fragmentation in dedifferentiating cells of *Nicotiana glauca* pith tissues grown *in vitro*. Cell Diff. 2: 77-85.

Nyman, L.P. and Arditti, J. (1984). Effects of 2,3,5-triiodobenzoic acid on plantlet formation from cultured tissues of Taro *Colocasia esculenta* (L.) Schott (*Araceae*). Ann. Bot. 54: 459-466.

Oelck, M.M. and Schieder, O. (1983). Genotypic differences in some legume species affecting the redifferentiation ability from callus to plants. Z. Pflanzenzüchtg. 91: 312-321.

Ogihara, Y. (1981). Tissue culture in *Haworthia*. IV. Genetic characterization of plants regenerated from callus. Theor. Appl. Genet. 60: 353-363.

Ogihara, Y. (1982). Tissue culture in *Haworthia*. V. Characterization of chromosomal changes in cultured callus cells. Jap. J. Genet. 57: 499-511.

Ogihara, Y. and Tsunewaki, K. (1979). Tissue culture of *Haworthia*. III. Occurrence of callus variants during subculture and its mechanisms. Jap. J. Genet. 54(4): 271-293.

Ogura, H. (1976). The cytological chimeras in original regenerates from tobacco tissue cultures and in their offspring. Jap. Jour. Genet. 51(3): 161-174.

Ogura, H. (1978). Genetic control of chromosomal chimerism found in a regenerate from tobacco callus. Jap. Jour. Genet. 53(2): 77-90.

Ogura, H. (1982). Studies on the genetic instability of cultured tissues and the regenerated plants. Effects of auxins and cytokinins on mitosis of *Vicia faba* cells. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 433-434.

Ohki, S. and Bigot, C. (1976). Organogenèse *in vitro* a partir d'hypocotyle de Tomate (*Lycopersicon esculentum* Miller). Comparaison des aptitudes au bourgeonnement pour deux lignées et leur hybrids. Actes du 101e Congrès National des Societés Savantes Lille Sciences fac. 1: 607-618.

Orshinsky, B.R., Swanson, E.B. and Tomes, D.T. (1983). Enhanced shoot regeneration from homogenized callus cultures of birdsfoot trefoil (*Lotus corniculatus* L.). Plant Cell Tissue Organ Culture 2: 341-347.

Orton, T.J. (1980). Chromosomal variability in tissue cultures and regenerated plants of *Hordeum*. Theor. Appl. Genet. 56: 101-112.

Orton, T.J. (1983). Experimental approaches to the study of somaclonal variation. Plant Molecular Biology Reporter 12: 67-76.

Orton, T.J. (1984a). Somaclonal variation: Theoretical and practical considerations. In: Gene Manipulation in Plant Improvement (Ed. J.P. Gustafson). Plenum Publishing Co., New York, pp. 427-469.

Orton, T.J. (1984b). Genetic variation in somatic tissues: Method or madness? Adv. in Plant Pathology 2: 153-189.

Orton, T.J. and Steidl, R.P. (1980). Cytogenetic analysis of plants regenerated from colchicine-treated callus cultures of interspecific *Hordeum* hybrids. Theor. Appl. Genet. 57: 89-95.

Osborne, D.J., Dell'Aquilla, A. and Elder, R.H. (1984). DNA repair in plant cells. An essential event of early embryo germination in seeds. Folia biologia (Praha) 1984: 155-169.

Owens, L.D. (ed.) (1983). Genetic Engineering: Applications to Agriculture. Rowman and Allaheld Publishers, Granada, London.

Ozeki, Y. and Komamine, A. (1981). Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture: Correlations of metabolic differentiation with morphological differentiation. Physiol. Plant. 53: 570-577.

Papes, D., Jelaska, S., Tomaseo, M. and Devidé, Z. (1978). Triploidy in callus culture of *Vicea faba* L. investigated by the Giemsa C-banding technique. Experientia 34: 1016-1017.

Partanen, C.R. (1963). Plant tissue culture in relation to developmental cytology. Int. Rev. Cytol. 15: 215-243.

Patau, K. and Das, N.K. (1961). The relation of DNA synthesis and mitosis in tobacco pith tissue cultured *in vitro*. Chromosoma 11: 553-572.

Patel, K.R. and Berlyn, G.P. (1981). Genetic instability of multiple buds of *Pinus coulteri* regenerated from tissue culture. Can. J. Forestry 12: 93-101.

Patel, K.R. and Thorpe, T.A. (1984). *In vitro* differentiation of plantlets from embryogenic explants of lodgepole pine (*Pinus contorta* Douglas ex Lond). Plant Cell Tissue Organ Culture 3: 131-142.

Pences, V.C. and Caruso, J.Z. (1984). Effect of IAA and four IAA conjugates on morphogenesis and callus growth from tobacco leaf discs. Plant Cell Tissue Organ Culture 3: 101-110.

Phillips, R., Arnott, S.M. and Kaplan, S.E. (1981). Antibiotics in plant tissue culture: Rifamyuin effectively controls bacterial contaminants without affecting the growth of short-term explant culture of *Helianthus tuberosus*. Plant Sci. Lett. 21: 235-240.

Piton, F. (1962). Contribution à l'etude cytologique des *Begoniacées*. I. Rev. Cyt. Biol. Vég. 24: 165-216.

Plunkett, A. (1984). *In vitro* culture of *Saintpaulia* and related genera. PhD thesis, National University of Ireland.

Prevot, P. (1939). Les facteurs de la morphogenèse des bourgeons de neoformations chez *Begonia rex*. Assoc. Franc. p. Avanc. des Sciences 63e session: 611-619 (cited from Bigot, 1971).

Prevot, P. (1948). Contribution à l'histologie des phenomènes de neoformation chez *Begonia rex* Putz. La revue scientifique 87: 275-285.

Pring, D.R., Conde, M.F. and Gengenbach, B.G. (1981). Cytoplasmic genome variability in tissue culture derived plants. Env. Expt. Bot. 21(3/4): 369-377.

Quorin, M. and Lepoivre, P.H. (1977). Etude de mileux adapte aux culture *in vitro* de *Prunus*. Acta Hortic. 78: 437-442.

Raju, M.U.S. and Mann, H.E. (1970). Regenerative studies on the detached leaves of *Echeveria elegans*. Anatomy and regeneration of the leaves in sterile culture. Can. J. Bot. 48: 1887-1891.

Razin, A. and Riggs, A. (1980). DNA methylation and gene function. Science 210: 604-610.

Razin, A., Cedar, H. and Riggs, A.D. (eds.) (1985). DNA Methylation. Biochemistry and Biological Significance. Springer Verlag, Berlin.

Rees, H. (1961). Genotypic control of chromosome form and behaviour. Bot. Rev. 27: 280-318.

Regel, F. (1876). Die vermehrung der Begoniaceen aux ihren Blättern. Iena Z. Naturwiss. 10: 447-492 (cited from Bigot, 1971).

Reinert, J. (1958). Untersuchungen uber die morphogenese an gewebekulturen. Ber. Dtsch. Bot. Ges. 71: 15 (cited from Tisserat *et al.*, 1979).

Reinert, J. and Backs, D. (1968). Control of totipotency in plant cells growing *in vitro*. Nature 220: 1340-1341.

Reinert, J. and Bajaj, Y.P.S. (eds.) (1977). Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture. Springer, Berlin.

Reinert, J., Bajaj, Y.P.S. and ZBell, B. (1977). Aspects of organization - organogenesis, embryogenesis, cytodifferentiation. In: Plant Tissue and Cell Culture (Ed. H.E. Street). Blackwell Scientific, London, pp. 389-429.

Reuther, G. (1978). Die Gewebekultur als phytosanitäre Maßnahme bei der vegetativen vermehrung. Gb + GW 36: 840-842.

Reuther, G. and Bandari, N.N. (1981). Organogenesis and histogenesis of adventitious organs induced on leaf blade segments of *Begonia elatior* hybrids (*Begonia* x *hiemalis*) in tissue culture. Gartenbauwissenshaft 46(6): 242-249.

Ringe, F. and Nitsch, J.P. (1968). Conditions leading to flower formation on excised *Begonia* fragments cultured *in vitro*. Plant and Cell Physiol. 9: 639-652.

Rivin, C.J., Zimmer, E.A., Cullis, C.A., Walbot, V., Huynh, T. and Davis, R.W. (1983). Evaluation of genomic variability at the nucleic acid level. Plant Molec. Biol. Rep. 1: 9-16.

Roest, S. (1976). *In vitro* culture and mutation breeding. In: Proc. Workshop Eur. Com - Israel. The Use of Ionizing Radiation. Wageningen 22-24 March. Assoc. Euratom - ITAL, Wageningen, pp. 427-433.

Roest, S. and Bokelmann, G.S. (1981). Vegetative propagation of Carnation *in vitro*. Scientia Hortic. 14: 357-366.

Roest, S., Van Berkel, M.A.E., Bokelmann, G.S. and Broertjes, C. (1981). The use of *in vitro* adventitious bud technique for mutation breeding of *Begonia* x *hiemalis*. Euphytica 30: 381-388.

Romberger, J.A. and Tabor, C.A. (1971). The *Piceas abies* shoot apical meristem in culture. I. Agar and autoclaving effects. Am. J. Bot. 58(2): 131-140.

Ross, J.G., Franzke, C.F. and Schuh, L.A. (1954). Studies on colchicine induced variants in *Sorghum*. Agron. J. 46: 10-15.

Rost, R.L. (1977). Responses of the plant cell cycle to stress. In: Mechanisms and Control of Cell Division (Eds. R.L. Rost, E.M. Gifford). Dowden Hutchinson and Ross Stroudsbury Pa., pp. 111-134.

Rubluo, A., Kartha, K.K., Mroginski, L.A. and Dyck, J. (1984). Plant regeneration from pea leaflets cultured *in vitro* and genetic stability of regenerates. J. Plant Physiol. 117: 119-130.

Rucker, W. (1982). Morphactin-induced changes in the cytokinin effect on tissue and organ cultures of *Nicotiana tabacum*. Protoplasma 113: 103-109.

Ruiz, M.L. and Vazquez, A.M. (1982a). Chromosome number evolution in stem derived calluses of *Hordeum vulgare* L. cultured *in vitro*. Protoplasma 111: 83-86.

Ruiz, M.L. and Vazquez, A.M. (1982b). Colchicine effect of the chromosome number of barley embryos cultured *in vitro*. Protoplasma 113: 237-240.

Rutischauser, A. (1956). Chromosome distribution and spontaneous chromosome breakage in *Trillium grandiflorum*. Heredity 10: 367-407.

Sacristan, M.D. (1971). Karyotypic changes in callus cultures from haploid and diploid plants of *Crepis capillaris* (L.) Wallr. Chromosoma 33: 273-283.

Sacristan, M.D. (1981). Regeneration of plants from long-term callus cultures of haploid *Brassica napus*. Z. Pflanzenzüchtg. 86: 248-253.

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

Sampson, D.R., Walker, G.W.R., Hunter, A.W.S. and Bragd, M. (1958). Investigations of the sporting process in greenhouse chrysnathemums. Can. J. Plant Sci. 38: 346-356.

Samyn, G.L., Debergh, P.C. and Vermaerke, D. (1984). Field performance and phenotypic stability of virus-free tissue-cultured *Begonia* x *tuberhybrida* 'Multiflora'. Scientia Hortic. 24: 185-191.

Sanders, M.E. and Franzke, C.J. (1962). Cytological studies of non true-breeding mutants in *Sorghum* obtained after colchicine treatment. Am. J. Bot. 49: 990-996.

Sanders, M.E. and Franzke, C.J. (1962). Somatic reduction of tetraploid *Sorghum* to diploid mutants following colchicine treatment. Nature 196: 696-698.

Sangwan-Noreel, B.S. (1983). Male gametophyte nuclear DNA content evolution during androgenic induction in *Datura innoxia* Mill. Z. Pflanzenphysiol. 111: 47-54.

Santos, R.F. and Handro, W. (1983). Morphological patterns in *Petunia hybrida* plants regenerated from tissue cultures and differing in their ploidy. Theor. Appl. Genet. 66: 55-60.

Schäffner, K.H. and Nagl, W. (1979). Differential DNA replication involved in transition from juvenile to adult phase in *Hedera helix*. Plant. Syst. Evol. Suppl. 2: 105-110.

Scharpe, A. and Van Parijs, R. (1973). The formation of polyploid cells in ripening cotyledons of *Pisum sativum* L. in relation to ribosome and protein synthesis. J. Exp. Bot. 24: 216-222.

Schneider, G. (1970). Morphactins physiology and performance. Ann. Rev. Plant Physiol. 21: 499-536.

Schneider-Moldrickx, R. (1983). The influence of light intensity on regeneration of *Kalanchoe blossfeldiana* hybrids *in vitro*. Acta Hortic. 131: 163-170.

Scowcroft, W.R. (1984). Genetic variability in tissue culture. Impact on germplasm conservation and utilization. IBPGR Report 1984: 1-30.

Sehgal, C.B. (1975). *In vitro* differentiation of foliar embryos and adventitious buds from the leaves of *Begonia semperflorens* Link and Otto. Ind. J. Expt. Biol. 13: 486-488.

Seibert, M. (1973). Light - The effect of wave length and intensity on growth and shoot initiation in tobacco callus. *In vitro* 8: 435 Abstract.

Sen, S. (1974). Differentiated nuclei and elucidation of chromosomal control of differentiation. The Nucleus 17(1): 40-51.

Shamina, Z.B. (1966). Cytogenetic studies of tissue culture of *Haplopappus gracilis*. In: Proc. Symp. The Mutational Process Mechanism of Mutation and Inducing Factors (Ed. Z. Lauda). Academia, Prague, pp. 337-380 (cited from Sunderland, 1977).

Shapiro, J.A. and Cordell, B. (1982). Eukaryotic mobile and repeated genetic elements. Biol. Cell. 43: 31-54.

Sharma, A.K. (1983). Additional genetic material in chromosomes. In: Kew Chromosome Conference (Eds. P.E. Brandham and M.D. Bennett). George Allen and Unwin Press, London, pp. 35-43.

Sharma, A.K. and Bhattacharyya, U.C. (1957). Cytological studies in *Begonia* I. La Cellule 58: 307-330.

Sharma, A.K. and Bhattacharyya, U.C. (1961). Cytological studies in *Begonia* II. Caryologia 14(2): 279-301.

Sharma, A.K. and Sharma, A. (1956). Fixity in chromosome number in plants. Science 4503: 335-336.

Sharma, A.K. and Sharma, A. (1959). Chromosomal alterations in relation to speciation. Bot. Rev. 25: 514-544.

Sharma, A.K. and Sharma, A. (1981). Chromosomes, Techniques, Theory and Practice - 4th edition. Butterworths, London.

Sharma, A.K. and Sharma, A. (1984). Trends of chromosome evolution in the plant kingdom. In: Chromosomes in Evolution in Eukaryotic Groups. Vol. II. CRC Press, New York, pp. 228-239.

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

Sharp, W.R. and Evans, D.A. (1982). Application of somatic embryogenesis to crop improvement. In: Plant Tissue Culture 1982 (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 759-762.

Shepard, J.F., Bidney, D. and Shahin, E. (1980). Potato protoplast improvement. Science 208: 17-24.

Sheridan, W.F. (1974). A long term callus culture of *Lilium*: relative stability of the karyotype. J. Cell. Biol. 63: 313 Abstract.

Sheridan, W.F. (1975). Plant regeneration and chromosome stability in tissue cultures. In: Genetic Manipulations with Plant Material (Ed. L. Ledoux). Plenum, New York, pp. 263-295.

Shimada, T. and Tabata, M. (1967). Chromosome number in cultured pith tissues of tobacco. Japan J. Genet. 42: 195-201.

Simantel, G.M. and Ross, J.G. (1963). Colchicine induced somatic chromosome reduction in *Sorghum*. Jour. Hered. 54: 277-284.

Sinden, S.L. and Shepard, J.F. (1983). Variation at the cellular level. HortScience 18(6): 837-840.

Singh, B.D. (1981). Origin of aneuploid variation in tissue cultures of *Haplopappus gracilis* and *Vicia hastastana*. Caryologia 39(3): 337-343.

Singh, B.D. (1975a). Effect of physical condition of medium on karyotypes of cell populations *in vitro*. Nucleus 18: 61-65.

Singh, B.D. (1975b). Evolution of dominant karyotypes of *Haplopappus gracilis* cells cultured *in vitro*. Carylogia 28: 29-37.

Singh, B.D. and Harvey, B.L. (1972). Cytogenetic studies on *Haplopappus gracilis* cells cultured on agar and in liquid medium. Cytologia 40: 347-354.

Singh, B.D. and Harvey, B.L. (1975). Selection for diploid cells in suspension cultures of *Haplopappus gracilis*. Nature 253: 453-

Singh, B.D., Harvey, B.L., Kao, K.N. and Muller, R.A. (1972). Selection pressure in cell populations of *Vicia hajastana* cultured *in vitro*. Canad. Jour. Genet. Cytol. 14: 65-70.

Skirvin, R. (1978). Natural and induced variation in tissue culture. Euphytica 27: 241-266.

Skirvin, R.M. and Janick, J. (1976). Tissue culture induced variation in scented *Pelargonium* spp. J. Amer. Soc. HortSci. 101(3): 281-290.

Shigematsu, K. and Matsubara, H. (1972). The isolation and propagation of the mutant plant from sectorial chimera induced by irradiation in *Begonia rex*. J. Japan Soc. Hort. Sci. 41(2): 196-200.

Skoog, F. and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultivated *in vitro*. Symp. Soc. Expt. Biol. 11: 118-131.

Skoog, F. and Tsui, C. (1948). Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. Am. J. Bot. 35: 782-787.

Skvirsky, R.C., Hanson, M.R. and Ausubel, F.M. (1982). A genetic approach for studying plant regeneration: Analysis of cytokinin-controlled shoot morphogenesis from tissue explants of *Petunia hybrida*. In: Variability In Plants Regenerated from Tissue Culture (Ed. E. Earle and Y. Demarly). Praeger, New York, pp. 101-119.

Skvirsky, R.C., Hanson, M.R. and Ausubel, F.M. (1984). Intraspecific genetic variation in cytokinin-controlled shoot morphogenesis from tissue explants of *Petunia hybrida*. Plant Sci. Lett. 35: 237-246.

Smith, S.H. and Oglevee-O'Donovan, W.A. (1979). Meristem tip culture from virus infected plant material and commercial implications. In: Plant Cell and Tissue Culture (Eds. W.R. Sharp, P.O. Larsen, G.F. Paddock and V. Raghaven). Ohio State University Press, Columbus, pp. 453-460.

Smith, R.H. and Norris, R.E. (1983). *In vitro* propagation of African violet chimeras. HortScience 18(4): 436-437.

Smith, S.M. and Street, H.E. (1974). The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. Ann. Bot. 38: 223-241.

Snir, I. (1981). Micropropagation of red raspberry. Scientia Hortic. 14(2): 139-143.

Sorenson, J.C. (1984). The structure and expression of nuclear genes in higher plants. Adv. Genet. 22: 109-144.

Sree Ramulu, K., Devreux, M., Ancora, G. and Laneri, U. (1976). Chimerism in *Lycopersicon peruvianum* plants regenerated from *in vitro* culture of anthers and stem internodes. Z. Pflanzenzüchtg. 76: 299-319.

Sree Ramulu, K., Dijkhuis, P. and Roest, S. (1984a). Genetic instability in protoclones of potato (*Solanum tuberosum* L. cv. 'Bintje'): new types of variation after vegetative propagation. Theor. Appl. Genet. 68: 515-519.

Sree Ramulu, K., Dijkhuis, P., Roest, S., Bokelmann, G.S. and De Groot, B. (1984b). Early occurrence of genetic instability in protoplasts cultures of potato. Plant Sci. Lett. 36: 79-86.

Standardi, A. (1982). Effetti di subcolture ripetute in germoglio di *Actinidia chinensis* (Pl.) coltivati *in vitro*. Riv. Ortoflorofrutt. It. 66: 419-429.

Stebbins, G.L. (1971). Chromosomal evolution in higher plants. Addison-Wesley, Reading, Massachusetts.

Stevenson, J.H. and Harris, A.E. (1980). *In vitro* plantlet formation from shoot tip explants of *Fuschia hybrida* cv. Springtime. Can. J. Bot. 58(20): 2190-2192.

Steward, F.C., Mapes, M.D., Kent, A.E. and Holstein, R.D. (1964). Growth and development of cultured plant cells. Science N.Y. 163: 20-27.

Stewart, R.N. (1979). Ontogeny of the primary body in chimeral forms of higher plants. In: The Clonal Basis of Development (Eds. S. Subtleny and I.M. Sussex). Academic Press, New York, pp. 131-166.

Stewart, R.N. and Dermen, H. (1975). Flexibility in ontogeny as shown by the contribution of the shoot apical layer to leaves of periclinal chimeras. Amer. J. Bot. 62(9): 935-947.

Stoutemeyer, V.V. and Britt, O.K. (1965). The behaviour of tissue cultures from English and Algerian ivy in different growth phases. Amer. J. Bot. 52: 805-810.

Street, H.E. (1979). Embryogenesis and chemically induced organogenesis. In: Plant Cell and Tissue Culture (Eds. W.R. Sharp, P.O. Larsen, E.F. Paddock and V. Raghaven). Ohio State University Press, Columbus, pp. 123-155.

Sunderland, N. (1977). Nuclear cytology. In: Plant Tissue and Cell Culture (Ed. H.E. Street). Blackwell Scientific, London, pp. 177-207.

Sutters, E. and Langhans, R.W. (1979). Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hortic. Sci. 104: 493-496.

Sutters, E. and Langhans, R.W. (1982). Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot tip culture. Can. J. Bot. 60: 2896-2902.

Sutters, E.G. and Hutzell, M. (1984). Use of humidity tents and antitranspirants in the acclimatization of tissue cultured plants to the greenhouse. Scientia Hortic. 23: 303-312.

Swartz, H.J. (1981). Field performance and phenotypic stability of tissue cultured propagated strawberries. J. Amer. Soc. Hort. Sci. 106(5): 667-673.

Swedland, B. and Vasil, I.K. (1985). Cytogenetic characterization of embryogenic callus and regenerated plants of *Pennisetum americanum* (L.) K. Schum. Theor. Appl. Genet. 69: 575-581.

Sybenga, J. (1983). Genetic manipulation in plant breeding: somatic versus generative. Theor. Appl. Genet. 66: 179-201.

Tabachnik, L. and Kester, D.E. (1977). Shoot culture for almond and almond-peach hybrid clones *in vitro*. HortScience 12: 545-547.

Takayama, S. and Misawa, M. (1981). Mass propagation of *Begonia* x *hiemalis* plantlets by shake culture. Plant and Cell Phys. 22(3): 461-467.

Takayama, S. and Misawa, M. (1982a). Factors affecting differentiation and growth *in vitro* and a mass propagation scheme for *Begonia* x *hiemalis*. Scientia Hortic. 16: 65-75.

Takayama, S. and Misawa, M. (1982b). Mass propagation of ornamental plants through tissue culture. In: Plant Tissue Culture (Ed. F. Fujiwara). Maruzen, Tokyo, pp. 681-682.

Takayama, S. and Misawa, M. (1983). A scheme for mass propagation of *Lilium in vitro*. Scientia Hortic. 18: 353-362.

Tamura, Y., Nakamura, S., Fukui, H. and Tabata, M. (1984). Comparison of *Stevia* plants grown from seeds, cuttings and stem-tip culture for growth and sweet diterpene glucosides. Plant Cell Reports. 3: 180-182.

Tanimoto, S. and Harada, H. (1979). Influence of environmental and physiological conditions of floral bud formation of *Torenia* stem segments cultured *in vitro*. Z. Pflanzenphysiol. 95: 33-41.

Tanimoto, S. and Harada, H. (1982). Effect of cytokinin and anti-cytokinin on the initial stage of adventitious bud differentiation in the epidermis of *Torneia* stem segments. Plant and Cell Physiol. 23: 1371-1376.

Thiebaut, J. and Kasha, K.J. (1978). Modification of the colchicine technique for chromosome doubling of barley haploids. Can. Jour. Genet. Cytol. 20: 513-521.

Thomas, E. and Davey, M. (1975). From single cells to plants. Wykeman Press, London, 172 pp.

Thomas, E., King, P.J. and Potrykus, I. (1979). Improvement of crop plants via single cells *in vitro* - an assessment. Z. Pflanzenzüchtg. 82: 1-30.

Thomas, D.S. and Murashige, T. (1979). Volatile emissions of plant tissue culture. I. Identification of the major components. *In Vitro* 15(9): 649-653.

Thompson, M.L. and Thompson, E.J. (1981). Begonias. The complete reference guide. NYT Times Books, New York.

Thorpe, T.A. and Meir, D.D. (1972). Starch metabolism respiration and shoot formation in tobacco callus cultures. Physiol. Plant. 27: 365-369.

Thorpe, T.A. (1974). Carbohydrate availability and shoot formation in tobacco callus cultures. Physiol. Plant. 30: 77-81.

Thorpe, T.A. (ed.) (1981). Plant Tissue Culture Methods and Applications in Agriculture. Academic Press, London.

Thorpe, T.A. (1982). Callus organization and *de novo* formation of shoots, roots and embryos *in vitro*. In: Applications of Plant Cell and Tissue Culture to Industry (Eds. D.T. Tomes, B.E. Ellis, P.M. Harney, K.J. Kasha and R.L. Peterson). University of Guelph, Guelph, pp. 109-138.

Thorpe, T.A. and Brown, D.C.W. (1980). Osmotic relations during shoot formation in tobacco callus. *In Vitro* 16(3): 217-221.

Tisserat, B., Esau, E.B. and Murashige, T. (1979). Somatic embryogenesis in Angiosperms. Hort. Reviews. 1: 1-78.

Tomes, D.T. (1979). Tissue culture procedures for propagation and maintenance of *Lotus corniculatus* genotypes. Can. J. Bot. 57(2): 137-140.

Tomes, D.T. (1984). An assessment of biotechnology in plant breeding. IAPTC Newsletter 42: 2-9.

Torrey, J.G. (1961). Kinetin as a trigger for mitosis in mature endomitotic plant cells. Exp. Cell. Res. 23: 281-299.

Torrey, J.G. (1965). Cytological evidence of cell selection by plant tissue culture media. In: Proc. Int. Conf. Plant Tissue Cult. (Eds. P.R. White and A.R. Grove). McCutchan, Berkeley, pp. 473-484.

Torrey, J.G. (1966). The initiation of organized development in plants. In: Advances in Morphogenesis (Eds. M. Abercrombie and J. Brachet). Academic Press, London, pp. 39-87.

Tran Thanh Van, K. (1980). Control of morphogenesis by inherent and exogenously applied factors in thin cell layers. Int. Rev. Cytol. Supplement 11a: 175-194.

Tran Thanh Van, K. and Trinh, T.H. (1978). Plant propagation: non-identical and identical copies. In: Propagation of Higher Plants Through Tissue Culture (Eds. K.W. Hughes, R. Henke and M. Constantin). Springfield, Natl. Tech. Inf. Serv., pp. 134-158.

Vaarma, A. (1949). Spindle abnormalities and variation in chromosome number in *Ribes nigrum*. Hereditas 35: 136-162.

Van Harten, A.M., Bouter, H. and Broertjes, C. (1981). *In vitro* adventitious bud technique for vegetative propagation and mutation breeding of potato (*Solanum tuberosum* L.). II. Significance of mutation breeding. Euphytica 30: 1-8.

Van't Hof, J. and McMillan, B. (1969). Cell population kinetics in callus tissues of cultured pea root segments. Amer. Jour. Bot. 56(1): 42-51.

Van't Hof, J. and Kovacs, L.J. (1972). Mitotic cycle regulation in the meristem of cultured roots: the principle control point hypothesis. In: The Dynamics of Meristem Cell Populations (Eds. M.W. Miller and C.C. Kuehnert). Plenum, New York, pp. 15-32.

Vanzulli, L., Magnien, E. and Olivi, L. (1980). Caryological stability of *Datura innoxia* calli analysed by cytophotometry for 22 hormonal combinations. Plant Sci. Lett. 17: 181-197.

Vasil, V. and Vasil, V. (1982). The ontogeny of somatic embryos of *Pennisetum americanum* (L). K. Schum. I. In cultured immature embryos. Bot. Gaz. 143(4): 454-465.

Vasil, I.K. (1983). Regeneration of plants from single cells of cereals and grasses. In: Genetic Engineering in Eukaryotes (Eds. P. Lurquin and A. Kleinhofs). Plenum, New York, pp. 233-252.

Vasil, I.K. (1985). Somatic embryogenesis and its consequences in the *Graminaceae*. In: Tissue Culture in Forestry and Agriculture (Eds. R.R. Henke, K.W. Hughes, M.R. Constantin and A. Hollaender). Plenum, New York, pp. 31-47.

Vaughn, K.C. (1983). Chimeras and variegation: problems in propagation. HortScience 18(6): 17-20.

Vaughn, K.C., Kimpel, D.L. and Wilson, K.C. (1980). Investigations of the plastome mutant of *Chlorophytum*. Jour. Heredity 71: 154-157.

Venis, M.A. (1967). Effects of antibiotics on protein and ribonucleic acid synthesis in relation to the control of bacterial growth in excised tissues. Phytochem. 6: 799-806.

Venketeswaran, S. and Spiess, E.B. (1964). Tissue culture studies on *Vicia faba*. IV. Effect of growth factors on mitotic activity. Cytologia 29: 298-310.

Vieitez, A.M. and Vieitez, M.C. (1983). *Castanea sativa* plantlet proliferation from axillary buds cultivated *in vitro*. Scientia Hortic. 18: 343-351.

Villerts, A. (1938). Uber die Regeneration von Begonien blättern. Acta Biol. Latvica 8: 125-138 (cited from Bigot, 1971).

Von Arnold and Eriksson (1983). Effects of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. Plant Cell Tissue Organ Culture 3: 237-264.

Von Wangenheim, K.H. (1976). A mechanism for the endocellular control of cell differentiation and cell proliferation. J. Theor. Biol. 59: 205-222.

Walbot, V. and Cullis, C. (1985). Rapid genomic change in higher plants. Ann. Rev. Plant Phys. 36: 367-441.

Walker, K.A., Wendeln, M.L. and Jaworski, E.G. (1979). Organogenesis in callus tissues of *Medicago sativa*. The temporal separation of induction processes from differentiation processes. Plant Sci. Lett. 16: 23-30.

Walvaert, W., Samyn, G. and Van Wymersch, E. (1980). On the production of virus-free *Begonia multiflora* Voss Cv. multiflora variety. Acta Hortic. 110: 253-257.

Wang, S.Y., Bijun, J.K. and Steffens, G.L. (1985). Controlling plant growth via the gibberellin biosynthesis system. II. Biochemical and physiological alterations in apple seedlings. Physiol. Plant. 63: 169-175.

Wardell, W.L. and Skoog, F. (1973). Flower formation in excised tobacco stem segments. III. DNA content in stem tissue of vegetative and flowering tobacco plants. Plant Physiol. 52: 215-220.

Wareing, P.F. (1978). Determination in plant development. Bot. Mag. Tokyo Special Issue 1: 3-17.

Wareing, P.F. and Phillips, I.D. (1978). The Control of Growth and Differentiation in Plants. 2nd ed. Pergamon, Oxford.

Watts, J.W. and King, J.M. (1973). The use of antibiotics in the culture of nonsterile plant protoplasts. Planta 113(3): 271-277.

Webb, K.J., Osifo, E.O. and Henshaw, E.I. (1983). Shoot regeneration from leaflets discs of six cultivars of potato. Plant Sci. Lett. 30(1): 1-8.

Wehner, T.C. and Long, R.D. (1981). *In vitro* adventitious shoot and root formation of cultivars and lines of *Cucumis sativus* L. HortScience 16(6): 759-780.

Weis, J.S. and Jaffe, M.J. (1969). Photoenhancement by blue light of organogenesis in tobacco pith cultures. Physiol. Plant. 22: 171-176.

Welander, T. (1977). *In vitro* organogenesis in explants from different cultivars of *Begonia* x *hiemalis*. Physiol. Plant. 41: 142-145.

Welander, T. (1978a). *In vitro* propagation of clones from different cultivars of *Begonia* x *hiemalis*. Swedish J. Agric. Res. 8: 181-186.

Welander, T. (1978b). Influence of nitrogen and sucrose in the medium and of irradiance of stock plants on root formation in *Pelargonium* petioles grown *in vitro*. Physiol. Plant. 43: 136-141.

Welander, T. (1981). Effect of polarity on and origin of *in vitro* formed organs in explants of *Begonia elatior* hybrids. Swedish J. Agric. Res. 11: 77-83.

Werner, E.M. and Boe, F. (1980). *In vitro* propagation of Malling-7 apple rootstock. HortScience 15: 509-510.

Wernicke, W., Polrykus, I. and Thomas, E. (1982). Morphogenesis from cultured leaf tissue of *Sorghum bicolor*. The morphogenic pathways. Protoplasma 111(1): 53-62.

Westcott, R.J. (1981). Tissue culture of potato. Use of growth retardants. Potato Res. 24: 343-352.

Westerhof, J., Hakkaart, F.A. and Versluijs, D.M.A. (1984). Variation in two *Begonia* x *hiemalis* clones after *in vitro* propagation. Scientia Hortic. 24: 67-74.

Wetherell, D.F. (1978). *In vitro* embryoid formation in cells derived from somatic plant tissues. In: Propagation of Higher Plants Through Tissue Culture. A Bridge Between Research and Application (Eds. K.W. Hughes, R. Henke and M. Constantin). Technical Information Center, United States Department of Energy, Oak Ridge, pp. 102-124.

Wetherell, D.F. (1984). Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. Plant Cell Tissue Organ Culture 3: 221-227.

Whitham, T.G. and Stobodchikoff, C.N. (1981). Evolution by individuals, plant herbivore interactions and mosaics of genetic variability: The adaptive significance of somatic mutations in plants. Oecologia 49: 287-295.

Williams, L. and Collins, H.A. (1976). Growth and cytology of celery plants derived from tissue cultures. Ann. Bot. 40: 333-338.

Wilson, V.L. and Jones, P.A. (1983). DNA methylation decreases in aging but not in immortal cells. Science 220: 1055-1057.

Withers, L.A. (1980). Preservation of germplasm. Int. Rev. Cytol. Suppl. 11B: 101-136.

Withers, L.A. (1983). Germplasm storage in plant biotechnology. Semin. Ser. Soc. Exp. Bot. 18: 187-218.

Withers, L.A. (1984). Freeze preservation of cells. In: Cell Culture and Somatic Cell Genetics of Plants (ed. I.K. Vasil). Academic Press, New York, pp. 608-620.

Withers, L.A. and King, P.J. (1979). Proline - a novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L. Plant Physiol. 64: 675-678.

Yeoman, M.M. (1970). Early development in callus cultures. Int. Rev. Cytol. 29: 303-409.

Yeoman, M.M. and Aitchinson, P.A. (1973). Changes in enzyme activities during the division cycle of cultured plant cells. In: The Cell Cycle in Development and Differentiation. Br. Soc. Dev. Biol. Symp. I (Eds. M. Baus and F.R. Billet). Cambridge University Press, pp. 185-201.

Yeoman, M.M. and Forsche, E. (1980). Cell proliferation and growth in callus cultures. Int. Rev. Cytol. Suppl. 11A: 1-24.

Zelcer, A., Soferman, O. and Izhar, S. (1984). An *in vitro* screening for tomato genotypes exhibiting efficient shoot regeneration. J. Plant. Physiol. 115: 211-215.

Zieslen, N., Kopperudg, C., Heide, O. and Moe, R. (1984). Effects of temperature and gibberellin on the activity of endogenous cytokinin-like substances in leaves of *Begonia* x *cheimantha*. Physiol. Plant. 60: 92-97.

Zimmerman, R.H. (1983). Rapid clonal propagation by tissue culture. In: Genetic Engineering Application to Agriculture (Ed. L.D. Owens). Rowman and Allanheld, Granada, London, pp. 305-316.

Ziv, M., Meir, G. and Halevy, A.H. (1983). Factors influencing the production of hardened glaucous carnation plants *in vitro*. Plant Cell Tissue Organ Culture 2: 55-65.

SECTION I. APPENDIX

I.1. Chloroplast counts and chromosome counts from plants produced from colchicine treatments

I.1.1. Results and discussion

In the sample of plants assessed with polyploid morphologies, varying levels of ploidy were observed (see Figure I.1.). Chloroplast number was also found to fluctuate (Figure I.2.). Of the plants evaluated 2 plants (5565 and 5555), both produced on high levels of colchicine (1,800 mg/l), showed a wider range of chromosome numbers when compared with the other plants assessed. This fluctuating chromosome number may be associated with the presence of a high frequency of mitotic abnormalities in these plants. These included anaphase bridges, lagging chromosomes at anaphase, segregating genomes and asynchronous division of constituent genomes (see Plate I.1.). These abnormalities were also observed in the other plants under study but were not as frequent.

The high level of ploidy found in some cells of these colchicine derived plants (see Figure I.1.) may be a result of restitution cycles caused by colchicine (see Section *B.3.5.*). These cells would appear to be maintained at a low frequency, but may breakdown on division by somatic reduction (Huskin, 1948) to give cells of lower ploidy.

Of the plants observed variant 5265 appeared the most stable polyploid with a high frequency of cells between 40–48 in chromosome number. Plant 5304 was diploid and contrasts markedly in chromosome distribution when compared with the remaining population. This variant did have a polyploid morphology. This observation however is based on the more developed leaves of the plants and the diploid nature of the young leaf meristem taken for chromosome counting suggests diplontic selection or a chimeral origin (Derman and Bain, 1944).

Results from chloroplast counts (see Figure I.2.) again illustrate the variation which exists within this population of putative polyploids. These results demonstrate that chloroplast counts may be used in *B. rex* as a measure of the presence of polyploid cells and are particularly useful for assessing breakdown in putative polyploids during development where chromosome counts cannot be taken from large leaves.

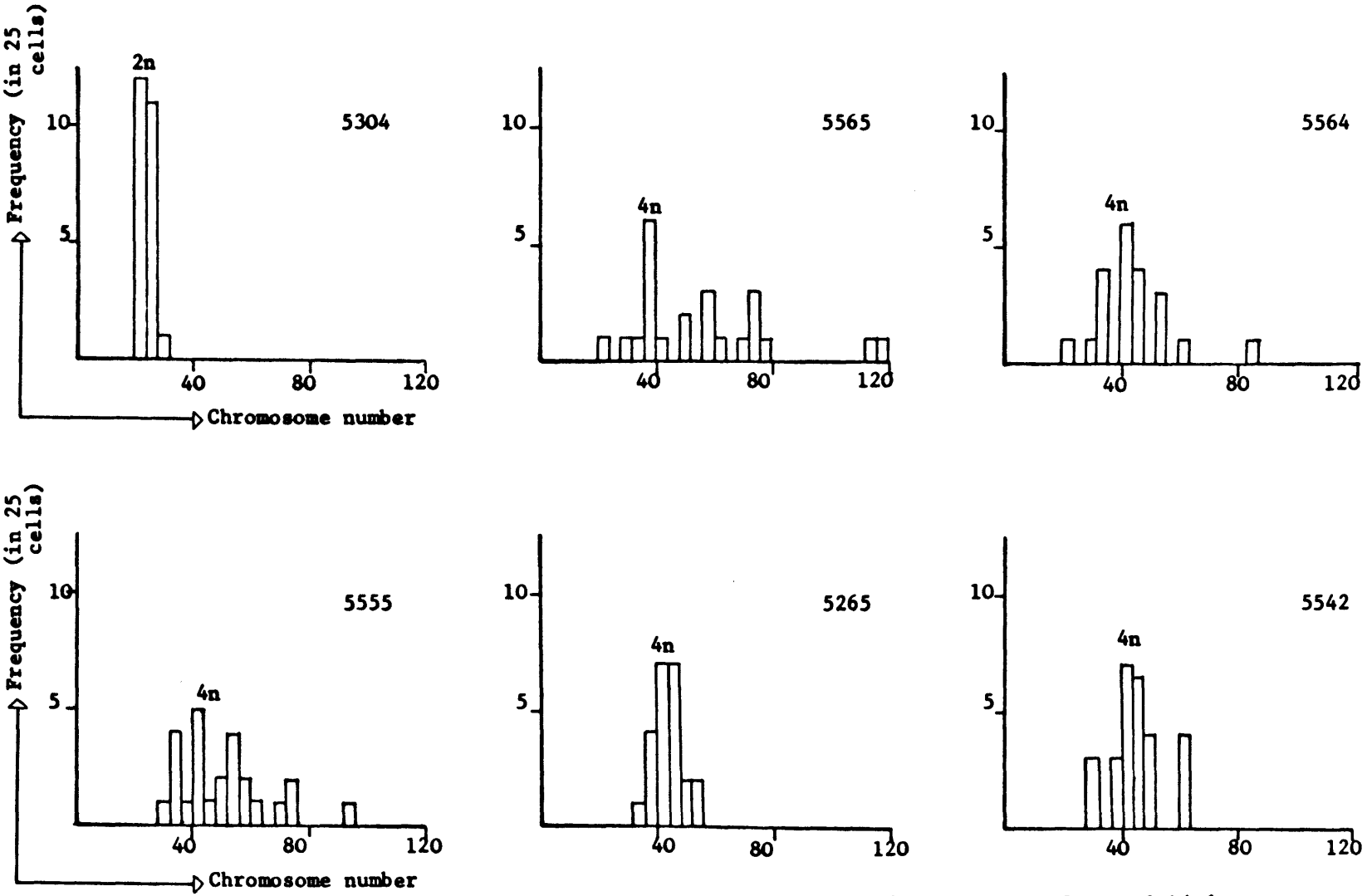


Figure I.1. Frequency plots for chromosome numbers in putative polyploids produced after colchicine treatment of petioles and callus. 5304, 5265 = 300 mg/l; 5555, 5542 = 1,800 mg/l (petiole progeny); 5564, 5565 = 1,000 mg/l (callus progeny).

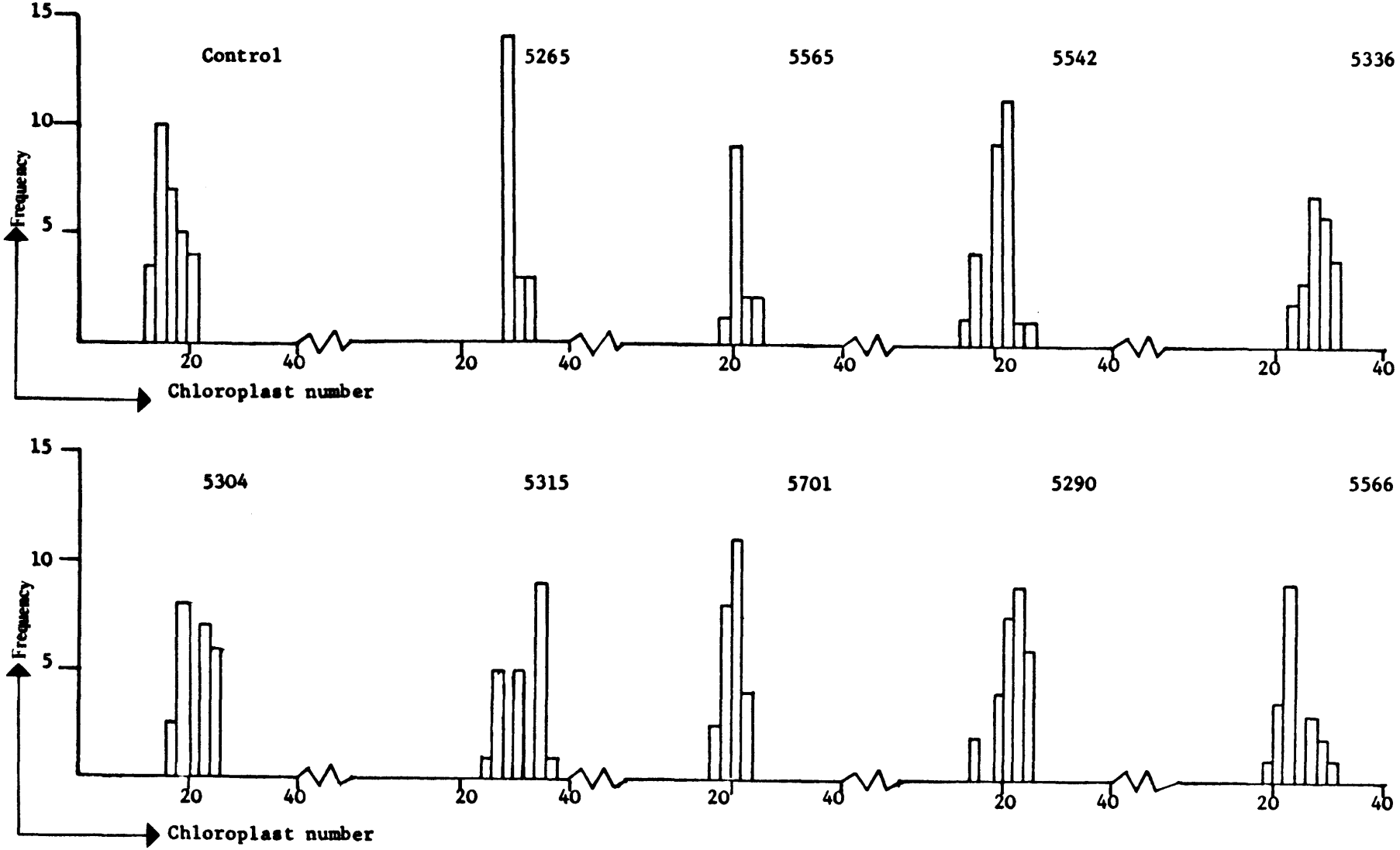
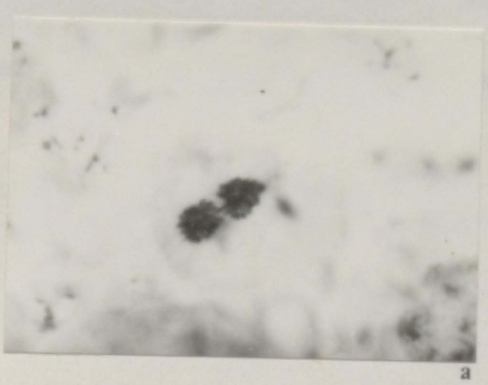
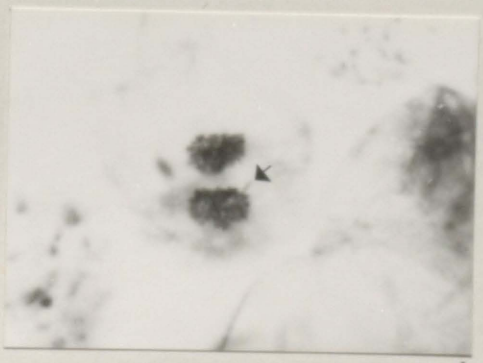


Figure I.2. Frequency plots of chloroplast numbers in guard cells of putative polyploids produced from colchicine treatments of petioles and callus. (5265, 5304, 5315 = 300 mg/l, 5701, 5209 = 500 mg/l treatments of petioles; 5566, 5542, 5565, 5336 = 1,000 mg/l treatments of callus).



a



b

Plate I.1. Mitotic abnormalities observed in putative polyploids. (a) anaphase bridge, (b) straying chromosome at telophase. Enlargement x 1,000.

These results indicate that most of the plants produced using colchicine were mixoploids of varying levels of stability. Those produced on high colchicine produced the widest range of chromosome numbers which suggests that the high concentration of colchicine used may have significantly affected the mitotic apparatus. The nature of change in the phenotype, which may result from this diversity of chromosome numbers can only be assessed when these plants have grown to maturity.