DEVELOPMENT OF TISSUE AND PROTOPLAST CULTURE TECHNIQUES FOR *IMPATIENS WALLERIANA* Hook. f. WITH A VIEW TO THE TRANSFER OF YELLOW FLOWER COLOUR.

by

Susan J. Delafield

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Susan J. Delafield ©
Department of Plant Science
Macdonald College
McGill University
Montreal

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SHORT TITLE

TISSUE AND PROTOPLAST CULTURE OF *IMPATIENS WALLERIANA*.

ABSTRACT

Impatiens walleriana Hook.f. is a popular bedding plant with a variety of flower colours excepting yellow. Transfer of this characteristic could be achieved through somatic hybridization, thus this study was an attempt to establish the basic parameters of tissue and protoplast culture. The condition of the stock plants and surface sterilisation techniques are important for this species. Most tissues responded to trials for callus induction with rhizogenesis. Evaluation of media and plant growth regulator combinations revealed that axillary shoot proliferation was most efficient on MS medium with 3.0 mg/L BAP and 0.1 mg/L IBA. Protoplast isolation was most effective in 0.5% w/v cellulase and 0.25% w/v pectinase with 0.25 M mannitol and 0.25 M glucose yielding 2.70 x 10^5 protoplasts per gram of leaf tissue. The presence of mucous, raphids and excessive debris inhibited successful culture of leaf mesophyll protoplasts. Cotyledon protoplasts show promise for such investigations.

RESUME

Impatiens walleriana Hook. f. est une plante annuelle horticole recherchée qui présente une grande variétée de couleurs à l'exception remarquable de la couleur jaune. Le transfert de ce caractère poura être accomplit par l'hybridation des cellules somatiques, alors cet étude était un essai à établir les paramètres de base de culture des tissus et des protoplastes. La nature des plantes ainsi que les techniques de stérélisation superficielle sont déterminantes chez cette espèce. La plupart des tissus soumis à l'induction de cals réagirent en produisant des racines. L'évaluation des milieux de culture et des régulateurs de croissance a révélée que le développement des bourgeons axillaires était optimale en milieu de culture MS à des concentrations de 3.0 mg/L d'adénine benzylique et 0.1 mg/L d'acide indole butyrique. L'isolation des protoplastes était, quant à elle, optimale à des concentrations de 0.5% de cellulase par volume, de 0.25% de pectinase par volume, de mannitol 0.25 M et de glucose 0.25 M. Jusqu'à 2.70 X 10^5 protoplastes par gramme de tissu foliaire furent ainsi produits. La présence de mucus et d'artéfacts a entravée la culture des protoplastes de mésophylle foliaire. Les protoplastes de cotylédons sont voués à un avenir plus prometteur.

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TABLE OF CONTENTS

Page

Short Title..... ii

Abstract..... iii

Résumé..... iv

Acknowledgements..... v

Table of Contents..... vi

List of Tables..... xvi

List of Figures..... xviii

List of Appendices..... xx

List of Abbreviations..... xxi

1. Introduction..... 1

2. Literature Review..... 4

2.1 Economic Importance..... 4

2.2 *Impatiens walleriana* Hook.f..... 4

2.3 The Genus *Impatiens*..... 5

2.4 The New Guinea Impatiens..... 6

2.5 Breeding Behaviour..... 8

2.5.1 *I. walleriana*..... 8

2.5.2 New Guinea Impatiens..... 9

2.6 Flower Colour..... 10

2.6.1 The Biochemistry of Flower Colour..... 11

2.6.2 Floral Pigments of the Genus *Impatiens*..... 13

Page

2.6.3 The Genetics of Flower Colour..... 14

2.6.3.1 The Inheritance of Flower Colour in the Genus *Impatiens*..... 15

2.6.3.2 The Inheritance of Flower Colour in *I. walleriana*..... 15

2.6.3.3 The Inheritance of Flower Colour in *I. balsamina*..... 17

2.6.4 Yellow Flowering Species in the Genus *Impatiens*..... 17

2.6.5 Breeding for a Yellow Flowering *I. walleriana*..... 18

2.7 Somatic Cell Hybridization..... 19

2.7.1 Protoplast Isolation and Culture..... 20

2.7.2 Protoplast Fusion..... 23

2.8 Tissue Culture..... 24

2.8.1 Callus Culture..... 25

2.8.2 Micropropagation..... 26

2.8.3 Surface Sterilisation of Donor Tissue..... 27

2.8.4 Tissue Culture of Impatiens..... 28

3. Materials and Methods..... 31

3.1 Plant Material..... 31

3.1.1 Cultural Conditions..... 31

3.2 Crossing Procedure..... 33

3.2.1 Crosses..... 34

3.3 Culture Media and Procedure..... 34

Page

3.3.1 Cultural Conditions..... 34

3.3.2 Preliminary Experiments-Tissue Culture..... 35

3.3.2.1 Experiment 1. Evaluation of the Effect of Plant Growth Regulators on *in vitro* Growth of *I. repens* and *I. walleriana* var. *variegata*..... 35

3.3.3 Surface Sterilisation Experiments..... 35

3.3.3.1 Experiment 1. Determination of Appropriate Surface Sterilisation Techniques on Stem and Leaf Tissue..... 36

3.3.3.2 Experiment 2. Comparison of Surface Sterilants on Stem and Leaf Tissue..... 37

3.3.3.3 Experiment 3. Comparison of Surface Sterilisation Techniques on Nodes..... 37

3.3.3.4 Experiment 4. Surface Sterilization and Germination of Seeds..... 38

3.3.3.5 Experiment 5. Surface Sterilisation of Leaves for Protoplast Culture..... 39

3.3.3.6 Statistical Analysis of Surface Sterilisation Experiments..... 39

3.3.4 Callogenesis Experiments..... 40

3.3.4.1 Experiment 1. Evaluation of the Effect of Media and 2,4-D on Leaf and Stem Tissue..... 40

3.3.4.2 Experiment 2. Evaluation of the Effect of 2,4-D and Kinetin on Stem Tissue..... 40

3.3.4.3 Experiment 3. Evaluation of the Effect of IAA and BAP on Seedling Stem Tissue... 41

3.3.4.4 Experiment 4. Evaluation of the Effect of Media and Carbon Source on Callogenesis of Axillary Buds..... 42

Page

3.3.4.5 Statistical Analysis of Callogenesis Experiments..... 43

3.3.5 Micropropagation..... 43

3.3.5.1 Experiment 1. Evaluation of the Effect of BAP on Shoot Proliferation..... 44

3.3.5.2 Experiment 2. Evaluation of the Effect of Media Concentrations and Growth Regulator Ratios on Shoot Proliferation... 46

3.3.5.3 Experiment 3. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation..... 47

3.3.5.4 Experiment 4. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation..... 47

3.3.5.5 Experiment 5. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation..... 48

3.3.5.6 Experiment 6. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation..... 48

3.3.5.7 Experiment 7. Evaluation of the Effect of IAA and BAP Concentrations on Shoot Proliferation..... 49

3.3.5.8 Statistical Analysis of Micropropagation Experiments..... 49

3.3.6 Acclimation and Rooting of Micropropagated Shoots..... 49

3.4 Protoplast Isolation - Basic Procedure..... 50

3.4.1 Preliminary Experiments..... 54

3.4.1.1 Experiment 1. Evaluation of Enzymes..... 54

3.4.1.2 Experiment 2. Evaluation of Enzymes..... 54

Page

3.4.1.3 Experiment 3. Evaluation of Osmotic Levels..... 55

3.4.1.4 Experiment 4. Evaluation of Pretreatment of Donor Tissue.... 55

3.4.1.5 Experiment 5. Evaluation of Protoplast Isolation Procedures..... 55

3.4.1.6 Experiment 6. Evaluation of Protoplast Separation Techniques..... 56

3.4.2 Quantification of Protoplast Protocol. Phase 1..... 57

3.4.2.1 Experiment 1. Comparison of the Effect of Surface Sterilisation Techniques on Protoplast Yield and Viability..... 58

3.4.2.2 Experiment 2. Comparison of the Effect of Several Enzyme Mixtures on Protoplast Yield and Viability..... 59

3.4.2.3 Experiment 3. Evaluation of the Effect of Incubation Time on Protoplast Yield and Viability..... 59

3.4.2.4 Experiment 4. Comparison of the Effect of Sorbitol, Glucose and Mannitol, as Osmotic Regulators on Protoplast Yield and Viability..... 60

3.4.2.5 Experiment 5. Comparison of the Effect of Pretreatment of the Donor tissue on Protoplast Yield and Viability..... 60

3.4.2.6 Experiment 6. Evaluation of the Effect of Leaf Size and Age on Protoplast Yield and Viability..... 61

3.4.2.7 Experiment 7. Evaluation of the Effect of Nylon Mesh Filter Size on Protoplast Yield and Viability..... 61

3.4.2.8 Experiment 8. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability..... 62

3.4.2.9 Experiment 9. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability..... 64

3.4.3 Quantification of Protoplast Protocol. Phase 2..... 64

3.4.3.1 Experiment 1. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability..... 65

3.4.3.2 Experiment 2. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability..... 66

3.4.3.3 Experiment 3. Evaluation of the Effect of Centrifugation Speed on Protoplast Yield and Viability..... 66

3.4.3.4 Experiment 4. Comparison of the Effect of Media on Protoplast Survival..... 66

3.4.4 Preliminary Evaluation of Protoplast Isolation from Cotyledons..... 67

3.4.5 Statistical Analysis of the Protoplast Experiments..... 68

4. Results..... 69

4.1 Crosses..... 69

4.2 Preliminary Experiments - Tissue Culture..... 69

4.2.1 Experiment 1. Evaluation of the Effect Plant Growth Regulators..... 69

4.3 Surface Sterilisation Experiments..... 69

Page

4.3.1 Experiment 1. Determination of Appropriate Surface Sterilisation Techniques on Stem and Leaf Tissue..... 69

4.3.2 Experiment 2. Comparison of Surface Sterilants on Stem and Leaf Tissue..... 73

4.3.3 Experiment 3. Determination of Appropriate Surface Sterilisation Techniques on Nodes..... 76

4.3.4 Experiment 4. Surface Sterilisation and Germination of Seeds..... 77

4.3.5 Experiment 5. Surface Sterilisation of Leaves for Protoplast Culture..... 79

4.4 Callogenesis Experiments..... 79

4.4.1 Experiment 1. Evaluation of the Effect of 2,4-D and Media on Leaf and Stem Tissue..... 79

4.4.2 Experiment 2. Evaluation of the Effect of 2,4-D and Kinetin on Stem Tissue..... 81

4.4.3 Experiment 3. Evaluation of the Effect of IAA and BAP on Seedling Stem Tissue..... 81

4.4.4 Experiment 4. Evaluation of the Effect of Media and Carbon Source on Callogenesis of Axillary Buds..... 85

4.5 Micropropagation..... 85

4.5.1 Experiment 1. Evaluation of the Effect of BAP on Shoot Proliferation..... 85

4.5.2 Experiment 2. Evaluation of the Effect of Media Concentrations and Growth Regulator Ratios on Shoot Proliferation..... 88

4.5.3 Experiment 3. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation..... 88

Page

4.5.4 Experiment 4. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation..... 91

4.5.5 Experiment 5. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation..... 91

4.5.6 Experiment 6. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation..... 95

4.5.7 Experiment 7. Evaluation of the Effect of IAA and BAP Concentrations on Shoot Proliferation..... 99

4.6 Acclimation and Rooting of Micropropagated Shoots.....101

4.7 Protoplast Isolation 101

4.7.1 Preliminary Experiments.....101

4.7.1.1 Experiment 1. Evaluation of Enzymes.....101

4.7.1.2 Experiment 2. Evaluation of Enzymes.....101

4.7.1.3 Experiment 3. Evaluation of Osmotic Levels.....103

4.7.1.4 Experiment 4. Evaluation of Pretreatment of Donor Tissue.....103

4.7.1.5 Experiment 5. Evaluation of Protoplast Isolation Procedures.....104

4.7.1.6 Experiment 6. Evaluation of Protoplast Separation Techniques.....104

4.7.2 Quantification of Protoplast Protocol. Phase 1.....106

4.7.2.1 Experiment 1. Comparison of the Effect of Surface Sterilisation Techniques on Protoplast Yield and Viability.....106

4.7.2.2 Experiment 2. Comparison of the Effect of Several Enzyme Mixtures on Protoplast Yield and Viability.....109

4.7.2.3 Experiment 3. Evaluation of the Effect of Incubation Time on Protoplast Yield and Viability.....109

4.7.2.4 Experiment 4. Comparison of the Effect of Sorbitol, Glucose and Mannitol, as Osmotic Regulators on Protoplast Yield and Viability.....112

4.7.2.5 Experiment 5. Comparison of the Effect of Pretreatment of the Donor tissue on Protoplast Yield and Viability.....112

4.7.2.6 Experiment 6. Evaluation of the Effect of Leaf Size and Age on Protoplast Yield and Viability.....115

4.7.2.7 Experiment 7. Evaluation of the Effect of Nylon Mesh Filter Size on Protoplast Yield and Viability.....115

4.7.2.8 Experiment 8. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability.....118

4.7.2.9 Experiment 9. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability.....120

4.7.3 Quantification of Protoplast Protocol. Phase 2.....120

4.7.3.1 Experiment 1. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability.....120

4.7.3.2 Experiment 2. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability.....123

4.7.3.3 Experiment 3. Evaluation of the Effect of Centrifugation Speed on Protoplast Yield and Viability.....123

4.7.3.4 Experiment 4. Comparison of the Effect of Media on Protoplast Survival.....123

4.7.4 Preliminary Evaluation of Protoplast Isolation from Cotyledons.....124

5. Discussion.....128

5.1 Crosses.....128

5.2 Surface Sterilisation of *I. walleriana*.....128

5.3 Callogenesis of *I. walleriana*.....130

5.4 Micropropagation of *I. walleriana*.....132

5.5 Protoplast Isolation.....136

5.6 Protoplast Viability.....142

5.7 Protoplast Culture.....143

6. Conclusions.....144

7. References.....148

Appendices.....164

LIST OF TABLES

Table		Page
Table 4.1	Rooting response of *I. repens* and *I. walleriana* to various plant growth regulator combinations	70
Table 4.2.1	Surface sterilisation techniques performed on leaves	72
Table 4.2.2	Surface sterilisation techniques performed on stems	74
Table 4.3	Comparison of surface sterilants for stem and leaf tissue	75
Table 4.4	Surface sterilisation techniques performed on nodes	77
Table 4.5	Surface sterilisation techniques performed on seeds	78
Table 4.6	Main effect of 2,4-D and media on leaf and stem tissue	80
Table 4.7	Main effect of 2,4-D and kinetin on stem tissue	83
Table 4.8	Main effect of growth regulators and light on tissue growth	84
Table 4.9	The effect of media and carbon source on axillary buds	86
Table 4.10	The effect of growth regulator ratios and media strength on shoot proliferation	89
Table 4.11	Main effect on kinetin, NAA and media on shoot proliferation	90
Table 4.12	Main effect of kinetin, NAA and media on shoot height	93
Table 4.13	Main effect of IBA and BAP on shoot proliferation	94

Table Page

Table 4.14 Main effect of IBA and BAP on shoot height..... 97

Table 4.15 Main effect of BAP and IAA on shoot proliferation.....100

Table 4.16 The effect of surface sterilisation techniques on protoplast yield and viability.....107

Table 4.17 The effect of different enzyme mixtures on protoplast yield and viability.....110

Table 4.18 The effect of incubation time on protoplast yield and viability.....111

Table 4.19 The effect of osmotic regulators on protoplast yield and viability.....113

Table 4.20 The effect of pretreatment on the donor tissue on protoplast yield and viability...114

Table 4.21 The effect of leaf size on protoplast yield and viability.....116

Table 4.22 The effect of nylon mesh filter size on protoplast yield and viability.....117

Table 4.23 The effect of different protoplast wash protocols on protoplast yield and viability (Experiment 8).....119

Table 4.24 The effect of different protoplast wash protocols on protoplast yield and viability (Experiment 9).....121

Table 4.25 The effect of enzyme mixtures on protoplast yield and viability (Experiment 1)...122

Table 4.26 The effect of enzyme mixtures on protoplast yield and viability (Experiment 2)...124

Table 4.27 The effect of centrifugation speed on protoplast yield and viability.....125

LIST OF FIGURES

Figure		Page
1.	*Impatiens walleriana* var. *variegata*	32
2.	Shoot tips of *Impatiens walleriana* var. *variegata* on screening medium	45
3.	*Impatiens walleriana* var. *variegata* showing axillary growth after pruning	45
4.	Peeling of the lower epidermis off leaves of *I. walleriana*	51
5.	Filtration of the enzyme-tissue mixture after incubation	53
6.	Comparison of leaf maturity of *I. walleriana* var. *variegata*	63
7.	Surface sterilisation of *I. walleriana* for protoplast isolation	63
8.	Root proliferation on B5 medium with 1.0 mg/L IBA.	71
9.	Abnormal root growth on B5 and MS media with 8 mg/L 2,4-D	71
10.	Root formation on leaf sections and root sections on B5 medium with no plant growth regulators	82
11.	The effect of BAP on shoot proliferation at three transfer dates	87
12.	The simple effects of NAA and kinetin on shoot number	92
13.	The simple effects of IBA and BAP on shoot number and shoot weight	96
14.	The effects of IBA and BAP on shoot proliferation.	98
15.	The effects of IAA and BAP on shoot proliferation.	98

Figure Page

16. Leaves after incubation for 16 hours in an enzyme solution of 2% w/v cellulase and 0.5% pectinase.....102

17. Raphids and debris in the enzyme solution after tissue digestion.....102

18. Protoplasts isolated after flotation on 40% sucrose.....105

19. Protoplasts isolated after transferring the tissue from the enzyme solution to the funnel for separation by teasing with tweezers.....105

20. Fluorescence of viable protoplasts stained with FDA.....108

21. Protoplasts stained with Calcofour White showing no cell wall.....108

22. Protoplasts after one week of culture on Kao's medium.....127

23. Protoplasts from cotyledons of *I. walleriana* 'economy mix'.....127

24. Protoplasts of *Impatiens walleriana* var. *variegata*, isolated with the optimized protocol...147

LIST OF APPENDICES

Appendix		Page
1	Impatiens Chromosome Numbers and Native Country....	164
2	List of Micro Molar Equivalents.....	166
3	Media Composition - Tissue Culture.....	168
4	Media Composition - Plant Protoplast Culture.....	170

LIST OF ABBREVIATIONS

BAP = 6-benzylamino purine

2,4-D = 2,4-dichlorophenoxyacetic acid

IAA = indole-3-acetic acid

IBA = 3-indole butyric acid

2iP = 2-isopentenyladenine or 6-γ,γ-dimethylallylamino purine

NAA = α-naphthalene acetic acid

1. INTRODUCTION

Impatiens walleriana Hook.f. is a popular annual bedding plant that has a great variety of flower colours with the notable exceptions of yellow and true blue. Gardeners, always in search of novelties, would welcome such additions to their plant selection. The large genus, *Impatiens*, encompasses a great variety of germplasm with characteristics that show great horticultural potential. Unfortunately, the horticultural species does not cross readily with many other species of the genus and no source of yellow pigment exists in the species. Blue flowers can be achieved by the selection for intensification of the pigment, cyanidin, which already is found in the species, but the yellow flower colour must be introduced from another species. Thus, an alternative approach to the transfer of yellow pigment, such as somatic cell hybridization, must be considered. *Impatiens* are good candidates for this technique, since polyploids with diverse genomes are known in the genus.

In order to apply this technique to *I. walleriana* a foundation of basic tissue culture and protoplast techniques must be established. Little work of this nature has been done with the species and the research within the genus has been restricted to the New Guinea hybrids.

Ultimately the goal of this project was to develop a horticulturally acceptable yellow-flowered *I. walleriana* but conservatively the goals of my thesis were to develop techniques for callus culture, micropropagation and protoplast isolation and culture of *I. walleriana*.

These goals were to be achieved by first doing a series of crosses to confirm the findings of previous researchers that *I. repens* Moon, a yellow-flowered species, and *I. walleriana* are incompatible (Arisumi, 1980a) and with hopes of producing seed for *in vitro* work. The next step was to be a preliminary evaluation of the effect of the common auxins and cytokinins on the *in vitro* growth of *I. repens* and *I. walleriana*. This experiment revealed certain difficulties with the establishment and growth of both species *in vitro* and thus the need for detailed evaluation of surface sterilisation techniques. At this point it was decided that it would be wise to concentrate efforts on the successful culture of one species first, so *I. walleriana* was chosen for all further experimental work. With the selection of an acceptable method of surface sterilisation the third stage of the project was feasible. This stage was a detailed evaluation the effects of several types of basal media, carbon sources and different plant growth regulators on callogenesis of *I. walleriana*. Subsequently a series of experiments with several types of basal media and different plant growth regulators were to be performed to find the highest shoot proliferation rate for micropropagation.

A protocol for protoplast isolation from *I. walleriana* was to be developed by adapting appropriate techniques from preliminary experiments, then optimized by two phases of quantification. The first phase was to involve the use of whole leaves, larger enzyme solution volumes and repetition of experiments for statistical evaluation. The second phase was to be achieved by using smaller tissue pieces, smaller enzyme solution volumes and repetitions within the experiments for more valid treatment comparisons.

I. walleriana var. *variegata* was selected for this study since this variety is composed of both green (cnloroplast-present) and white (chloroplast-absent) tissue layers, and could therefore supply a means of visual detection of heterokaryons if somatic cell hybridization was to be attempted.

2. LITERATURE REVIEW

2.1 Economic Importance

During the last decade Impatiens plants have become increasingly popular in North America, representing a full 43% of a $239,844,000 American wholesale bedding plant market (Tayama *et al.*, 1987). They have moved from the rank of the third most popular bedding plant in 1977 to the first in 1986 (Voight, 1980). Although the Canadian statistics are not as specific, interviews of Toronto growers, by C. Anne Whealy (1988), suggest that this genus represents a similar proportion of the $160,403,996.00 Canadian wholesale bedding plant market (Statistics Canada, 1989). These are statistics from 1987 which have doubled since 1977. In Quebec alone 48,329,195 bedding plants are marketed annually. This represents 70% of the total greenhouse ornamental plants sold in Quebec, at a value of $36,859,265.00. This is a truly dynamic industry and in which Impatiens play a very important part.

2.2 *Impatiens walleriana* Hook.f.

For many years the most prominent commercial species was *Impatiens walleriana* and this species still occupies many gardens. This delightful plant is available in a full range of sizes, from the 20-25 cm Super-Elfin^{R} to the 30-35 cm Blitz series (Hamrick, 1989). Most colours, including white, pink, salmon, coral, red, orange, violet, mauve and bicolours can be found, except yellow and a true blue (Anon., 1989b). The ability of this plant to thrive in shady situations and its tolerance of air pollution has

contributed to its popularity (Adedipe *et al.*, 1972). Development of compact varieties and the improvement of production qualities has continued to endear this plant to both gardeners and growers. The new varieties will remain compact through the heat of the summer and sport lovely colourful flowers against dark foliage from bedding out until frost (McBride, 1982).

2.3 The genus *Impatiens*

Impatiens is a cosmopolitan genus with 1000 described species, of which only 500 should be recognized, depending on the system of classification (Clevenger, 1971). The ambiguity of the number of species in the genus can be attributed to systematic studies of the genus either combining or dividing species, which clouds the true identity of the cultivated species. Although several recent publications have clarified the situation (Bhaskar, 1975; Grey-Wilson, 1980a; Zinov'eva-Stahevitch, 1981). The genus is found primarily in the Old World Tropics, with species throughout much of tropical Africa, India, southern China and Japan, a few in south-east Asia and the occasional species in the temperate regions; northern Asia, Europe and North America (Grey-Wilson, 1980a; Chinnappa and Gill, 1974). Most of the plants are semi-succulent herbs, however, a few are small shrubs. Their chief attraction though, is the great diversity of the showy and often brightly coloured flowers. The other member of the family, Balsaminaceae, is the monotypic genus *Hydrocera* which is of little economic importance (Grey-Wilson, 1980d). *Impatiens* is characterised by a complex floral anatomy and makes poor

herbarium specimens, which have caused endless taxonomic confusion (Zinov'eva-Stahevitch, 1981).

Both *I. sultanii* Hook.f. and *I. holstii* Engl. et Warb. are synonyms for *I. walleriana* and are used interchangeably in the literature. The name *I. petersiana* can be found in the horticultural literature and also refers to *I. walleriana* (Grey-Wilson, 1980c). This species came from East Africa at the end of the eighteenth century and is one of the almost thirty that have been in cultivation at one time or another (Russell, 1980). Until the introduction of *I. walleriana*, *I. balsamina* L. was the only species that was widely grown but its leggy nature and sparse, hidden flowers have caused a declining interest in this plant (McBride, 1982). This species, the garden balsam, has been cultivated for almost 300 years. The place for *I. balsamina* in modern gardens will be guaranteed for years to come, due to the recent development of dwarf bushy varieties. This plant has not only been a great favourite of gardeners but has been used in a number of physiological and biochemical studies such as those by Kumar and Nanda (1981) and Bhattacharya and co-workers (1976).

2.4 The New Guinea Impatiens

A plant exploration expedition to New Guinea in 1970 brought back many new species of *Impatiens* which encouraged a great interest in the breeding of new varieties in the United States. Joseph Higgins and Harold Winters from the Agricultural Research Service, United States Department of Agriculture, went to the subtropical highlands of Australian New Guinea to seek out new ornamental germplasm (Arisumi and Cathey, 1976). The collectc · noticed many large-flowered,

brightly coloured *Impatiens* being cultivated along the roadside in native villages (Winters, 1970). That the native people would go to the trouble of cultivating these unknown species indicates their striking appearance (Winters, 1973). Despite their perishable nature and chronic transportation difficulties, 23 accessions of both seeds and cuttings, representing six to eight species (depending on classification) were sent back to the United States (Murphy, 1978). One species was picked up in Java (*I. platypetala* Lindl.) on the way back and two more came from the Celebes (*I. platypetala* var. *aurantiaca* Steen and an unidentified species) the following year (McBride, 1982).

After a year of tenuous existence in quarantine the plants were sent to Longwood Gardens in Pennsylvania. The plants didn't look very promising, spindly, and with few flowers, but they were planted outside in a last attempt to revive the introductions (Martin, 1984). Surprisingly the plants burst into flower and displayed exciting variegated foliage, especially in full sun (Armstrong, 1974). This was quite unlike their cousin, *I. walleriana*, which thrives in shady areas. Those plants that didn't flower during the summer soon did so in the greenhouses that winter and as many crosses as possible were performed. Seven hundred F_1 progeny were planted out during the summer of 1972 and 3000 the following year. From these hybrids many new and exciting cultivars, possessing large flowers, unusual flower colours, foliar variegation and a tolerance of full sun, have been released. The first varieties were the Circus collection from Longwood and the Cyclone hybrids from Iowa State University (Woodroffe, 1975). Although these plants were quite a disappointment to the public, ongoing breeding

programs have produced outstanding new varieties that are much easier to maintain. Examples of the most recent releases are the Celebration series from Ball Seed, the Sunshine Series from Mikkelsens, and the Kientzler series from Paul Ecke Ranches (Anon., 1989a).

2.5 Breeding Behaviour

Plants of the genus *Impatiens* are generally outbreeding due to the plants morphological characteristics (Grey-Wilson, 1980a). The flowers are markedly protandrous with a fused column of stamens surrounding the ovary and the stigma. The stigma is actually blocked by the staminal unit. This prevents the pollen from coming in contact with the stigma. Also the stigma is not receptive until well after the anther has dehisced. *I. balsamina* appears to be an exception to this rule since the staminal unit hangs in such a way as to leave pollen on the stigma (Watts, 1980). Cleistogamy does exist in the genus but is restricted to the north temperate species, which are of little horticultural interest (Russell, 1976; Waller, 1984).

2.5.1 *I. walleriana*

This large genus is full of promising germplasm that can potentially be used to improve and enlarge the scope of existing *I. walleriana* varieties. This is exactly what the sponsors of the Longwood Foundation-USDA collecting trip had in mind. Early attempts at crossing the New Guinea, Java and Celebes introductions with *I. walleriana* met with failure (Beck *et al.*, 1974). Arisumi (1980a) found that *I. walleriana* was only compatible with *I. thomassettii* Hook.(synonym for *I. gordonii* Horne ex Bak.), an African

species among a group of 21 African, Indian and New Guinea species. *I. uguenensis* Warb. (synonym for *I. sodenii* Warb. et Engl. ex Engl.) set seed but failed to mature, when crossed with *I. walleriana*. Arisumi (1980b) continued his hybridization program by developing *in vitro* embryo and ovule culture techniques for *Impatiens*. Crosses between *I. walleriana* 'Elfin White' and *I. campanulata* Wight, *I. marianae* Rchb.f. ex Hook.f., *I. repens* Moon, *I. tuberifera* Humbert and *I. verticillata* Wight did not produce embryos that were viable in culture (Arisumi, 1985). On the other hand crosses between *I. walleriana* and *I. auricoma* Baill., *I. epiphytica* G.M. Schulz, *I. niamniamensis* Gilg. and several New Guinea hybrids produced culturable ovules, that grew successfully into plants. Arisumi (1987) has also raised hybrids of *I. walleriana* and *I. flaccida* Arn. and an unidentified species from the Celebes. Natural hybridization has been observed between *I. walleriana* and *I. usambarensis* Grey-Wilson (Grey-Wilson,1980c).

2.5.2 New Guinea Impatiens

Although this apparent prevention of the incorporation of new characteristics in the popular horticultural species was disappointing, the breeders produced many hybrids within the introduced species. Almost immediately Arisumi (1973), Armstrong (1973) and Beck and associates (1974) found fertile hybrids among the New Guinea introductions. Crosses between the New Guinea species and the introductions from Java (*I. platypetala*) and Celebes (*I. platypetala* var. *aurantiaca*) were successful but produced sterile progeny (Arisumi, 1974). In order to create fertile plants Arisumi (1975) treated these hybrids and their parents with

colchicine. Artificial and "natural" amphidiploids were produced that were fertile if the parental genomes were in equal combinations (Arisumi, 1978). One of the fertile seedlings was released as the first seed propagated New Guinea variety, 'Sweet Sue' (Anon., 1977). These amphidiploids showed the lack of variability characteristic of polyploids (Sparnaaj, 1979). Since their high number of chromosomes would require extensive breeding work, Pasutti (1976) proposed to use chromosome random assortment to produce offspring from hybrids of *I. platypetala* and a commercial clone 'Tangerine' of *I. platypetala* var. *aurantiaca*. Eight viable seeds resulted from backcrosses to the parents. Success was limited to this hybrid and Pasutti and Weigle (1980a) decided to look for naturally occurring pollen fertility in the New Guinea and Java hybrids. Two hybrids were made using this technique, one of which was fertile and which combined the chromosomes of the New Guinea, Java and Celebes introductions, allowing the transfer of such a desirable characteristic as heat tolerance. Now, many cutting-propagated varieties and another seed-propagated variety, 'Tango' are available, resulting from both public and private breeding programs (Hamrick, 1988).

2.6 Flower Colour

One possibility to explore for breeding *I. walleriana* is the extension of the available colour range. Neither a true blue nor a yellow flower has been found in this species. Seed companies and breeders are developing cultivars, such as Blue Moon (Weigle and Pasutti, 1979), Garden Blues and Blue Lilac, with flowers closer and closer

to a true blue. Selection for the intensification of blue should yield a blue variety in a few years. Yellow, on the other hand must be transferred to the species. A little background on the nature of flower pigments would elucidate the problem.

2.6.1 The Biochemistry of Flower Colour

Colours in flowers are produced by either the water soluble flavonoids found in vacuoles or the fat soluble carotenoids found in plastids (Clevenger, 1964a). The former are red, blue or yellow pigments and the latter are red, orange and yellow. Flavonoids can be either anthocyanins, which are red (pelargonidin), blue (cyanidin) and purple (delphinidin) or flavones, flavonols, chalcones and aurones, which are yellow and ivory. Anthocyanins are made of the colour producing molecule, anthocyanidin, and one or more molecules of sugar (Crane and Lawrence, 1952). The three main anthocyanidins, listed above only differ from each other in the number of substituent hydroxyl groups on the phenyl ring. As the number of hydroxyl groups increase the blueness of the tone does as well. The number and position of the sugar molecules also affects flower colour. Those with sugars in two positions are always bluer than the corresponding pigment with the sugar in one position. Methylation, in the same positions that hydroxylation occurs, will cause a reddening with increasing substitution. One of the most important factors involved in flower colour is the quantity of the chemicals, where flowers with low pigmentation will be light rose and those due to intense pigmentation would be deep purple-black (Harborne, 1976). Other factors involved in flower colour include co-

pigmentation with flavones, metal complexing, the pH of the cell sap and the colloidal nature of the pigments.

This second group of substances is similar to anthocyanins but has greater structural variability, although similar substitution patterns can occur. Hydroxylation of the flavone and flavonols will cause an intensification of yellowness. The common flavonols and flavones contribute little to flower colour. If however there is methylation or unusual glycosidic patterns in the flavonols, colours such as the yellow of the meadow pea, *Lathyrus pratensis* L., become apparent (Goodwin and Mercer, 1983). The colourless flavonols and flavones are the major source of body or ground colour in white, cream and ivory-coloured flowers and they often act as co-pigments. Aurones and chalones, the anthochlor pigments, are yellow pigments found in only about nine plant families. Although they can be the sole source of yellow colour such as the aurone auresin in yellow snapdragons, *Antirrhinum majus* L., they are often associated with carotenoids.

Carotenoids are long chain yellow pigments found in the plastids. They are so structurally different from flavonoids that there is no question of parallel synthesis and there is no interaction between these groups (Crane and Lawrence, 1952). Carotenoids can be the sole source of yellow pigment, which is the case in most yellow-flowered angiosperm species. They provide a background for anthocyanins to give colours that range from orange to brown or as mentioned above, associate with the anthochlor pigments. Thus yellow colour in plants can be due to specialized flavones, aurones or carotenoids and their combinations (Harborne, 1976).

2.6.2 Floral Pigments of the Genus *Impatiens*

Clevenger (1971) did a survey of the anthocyanidins of 24 *Impatiens* species and varieties. As expected she found the most common pigment to be cyanidin and the next most abundant was malvidin. Cyanidin was observed in the yellow-flowering species, *I. pallida* Nutt. and *I. scabrida* D.C. and the orange-flowering *I. capensis* Thunb., but only if there were reddish brown spots on the flowers (Nozzolillo, 1983). Upon analysis, the orange-flowered *I. platypetala* var. *aurantiaca* proved to have a previously unknown anthocyanidin (Clevenger, 1964b). The pigment was named aurantinidin and its structural differences from the anthocyanidins of the purple *I. platypetela* caused Clevenger (1971) to propose that the variety *aurantiaca* should be elevated to the species level as *I. aurantiaca* Teysm. Extensive work has been done with the inheritance and biosynthesis of floral pigmentation of *I. balsamina*. Malvidin is the main anthocyanidin of red and pink flowers, while the lavender and purple flowers are characterised by pelargonidin (Clevenger, 1958). No anthocyanins were found in white flowers (Alston and Hagen, 1958).

Nozzolillo (1983) examined the non-chloroplast pigments of seedlings of several North American *Impatiens* and found a deep yellow pigment similar to violaxanthin, a xanthophyll, in large quantities. Violaxanthin was reported to be found in *I. noli-tangere* (Gibbs, 1974). Carotenoids were also observed in petals of *I. noli-tangere* by Kleinig and Metsche (1968). The aurone test revealed no such pigments in the flowers of *I. pallida* or *I. biflora* Walter (synonym for *I. capensis*) (Gibbs, 1974). Thus, it is likely that the source of yellow pigment in the genus is

carotenoids and an entire biochemical pathway must be transferred to produce a yellow *I. walleriana*.

2.6.3 The Genetics of Flower Colour

Flower colour inheritance in plants is apparently governed by genes that show normal Mendelian segregation but the diversity of flower colour makes the matter very complex (Paris *et al.*, 1960). Genetic phenomena such as co-dominance, complementation, inhibition and epistasis may also be involved (Grant, 1975). As mentioned above many different factors contribute to flower colour but the majority of these factors are known to be controlled by genes (Crane and Lawrence, 1952). The action of these genes is highly specific and may determine the presence or absence of an anthocyanin, another of these may effect the degree of hydroxylation and while a third gene could control the production of a co-pigment. Work with *Streptocarpus* has shown that two genes control the methoxylate positions on the phenyl ring of the anthocyanidin molecule and a third controls the attachment of a second sugar molecule (Lawrence *et al.*, 1939). Paris and co-workers (1960) surveyed the genetics of flower colour in 75 plant species to attempt to derive a common inheritance scheme. They found that they could explain the inheritance in most species by using six genes; W, Iv, Y, B, P, and Dil, which designate colour/white, non-ivory/ivory, non-yellow/yellow, purple or violet/blue, purple or violet/pink, red or rose and intense colour/dilute colour respectively. The six genes form an epistatic series, the first being the top epistatic member. Clevenger (1975) used flower colour inheritance as a model

system for a discussion of epistasis. Then she and Hagen (1975) used *I. walleriana* for the application of the method.

2.6.3.1 The Inheritance of Flower Colour in the Genus *Impatiens*

Most of the work on flower colour in this genus has been done with *I. balsamina* and *I. walleriana* and will be discussed below. However, it became obvious from observations of breeding programs of the New Guinea *Impatiens* that aurantinidin was dominant to all the other flower pigments of the group, including the malvidin of the magenta *I. platypetala* (Stephens *et al.*, 1988). Arisumi (1973) noticed that crosses between white and red or lavender New Guinea plants yielded offspring with flowers of varying shades of lavender. The white flower colour of *I. flaccida alba* is recessive to the lavender flower colour of *I. flaccida* (Arisumi, 1986). The offspring of the cross of *I. flaccida alba*, a white-flowered species and *I. repens*, a yellow-flowered species had light pale pink violet flowers with a yellow throat (Arisumi, 1980b). Although white appears to be recessive or co-dominant, yellow colour is unable to penetrate or it is not easy to transfer its biosynthetic pathway.

2.6.3.2 The Inheritance of Flower Colour in *I. walleriana*

Clevenger and Hagen (1975) found that *I. walleriana* was a difficult species for the study of flower colour since it is an outbreeder, with explosive seed pods, a wide variety of colours and probably has pollen sterility. There are approximately six genes for colour type and others that modify intensity and background colours. They divided their

plants into two colour groups and attributed genes to each group. The first group had mainly white, purple, prawn, lavender and red flowers with the genes W and I for white, P for purple and R for red. The genes in the second group, D and E for red, F for purple and S for salmon, were responsible for the more intense flower colours; fuschia, orange, red and salmon. Since both P and F are for purple and D and E are for red they are likely to be the same genes. The flower colours of the second group were more intense and if combined with any of the first group they would probably be dominant or epistatic. Often the number of progeny of the crosses was too small to give significant results and there were only subtle differences between floral colour that could have been effected environmentally or physiologically. However, this study was a valuable contribution to breeding for flower colour in *I. walleriana*.

Gray and Myers (1977) looked at the progeny of a cross between *I. walleriana* 'Futura' burgundy and 'Shade King' white and found indications for the existence of seven independently segregating loci for flower colour. Genes I, C_1 and C_2 are responsible for the presence or absence of pigmentation. 'I' seems to be a temperature sensitive dominant colour inhibitor gene transmitted at temperatures below 20°C. The two C genes are two dominant genes required for colour in the absence of I. The gene P, if at two loci, is responsible for purple flowers and O will give an orange colour with one P. The two additive dilution genes, D_1 and D_2, give such colours as red, pink, orange-pink and light pink. Thus a white-flowered *I. walleriana* parent, without the genes I, C_1 or C_2, would be the best parent for a cross

with a yellow-flowered parent to transmit the manifestation of the yellow pigment.

2.6.3.3 The Inheritance of Flower Colour in *I. balsamina*

This species has been the target of many studies on the inheritance and biosynthesis of anthocyanins. Davis and co-workers (1958) established that five gene loci are responsible for flower colour; W,w-white/cream, L-purple or lavender, H-red or pink, P^i-intense colour, P^g-lighter colours and p-lightest colours. The L and H genes lead to the production of pelargonidin and malvidin respectively, controlling specific substitutions on the ring structure, while the P alleles control the quantity of pigments produced by regulating a general reaction in anthocyanin synthesis (Alston and Hagen, 1958). The white/cream locus controls background colour, apparently not involved specifically with the other three genes and so is probably a gene for a flavone. Many studies have been done with the detached petals of this species to elucidate the biosynthesis of anthocyanins (Hagen, 1966; Bibb and Hagen, 1972).

2.6.4 Yellow Flowering Species in the Genus *Impatiens*

There are ten species in the genus that show yellow pigment in their flowers. *I. repens* has a creeping growth habit, well suited to hanging baskets. Large bright yellow campanulate flowers are borne among roundish green leaves for only a few months of the year (Russell, 1980). *I. auricoma* Baill. is an upright plant with creamy yellow campanulate flowers. These two species have the most potential for a breeding program, since they are available

and have chromosome numbers similar to *I. walleriana* (see Appendix 1). A third species, *I. onciodes* Ridley ex. Hk. f., with an attractive flat golden yellow flower is a tetraploid, but was suggested to me as a potential parent (for a breeding program) by Dr. A Stahevitch. It was only available for one season, at great cost, from the Thomson and Morgan seed company in 1988. Other species include *I. scabrida* DC, with a creamy yellow campanulate flower, *I. mirabilis* Hook.f., the only truly arboreal form in the genus, *I. dalzellii* Hook.f. & T, with a deep yellow campanulate flower and *I. acmanthera* L. (Zinov'eva-Stahevitch, 1981; Russell, 1980). These species are all either inaccessible or they are unsuitable parents, morphologically or genetically. The three temperate species, *I. capensis*, *I. noli-tangere* and *I. pallida* all have varying shades of yellow in their flowers but they are very weedy plants and would be unsuitable in an ornamental breeding program. Their morphological and genetic differences would make even back crossing virtually impossible.

2.6.5 Breeding for a Yellow Flowering *I. walleriana*

The transfer of yellow pigment to a horticultural variety of *I. walleriana* will be quite a challenge since there is none observed in the species or in any of its close relatives. Thus it is necessary to make wide crosses with one of the above described yellow flowered species. Arisumi (1980a) had no success with the cross between *I. walleriana* and *I. repens*. The sterile progeny of the cross between *I. walleriana* and *I. auricoma* were rescued by embryo culture and their flowers resembled the former parent (Arisumi,

1985). The hybrids had pale to medium yellow flowers that bleached and faded with age or hot weather (Arisumi, 1987). Doubling the chromosomes of the plants with colchicine did not restore fertility suggesting that the sterility has genetic and cytological origins. The genes for yellow pigment can be transferred but the creation of a yellow-flowered *I. walleriana* has only just begun.

Another approach to the transfer of yellow pigment is to use 'bridging' species between the yellow-flowered species and the target species. Possible candidates for this role are *I. sodenii* and *I. flaccida alba*, since they are compatible with both *I. repens* and *I. auricoma*, although the problem of sterile progeny still exists (Arisumi, 1987). There are several other species that cross with *I. walleriana* and produce fertile progeny, including *I. usambarensis* and *I. gordonii* (Merlin and Grant, 1986). Obviously one must search farther in the genus and in the world to find candidates for such a breeding program or one must look to alternative techniques. Somatic cell hybridization is a technique that is potentially applicable to this situation.

2.7 Somatic Cell Hybridization

Somatic cell hybridization involves the fusion of protoplasts, cells from which the cell walls have been removed, of vegetative cells by chemical or physical means. The cytoplasm and chromosomes of both parental cells then co-exist as a heterokaryon. Since natural polyploidy is known to exist in the genus *Impatiens* (Zinov'eva-Stahevitch and Grant, 1985) and the co-existence of visibly different chromosomes in the nuclei of interspecific hybrids have been

observed in karyological studies (Pasutti *et al.*, 1977), *Impatiens* should be excellent organisms for the application of this technique. Unfortunately there have been no reports of either protoplast isolation or fusion in the genus.

The development of protoplast fusion techniques has advanced rapidly in the last 30 years and the subject is well-documented in a number of reviews (Davey and Power, 1988; Griesbach, 1988; Cocking, 1983). The regeneration of hybrid plants requires the successful completion of a number of steps; 1) the isolation and culture of protoplasts, 2) the fusion of protoplasts and the selection of fusion products, and 3) the culture and regeneration of the fusion products (Bhojwani and Razdan, 1983).

2.7.1 Protoplast Isolation and Culture

Protoplasts can be isolated either mechanically or enzymatically (Gamborg, 1976). The former method is restricted to specific tissues and usually has a very low yield, while the latter method has been successfully applied to many plants. Hydrolytic enzymes such as cellulase, Driselase^{R}, pectinase, pectolyase and Rhozyme^{R} are used in either a one or two step incubation to remove the plant cell walls. Enzymes such as Onozuka R10^{R}, a cellulase, and Macerozyme^{R}, a pectinase, have been developed specifically for this purpose and usually provide the best results. The standard treatment is a mixture of 0.5-2.0% w/v of a cellulase and a pectinase (Potrykus and Shillito, 1988). Protoplasts are very fragile, so the enzymes must be included in an isolation mixture that contains an osmotic stabilizer, a calcium salt, phosphate and a buffer (Gamborg, 1976). The osmoticum can be either a salt such as NaCl, one

of several sugars such as mannitol, sorbitol, glucose or sucrose or any suitable mixture within the molar range of 0.3 M to 0.8 M. The explant tissue often responds best if the donor plants are given a low or no light treatment prior to protoplast isolation and if the tissue is preplasmolyzed in a mixture similar to the isolation mixture, but without the enzymes (Ericksson *et al.*, 1974). Every stage in the procedure will affect the yield of viable protoplasts, including the physical preparation of the donor tissue, the nature and concentration of the enzymes and the osmoticum, the duration of incubation and the incubation environment (Keller *et al.*, 1982).

Virtually any plant organ or tissue can be a source of protoplasts. Among the most popular and the most convenient sources are leaf mesophyll tissue and callus or suspension cultures (Vasil and Vasil, 1980). The yield and viability of protoplasts are closely related to the conditions under which the donor plant or callus has been grown. Donor tissue must be surface sterilised and the minimum exposure time and sterilant concentration must be established for each case (Potrykus and Shillito, 1988). One of the best sources of plant material is from axenic culture of both plants and callus, since these are grown under aseptic and controlled conditions, thus eliminating injury from surface sterilisation.

After the plant tissue has been incubated in the enzyme preparation for the appropriate period of time, any undigested tissue is removed by filtration and the protoplasts are collected and washed by centrifugation, the duration and speeds of which must be evaluated for each experimental tissue type and species (Gamborg, 1976). Often

the finer debris is hard to separate from the protoplasts and techniques such as sucrose or Ficoll flotation must be adapted for use (Larkin, 1976). The viability of the protoplasts is then tested with vital stains such as, Evans blue or flourescein diacetate (Kanai and Edwards, 1973). Protoplasts are then plated at a critical density of 2 x 10^4 to 2 x 10^5/ml, in thin layer liquid cultures, drop cultures or agar-solidified cultures in small petri dishes or multiwell plates (Vasil and Vasil, 1980).

Many different media have been used for protoplast culture and are usually quite similar to those used for cell cultures, although many minor adaptations have been made to improve protoplast survival (Ericksson, 1985). The media range from the fairly simple salt, vitamin and carbohydrate mixtures, similar to that developed by Nagata and Takebe (1971) to complex mixtures that also include organic acids and additional vitamins and sugars such as that created for the culture of very low density protoplasts (Kao and Michayluk, 1975). Very few plant protoplasts will grow without the inclusion of plant growth regulators. At first relatively high amounts of 2,4-D or NAA (1-3 mg/L) and lower amounts of BAP or zeatin (0.1-1.0 mg/L) are needed (Ericksson, 1985). Protoplasts are generally cultured with low light intensity and in a temperature range of 20 to 28°C.

The cultures are observed regularly to evaluate cell wall formation, karyokinesis, cytokinesis, colony formation and finally microcallus formation. Cell wall regeneration usually occurs within the first 24 to 48 hours of culture on a suitable medium and the protoplasts loose their characteristic spherical shape (Vasil and Vasil, 1980).

Mitosis and cytokinesis should follow, two to seven days after culture initiation. Multicellular clumps or colonies are formed within one to three weeks, and are often transferred to agar medium for further growth. Careful addition of media with a lower osmotic strength must be done during this culture period to dilute the high molar levels of salt or sugar required to maintain intact protoplasts. The timing and dilution steps of this procedure are critical and almost culture specific. Regeneration of plants can occur via shoot formation from callus or directly from embryoids (Binding, 1986).

2.7.2 Protoplast Fusion

Fusion can, but rarely does, occur spontaneously. In most cases fusion must be induced, by causing the protoplasts to agglutinate and establish large areas of membrane contact (Gamborg, 1976). The high calcium and pH method of Keller and Melchers (1973) and the polyethylene glycol method of Kao and Michayluk (1974) are both very effective chemical techniques for protoplast fusion. A third method that has recently been developed is electrofusion, a technique that induces fusion without the introduction of potentially cytotoxic chemicals (Gaynor, 1986).

After fusion the hybrid cells must be selected from the mixture of parental cells, homokaryons and heterokaryons (Bhojwani and Razdan, 1983). One approach to this critical step, is visual selection, which involves the use of a visual marker such as pigmentation or the presence of chlorophyll and mechanical removal of the heterokaryons with micro-pipettes, and growing them individually (Schieder,

1982). Special cultural procedures such as nurse cultures or media-conditioning are necessary to successfully maintain the hybrid cells (Cocking, 1986). If there are no visual markers available other selection systems must be used, such as complementation for natural or induced sensitivities, complementation of recessive genes or mutant lines with biochemical modifications (Keller *et al.*, 1982).

Once the heterokaryons are selected they must be carefully nurtured and encouraged to regenerate into hybrid plants-the limiting but vital step in the procedure (Evans and Sharp, 1982). To confirm the hybrid nature of the plants morphological and karyological observations must be done, and isozyme and DNA hybridization techniques are recommended as well (Bhojwani and Razdan, 1983).

2.8 Tissue Culture

Among the best tissue sources for protoplast isolation are callus and shoot cultures. The ability to culture callus and shoots of the species from which hybrids are desired would also be useful for the completion of the breeding project. Very little *in vitro* work has been done with plants from the genus *Impatiens* and only one brief abstract has been published about culture work of *I. walleriana* (Stephens, 1985). Thus it is necessary to evaluate the media and growth regulator requirements of this species.

Many different media are available for tissue culture work, but they are essentially subtle variations of the four major media constituents (Bhojwani and Razdan, 1983). A tissue culture medium contains; 1) inorganic nutrients, salts that are the source of macro- and micronutrients, 2)

organic nutrients, which include the essential thiamine and other vitamins, usually pyridoxine, nicotinic acid and glycine and the sugar alcohol, inositol, 3) a carbon source, such as sucrose and 4) plant growth regulators, auxins, cytokinins and occasionally gibberellic acid and abscissic acid. It is best to start with a well known basal medium, such as MS (Murashige and Skoog, 1962) or B5 (Gamborg *et al.*, 1968) and through a series of minor adjustments, adapt it to the plant material in question (Gamborg and Shyluk, 1981). The first target of investigation should be the growth regulators. Appropriate auxin and cytokinin levels must be established for every species and possibly each variety studied (Hughes, 1981). Carbohydrate levels are usually in the range of two to five percent sucrose, but the concentration range and the use of gl cose or fructose could be considered (Bhojwani and Razdan, 1983). The basal media are usually satisfactory, but some cultures grow better at lower salt concentrations, so half and quarter strength MS or B5 salts should also be evaluated.

2.8.1 Callus Culture

Auxins and usually a cytokinin are required in callus culture. Generally a high auxin to cytokinin ratio is needed to induce callus (Evans *et al.*, 1981). Gamborg and Shyluk recommend a range of 1-5 mg/L 2,4-D and 1 mg kinetin for callus production. 2,4-D is the most popular growth regulator for this purpose, but success has been observed with all the common auxins with or without any cytokinins (Dixon, 1985). Solid medium is recommended for the initiation and maintenance of callus, although actively growing callus can be transferred to liquid medium for cell

suspensions. The cultures can be grown in darkness but the fact that many morphogenetic processes require light must be considered (Hughes, 1985). Most viable plant cells will divide on the appropriate medium but the best explant source contains actively dividing cells as might be found in seedlings or meristematic tissue (Evans *et al.*, 1981). Callus cultures have been derived from a wide variety of juvenile and mature plant organs.

2.8.2 Micropropagation

Micropropagation of plants *in vitro* can occur by one of several pathways, via axillary shoot proliferation, adventitious bud formation, organogenesis from callus or somatic embryogenesis (Zimmerman, 1983). Both organogenesis and embryogenesis would be useful for the regeneration of somatic hybrids. This would involve the transfer of the microcalli to media with less plant growth regulators or different auxin to cytokinin ratios. In some cases this may "shock" the callus into regeneration (Potrykus and Shillito, 1988). Generally though, morphogenesis requires low levels of auxin and higher levels of cytokinin. The key factors to somatic embryogenesis seem to be the need for 2,4-D, a reduced source of nitrogen in the initial medium and the transfer of protoplast-derived calli to media with low concentrations of or no growth regulators (Binding, 1985). Axillary shoot proliferation, although the slowest method of *in vitro* propagation, would be the most useful for the production of donor plant tissue and the proliferation of the hybrid, once it is produced, because the genetic integrity of plants propagated in this manner is usually maintained (Hussey, 1984). A relatively high level of

cytokinin and often a low level of auxin induces the production of axillary buds (George and Sherrington, 1984). After proliferation of shoots, rooting and acclimation must be done. Microcuttings can either be rooted directly in propagation facilities in the greenhouse or transferred to media with auxins to induce root formation (de Fossard, 1986). Acclimation of the tissue cultured plants is often a difficult stage that requires strict hygiene to avoid "damping off" diseases and high humidity to prevent the desiccation of the plantlets, which often have thin cuticles and malfunctioning stomata (Constantine, 1986).

2.8.3 Surface Sterilisation of Donor Tissue

One of the most important steps in tissue culture is the establishment of an axenic culture. Since the source plants generally live in an environment full of organisms that would thrive on the nutritious media used for tissue culture, the donor tissue must be carefully cleaned or surface sterilised. A ten to twenty minute exposure to sodium hypochlorite (0.5 to 2.5%), the active component of commercial bleach preparations, is usually adequate, but if not, an effective system that causes minimum damage to the tissue must be developed (Torres, 1989). Debergh and Maene (1981) described a system that included washing of the donor tissue in running water and treatment with seventy percent ethanol and mercuric chloride prior to exposure to the diluted bleach. However, they stress that growing clean and healthy stock plants (Debergh and Maene, 1985) and using effective surface sterilisation techniques will prevent the interference of contamination and avoid such problems as latent infection appearing after several subcultures.

2.8.4 Tissue Culture of Impatiens

The first record of tissue culture in this genus is due to Arisumi's (1977) efforts to save ovules and embryos of interspecific crosses. A modified Nitsch and Nitsch (1969) medium with 1 mg/L IAA and 1 mg/L kinetin or zeatin supported the growth of both ovules and embryos. Very few of the ovules or embryos responded to culture and examination of the dead ovules indicated that no endosperm or embryo development took place in culture (Arisumi, 1980b). Only twenty-three seedlings were established in soil from approximately 2000 repetitions of eleven crosses. In the second experiment about half the crosses produced embryos that could survive *in vitro*, of which 8.5% germinated and then only 23.4% of the seedlings were rescued (Arisumi. 1985). Most of the post germination losses were due to the development of hard callus-like masses of which 3% developed shoots that grew into whole plants. Obviously the success of embryo and ovule culture reflects the genetic composition of the crosses, as well as the suitability of the medium but the low numbers of plants even from self-fertilized progeny suggests the necessity for medium improvement. However the rescued hybrids were valuable contributions to *Impatiens* breeding programs (Arisumi, 1987).

Koenigsberg (1978) attempted to develop a system for the *in vitro* propagation of several New Guinea *Impatiens*. He found that the varieties responded with the greatest fresh weight increase, to shoot-tip culture on a combination of 6 mg/l kinetin and 0.6 mg/L NAA, and grew best at 380 foot candles (79.9 $\mu mol/m^2/sec$) of light. He also investigated an apparent toxicity of the full strength LS

(Linsmaier and Skoog) medium and concluded that 3% sucrose had too high an osmotic potential for New Guinea *Impatiens*. Propagation was unsuccessful in liquid medium in a rotating drum, but shoot clumps from a previous experiment were broken up into the maximum number of propagules, producing an average of 13 per shoot after nine weeks. Since *Impatiens* propagate so easily by cuttings, the system developed by Koenigsberg could only be justified for valuable plant material.

Stephens (1985) compared the effect of 0 to 140 uM kinetin with 0 and 0.5 uM NAA and IBA (see Appendix 2) on organogenesis of cotyledon and hypocotyl explants of *I. walleriana* and *I. balsamina*. The later produced more roots and callus than the former and no shoots were regenerated from either species. Stephens and co-workers (1985) did the same study with shoot tips of Java, New Guinea and Java x New Guinea hybrid *Impatiens* and found a maximum multiplication rate of 8.5 shoots per culture per culture on 140 um kinetin with no NAA, which inhibited shoot proliferation. The hybrids showed a much greater multiplication rate in culture than either of their parents. Han and Stephens (1987) continued the work with *in vitro* propagation of the New Guinea *Impatiens*, by comparing several cytokinins (kinetin, BAP and 2iP) using several concentrations of NAA. They found that a range of 10 to 40 um enhanced shoot multiplication but one hybrid responded better to 2iP and the other to BAP. The latter also had higher multiplication with 2 um NAA but the former was inhibited at all levels of NAA tested. These results stress the variation of tissue culture responses of different genotypes and to diffe· nt plant growth regulators. It is

interesting to note that the highest proliferation rate, 5.3 shoots per month, was considerably lower than that of the previous experiment.

Warren-Wilson and co-workers (1986) developed an *in vitro* system to study the abscission sites in internodal explants of *I. walleriana*. The internodes were cultured on 1% agar with 3% sucrose and 1.0 mg/L IAA and survived for several weeks in culture with the production of some roots and little callus. They continued their studies with nodal explants and the use of IAA-conjugates (Warren-Wilson *et al.*, 1987a: Warren-Wilson *et al.*, 1987b). These conjugates are similar to endogenous auxin and are more stable in culture since they are immune to photo-oxidative degradation and catabolism. Their effects were similar but slightly less than IAA at the same concentrations, so these conjugates are possible alternatives to the unstable IAA in culture.

Several studies have been done with *in vitro* germination and growth of pollen of *Impatiens*. Bilderback (1981) proposed to use pollen germination and tube growth of *I. walleriana* as a bioassay for toxic substances and defined the criteria for growth in a simple liquid medium. Kordan (1981) evaluated the germination of *I. walleriana* pollen on non-nutrient agars and suggested that each of the agars supplied chemical factors capable of inducing *in vitro* germination. Hicks and co-workers (1987) found that they could use the germination of pollen *in vitro* as a suitable indicator of the pollen fertility of New Guinea by Java hybrids to evaluate their potential as parents. Pollen germination and tube growth is a brief phenomenon providing little indication of the applicability of media for tissue culture purposes.

3. MATERIALS AND METHODS

3.1 Plant Material

A green and white variegated form of *Impatiens walleriana*, which is often referred to as *I. walleriana* var. *variegata* (Everett, 1981), was used for the major portion of this study and was acquired from an unknown source (see Fig. 1). Plants of *I. walleriana* cv. Blitz Orange (Stokes Seeds Inc., St. Catherines, Ont.) and cv. Harlequin (Cruickshank, Ltd., Toronto, Ont.) were used for crossing studies. Blitz Orange is a large-flowered vigorous variety that often sets seed in the garden (pers. obs.). Harlequin is a compact variety with white and orange striped flowers, thus representing two diverse *I. walleriana* genotypes. Preliminary studies were also done with *I. repens*, a yellow-flowered species, which was collected by Dr. A. Stahevitch from Sri Lanka. All seed of *I. walleriana*, was the hybrid "F_2 economy" mixture #992 from Stokes Seeds Inc.

3.1.1 Cultural Conditions

I. repens was grown in the greenhouse with natural daylength, summer shading and temperature within the range of 21°C to 25°C. *I. walleriana* was originally grown in the greenhouse as well, until it became obvious that these conditions were too conducive to contamination *in vitro*. Plants were then transferred to a growth chamber with a constant 23°C temperature, 16 hour daylength and a light intensity of 284 $\mu mol/m^2/sec$ and which was later lowered to 200 $\mu mol/m^2/sec$. Rooted cuttings were planted in Premier

Figure 1. *Impatiens walleriana* var. *variegata*

Brands Promix^{R} supplemented with 30 g/0.0086m^{3} dolomitic lime and 100 g/0.0086m^{3} MegAmp^{R}, a three-to-four month slow-release fertilizer with 7-40-6 (% N-P_2O_5-K_2O) composition. The plants were started in 10 cm pots and repotted when necessary. Water was supplied as required, without wetting the foliage, and 20-20-20 (% N-P_2O_5-K_2O), dissolved at 3 g/L was provided every two weeks. Pruning was done on a regular basis to prevent flowering and promote vigorous vegetative growth. Plants were checked regularly for insect pests, especially thrips, mites and mealy bugs, which could be controlled by Trumpet^{R}, Pentac^{R} or Kelthane^{R} or by swabbing the plants with 70% ethanol, respectively, and careful pruning. Cuttings, rooted in water, provided replacement plants when necessary.

3.2 Crossing Procedure

Impatiens is generally an outcrossing genus but occasionally, when the fused anthers dehisce, pollen might be left on the stigma. It was therefore necessary to carefully remove the anthers and cover the potential female parent flowers with a light gauze crossing bag. After a few hours the stigma was receptive to pollen, manifested by an open star-like appearance. Pollen was then transferred with a small paint brush to the uncontaminated stigmas and the flower was again protected with a crossing bag to prevent further pollination. After a few days the bag was removed and if the cross was successful the maturing seed pod was enclosed in clear plastic wrap to catch the seeds when the pod exploded. This method was perfected by C. Merlin (Merlin and Grant, 1986).

3.2.1 Crosses

Twenty reciprocal crosses were done with *I. repens* and each of *I. walleriana* cv. Blitz Orange, cv. Harlequin and var. *variegata*. Crosses were also made between the variegated variety and Blitz Orange and Harlequin.

3.3 Culture Media and Procedure

The three basic types of tissue culture media used were those according to Murashige and Skoog (1962), Linsmiaer and Skoog (1965) and Gamborg *et al.* (1965) and will be referred to respectively as MS, LS, and B5 (see Appendix 3). The macro- and micronutrients, vitamins and sucrose for these media, unless otherwise specified, were prepared at ten times concentration and frozen until required, as described in Gamborg and Shyluk (1981). At the time of use the concentrates were thawed, appropriate amounts of plant growth regulator stock solutions were added, as indicated in each experiment, and the pH adjusted with NaOH or HCl to 5.7, 5.7 and 5.5 in MS, LS, and B5 respectively. Eight g/L agar (TC agar^{R}, Hazelton) was added to the media, which was then autoclaved at 1 kg/cm^2 and 121°C for 15 minutes and dispensed as specified in each experiment. All cultural procedures and media dispensation were carried out in a laminar flow hood using aseptic technique.

3.3.1 Cultural Conditions

Tissue cultures were grown in a culture room with 25+/-1°C temperature and a 16 hour photoperiod. Cool white fluorescent tubes provided light at the intensity of 24 to 30 µmol/m^2/sec.

3.3.2 Preliminary Experiments-Tissue Culture

3.3.2.1 Experiment 1. Evaluation of the Effect of Plant Growth Regulators on *in Vitro* Growth of *I. repens* and *I. walleriana* var. *variegata*

Twenty-five combinations of four auxins (NAA, IAA, IBA and 2,4-D) and four cytokinins (kinetin, zeatin, 2iP and BAP) at 0 and 1 mg/L were incorporated into the basal B5 medium. Twenty ml of media were dispensed into 100 by 15 mm plastic disposable petri dishes.

Plant material was washed briefly in running water and 10-15 cm cuttings were submersed for 15 minutes in agitated 10% v/v commercial bleach (0.6% NaOCl) containing a drop of Tween 20^{R}, then rinsed in sterile distilled water three times. Each cutting was placed on a sterile glass petri dish and 3 mm internodal slices and 10 mm leaf squares, without the central vein, were excised. Ten explants plated, proximal end or abaxial surface down, and with five plates of each tissue type per treatment.

The experimental design was a randomized complete block design and one petri dish was a replicate. The material was found to be seriously contaminated after one week of culture, so all clean material was transferred to fresh media as soon as possible. The cultures were evaluated for growth at two and four weeks. Thus the necessity for an improved surface sterilisation technique became obvious.

3.3.3 Surface Sterilisation Experiments

All of the following experiments were done with the variegated *I. walleriana*, except experiment 4. For each experiment a randomized complete block design was used and

one petri dish was considered a replicate.

3.3.3.1 Experiment 1. Determination of Appropriate Surface Sterilisation Techniques for Stem and Leaf Tissue

Leaves were dipped in 70% ethanol and exposed to agitated 10% bleach with a few drops of Tween 20, for different periods of time. The five treatments were:

1) Ethanol dip-0 min bleach
2) Ethanol dip-5 min bleach
3) Ethanol dip-10 min bleach
4) Ethanol dip-15 min bleach
5) Ethanol dip-5 min bleach with vacuum infiltration (30 cm of mercury)

After the appropriate exposure to bleach the leaves were rinsed in sterile distilled water three times. Ten 10 x 10 mm sections were excised and plated on B5 medium with 8 mg/L 2,4-D, in 100 x 15 mm petri dishes, four petri dishes per treatment. The central vein was not included in the explant which was placed abaxial surface down on the medium. After two weeks the leaf sections were visually evaluated for contamination and response to the surface sterilisation technique.

Ten to fifteen cm stem pieces were exposed to three different surface sterilisation techniques:

1) 70% ethanol dip and 15 min in 10% bleach, with a few drops of Tween 20
2) One hour wash in running water followed by the above treatment
3) As number one with vacuum infiltration (30 cm of mercury)

Three mm long internode sections were excised, plated, 10 per plate, four plates per treatment, with the cut proximal end inserted into the medium described above and evaluated in the same manner as the leaf tissue.

3.3.3.2 Experiment 2. Comparison of the Effect of Different Surface Sterilants on Stem and Leaf Tissue

Ten to fifteen cm stem sections, with or without leaves, were dipped in 70% ethanol and exposed to four different surface sterilisation techniques, for 15 minutes each.

1) leaves and stems separately in 7% CaOCl
2) leaves and stems together in 7% CaOCl
3) leaves and stems separately in 10% bleach
4) leaves and stems together in 10% bleach

The tissue was rinsed three times in sterile distilled water. The leaves and stems were plated separately, 10 per plate, on B5 with 2 mg/L NAA, four replicates per treatment. 10 x 10 mm leaf sections, excluding the central vein and three mm long internodal sections, were placed abaxial surface and proximal end down, respectively, on the medium. The plates were evaluated visually for contamination two weeks after preparation.

3.3.3.3 Experiment 3. Comparison of Surface Sterilisation Techniques on Nodes

Ten to fifteen cm stem tip cuttings with leaves removed, were washed in running tap water for one hour, dipped into 70% ethanol and exposed to seven different surface sterilisation treatments:

1) Fifteen minutes in 10% bleach, rinsed in sterile distilled water, petioles excised, five minutes in fresh 10% bleach.
2) Ten minutes in 10% bleach
3) Ten minutes in 10% bleach, with vacuum infiltration (30 cm of mercury)
4) Fifteen minutes in 5% bleach
5) Fifteen minutes in 5% bleach, with petioles removed
6) Five minutes in 10% bleach, with vacuum infiltration (30 cm of mercury)
7) Fifteen minutes in 10% bleach, with vacuum infiltration (30 cm of mercury)

Treatment one was modified from Debergh and Maene (1981). In all of the treatments, except #6, approximately two cm of the petioles were left on the stem to protect the axillary bud. A few drops of Tween 20 was added to the bleach in each case and the bleach was agitated. After exposure to the surface sterilant all the plant material was rinsed three times with sterile distilled water. The nodal explants were three mm long and included an axillary bud. Ten nodes were put on each 100 x 15 mm petri dish of B5 medium (20 ml/plate, solidified), with six plates per treatment. The plates were evaluated visually at two weeks for contamination and healthiness of tissue.

3.3.3.4 Experiment 4. Surface Sterilisation and Germination of Seeds

Seeds of *I. walleriana* "F_2 economy" mixture were surface sterilised with three treatments.

1) 33% bleach for 5 min
2) 10% bleach for 15 min

3) washed in running water for one hour followed by the above treatment

Each of the bleach solutions contained a few drops of Tween 20 and were agitated. Seeds were rinsed three times in sterile distilled water and placed five per plate on water agar (0.8%) with two g/L casein hydrolysate and B5 medium. Five plates were prepared for each of the six treatments. The plates were examined for contamination and germination after two weeks.

3.3.3.5 Experiment 5. Surface Sterilisation of Leaves for Protoplast Culture

Leaves were exposed to five different surface sterilisation treatments, listed below:

1) 2 min in 70% ethanol
2) 2 min in 70% ethanol and 2 min in 10% bleach
3) 2 min in 10% bleach
4) 4 min in 10% bleach
5) 6 min in 10% bleach

A few drops of Tween 20 were added to the bleach. After treatment the leaves were rinsed three times in sterile distilled water. Five leaves were placed on solidified MS medium in 100 x 15 mm petri dishes with four plates per treatment. The plates were evaluated for contamination after one week.

3.3.3.6 Statistical Analysis of Surface Sterilisation Experiments

The data were analysed using the general linear model procedure of Statistical Analysis Systems (SAS Institute) and separation among means was done using Duncan's Multiple

Range Test (Steele and Torrie, 1980)

3.3.4 Callogenesis Experiments

For each experiment a randomized complete block design was used and a petri dish or test tube was considered a replicate within a block that consisted of one of each treatment.

3.3.4.1 Experiment 1. Evaluation of the Effect of Media and 2,4-D on Leaf and Stem Tissue

Leaves and stems of the variegated *Impatiens* were washed in water with a few drops of Tween 20 and then surface sterilised separately in 10% bleach for 15 minutes and rinsed three times in sterile distilled water. Leaf and stem sections (as described in section 3.3.3.1) were plated in 100 x 15 mm petri dishes of B5 and MS with three levels of 2,4-D, 0, 2 and 4 mg/L, 20 ml per plate. There were ten sections per plate and four plates per treatment. The tissue was evaluated for root and callus growth after four weeks. The experiment had a 2 x 3 factorial arrangement with four replicates. There was a considerable amount of contamination, so the data was presented as a percent of uncontaminated sampling units. Although the sample size was rather small for data expression as percentage, it allowed some information to be derived from the experiment.

3.3.4.2 Experiment 2. Evaluation of the Effect of 2,4-D and Kinetin on Stem Tissue

Stem cuttings of the variegated *Impatiens* were surface sterilised by washing them in running water for an hour, dipping them into 70% ethanol and stirring them in 10%

bleach, with a few drops of Tween 20, for 15 minutes. The cuttings were washed in sterile distilled water three times and three mm sections were excised. These sections were plated on all 25 combinations of 2,4-D (0, 1, 2, 4, 8 mg/L) and kinetin (0, 1, 2, 4, 8 mg/L) in B5 medium. Ten 3 mm stem sections were placed on each 100 x 15 mm plate with 20 ml of medium and five plates were done for each of the twenty-five treatments. The tissue was evaluated for callus and root growth at two and four weeks. There was a significant amount of contamination and the cultures had to be transferred at two weeks to save sufficient tissue for the evaluation of growth at four weeks. The original experiment had a 5 x 5 factorial arrangement with five replicates. The data were presented as a percentage of uncontaminated sampling units at two weeks and a percentage of transferred sampling units at four weeks. These data, although no longer a complete representation of the experiment gave an indication of the reaction of the stem tissue to 2,4-D and kinetin.

3.3.4.3 Experiment 3. Evaluation of the Effect of IAA and BAP on Seedling Stem Tissue

Seeds of *I. walleriana* "F_2 economy" mixture were surface sterilised by dipping them into 70% ethanol and then transferring them into 10% bleach, containing a few drops of Tween 20, for 15 minutes. The seeds were plated on 15 ml of solid B5 medium in 60 x 20 mm disposable plastic petri dishes.

After two weeks the one cm tall seedlings were transferred to 240 ml jars containing 30 ml of solid B5 medium. At six weeks of age three mm long stem sections

were plated, proximal side down, ten per plate, ten plates per treatment on 20 ml of solid B5 medium in 100 x 15 mm plastic disposable petri dishes with the following plant growth regulator combinations:

1) 0.5 mg/L IAA and 2.0 mg/L BAP
2) 1.0 mg/L IAA and 1.0 mg/L BAP
3) 2.0 mg/L IAA and 0.5 mg/L BAP

One-half of the replicates were put in the dark and the other half under lights. The experiment was evaluated for callus and root growth after six weeks of culture. The experiment had a 2 x 3 factorial arrangement with five replicates.

3.3.4.4 Experiment 4. Evaluation of the Effect of Media and Carbon Source on Callogenesis of Axillary Buds

Stem cuttings of the variegated *Impatiens* were surface sterilised by washing them in running water for an hour, removing all the leaves and petioles, submersing them in agitated 10% bleach, containing a few drops of Tween 20, for 15 minutes and rinsing them three times in sterile distilled water. Axillary buds were excised and placed on the experimental media in test tubes, ten replicates per treatment. Ten ml of the following media and carbon source combinations were prepared in test tubes in the solid phase, with 0.5 mg/L NAA:

1) MS with 5 g/L glucose
2) MS with 10 g/L glucose
3) MS with 15 g/L glucose
4) MS with 20 g/L glucose
5) MS with 10 g/L sucrose
6) MS with 20 g/L sucrose

7) MS with 30 g/L sucrose
8) MS with 50 g/L sucrose
9) B5 with 5 g/L glucose
10) B5 with 10 g/L glucose
11) B5 with 15 g/L glucose
12) B5 with 20 g/L glucose
13) B5 with 10 g/L sucrose
14) B5 with 20 g/L sucrose
15) B5 with 30 g/L sucrose
16) B5 with 50 g/L sucrose

The axillary buds were evaluated for survival and transferred after six weeks, then evaluated for callogenesis after 12 weeks. The experiment had a 2 x 8 factorial arrangement with ten replicates. The data were expressed as percent of the replicates that were uncontaminated and were not analysed statistically due to the lack of growth response.

3.3.4.5 Statistical Analysis of Callogenesis Experiments

The data were first analysed with the general linear model procedure of Statistical Analysis Systems (SAS Institute) and then the factorial experiments were analysed by using single degree of freedom contrasts on the main effects when there was no interaction between the factors. Means of unrelated treatments were analysed with Duncan's Multiple Range Test (Steele and Torrie, 1980).

3.3.5 Micropropagation

The variegated *Impatiens* was used for all the experiments in this section. The plant material for the following experiments was surface sterilised according to

treatment 1 of section 3.3.3.3 but for the second experiment the plant material was surface sterilised as described in section 3.3.4.4. To minimize contamination problems axillary buds and shoot tips (2-5 mm long) were plated on a screening medium for 7 to 10 days prior to the initiation of the experiment (see Fig. 2). The screening medium consisted of the experimental medium with no plant growth regulators and was solidified in 60 x 20 mm plastic disposable petri dishes. Thus healthy, apparently uncontaminated plant material was available for experimental use. The shoot tips were pruned off the plants to provide large healthy axillary buds and shoot tips (see Fig. 3). This was done for experiments 4 to 7. Except for experiment 1, ten ml of experimental medium was dispensed into 150 x 25 mm glass tubes that were covered with translucent plastic Kimkaps^{R}. The medium was allowed to solidify at an approximately 45 degree angle, so that slants were formed. For each experiment up to ten replicates were prepared, depending on the availability of plant material. Each experiment was a randomized complete block design. One jar or tube represented a replicate and each block had one of each treatment.

3.3.5.1 Experiment 1. Evaluation of the Effect of BAP on Shoot Proliferation

Cuttings were surface sterilised by washing them in running water for one hour, dipping them in 70% ethanol and submersing them in agitated 10% bleach for 15 minutes. Shoot tips two to five mm long were excised and placed on 60 x 20 mm plastic disposable petri dishes of solid B5 medium with 3 mg/L BAP. After one month on this medium there was

Figure 2. Shoot tips of *Impatiens walleriana* var. *variegata* on screening medium.

Figure 3. *Impatiens walleriana* var. *variegata* showing axillary growth after pruning.

not much obvious growth so the shoot tips were transferred to 15 ml solid B5 medium in 120 ml jars with four different levels of BAP (0, 1, 2, 3 and 4 mg/L). There was one shoot per jar and five jars per treatment. The cultures were evaluated for shoot number and transferred, at four, eight and twelve weeks. The experiment had a single factor concentration gradient and was analysed with the regression procedure of Statistical Analysis Systems (SAS Institute).

3.3.5.2. Experiment 2. Evaluation of the Effect of Media Concentrations and Growth Regulator Ratios on Shoot Proliferation

Axillary buds were excised from surface sterilised cuttings and introduced to ten different media growth regulator combinations. The two media were MS and half strength MS. The growth regulator combinations were:

1) 0.1 mg/L NAA and 0.3 mg/L kinetin
2) 0.1 mg/L NAA and 3.0 mg/L kinetin
3) 0.5 mg/L NAA and 5.0 mg/L kinetin
4) 0.1 mg/L IBA and 1.0 mg/L BAP
5) 0.1 mg/L IBA and 3.0 mg/L BAP

Each medium was combined with all of the growth regulator combinations to yield the ten treatments. There was one axillary bud per test tube and eight test tubes per treatment. The buds were subcultured after four weeks and evaluated for shoot number, height and fresh weight after eight weeks. Separation among the means of the data was done with a common Least Significant Difference Test after the data were analysed with general linear models procedure of Statistical Analysis Systems (SAS Institute).

3.3.5.3 Experiment 3. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation

The three experimental media were B5, MS and half-strength LS. They were used with each of the growth regulator combinations given below:

1) 0.0 mg/L NAA and 0 mg/L kinetin
2) 0.0 mg/L NAA and 3 mg/L kinetin
3) 0.0 mg/L NAA and 30 mg/L kinetin
4) 0.5 mg/L NAA and 0 mg/L kinetin
5) 0.5 mg/L NAA and 3 mg/L kinetin
6) 0.5 mg/L NAA and 30 mg/L kinetin

One axillary bud was placed in each test tube with four test tubes for each of the eighteen treatments. The buds were subcultured after four weeks and evaluated for shoot number and height after eight weeks. The experiment had a 3 x 2 x 3 factorial arrangement with four replicates.

3.3.5.4 Experiment 4. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation

The three experimental media were B5, MS and half-strength LS. They were used with each of the growth regulator combinations given below:

1) 0.0 mg/L NAA and 0 mg/L kinetin
2) 0.0 mg/L NAA and 5 mg/L kinetin
3) 0.0 mg/L NAA and 20 mg/L kinetin
4) 0.2 mg/L NAA and 0 mg/L kinetin
5) 0.2 mg/L NAA and 5 mg/L kinetin
6) 0.2 mg/L NAA and 20 mg/L kinetin

One axillary bud was placed in each test tube with seven tubes for each of the eighteen treatments. The buds were subcultured after four weeks and evaluated for shoot number and height after eight weeks. The experiment had a 3 x 2 x 3 factorial arrangement with seven replicates.

3.3.5.5 Experiment 5. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation

Axillary buds were placed in test tubes with solid, slanted MS medium and sixteen combinations of IBA (0, 0.01, 0.1 and 1 mg/L) and BAP (0, 0.1, 1 and 10 mg/L), in all combinations. There was one axillary bud per test tube and eight test tubes per treatment. The cultures were subcultured and evaluated for shoot number, height and fresh weight after four weeks and evaluated again after eight weeks. The experiment had a 4 x 4 factorial arrangement with eight replicates.

3.3.5.6 Experiment 6. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation

Axillary buds were placed on solid, slanted MS medium in test tubes with sixteen combinations of IBA (0, 0.03, 0.3 and 3 mg/L) and BAP (0, 0.5, 2.5 and 5.0 mg/L), in all combinations. There was one bud per test tube and seven replicates for each treatment. The cultures were evaluated for shoot number, height and fresh weight after six weeks. The experiment had a 4 x 4 factorial arrangement with seven replicates.

3.3.5.7 Experiment 7. Evaluation of the Effect of IAA and BAP Concentrations on Shoot Proliferation

Axillary buds were placed on solid, slanted MS medium in test tubes with fifteen combinations of IAA (0, 0.017,and 0.17 mg/L) and BAP (0, 2.5, 3.5, 6.25 and 7.5 mg/L), in all combinations. There was one bud per test tube and ten tubes for each treatment. Han and co-workers (1987) found that 8.13 mg/L 2iP (or 40 μM, see Appendix 2) was the most effective cytokinin for proliferation of the New Guinea hybrid 'Starfire', so it was included, with 0.017 mg/L IAA as the sixteenth treatment. The cultures were evaluated for shoot number, height and fresh weight after six weeks. The experiment had 3 x 5 factorial arrangement with ten replicates.

3.3.5.8 Statistical Analysis of the Micropropagation Experiments

Data were first analysed using the general linear model procedure of Statistical Analysis Systems (SAS Institute) and if there were no interactions between the factors, analysis was done using single degree of freedom orthogonal polynomials and contrasts on the main effects, unless otherwise specified. When there was a significant interaction between the factors the data were presented as graphs and separation of the means was done by a common Least Significant Difference Test.

3.3.6 Acclimation and Rooting of Micropropagated Shoots

Often the propagules did not require rooting, so only 20 microcuttings of 5-10 mm from the treatments with the highest cytokinin levels (5.0 mg/L) of experiment 6 were

transferred to 30 ml of agar-solidified MS medium, with no plant growth regulators, in 240 ml jars. Four microcuttings, one from each treatment, were put in each of five jars. The jars were placed in the culture room and the plants observed regularly for rooting.

Rooted and unrooted propagules were acclimatized in the growth chamber, covered with plastic, or in an intermittent mist propagation bench in the greenhouse. Twelve cuttings per treatment from experiment 6, six rooted and six unrooted, of short, medium and tall stature (two per size rooted and unrooted) were planted in pasteurized Promix^{R} amended as described in section 3.1.1 in 50 by 55 mm cell-paks and placed in the growth chamber and the mist frame. The plants were observed regularly to evaluate establishment.

3.4 Protoplast Isolation - Basic Procedure

The basic protoplast protocol was derived from Constabel (1982) and Dodds and Roberts (1982). Leaves were surface sterilised in 10% bleach for 15 minutes and rinsed three times with sterile distilled water. The lower epidermis was peeled from the leaves, while submersed in CPW, by using fine forceps (Freason *et al.*, 1973) (see Fig. 4). This solution contains 0.0544 g/L KH_2PO_4, 0.202 g/L KNO_3, 2.969 g/L $CaCl_2.2H_2O$, 0.480 g/L, $MgSO_4.7H_2O$, 0.320 mg/L KI and 0.050 mg/L $CuSO_4.7H_2O$ when used full strength for peeling and pre-plasmolysis and half those concentrations, with 0.1 g/L $NaH_2PO_4.H_2O$ and 0.275 g/L MES (2-(N-morpholino)-ethanesulfonic acid) when included with the enzyme mixture. In all the solutions the osmotic level was adjusted with mannitol to a molarity of 0.5 and the pH of the solutions, media, CPW and enzymes was 5.7. The leaves were placed with

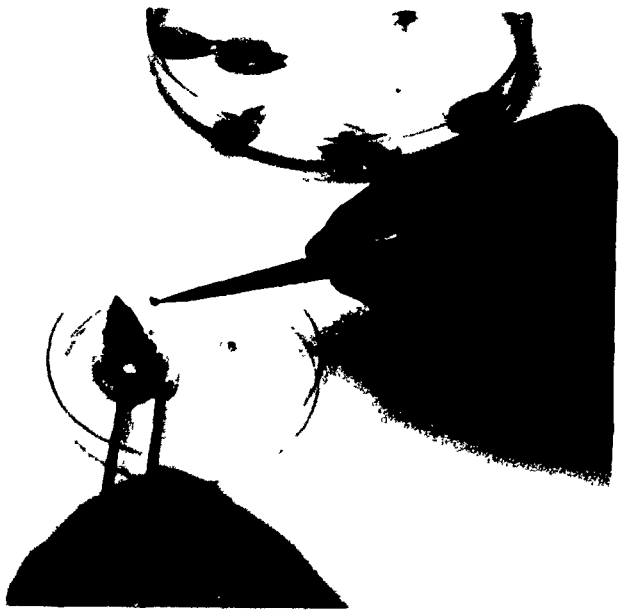

Figure 4. Peeling the lower epidermis off leaves of *I. walleriana*.

the peeled surface down in contact with the enzyme, six leaves in 10 ml of enzyme in 100 x 15 mm plastic disposable petri dishes. The enzyme mixtures consisted of various combinations and concentrations of cellulase Onazuka R10^{R} (Yakult Honsha Ltd, Japan), pectinase Macerase^{R} (Calbiochem, California), Driselase^{R} (Plenum Scientific Research Inc., N. J.), Rhozyme^{R} (Rohm and Haas Co., Ontario) and pectolyase (Sigma, Missouri). The tissue was incubated in the enzyme solutions for 14 to 16 hours at ambient temperature (23°C).

After incubation the mixtures of enzymes and tissue were filtered through 88 um nylon mesh in a small funnel sitting in a 50 ml erlenmyer flask (see Fig. 5). The solutions of enzymes and protoplasts were transferred to 15 ml graduated, conical polypropylene centrifuge tubes and spun at 100 x g in an IEC bench top centrifuge, using a swing-bucket rotor for five minutes to pellet the protoplasts. The supernatants were removed, fresh CPW added and each pellet was resuspended. The mixtures were spun again, for three minutes and washed for the third time with Kao's medium with 0.1 mg/L BAP. 1.0 mg/L NAA, 1.0 mg/L 2,4-D and 0.5 M mannitol (see Appendix 4). The solutions containing protoplasts had to be handled very gently, using only wide bore glass Pasteur pipettes.

The protoplasts were cultured in drops in 30 x 10 mm plastic disposable petri dishes and the plates were kept in plastic boxes with moistened Kimwipe^{R} in dim light (6 µmol/m^{2}/sec) and 25 +/- 1°C. The cultures were observed regularly with an inverted microscope and media added if necessary. Since the goal of this project was to develop a successful technique for the isolation and culture of *I. walleriana* protoplasts, each experiment was a

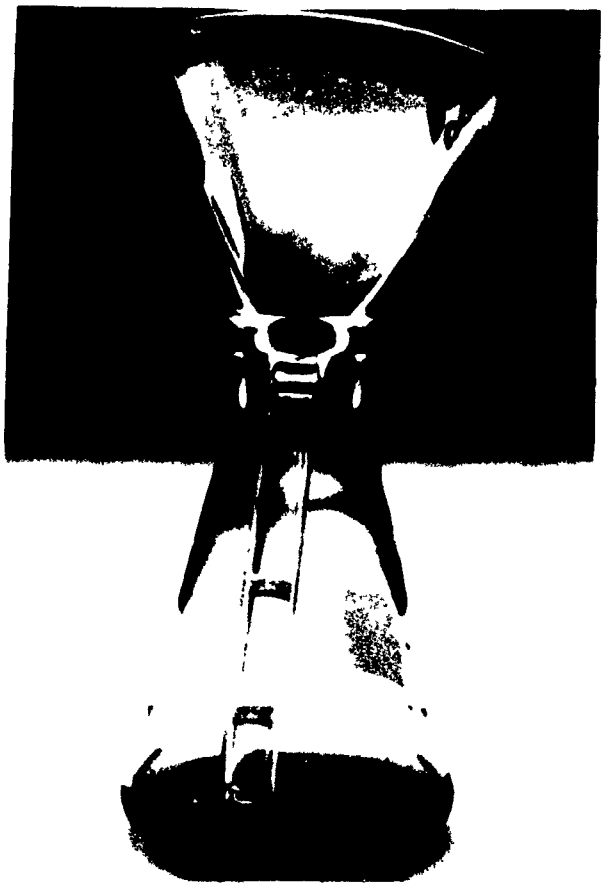

Figure 5. Filtration of the enzyme-tissue mixture after incubation.

modification of this basic procedure. The protocol was modified for each subsequent experiment if improvements were observed in previous experiments. All manipulations were carried out in the laminar flow hood using aseptic technique.

3.4.1 Preliminary Experiments

These experiments were done to establish the basic parameters of protoplast isolation and visual comparison was used to evaluate the efficacy of the techniques.

3.4.1.1. Experiment 1. Evaluation of Enzymes

Surface Sterilisation: 70% ethanol rinse
15 minutes in 10% bleach.

Enzyme mixture:

Enzymes (w/v)	1	2
Cellulase	1.0%	1.0%
Macerozyme	-	0.5%
Driselase	1.0	0.5%
Rhozhyme	1.0	1.0%

Vacuum infiltrated for 5 minutes (30 cm of mercury).

Incubation Time: 16 hours.

3.4.1.2. Experiment 2. Evaluation of Enzymes

Surface Sterilisation: 70% ethanol rinse.
30 minutes in 10% bleach.

Enzyme mixture:

Enzyme w/v	1	2
Cellulase	0.5%	2.0%
Macerozyme	0.5%	0.5
Driselase	0.125%	-

Vacuum infiltrated for 5 minutes (30 cm of mercury).

Incubation Time: 16 hours.

3.4.1.3 Experiment 3. Evaluation of Osmotic Levels

The surface sterilisation was done as described for experiment one and the enzyme mixture consisted of 1% cellulase and 0.5% macerozyme and 0.3, 0.4, 0.5, 0.6 or 0.7 M mannitol. The incubation period was sixteen hours. The protoplasts were washed and plated as in section 3.4.1 and evaluated visually.

3.4.1.4 Experiment 4. Evaluation of Pretreatment of Donor Tissue

Three pretreatments were evaluated. Potted plants and cuttings in distilled water were placed in the dark twenty-four hours before the leaves were needed. After surface sterilisation as described in section 3.4.1.2 was performed, some leaves from the untreated plants were soaked in CPW with 0.6 M mannitol for an hour. Then the epidermis was peeled from the leaves of the dark-treated cuttings and plants, those soaked in CPW and the untreated control and the leaves put into the enzyme mixture described in the previous section with 0.5 M mannitol. The protoplasts were washed and plated as described above and evaluated visually.

3.4.1.5 Experiment 5. Evaluation of Protoplast Isolation Procedures

Three isolation procedures were compared to the usual pelleting technique of washing protoplasts. The protoplasts were isolated as described in experiment four, with a dark

pretreatment of cuttings and preplasmolysis in 0.6 M mannitol in CPW were included. Three petri dishes of leaves were incubated and the resulting enzyme-protoplast mixture was divided into six aliquots and the protoplasts were washed with the following treatments:

1) Layered on 20% sucrose (w/v)
2) Layered on 40% sucrose (w/v)
3) Layered on 10% Ficoll (w/v)
4) Layered on 20% Ficoll (w/v)
5) Mixed with CPW with 20% sucrose (w/v) for flotation
6) Pelleted - usual procedure

After centrifugation the layers were collected and washed in the usual manner with two more sedimentations. The protoplasts were plated and observed.

3.4.1.6 Experiment 6. Evaluation of Protoplast Separation Procedures

Several methods of separating the protoplasts from the undigested tissue matrix were evaluated. Three plates of leaves were incubated as described in experiment five, section 3.4.1.5, and the digested tissue was treated in three different ways, as listed below:

1) Enzyme removed from the plate and replaced with CPW and the tissue gently squeezed with forceps.
2) The tissue carefully moved from the plate to the funnel with fine forceps and gently teased apart, then washed through the nylon mesh with CPW.
3) The enzyme-tissue mixture pipetted into the funnel - usual procedure.

The protoplasts were washed and plated as described in section 3.4.1 and then observed.

3.4.2. Quantification of Protoplast Protocol. Phase 1

A combination of the most effective methods from section 3.4.1, as outlined below were used to quantify the various parameters of isolation and recovery protocol. Fresh non-flowering cuttings of *I. walleriana* were put in distilled water in a dark place for 24 hours prior to protoplast incubation. Fully expanded leaves from the third or fourth node were surface sterilised with a dip in 70% ethanol and submersion in 10% bleach for 15 minutes, unless otherwise specified, then transferred to preplasmolysis in CPW with 0.6 M mannitol for an hour. The leaves were incubated in 1% w/v cellulase and 0.5% w/v macerase for 16 hours. There were 6 leaves in 10 ml of enzyme in a 100 x 15 mm petri dish. One plate was done for every treatment and the treatments were repeated as mentioned in each experiment. The digested tissue was transferred to a small funnel, sitting in a 50 ml erlenmyer and gently teased apart with tweezers. Protoplasts were washed out of the tissue with liberal amounts of CPW and pelleted by centrifugation three times, as described in section 3.4.1. The pellet was resuspended with a small volume (less than 1 ml) of Kao's medium, amended as above, and the protoplasts were counted in a Fusch-Rosenthal modified cytometer with a depth of 0.2 mm. The equation (number of protoplasts counted x 5 x 1000 x volume x dilution)/number of three lined squares counted, from Power and Chapman (1981), was used to calculate the number of protoplasts per ml and then divided by the tissue weight to give protoplasts per gram of tissue. If necessary the protoplast density was adjusted to within the range of 2 x 10^4 and 2 x 10^5 prior to plating in drops on 30 x 10 mm plastic disposable petri dishes (Potrykus and

Shillito, 1988).

Viability of the protoplasts was evaluated with the vital stain, fluorescein diacetate (FDA) (Jones and Senft, 1985). The stain was prepared in a stock of five mg of FDA in 1 ml of acetone and diluted 10 ul of stock in 490 uM of Kao's medium, then mixed one to one with the protoplast suspension for counting. The barrier filter was number 41 and the excitor filter was UG1 on a Zeiss microscope with ultraviolet lighting, to cause fluorescence in the viable cells. Viability, evaluated by counting the number of cells fluorescing, was expressed as a percent of the total number of cells.

3.4.2.1 Experiment 1. Comparison of the Effect of Surface Sterilisation Techniques on Protoplast Yield and Viability

Seven methods of surface sterilisation were compared, as listed below:

1) No surface sterilisation - control
2) 5 minutes in 70% ethanol
3) 5 minutes in 10% bleach
4) 10 minutes in 10% bleach
5) 15 minutes in 10% bleach
6) 15 minutes in 10% bleach, after a wash in 70% ethanol
7) 15 minutes in 10% bleach, after a 30 minute wash in running water

After surface sterilisation the leaves were rinsed three times in sterile distilled water, the epidermis removed and the leaves transferred to the enzyme solutions. The protoplasts were washed, counted, assessed for viability and

plated as described in section 3.4.2. The results were pooled from four separate experiments.

3.4.2.2 Experiment 2. Comparison of the Effect of Several Enzyme Mixtures on Protoplast Yield and Viability

Leaves were surface sterilised in 10% bleach, with a few drops of Tween 20, for 15 minutes. After rinsing three times in sterile distilled water the leaves were transferred to three different enzyme treatments, as described below:

Enzyme w/v	1	2	3
Cellulase	1%	1%	1%
Pectinase	0.25%	0.25%	-
Rhozyme	-	0.25%	-
Pectolyase	-	-	0.1%

After incubation and processing, the protoplasts were counted, assessed for viability and plated as described in section 3.4.2. The results were pooled from three separate experiments.

3.4.2.3 Experiment 3. Evaluation of the Effect of Incubation Time on Protoplast Yield and Viability

After surface sterilisation as described in experiment two, section 3.4.2.2, the peeled leaves were incubated in 1% w/v cellulase and 0.25% w/v pectinase and observed from two to 16 hours, at two hour intervals. The tissues were processed as described in section and the protoplasts were only observed at 2, 4, 6 and 16 hours. The protoplasts were counted and their viability was assessed at 8, 10, 12, and 14 hours. Calcofluor White M2R was used to determine if the cell wall was completely digested (Constabel, 1982). Calcofluor White (or Fluorescent Brightener) was made into a

0.1% stock with 0.4 M sorbitol and then some of the stock was mixed one to one with the protoplast suspension. The mixture was observed through a Zeiss ultraviolet microscope with the excitor filter numbers KP500 and F1546 and no barrier filters to cause fluorescence of cellulose. The results were pooled from two experiments.

3.4.2.4 Experiment 4. Comparison of the Effect of Sorbitol, Glucose and Mannitol, as Osmotic Regulators, on Protoplast Yield and Viability

After surface sterilisation, as described in experiment two, section 3.4.2.2, the leaves were incubated in 1% w/v cellulase and 0.25% w/v pectinase with 0.5 M sorbitol, 0.5 M glucose or 0.5 M mannitol for 14 hours. The protoplasts were washed, counted, assessed for viability and plated as described in section 3.4.2. The results were pooled from three experiments.

3.4.2.5 Experiment 5. Comparison of the Effect of Pretreatment of the Donor Tissue on Protoplast Yield and Viability

Three pretreatments were compared to the usual method of treating the donor tissue, which was placing cuttings in the dark 24 hours prior to use for isolation of protoplasts and preplasmolysis of the leaves. Leaves were also harvested from six week old *in vitro* grown plants that were prepared from seeds of *I. walleriana* "F_2 economy" mixture as described in section 3.3.4.3. Cuttings were also placed in the dark 48 hours prior to use. Leaves from the cuttings were surface sterilised and all except six leaves from the 24 hour dark treatment (#4) were preplasmolysized in CPW

with 0.3 M mannitol and 0.3 M glucose. Thus the four treatments were:

1) 24 hours dark pretreatment
2) 48 hours dark pretreatment
3) 24 hours dark treatment, *in vitro* grown donor tissue
4) 24 hours dark treatment and no preplasmolysis of the donor tissue

After a 14 hour incubation, in 1% w/v cellulase, 0.25% w/v pectinase and 0.5 M mannitol, the protoplasts were processed as described above. The results were pooled from three experiments.

3.4.2.6 Experiment 6. Evaluation of the Effect of Leaf Size and Age on Protoplast Yield and Viability

Protoplasts from leaves of small (less than 40 mm long and 30 mm wide, but still expanded), medium (40 - 48 mm long and 30 - 35 mm wide) and large (longer than 48 mm and wider than 35 mm) sizes from non-flowering cuttings (juvenile), medium sized leaves from flowering cuttings (mature) and leaves from *in vitro* grown plants from section 3.3.5.6 were compared (see Fig. 6). The procedure was the same as that of experiment five, section 3.4.2.5, but all the cuttings had a 24 hour dark pretreatment and the leaves were preplasmolysed in CPW with 0.3 M mannitol and 0.3 M glucose. The results were pooled from three different experiments.

3.4.2.7 Experiment 7. Evaluation of the Effect of Nylon Mesh Filter Size on Protoplast Viability and Yield

Three sizes of mesh were compared for the filtration of the digested tissue; 88 um (usual size), 62 um and 44 um.

The protoplasts were prepared as described in experiment six, section 3.4.2.6, using medium sized leaves. The results were pooled from three different experiments.

3.4.2.8 Experiment 8. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability

Five different procedures of separating the protoplasts from the enzyme and washing them were compared. The protoplasts, from medium sized leaves, were prepared according to the procedure in experiment six, section 3.4.2.6, using 62 um nylon mesh for filtering, and washed in one of the methods described below:

1) filtered with a layer of glass wool (as well as the nylon mesh)
2) gravitational sedimentation of the protoplasts for two hours, resuspended with fresh CPW and allowed to settle again
3) sedimentation at slow speed, 50 x g
4) protoplast-enzyme solution layered on 30% w/v sucrose, for the first spin
5) protoplast-enzyme solution layered on 15% w/v Ficoll, for the first spin

These modifications were incorporated into the regular procedure, as described in section 3.4.2, and the protoplasts were processed in the usual manner. The treatments were always paired with a control - the normal procedure, which means that each petri dish of protoplasts was divided in half and only half was treated differently. The results were pooled from three experiments and expressed as percent of the control or regular treatment.

Figure 6. Comparison of leaf maturity of *I. walleriana* var. *variegata*. Mature, transitional and juvenile from left to right.

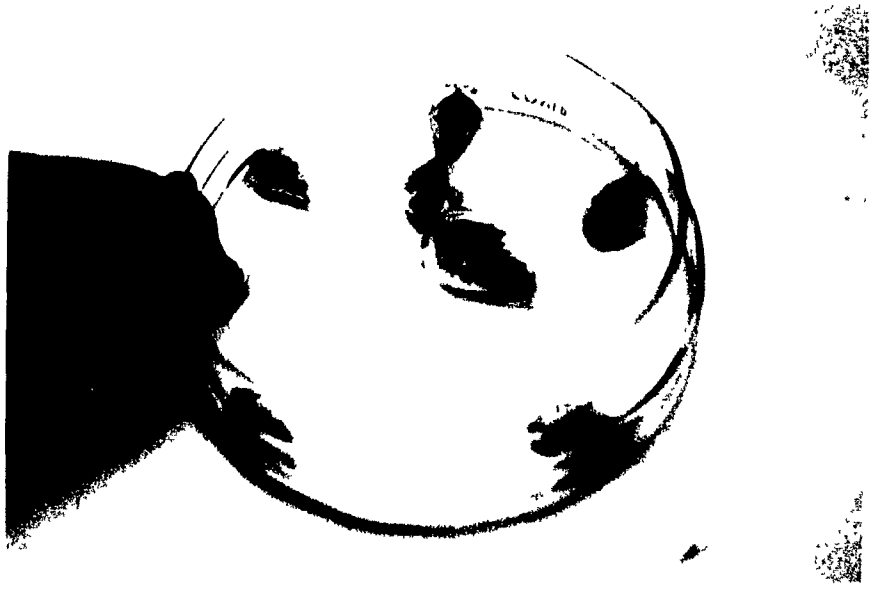

Figure 7. Surface sterilisation of *I. walleriana* leaves for protoplast isolation.

3.4.2.9 Experiment 9. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability

Two more washing protocols were tested in this experiment. The procedure was the same as experiment eight, section 3.4.2.8, but the treatments were not paired from the same petri dish. The first treatment was a layering on a mixture of 5.6% w/v Ficoll and 9.6% w/v sodium metrizoate (Larkin, 1976). The second treatment was the use of round bottom centrifuge tubes and the third was layering on 30% w/v sucrose. The layering was done for the first centrifugation and two pellet sedimentations followed. The second treatment was handled in the usual manner. The protoplasts were counted, the viability assessed and plating was done in the usual manner.

3.4.3 Quantification of Protoplast Protocol. Phase 2

The leaves were surface sterilised with 10% bleach, with a few drops of Tween 20, for 15 minutes in a 150 x 20 mm glass petri dish. Full contact of the leaves with the bleach was enforced by placing the bleach in the lid of the dish and submersing the leaves with the bottom of the petri dish (see Fig. 7). The leaves were rinsed three times with sterile distilled water and transferred to CPW with 0.3 M mannitol and 0.3 M glucose for preplasmolysis. These experiments were done with 20 mm diameter disks of leaf tissues, cut with a number 12 cork borer after peeling, that weighed an average of 90 mg. The pieces of tissue were almost identical, all without the lower epidermis, cut from leaves of similar size. One disk was incubated per 1 ml of enzyme, as specified for each experiment. Garamycin^{R}

(gentamycin sulfate), an antibiotic suitable for *in vitro* use, was added to the CPW, enzymes and culture media at 10 mg/L. Incubation was 14 to 16 hours and the protoplasts were washed out of the tissue with five ml CPW per disk (after the tissue was transferred to a small funnel sitting in a 50 ml erlenmyer, with fine tweezers). Unless otherwise specified the protoplasts were centrifuged at 100 x g for four minutes for the first spin and three minutes for the second spin. At that dilution a third centrifugation was unnecessary.

3.4.3.1 Experiment 1. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability

Four enzyme mixtures were compared using the above protocol:

Enzyme w/v	1	2	3	4
Cellulase	1%	1%	1%	1%
Pectinase	0.25%	0.5%	0.25%	-
Rhozyme	-	-	0.25%	-
Pectolyase	-	-	-	0.1%

One leaf disk was incubated in 1 ml of each enzyme mixture, in 10 ml glass beakers, for 15 hours. After incubation the treatments were processed separately and the protoplasts were counted and the viability assessed as described in section 3.4.2. The protoplasts were plated in 24 cell multiwell plates, in 0.2 ml of Kao's media amended as in section 3.4. The results were pooled from four experiments.

3.4.3.2 Experiment 2. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability

Four enzyme mixtures were compared using the protocol described in experiment one (section 3.4.3.1), including half strength concentrations of mixtures one and two, shown below:

Enzyme	1	2	3	4
Cellulase	1%	0.5%	0.5%	1%
Pectinase	0.25%	0.125%	0.25%	-
Pectolyase	-	-	-	0.1%

The results were pooled from six experiments.

3.4.3.3 Experiment 3. Evaluation of the Effect of Centrifugation Speed on Protoplast Yield and Viability

The general protocol was used to compare the effect of four centrifugation speeds. Four leaf disks were incubated for 15 hours in 4 ml of enzyme mixture three from experiment two (section 3.4.3.2) and one-quarter of the protoplast suspension, after filtering, was used for each centrifugation speed (100 x g, 60 x g, 45 x g, 30 x g). The results were pooled from four separate experiments.

3.4.3.4 Experiment 4. Comparison of the Effect of Media on Protoplast Survival

Kao's (Kao and Michayluk, 1975), Nagata and Takebe (1971) and Uchimiya and Murashige (1974) media were tested for their effect on the survival of protoplasts in culture (see Appendix 4), with the protocol from experiment one (section 3.4.3.1). Three leaf disks were incubated in 3 ml of enzyme mixture three of experiment two (section 3.4.3.2)

in a 30 x 10 mm plastic disposable petri dish. The protoplast suspension was divided in three, after the first centrifugation and rediluted with the appropriate medium for the second spin. After counting and viability assessment the protoplasts were cultured in 0.2 ml of the appropriate medium in 24 well multiwell plates. The media were supplemented with the growth regulators described in section 3.4 (0.1 mg/L BAP, 1.0 mg/L 2,4-D and 1.0 mg/L NAA). The plates were observed regularly for two weeks and the presence or absence of cellulose was evaluated with Calcofluor White M2R, as described in section 3.4.2.3, at one week.

3.4.4 Preliminary Evaluation of Protoplast Isolation from Cotyledons

Seeds of the "F_2 economy" mixture were prepared as described in section 3.3.4.3 and cotyledons were harvested from 16 day old seedlings. Fifty cotyledons were floated, lower surface down, on 5 ml of an enzyme mixture, modified from Vessabutr and Grant (1989), that contained 2% w/v cellulase, 1% w/v macerase and 0.1% w/v pectolyase in CPW with 0.65 M mannitol, in 30 x 10 mm plastic disposable petri dishes. Two dishes were prepared and incubated for eight hours, for the first experiment and two dishes were incubated for six hours in the second experiment. The protoplasts were released from the cotyledons by gentle squeezing with forceps and the enzyme-protoplast solution was centrifuged in 15 ml conical centrifuge tubes for four minutes at 100 x g for the first sedimentation. The supernatant was replaced with Kao's medium amended as described in section 3.4.3.4 and the pellet resuspended.

The protoplast suspension was centrifuged at 100 x g for three minutes and repeated to complete the washing. The protoplasts were counted and the viability assessed as described in section 3.4.2 and the concentration adjusted to 1 x 10^5. The suspension was plated in 0.2 ml of media in 24 well multiwell plates and observed regularly.

3.4.5 Statistical Analysis of the Protoplast Experiments

The separate experiments were treated as blocks and the experiments were in a randomized complete block design. The results were analysed using the general linear models procedure of the Statistical Analysis System (SAS Institute) and the separation among means was done with Duncan's Multiple Range Test (Steele and Torrie, 1980).

4. RESULTS

4.1 Crosses

None of the manually performed crosses between *I. walleriana* and *I. repens* and were successful, nor were any of the crosses among the varieties of *I. walleriana*.

4.2 Preliminary Experiments - Tissue Culture

4.2.1 Experiment 1. Evaluation of the Effect of Plant Growth Regulators

The results are summarized on Table 4.1 for both species, *I. walleriana* and *I. repens*. Although there was a significant amount of contamination, it is obvious that the tissue responded to almost all the growth regulators with roots, especially to the auxins, 2,4-D, IBA and NAA. *I. repens* stem sections had roots that covered the plates from treatments with IAA and IBA alone (see Fig. 8). The leaf tissue was less responsive than the stem tissue and the stem tissue grew roots with no growth regulators present at all. However the desired response was callus production and none was observed.

4.3 Surface Sterilisation Experiments

4.3.1 Experiment 1. Determination of Appropriate Surface Sterilisation Techniques on Stem and Leaf Tissue

From Table 4.2.1 it may be seen that there was very little contamination on the leaf tissue but a dip in 70%

Table 4.1 Rooting response of *I. repens* and *I. walleriana* to various plant growth regulator combinations.

Plant growth regulator	Rooting response			
	I. repens		*I. walleriana*	
	stems	leaves	stems	leaves
IAA and BAP	o	o	-	-
IAA and K	r	-	-	-
IAA and 2iP	r	o	r	o
IAA and Z	r	o	-	o
IAA	rr	-	r	r
24D and BAP	rr	-	r	o
24D and K	rr	-	r	r
24D and 2iP	rr	-	r	r
24D and Z	r	-	r	r
24D	rr	-	r	r
IBA and BAP	r	-	r	-
IBA and K	rr	-	r	-
IBA and 2iP	rr	o	r	-
IBA and Z	rr	o	-	-
IBA	rr	r	r	-
NAA and BAP	o	-	r	o
NAA and K	r	o	r	r
NAA and 2iP	rr	o	r	r
NAA and Z	r	-	r	r
NAA	r	-	r	r
BAP	r	-	o	o
K	r	-	o	o
2iP	r	-	r	-
Z	-	-	-	-
none	r	-	r	-

Z=zeatin, 24D=2,4-D, K=kinetin, r=few roots, rr=many roots, o=occasional roots, -=no roots.

Figure 8. Root proliferation on B5 medium with 1.0 mg/L IBA.

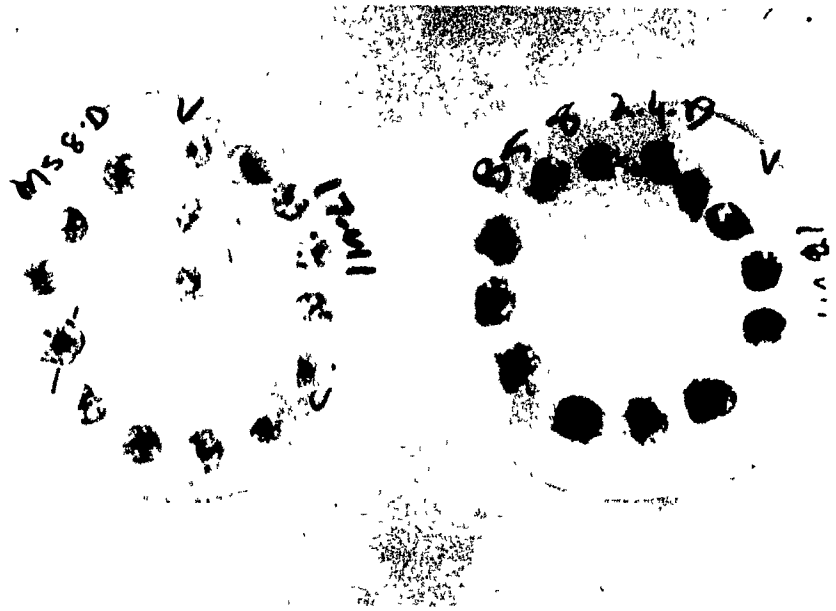

Figure 9. Abnormal root growth on B5 (left) and MS (right) media with 8 mg/L 2,4-D

Table 4.2.1 Surface Sterilisation Techniques Performed on Leaves.

Treatment #	Time (min)++	% Uncont'd+	% Unbleached+
1	0	55.0 a	100.0 a
2	5	100.0 b	100.0 a
3	10	100.0 b	100.0 a
4	15	87.5 b	100.0 a
5	5+++	100.0 b	52.5 b
Analysis of variance			
Treatment		*	**

\+ : % uncontaminated and unbleached leaf tissue sections - values based on the mean of 4 replicates.
++ : in 10% bleach.
+++: with vacuum infiltration.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,**: significant at $p < 0.05$ and 0.01, respectively.

ethanol was insufficient to remove all the micro-organisms. Although vacuum infiltration was an effective method of surface sterilisation, the tissues were killed. The lower percentage of uncontaminated tissue at 15 minutes is an indication of the ongoing presence of contamination. Surface sterilisation of tissue done in small amounts is quite effective, but as soon as the volume increases, contamination is always a problem, as will be seen in the following experiments.

There was no completely successful treatment for the surface sterilisation of stem tissue as shown in Table 4.2.2 which was a real problem for experimental work. Washing in running water prior to surface sterilisation showed the most uncontaminated tissue but the treatments are not significantly different. Also the strongest treatments caused tissue bleaching.

The tissues were plated on B5 with 8 mg/L 2,4-D to see if there was any callus production. None was observed after two weeks but the stem sections started to form short contorted roots (see Fig. 9).

4.3.2 Experiment 2. Comparison of Surface Sterilants on Stem and Leaf Tissue

Calcium hypochlorite was compared to 10% bleach to see if it was more effective. From Table 4.3 it may be seen that this is not the case for both leaves and stems. The treatments are not significantly different except for the leaves that were surface sterilised with the stems in 10% bleach. The combined stems in 7% $CaOCl_2$ were totally contaminated. Neither of the surface sterilants were better, but the importance of separating tissue can be seen.

Table 4.2.2 Surface Sterilisation Techniques Performed on Stems.

#	Treatment	% + Uncont'd	% + Unbleached
1	70% ethanol dip and 15 min. in 10% bleach.	82.5 a	100.0 a
2	1 hour wash preceeding treatment #1.	97.5 b	85.0 a
3	Treatment #1 with vacuum infiltration.	85.0 a	90.0 a
Analysis of variance			
Treatment		*	ns

+ : % uncontaminated and unbleached stem sections - values based on the mean of 4 replicates.
a,b : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,ns: significant at $p < 0.05$ and not significant, respectively.

Table 4.3 Comparison of Surface Sterilants for Stem and Leaf Tissue.

Treatment #	Sterilant	Stems & Leaves	% Uncontaminated+ Stems	Leaves
1	7% CaOCl	separate	22.5 a	95.0 a
2	7% CaOCl	combined	0.0 a	90.0 a
3	10% bleach	separate	25.0 a	85.0 a
4	10% bleach	combined	30.0 a	55.0 b
Analysis of variance				
Treatment			ns	*

+ : values based on the mean of 4 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,ns: significant at $p < 0.05$ and not significant, respectively.

The stems that were uncontaminated started to produce roots.

4.3.3 Experiment 3. Determination of Appropriate Surface Sterilisation Techniques on Nodes

A number of different surface sterilisation techniques were compared as shown in Table 4.4. No technique was totally effective but the best was the first treatment, which caused minimal damage to the tissue. As the period of exposure to bleach increased the amount of uncontaminated tissue did also (treatments 3, 6 and 7). The use of vacuum infiltration had the same effect (treatments 2 and 3) and the removal of excess tissues, such as petioles, improved the efficacy of the treatment (1 and 5). Although the amount of healthy tissue was not significantly different, it was noticeable that the more intense the treatment the more damaging it was to the tissues (treatment 7).

4.3.4 Experiment 4. Surface Sterilisation and Germination of Seeds

Seeds of *I. walleriana* germinated equally as well on water agar as they did on B5 medium (see Table 4.5). Although there was a significant difference between the water agar and the B5 medium for the 33% bleach treatment, the difference was due to contamination, not the media. Germination of the seeds was more affected by the surface sterilisation technique. Washing the seeds in running water for an hour prior to the bleach treatment killed a significant percentage of the seeds. The highest concentration of bleach was also detrimental, even though the exposure was brief. The mildest treatment (number 1) was the most effective surface sterilisation method and was

Table 4.4 Surface Sterilisation Techniques Performed on Nodes.

Treatment #	Time (min)	% Bleach	Other details	Results+ % Uncont'd	% Unbleached
1	15	10	rinsed in dist. water, petioles excised and a second bleach dip	71.7 a	90.0 a
	5	10			
2	10	10		46.7 a	100.0 a
3	10	10	with vacuum infiltration	51.7 a	88.3 a
4	15	5		43.3 a	90.0 a
5	15	5	with petioles removed	60.0 a	86.7 a
6	5	10	with vacuum infiltration	43.3 a	96.7 a
7	15	10	with vacuum infiltration	65.0 a	81.7 a
Analysis of variance					
Treatment				ns	ns

+ : % uncontaminated and unbleached nodes and axillary buds - values based on the mean of 6 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns : not significant.

Table 4.5 Surface Sterilisation Techniques Performed on Seeds.

Treatment #	Time (min)	% Bleach	Media	Results+ % Germinated	% Not germ.	% Uncont'd
1	15	10	B5	80.0 a	16.0 a	96.0 a
2	15	10	water agar	68.0 a	24.0 a	92.0 a
3	5	33	B5	4.0 b	32.0 ab	36.0 b
4	5	33	water agar	16.0 b	64.0 bc	80.0 a
5	15++	10	B5	8.0 b	84.0 c	92.0 a
6	15++	10	water agar	12.0 b	80.0 c	92.0 a
Analysis of variance						
Treatment				**	**	**

+ : values based on the mean of 5 replicates.
++ : preceeded by a 1 hour wash under running water.
a,b,c: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
** : significant at $p < 0.01$.

the least damaging. Five minutes in 33% bleach was not sufficiently long enough to kill all the micro-organisms, but washing effectively removed the contaminants.

4.3.5 Experiment 5. Surface Sterilisation of Leaves for Protoplast Culture

With the exception of two plates treated in 10% bleach which were free of contamination, the other treatments were unsuccessful. Obviously six minutes in bleach was insufficient to kill all the micro-organisms and 70% ethanol was not effective either, with or without 10% bleach.

4.4 Callogenesis Experiments

4.4.1 Experiment 1. Evaluation of the Effect of 2,4-D and Media on Stem and Leaf Tissue

Unfortunately the presence of contamination interfered with the evaluation of this experiment, so the data were presented as percent of uncontaminated tissue sections. The analysis of variance in Table 4.6 shows that there is no interaction between the media, MS and B5, and 2,4-D. and the media had no effect on the tissue response. The goal of this experiment was to produce callus but none was observed. There was however, copious root growth which increased significantly with the amount of 2,4-D. In the case of leaves the effect was both linear and quadratic, since there was a higher percentage of rooting at 2 mg/L 2,4-D. The roots observed on 4 mg/L 2,4-D were shorter and wider, not resembling roots grown with no 2,4-D or *in vivo*, at all. The effect of the 2,4-D gradient on root growth in the stem sections was not significant b t more growth was observed in

Table 4.6 Main Effect[+] of 2,4-D and Media on Leaf and Stem Tissue.

2,4-D (mg/L)	% Rooted[++] Leaves	Stems
0.0	0.0	59.9
2.0	72.5	90.0
4.0	56.7	89.7
Nature of response	L** Q**	ns
Media		
B5	46.0	85.3
MS	38.3	70.2
Contrast	ns	ns
Analysis of variance		
2,4-D (D)	**	ns
Media (M)	ns	ns
D*M	ns	ns

+ : all values based on means of 4 replicates over 3 levels of 2.4-D or 2 types of media.
++ : as a percentage of uncontaminated tissues.
L,Q: linear and quadratic, respectively, based on single degree freedom orthogonal polynomials.
**,ns : significant at $p < 0.01$ and not significant, respectively.

the media with 2 and 4 mg/L 2,4-D. There was generally less rooting on the leaf tissue on both media and in all levels of 2,4-D (see Fig. 10).

4.4.2 Experiment 2. Evaluation of the Effect of 2,4-D and Kinetin on Stem Tissue

Again the presence of contamination interfered with the experiment and the data were presented as a percent of uncontaminated tissue sections at two weeks and as a percent of the transferred sections at four weeks. No callus production was observed in this experiment, but there was prolific root production. There was no interaction between 2,4-D and kinetin, while only 2,4-D had a significant effect on rooting at four weeks (Table 4.7). The response to the increasing levels of 2,4-D is significantly linear and quadratic, since the highest 2,4-D concentrations caused virtually one hundred percent rooting at four weeks. There was no significant effect of kinetin on root production but the highest level demonstrated inhibition at two weeks and at four weeks 2.0 mg/L kinetin had the least effect on root growth.

4.4.3 Experiment 3. Evaluation of the Effect of IAA and BAP on Seedling Stem Tissue

From Table 4.8, it can be seen that there were no significant effects of either light or IAA/BAP ratios on the rooting of seedling stem tissue, nor was there an interaction between the factors. The desired response from the tissue sections was callus production, but none was observed. The lowest ratio of IAA to BAP (1 to 4) had the least amount of rooting.

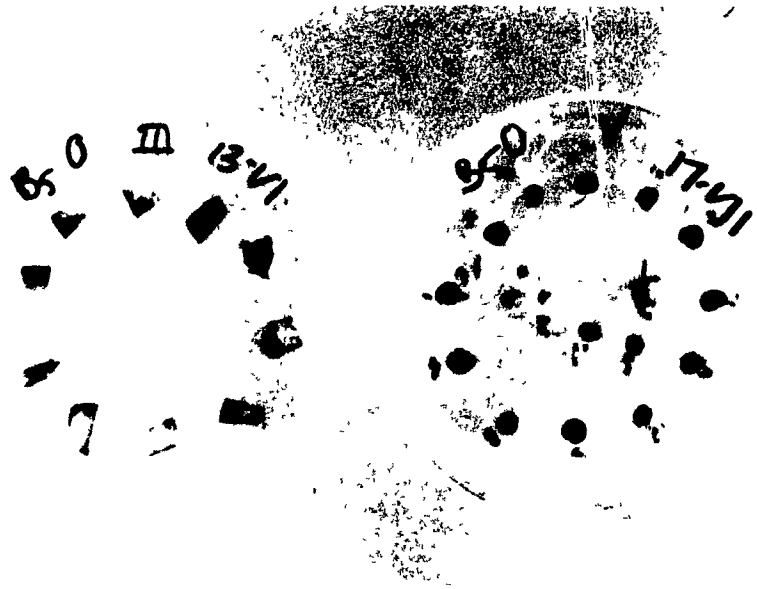

Figure 10. Root formation on leaf sections (left) and root sections (right) on B5 medium with no plant growth regulators.

Table 4.7 Main Effect[+] of 2,4-D and Kinetin on Stem Tissue.

2,4-D (mg/L)	2 weeks % rooted[++]	4 weeks % rooted[+++]
0.0	30.3	56.7
1.0	33.4	80.5
2.0	23.9	99.1
4.0	42.6	95.2
8.0	69.7	99.0
Nature of response	ns	L** Q**
Kinetin (mg/L)		
0.0	38.8	92.3
1.0	23.1	75.7
2.0	40.6	93.8
4.0	25.3	92.0
8.0	17.2	81.1
Nature of response	ns	ns
Analysis of variance		
2,4-D (D)	ns	**
Kinetin (K)	ns	ns
K*D	ns	ns

+ : all values based on means of 5 replicates over 5 levels of kinetin or 2,4-D.
++ : as a percentage of uncontaminated tissues.
+++: as a percentage of transferred tissues.
L,Q: linear and quadratic, respectively, based on single degree freedom orthogonal polynomials.
**,ns : significant at $p < 0.01$ and not significant, respectively.

Table 4.8 Main Effect[+] of Growth Regulators and Light on Tissue Growth.

IAA (mg/L)	BAP (mg/L)	% Rooted
0.5	2.0	58.0 a
1.0	1.0	70.0 a
2.0	0.5	75.0 a
Light		
Yes		71.3 a
No		64.0 a
Analysis of variance		
IAA:BAP (R) ratio		ns
Light (L) yes or no		ns
R*L		ns

+ : all values based on the mean of 5 replicates over 3 levels of IAA:BAP or 2 light regimes.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

4.4.4 Experiment 4. Evaluation of the Effect of Media and Carbon Source on Callogenesis on Axillary Buds

The results of this experiment are expressed merely as percent survival and then percent callusing of the total numbers of uncontaminated replicates because there was so little tissue response that no growth parameters could be measured. These results can be seen in Table 4.9 and it is noticeable that at six weeks the survival rate of the explants was effected by the level of carbohydrates in the medium. The highest concentration of glucose in MS actually totally inhibited survival and the remainder of the higher concentrations of carbohydrates decreased survival rate. At twelve weeks the percent callusing is a reflection of the survival rate, as well as an effect of the medium and carbon source. The callus that formed was either extremely hard or very soft and it had a beige brown colour. Callus formation was observed largely at the lower carbohydrate levels, except the high percentage found on B5 medium with 15 g/L glucose. Both the aforementioned treatment and 30 g/L sucrose in B5 medium caused the induction of callus but these levels did not do so in MS medium, otherwise the two media had similar effects.

4.5 Micropropagation

4.5.1 Experiment 1. Evaluation of the Effect of BAP on Shoot Proliferation

As can be seen from Figure 11, the effect of BAP on shoot proliferation was quadratic at all evaluation times, reaching a peak at 3 mg/L BAP and then decreasing. The highest level of shoot proliferation was 2.6 shoots per

Table 4.9 The Effect of Media and Carbon Source on Axillary Buds.

#	Media	Glucose (g/L)	Sucrose (g/L)	6 weeks % survived+	12 weeks % callused+
1	MS	5	-	77.8	55.6
2	MS	10	-	75.0	37.5
3	MS	15	-	30.0	0.0
4	MS	25	-	0.0	0.0
5	MS	-	10	50.0	25.0
6	MS	-	20	77.8	44.4
7	MS	-	30	28.6	0.0
8	MS	-	50	14.3	0.0
9	B5	5	-	75.0	12.5
10	B5	10	-	80.0	40.0
11	B5	15	-	70.0	60.0
12	B5	25	-	33.3	0.0
13	B5	-	10	88.9	44.4
14	B5	-	20	80.0	30.0
15	B5	-	30	33.3	11.1
16	B5	-	50	40.0	0.0

+: values represent the percent of the uncontaminated proportion of 10 replicates.

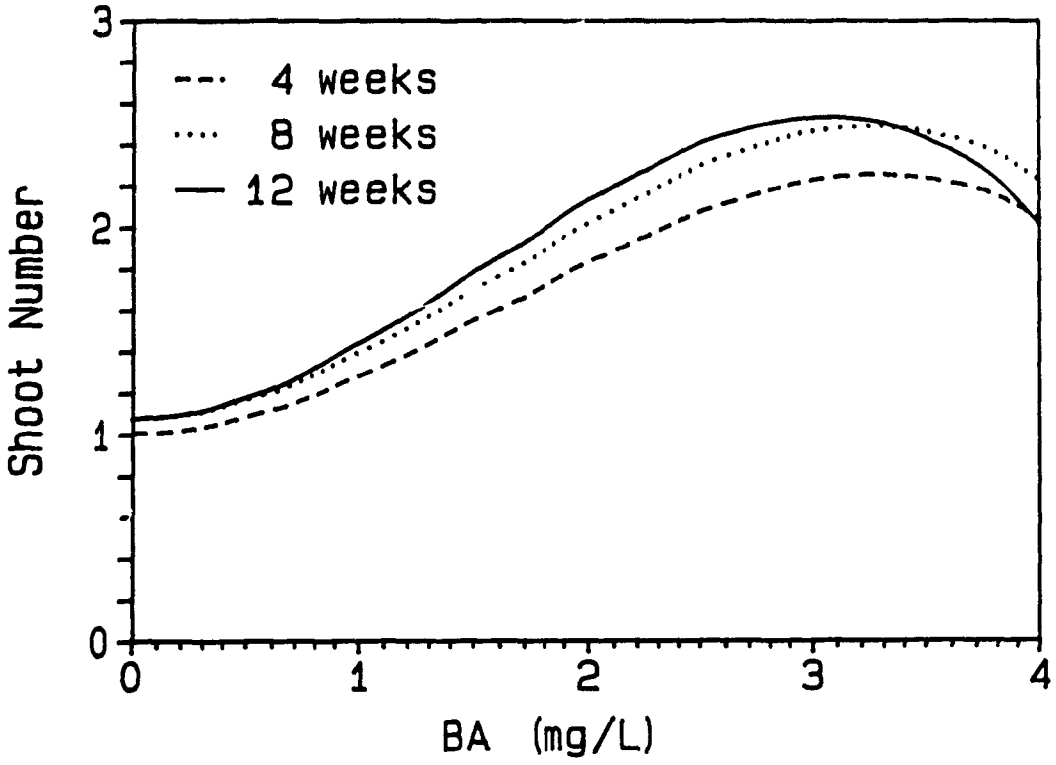

Figure 11. The effect of BAP on shoot proliferation at three transfer dates: Regression equations.

Week 4: $Y=1.0066 + (0.3462(T^2)) - (0.0711(T^3))$
$R^2 = 0.40$ $P < 0.01$

Week 8: $Y=1.0789 + (0.4011(T^2)) - (0.0823(T^3))$
$R^2 = 0.35$ $P < 0.01$

Week 12: $Y=1.0752 + (0.4717(T^2)) - (0.1032(T^3))$
$R^2 = 0.39$ $P < 0.01$

explant observed at 3 mg/L BAP during both the second and third transfer. Beyond that level shoot proliferation was inhibited.

4.5.2 Experiment 2. Evaluation of the Effect of Media Concentrations and Growth Regulator Ratios on Shoot Proliferation

Shoot proliferation was the only parameter significantly affected by the media growth regulator combinations, as seen in Table 4.10. BAP and IBA produced significantly more shoots in both media concentrations, than kinetin and NAA. The growth regulators were included in ratios of 3 to 1, 10 to 1 and 30 to 1 cytokinin to auxin and the largest ratio caused the greatest increase in shoot proliferation and decrease in shoot height. The statistical analysis indicates that there is no significant effect of media concentration on any of the parameters, but the mean shoot height was lower for the full strength media with the lower auxin/cytokinin ratios and was greater at the highest ratio. No trends can be discerned in total shoot fresh weights of the NAA/kinetin results, but those of the BAP/IBA were higher for the full strength MS medium than those of the half strength MS medium.

4.5.3 Experiment 3. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation.

In Table 4.11 it can be seen that only kinetin of the three factors, media, NAA and kinetin had a significant effect on shoot proliferation and on mean shoot height. There was no interaction between any of the factors. The response of shoot proliferation and shoot height to

Table 4.10 The Effect of Growth Regulator Ratios and Media Strength on Shoot Proliferation.

Treatment #	Media	Kinetin (mg/L)	NAA (mg/L)	Shoot number+	Mean shoot height+ (mm)	Total shoot fr. wt.+ (mg)
1	MS	0.3	0.1	1.0 a	7.3 a	14.2 a
2	1/2MS	0.3	0.1	1.0 a	19.8 ab	20.3 ab
3	MS	5.0	0.5	1.0 a	16.8 ab	29.7 ab
4	1/2MS	5.0	0.5	1.2 a	30.0 b	38.5 b
5	MS	3.0	0.1	1.0 a	30.7 b	38.5 b
6	1/2MS	3.0	0.1	1.0 a	14.0 ab	31.2 ab
#	Media	BAP (mg/L)	IBA (mg/L)			
7	MS	1.0	0.1	1.7 ab	16.3 ab	32.2 ab
8	1/2MS	1.0	0.1	1.8 b	13.2 ab	20.5 ab
9	MS	3.0	0.1	3.0 c	7.5 a	32.8 ab
10	1/2MS	3.0	0.1	3.0 c	7.8 a	19.8 ab
Analysis of variance						
Treatment				**	ns	ns

+ : each figure represents the mean of 6 replicates.
a,b,c: difference between means in a column with the same letter is not significant by T tests (LSD) at $p < 0.05$.
**,ns: significant at $p < 0.01$ and not significant, respectively.

Table 4.11 Main Effect of Kinetin, NAA and Media on Shoot Proliferation.

Kinetin (mg/L)	Shoot number[+]	Mean shoot height[+] (mm)
0.0	1.00	38.0
3.0	1.08	38.1
30.0	2.54	10.1
Nature of response	L**	L**
NAA (mg/L)		
0.0	1.53	26.4
0.1	1.56	31.1
Nature of response	ns	ns
Media		
MS	1.63	24.4
1/2LS	1.33	27.8
B5	1.67	34.0
Contrast	ns	ns
Analysis of variance		
Kinetin (K)	**	**
NAA (N)	ns	ns
Media (M)	ns	ns
K*N, K*M, N*M and K*N*M	ns	ns

+ : each figure represents the mean of 4 replicates over 3 levels of kinetin, 2 levels of NAA and/or 3 types of media.
L : linear, based on single degree freedom orthogonal polynomials.
**,ns : significant at $p < 0.01$ and not significant.

increasing levels of kinetin was linear and in the former case the parameter increased and the latter parameter decreased. Shoot height was slightly greater with 0.1 mg/L NAA and with the B5 medium, although not significantly.

4.5.4 Experiment 4. Evaluation of the Effect of Media, NAA and Kinetin Concentrations on Shoot Proliferation

The interaction of NAA and kinetin was significant for shoot proliferation in this experiment, so the simple effects of the growth regulators are graphed in Figure 12. Shoot proliferation was inhibited with 0.2 mg/L NAA and increased with the level of kinetin when there is no NAA. Mean shoot height was not affected by the interaction of NAA kinetin or media (see Table 4.12), but the main effects of the growth regulators were significant. The nature of response of shoot height to kinetin was both linear and quadratic in a decreasing fashion, since the decrease of mean shoot height was less proportional to the increase of kinetin and would have levelled off if kinetin had continued to increase. Mean shoot height increased in a linear manner with NAA, since only two levels of NAA were incorporated in the experiment.

4.5.5 Experiment 5. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation

IBA and BAP did not interact significantly, so the main effects of both growth regulators can be seen in Table 4.13. The effect of IBA was not significant on any of the growth parameters, although the total shoot weight appeared to be lower at 0.1 and 1.0 mg/L. Shoot number responded in either a linear or quadratic manner to BAP, since the increase was minimal at the lower levels and the greatest increase of

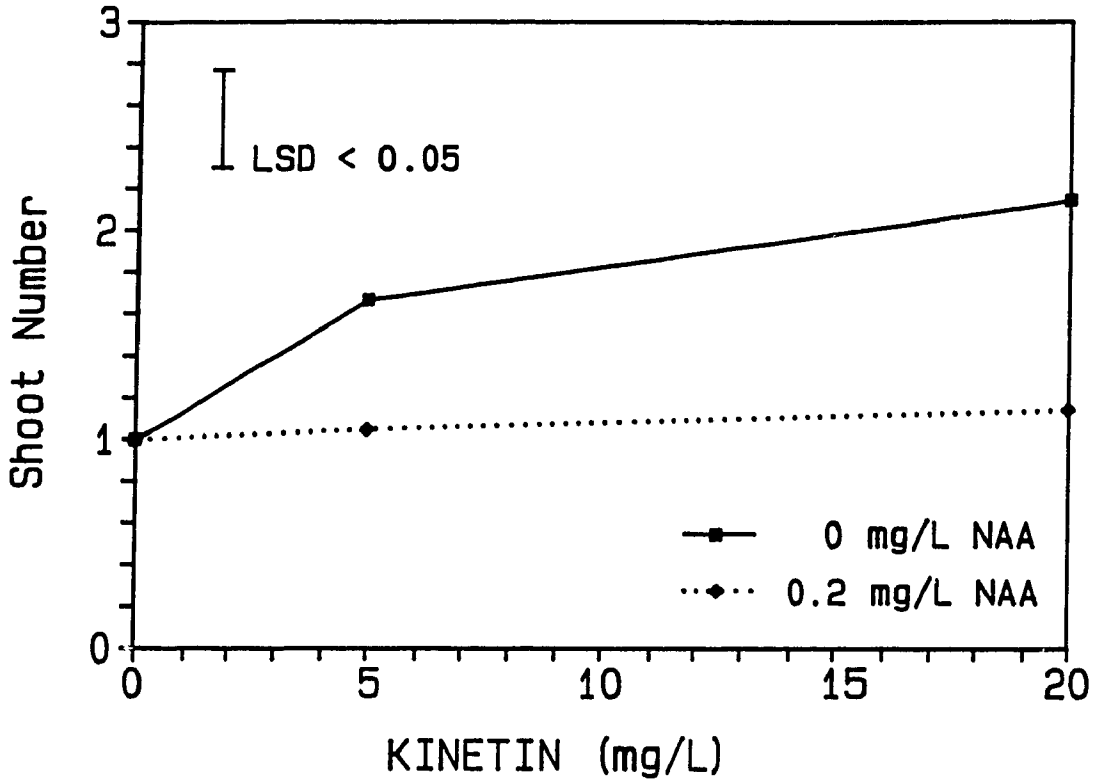

Figure 12. The simple effects of NAA and kinetin on shoot number.

Table 4.12 Main Effect of Kinetin, NAA and Media on Shoot Height.

Kinetin (mg/L)	Mean shoot height+ (mm)
0.0	51.9
5.0	28.9
20.0	9.0
Nature of response	L** Q**
NAA (mg/L)	
0.0	25.3
0.2	34.6
Nature of response	L**
Media	
MS	33.1
1/2LS	31.3
B5	25.3
Contrast	ns
Analysis of variance	
Kinetin (K)	**
NAA (N)	**
Media (M)	ns
K*N, K*M, N*M and K*N*M	ns

+ : each figure represents the mean of 4 replicates over 3 levels of kinetin, 2 levels of NAA and/or 3 types of media.
L,Q : linear and quadratic, respectively based on single degree freedom orthogonal polynomials.
**,ns : significant at $p < 0.01$ and not significant, respectively.

Table 4.13 Main Effect of IBA and BAP on Shoot Proliferation.

IBA (mg/L)	Shoot number+	Mean shoot height+ (mm)	Total shoot fr. weight+ (mg)
0.0	1.5	6.7	29.6
0.01	1.5	6.4	20.4
0.1	1.6	5.0	16.9
1.0	1.5	6.8	33.8
Nature of response	ns	ns	ns
BAP (mg/L)			
0.0	1.1	6.8	20.5
0.1	1.1	6.3	27.7
1.0	1.5	5.9	18.3
10.0	2.5	5.8	34.2
Nature of response	L** Q**	ns	ns
Analysis of variance			
IBA (I)	ns	ns	ns
BAP (B)	**	ns	ns
I*B	ns	ns	ns

+ : each figure represents the mean of 8 replicates over 4 levels of IBA or BAP.
L,Q : linear and quadratic, respectively based on single degree freedom orthogonal polynomials.
**,ns : significant at $p < 0.01$ and not significant.

shoot number was from 1.0 to 10.0 mg/L BAP. The highest shoot proliferation was observed with 10.0 mg/L BAP.

4.5.6 Experiment 6. Evaluation of the Effect of IBA and BAP Concentrations on Shoot Proliferation

Shoot number was influenced by the interaction of IBA and BAP and the simple effects are graphed in Figure 13. The lines of the three lowest levels of IBA overlap, but that of the fourth level is below them and almost level. There was virtually no shoot proliferation at 3.0 mg/L IBA. As the levels of BAP increased, so did the rate of shoot proliferation, the highest observed at 0 IBA and 5.0 mg/L BAP. IBA-BAP interaction had a significant effect on total shoot weight, as seen in Figure 13. The shoot weight at 3.0 mg/L IBA was considerably higher than the weights observed for the other IBA treatments and the parameter decreased with the level of BAP. The greatest weight was observed at 3.0 mg/L IBA and 0 mg/L BAP. With the other IBA levels total shoot weight was highest with no BAP, dropped to the lowest point with 0.5 mg/L BAP and then increased gradually with the level of BAP. The interaction of IBA and BAP had no effect on mean shoot height and the main effects of the growth regulators are found in Table 4.14. In both cases the nature of response was both significantly linear and cubic because shoot height decreased from 0 mg/l to 0.03 mg/L IBA and then increased, while the decrease was not in regular steps for BAP. The tallest shoots were found at the highest level of IBA and the lowest level of BAP. The effect of all the treatments can be seen in Figure 14. Rooting was noticeable at all the zero levels of either growth regulator and when there was no BAP, the explant

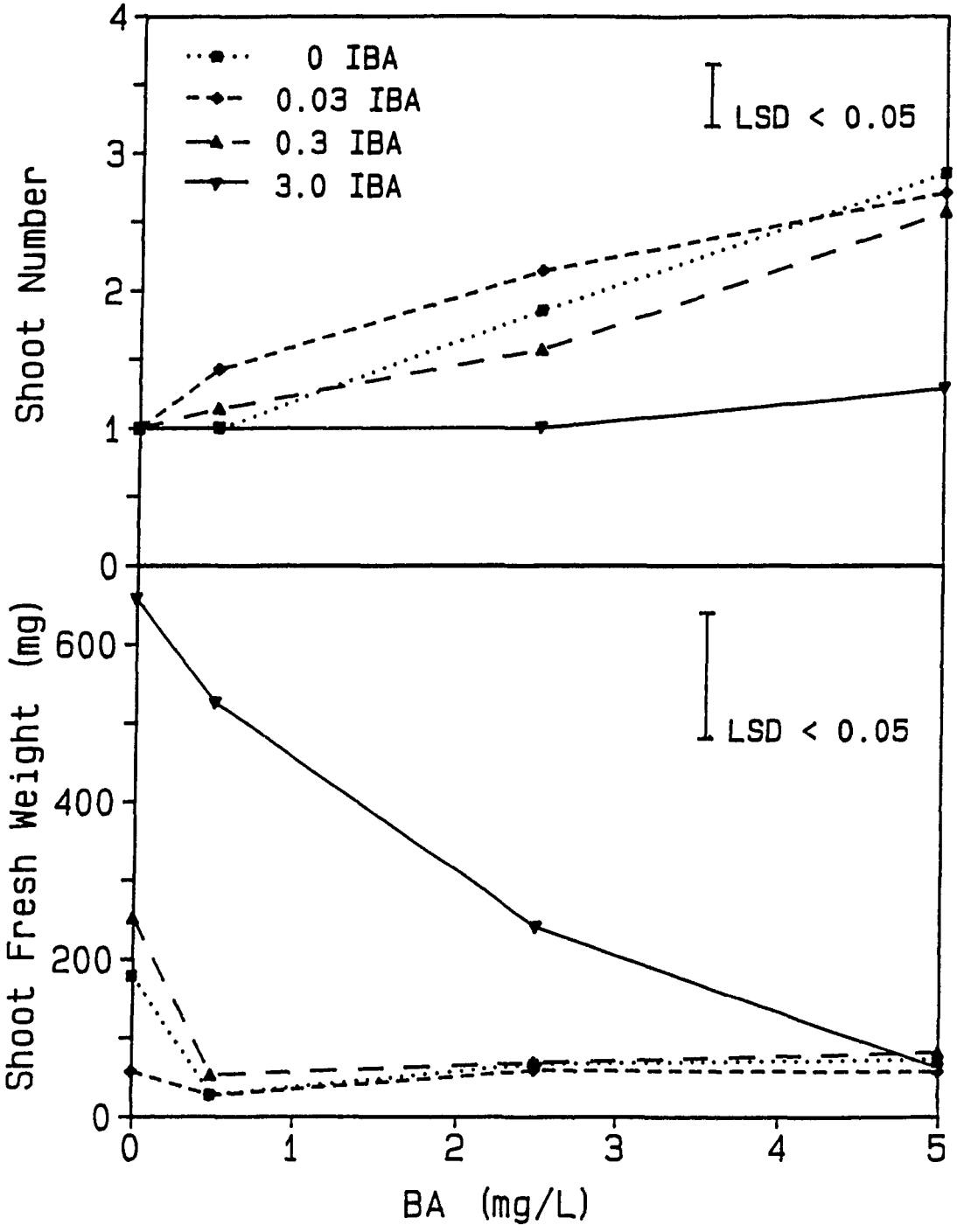

Figure 13. The simple effects of IBA and BAP on shoot number and shoot weight.

Table 4.14 Main Effect of IBA and BAP on Shoot Height.

IBA (mg/L)	Mean shoot height+ (mm)
0.0	10.1
0.03	6.5
0.3	9.8
3.0	18.0
Nature of response	L** C**
BAP (mg/L)	
0.0	19.4
0.5	10.6
2.5	8.9
5.0	5.5
Nature of response	L* C**
Analysis of variance	
IBA (I)	**
BAP (B)	**
I*B	ns

+ : each figure represents the mean of 7 replicates over 4 levels of IBA or BAP.
L,C : linear and cubic, respectively based on single degree freedom orthogonal polynomials.
**,*,ns : significant at $p < 0.01$, $p < 0.05$ and not significant, respectively.

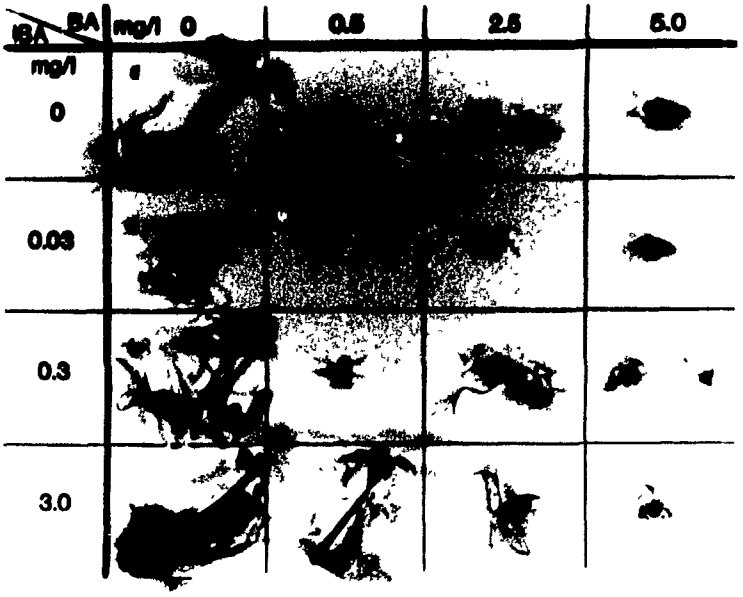

Figure 14. The effects of IBA and BAP on shoot proliferation.

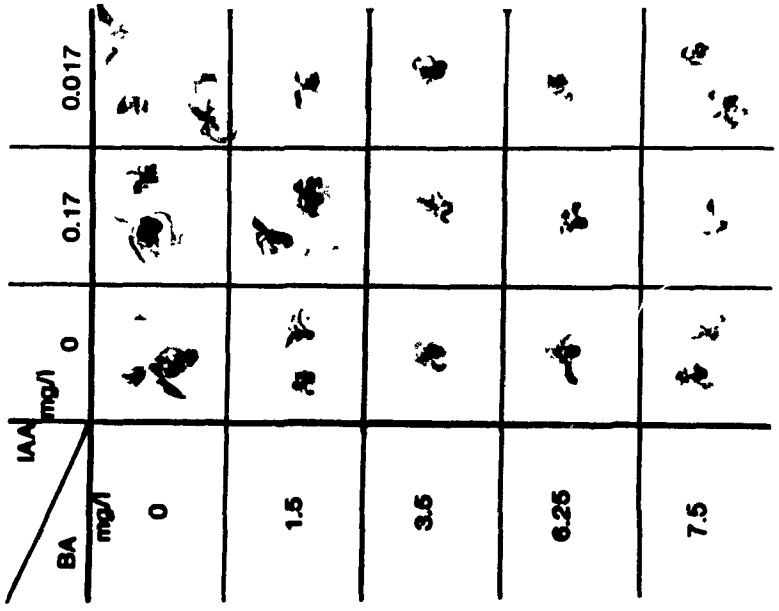

Figure 15. The effects of IAA and BAP on shoot proliferation.

either did not change or roots were formed and it grew very well.

4.5.7 Experiment 7. Evaluation of the Effect of IAA and BAP Concentrations on Shoot Proliferation

No interaction of IAA and BAP influenced any of the growth parameters, as seen in Table 4.15, nor did IAA have an effect, excepting the effect on total shoot weight. The effect of IAA on shoot weight was quadratic, since there was a large increase in shoot weight with the second increase of IAA. The greatest total shoot weight was observed at 0.17 mg/L IAA. BAP affected all the growth parameters and the nature of response was either linear or quadratic. The increase in shoot number levelled out between 6.25 and 7.5 mg/L of BAP and the decrease of mean shoot height was drastic at the lower levels of BAP, then increased slightly at 7.5 mg/L BAP. The greatest number of shoots was observed at both 6.25 and 7.5 mg/l BAP and the shoots were tallest with no BAP in the media. Total shoot weight increased linearly with the level of BAP and was greatest at 7.5 mg/L BAP. As can be seen in Figure 15, some rooting was observed when there was no BAP in the medium and those that rooted grew very well. When the explants were cultured on media with 0.017 mg/L IAA and 8.13 mg/L 2iP there was an average shoot number of 2.3, mean shoot height of 7.7 mm and total shoot weight of 104 mg which is lower than the best results with BAP alone.

Table 4.15 Main Effect of IAA and BAP on Shoot Proliferation.

IAA (mg/L)	Shoot number+	Mean shoot height+ (mm)	Total shoot fr. wt.+ (mg)
0.0	2.2	6.0	66.6
0.017	2.0	6.3	72.4
0.17	2.1	6.9	98.2
nature of response	ns	ns	Q**
BAP (mg/L)			
0.0	1.0	8.7	71.4
1.5	1.7	6.0	56.4
3.5	2.5	5.8	85.1
6.25	2.7	5.4	84.1
7.5	2.7	6.1	98.4
Nature of response	L** Q**	L** Q**	L**
Analysis of variance			
IAA (I)	ns	ns	**
BAP (B)	**	**	*
I*B	ns	ns	ns

+ : each figure represents the mean of 10 replicates over 5 levels of BAP or 3 levels of IAA.
L,Q : linear and quadratic, respectively, based on single degree freedom orthogonal polynomials.
*,**: significant at $p < 0.05$, $p < 0.01$ and not significant, respectively.

4.6 Acclimation and Rooting of Micropropagated Shoots

There was one hundred percent of rooting after ten days on the hormone-free media and shortly thereafter the microcuttings started elongating.

Only two microcuttings died during the acclimatization process and they were both from the group in the growth chamber. One short unrooted propagule and one medium rooted propagule deceased.

4.7 Protoplast Isolation

4.7.1 Preliminary Experiments

4.7.1.1 Experiment 1. Evaluation of Enzymes

After a 16 hour incubation the leaves were still intact, as if the digestion had been not completed, in both enzyme solutions (see Fig. 16). However, observation with the inverted microscope revealed that the cells had been destroyed, although still in the form of the leaf. Only raphids, chloroplasts, crushed protoplasts, vascular debris and bacteria were observed in the enzyme solution (see Fig. 17).

4.7.1.2 Experiment 2. Evaluation of Enzymes

After 16 hours the leaves were still intact but observation revealed that there were some protoplasts, as well as the debris described above, in the enzyme solution and more still in the leaf matrix. So the mixture was processed to wash and separate the protoplasts, revealing that the consistency of the solution resembled mucous. It

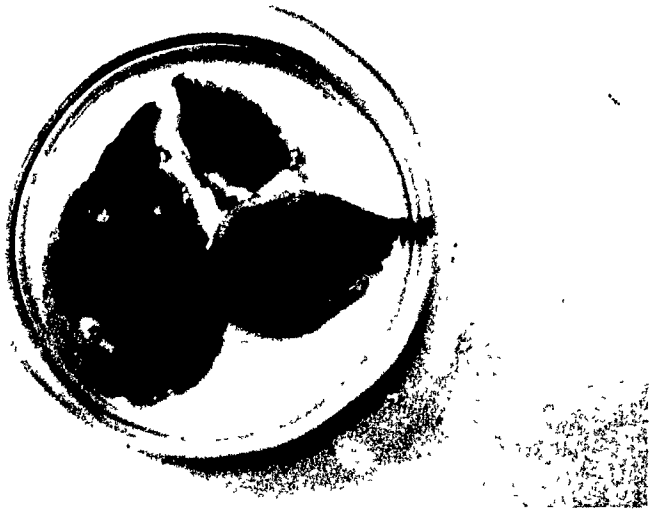

Figure 16. Leaves after incubation for 16 hours in an enzyme solution of 2% w/v cellulase and 0.5% pectinase.

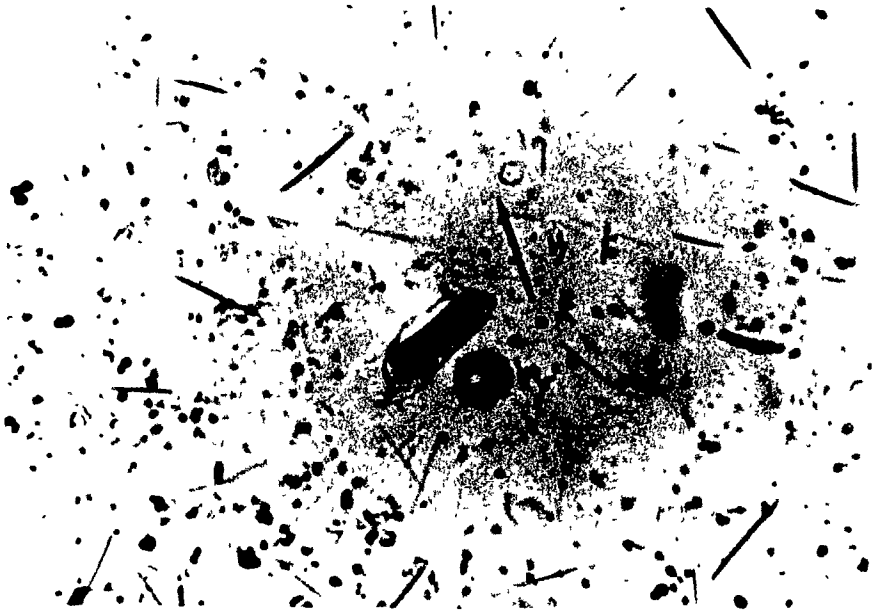

Figure 17. Raphids and debris in the enzyme solution after tissue digestion.

was very difficult to work with and caused great problems with washing of the protoplasts, since they all stuck together and they did not pellet well. At least half the protoplasts stayed in the suspension even after centrifugation. There were no protoplasts released by enzyme two and enzyme one produced a low yield. Observation of the protoplasts in the petri dish revealed that there was contamination and the same debris described in section 4.4.1.

4.7.1.3 Experiment 3. Evaluation of Osmotic Levels

Generally, the range of osmotic levels in the medium and the enzyme solution caused little obvious variation among the protoplasts from different treatments. However, there was nothing but debris and chloroplasts from the 0.3 M mannitol treatment and the protoplasts were shrunken in the highest osmotic level, 0.7 M mannitol. The osmotic level did not change the mucous-like nature of the protoplast-enzyme mixture, nor the presence of the copious amount of debris.

4.7.1.4 Experiment 4. Evaluation of Pretreatment of Donor Tissue

Leaves that were taken from cuttings placed in the dark produced dense, spherical protoplasts and less small granular debris, although placing the intact plants in the dark did not improve the protoplast quality and quantity. The preplasmolysis in CPW with 0.5 M mannitol improved the appearance of the protoplasts, which were no longer uncohesive and irregular. The yield increased slightly.

4.7.1.5 Experiment 5. Evaluation of Protoplast Isolation Procedures

Five different methods of protoplast isolation procedures were compared. When the protoplast-enzyme suspensions were layered on top of 20% sucrose or 10% Ficoll the solutions started to mix, so after centrifugation there was a small pellet and no layer. The protoplasts did not float when mixed with 20% sucrose in CPW, even after ten minutes of centrifugation. Layers formed at the junction of both 20% Ficoll and 40% sucrose. The layer on Ficoll was defined clearly and easy to remove, whereas the layer on sucrose was hazy. Observation of the plated protoplasts showed less of the larger debris and raphids, but large amounts of chloroplasts and crumpled protoplasts remained (see Fig. 18). The yield was greater from the sucrose layer and the protoplasts stayed greener for a few days longer than those of the Ficoll treatment did. Both treatments had a lower yield than the regular method of pelleting and neither method improved the mucous-like texture of the suspension.

4.7.1.6 Experiment 6. Evaluation of Protoplast Separation Techniques

When the digested leaves were lifted from the enzyme solution, many of the raphids were left on the bottom of the petri dish. Some of the mucous remained in the petri dish with the enzyme, although it is the mucous that holds the leaf shape after digestion. After the tissue was teased apart with tweezers in the funnel, the protoplasts were washed through with CPW, which diluted the remaining enzyme

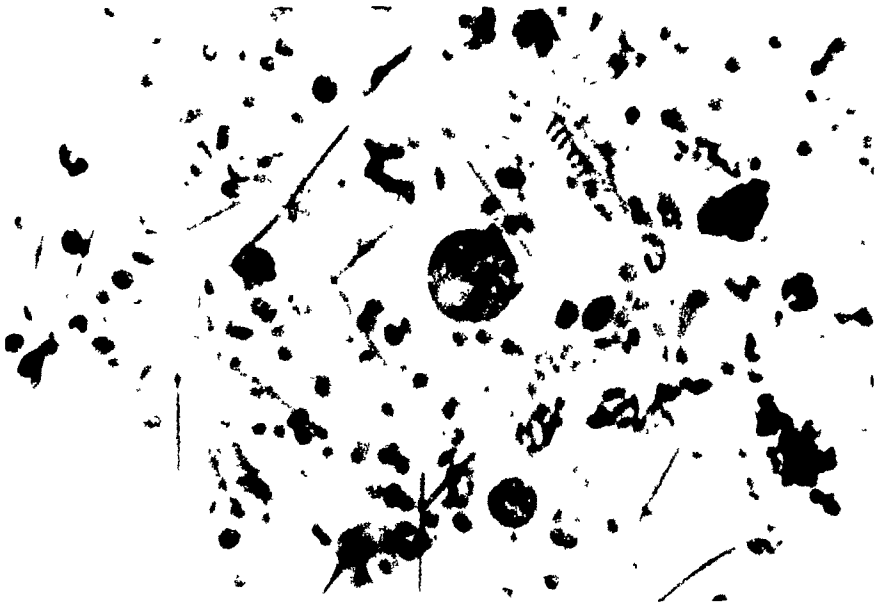

Figure 18. Protoplasts isolated after flotation on 40% sucrose.

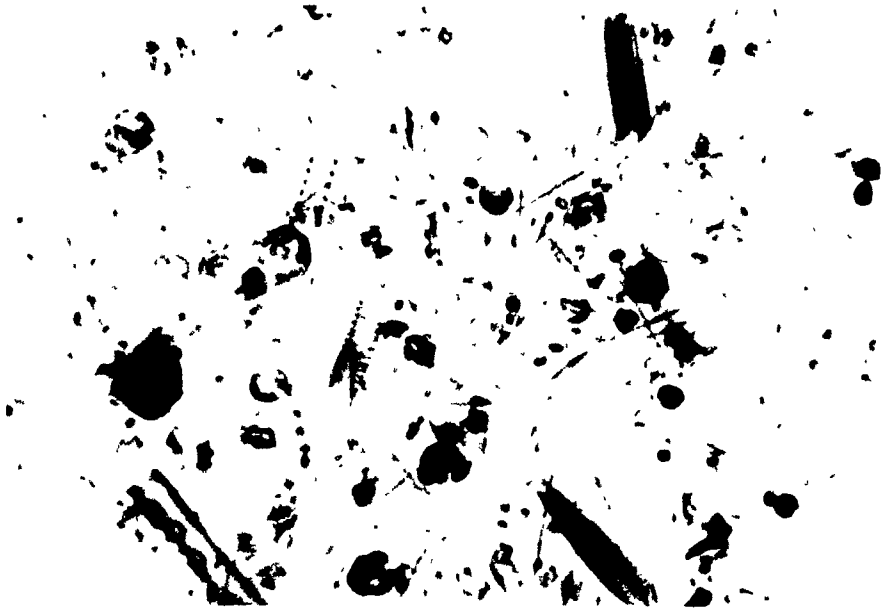

Figure 19. Protoplasts isolated after transferring the tissue from the enzyme solution to the funnel for separation by teasing with tweezers.

and the mucous, making washing the protoplasts much easier. The protoplast suspension still had a mucous-like texture but it was less of a problem and more protoplasts were rescued than with the conventional method. The other treatment, releasing the protoplasts by squeezing the tissue, was an improvement over the normal procedure but the raphids and the mucous that had settled to the bottom of the petri dish were incorporated into the protoplast suspension. Observation of the cultured protoplasts still revealed large quantities of debris (see Fig. 19).

4.7.2 Quantification of Protoplast Protocol. Phase 1

4.7.2.1 Experiment 1. Comparison of the Effect of Surface Sterilisation Techniques on Protoplast Viability and Yield

In Table 4.16 it can be seen that surface sterilisation techniques had a significant effect on the yield, but not the viability of protoplasts. All the treatments that involved the use of sterilisation techniques other than bleach had a significantly lower yield than that of the 15 minute exposure to bleach. However surface sterilisation with 10% bleach for 15 minutes did not lower the yield below that of the treatment that involved no surface sterilisation. Protoplast yield for this experiment ranged from 1.2×10^5 per gram of leaf tissue for the five minutes in 70% ethanol treatment to 3.89×10^5 per gram of leaf tissue for the 15 minutes in 10% bleach treatment. Viability of the protoplasts, determined with the FDA technique as shown in Figure 20, ranged from 69.0% for the ethanol treatment to 85% for 15 minutes in the 10% bleach treatment.

Table 4.16 The Effect of Surface Sterilization Techniques on Protoplast Yield and Viability.

#	Treatment	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	no surface sterilization	27.0 ab	82.2 a
2	5 min. in 70% ethanol	12.0 a	69.0 a
3	5 min. in 10% bleach	22.8 ab	84.4 a
4	10 min. in 10% bleach	26.9 ab	77.0 a
5	15 min. in 10% bleach	38.9 b	84.6 a
6	15 min. in 10% bleach, after a wash in 70% ethanol	19.8 a	82.4 a
7	15 min. in 10% bleach, after a 30 min. wash in running water	18.9 a	77.9 a
Analysis of variance			
Treatment		*	ns

+ : values based on the mean of 4 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,ns: significant at $p < 0.05$ and not significant, respectively.

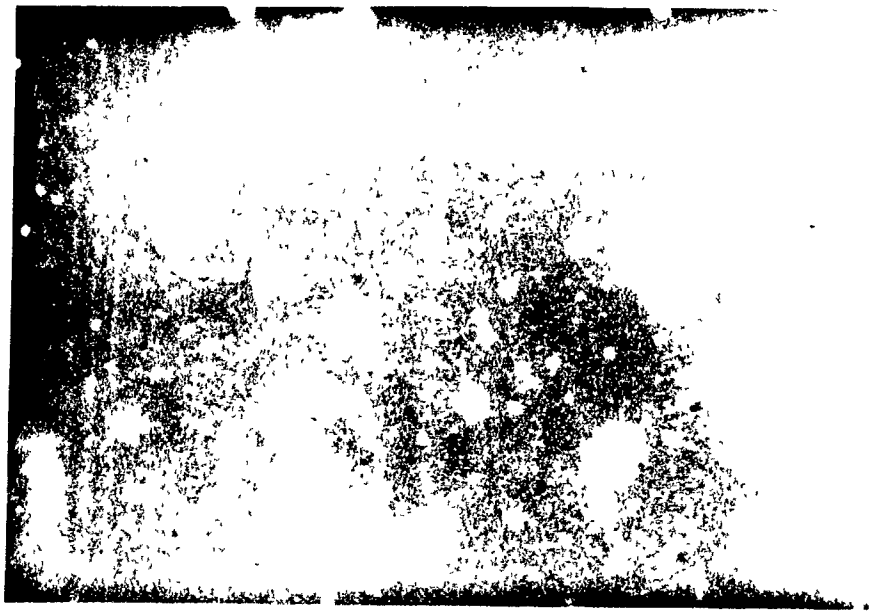

Figure 20. Fluorescence of viable protoplasts stained with FDA.

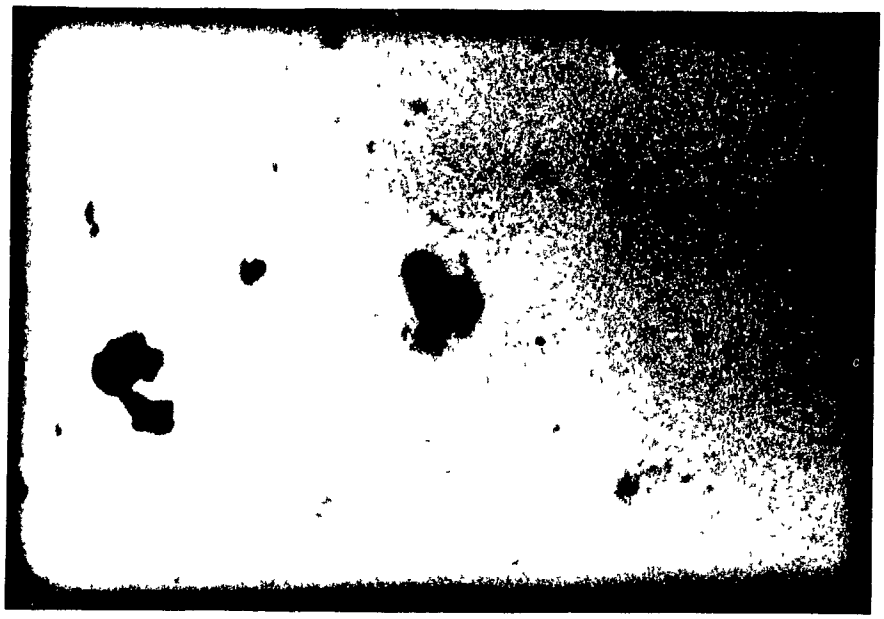

Figure 21. Protoplasts stained with Calcofluor White showing no cell wall.

Contamination was later observed in all of the cultures from treatments one, two and three, and half of the cultures from treatments four, five and six.

4.7.2.2 Experiment 2. Evaluation of the Effect of Several Enzyme Mixtures on Protoplast Yield and Viability

Statistical analysis of the results of this experiment showed significant differences between the treatments (see Table 4.17). The yield was in the usual range, the highest being that of treatment one with 1% cellulase and 0.25% pectinase, but viability was very low. The yield from 1% cellulase and 0.1% pectolyase was almost as high.

4.7.2.3 Experiment 3. Evaluation of the Effect of Incubation Time on Protoplast Yield and Viability

The results presented in Table 4.18 are not significantly different but there seems to be a trend of higher yield with longer incubation periods, with no effect on viability. The yield ranged from 1.033×10^5 to 4.57×10^5 per gram of leaf tissue, with high viabilities. Observations on the incubated leaves showed that there were no protoplasts at two hours and few at four hours. There were protoplasts at six hours but evaluation with Calcofluor White fluorescence showed the remains of the cell wall around the protoplasts. At eight hours of incubation no fluorescence was noticeable around the protoplasts (Fig. 21). There was still a high yield of protoplasts at sixteen hours but the protoplasts were starting to appear damaged and the viability was lower.

Table 4.17 The Effect of Different Enzyme Mixtures on Protoplast Yield and Viability.

Treatment #	Enzyme Mixture++	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	0.25% pectinase	16.5 a	58.0 a
2	0.25% pectinase + 0.25% rhozyme	11.5 a	58.1 a
3	0.10% pectolyase	15.6 a	54.9 a
Analysis of variance			
Treatment		ns	ns

+ : values based on the mean of 3 replicates.
++: 1.0% cellulase was included in all treatments.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

Table 4.18 The Effect of Incubation Time on Protoplast Yield and Viability.

#	Incubation time (hours)	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	8	10.6 a	82.2 a
2	10	14.2 a	91.8 a
3	12	38.0 a	87.5 a
4	14	45.7 a	93.1 a
Analysis of variance			
Incubation time		ns	ns

+ : values based on the mean of 2 replicates.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

4.7.2.4 Experiment 4. Comparison of the Effect of Sorbitol, Glucose and Mannitol, as Osmotic Regulators, on Protoplast Yield and Viability.

Sorbitol, glucose and mannitol were all equally effective osmotic regulators, since there was no significant difference between the treatments, as seen in Table 4.19. The mannitol treatment had the highest yield of protoplasts and the viability for all treatments was good. The type of osmotic regulator did not affect the consistency of the protoplast suspension - mucous was still present.

4.7.2.5 Experiment 5. Comparison of the Effect of Pretreatment of the Donor Tissue on Protoplast Yield and Viability

From the results in Table 4.20 it can be seen that *in vitro*-grown donor tissue had a significantly higher yield than all other pretreatments, although there were no differences among treatments on the viability of the protoplasts. The usual pretreatment of 24 hours in the dark had the next greatest protoplast yield. Two days pretreatment of the donor tissue was detrimental to protoplast yield and the lack of a CPW pretreatment decreased the yield as well. The *in vitro* grown tissue was, in this case, from a non variegated plant, so there was a much higher percent of green cells, but more crumpled green protoplasts were observed as well. The protoplasts from this treatment actually floated on 0.6 M CPW and so for the second centrifugation the suspension was mixed with 20% w/v sucrose for floatation. These protoplasts purified well with that treatment and produced a culture almost free from debris, raphids and mucous. The 48 hour pretreatment also

Table 4.19 The Effect of Osmotic Regulators on Protoplast Yield and Viability.

Treatment #	Osmotic regulator	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	0.5 M sorbitol	3.9 a	85.6 a
2	0.5 M glucose	4.0 a	84.8 a
3	0.5 M mannitol	5.7 a	83.5 a
Analysis of variance			
Treatment		ns	ns

+ : values based on the mean of 3 replicates.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

Table 4.20 The Effect of Pretreatment on the Donor Tissue on Protoplast Yield and Viability.

#	Pretreatment	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	24 hours in the dark	25.2 a	74.7 a
2	48 hours in the dark	12.3 a	74.5 a
3	24 hours in the dark using in vitro grown donor tissue	71.8 b	77.2 a
4	24 hours in the dark without preplasmolysis	19.1 a	72.3 a
Analysis of variance			
Pretreatment		**	ns

+ : values based on the mean of 3 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
**,ns: significant at $p < 0.01$ and not significant, respectively.

yielded a suspension with less debris and mucous, but not as clean as that of the *in vitro* grown tissue.

4.7.2.6 Experiment 6. Evaluation of the Effect of Leaf Size and Age on Protoplast yield and Viability

The comparison of the factors in this experiment shows that the highest yield was from *in vitro* grown tissue of the variegated *Impatiens* and as can be seen in Table 4.21, the result was significantly better than from the leaves of plants grown in the growth chamber. The yield was higher from the plants grown in test tubes, although some raphids and mucous were observed. There was little effect of the leaf sizes on the protoplast yield, although the largest leaves had the biggest yields. The yields from the mature leaves were not significantly different but the viability of the protoplasts was. Protoplasts from mature leaves had a lower viability and there was more debris in the suspension.

4.7.2.7 Experiment 7. Evaluation of the Effect of Nylon Mesh Filter Size on Protoplast Yield and Viability

As can be seen from Table 4.22 there was no statistically significant differences between the yields from the three mesh sizes tested in this experiment, nor was there an effect on viability of the protoplasts. The highest yield was from the 62 µm mesh treatment and this treatment removed a significant proportion of the larger vascular debris, but not the raphids.

Table 4.21 The Effect of Leaf Size on Protoplast Yield and Viability.

Description	Leaf size range Length (mm)	Width (mm)	Protoplast yield (x 10000) /g fr. wt.+	% viability+
small	< 40	< 30	9.7 a	68.2 a
medium	40 - 48	30 - 35	8.7 a	66.0 a
large	> 48	> 35	11.6 a	76.8 a
mature	40 - 48	25 - 30	8.0 a	45.6 b
in vitro	< 40	< 30	21.7 b	85.5 a
Analysis of variance				
Leaf size			**	*

+ : values based on the mean of 3 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,**: significant at $p < 0.05$ and $p < 0.01$, respectively.

Table 4.22 The Effect of Nylon Mesh Filter Size on Protoplast Yield and Viability.

Treatment #	Nylon mesh size (um)	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	88	13.7 a	85.3 a
2	62	17.9 a	73.1 a
3	44	16.4 a	76.1 a
Analysis of variance			
Treatment		ns	ns

+ : values based on the mean of 3 replicates.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

4.7.2.8 Experiment 8. Comparison of the Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability

The comparison of these five different washing protocols showed no significant difference on the protoplast yield, viability or percent of control (see Table 4.23). The actual yields for each treatment were misleading, since each treatment came from a separate petri dish, although pairing the treatments with a control from the same petri dish compensated for this problem. From these results the variability of yields from different petri dishes can be deduced. The lowest protoplast yield and percent of control was from layering on 30% sucrose, since there was some mixing between the sucrose layer and the protoplast suspension. The floatation on Ficoll had a reasonably good yield but the yield was only 67.2% of its control. The floatation treatments produced protoplast isolations with less large debris than their controls. Both the glass wool filtration and the low speed centrifugation had yields that were approximately 70% of their controls but there was not significantly less debris in either treatment. Protoplast yield from the gravitational sedimentation of the suspension represented the highest percentage of the control of all the treatments, but there was just as much debris present and the lack of a concentrated pellet made washing the protoplasts difficult. Observation in culture revealed that the protoplasts from the Ficoll floatation lost their green colour much sooner than the protoplasts from the other treatments. Viability was good in this experiment.

Table 4.23 The Effect of Different Protoplast Wash Protocols on Protoplast Yield and Viability (Experiment 8).

Treatment #	Wash protocol	Protoplast yield (x 10000) /g fr. wt.+	% of control+	% viability+
1	filtration through glass wool and nylon mesh	11.1 a	70.2 a	93.1 a
2	2 hr. gravity sedimentation, resuspension with fresh CPW and further sedimentation	11.1 a	88.0 a	85.2 a
3	sedimentation at 50 x g	9.2 a	71.1 a	82.2 a
4	initial centrifugation of protoplast-enzyme solution layered on 30% w/v sucrose	8.9 a	58.6 a	92.3 a
5	as in protocol 4 above, except with 15% w/v ficoll	11.4 a	67.2 a	84.7 a
Analysis of variance				
Treatment		ns	ns	ns

+ : values based on the mean of 3 replicates.
a : difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns: not significant.

4.7.2.9 Experiment 9. The Effect of Different Protoplast Washing Protocols on Protoplast Yield and Viability

There was no control included in this experiment but the significant difference between the treatments shows that the yield in the layering treatments was lower and the use of the round bottomed tube is similar to the usual procedure (see Table 4.24). The layer on the mixture of Ficoll and sodium metrizoate was crisp and easy to separate, while that on the sucrose is quite hazy but an improvement on the 30% sucrose from the previous experiment. Less large debris was observed in the isolations from the layered treatments but there was still many crumpled protoplasts and chloroplasts and the yield in both cases was below that of the regular treatment of the previous experiment. The use of the rounded centrifuge tube did not decrease the amount of observed debris.

4.7.3 Quantification of the Protoplast Protocol. Phase 2

4.7.3.1 Experiment 1. Evaluation of the Effect of Enzyme Mixtures on Protoplast Yield and Viability

As can be seen in Table 4.25 there was no significant difference between the four enzyme recipes but several of the replicates of treatments two and three had no protoplast yields at all. The short coming of the protocol is highlighted in this case since the failure of one leaf to produce protoplasts biases the results and causes such variation in the data that the differences between the treatments are insignificant. Treatments two and three had low yields and low viability as well, probably because the

Table 4.24 The Effect of Different Protoplast Wash Protocols on Protoplast Yield and Viability (Experiment 9).

Treatment #	Wash protocol	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	centrifugation of protoplast-enzyme solution layered on 5.6% w/v Ficoll plus 9.6% w/v sodium metrizoate	8.7 a	60.3 a
2	centrifugation using round bottomed centrifuge tubes	13.5 b	71.4 a
3	as in protocol 1 above, except with 40% w/v sucrose	7.2 c	76.6 a
Analysis of variance			
Treatment		**	ns

+ : values based on the mean of 3 replicates.
a,b,c: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
**,ns: significant at $p < 0.01$ and not significant, respectively.

Table 4.25 The Effect of Enzyme Mixtures on Protoplast Yield and Viability (Experiment 1).

Treatment #	Enzyme Mixture++	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	0.25% pectinase	8.6 a	71.6 a
2	0.50% pectinase	3.3 a	16.4 b
3	0.25% pectinase + 0.25% rhozyme	1.7 a	36.6 ab
4	0.10% pectolyase	12.8 a	72.8 a
Analysis of variance			
Treatment		ns	*

+ : values based on the mean of 4 replicates.
++ : 1.0% cellulase was included in all treatments.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,ns: significant at $p < 0.05$ and not significant, respectively.

incubation was too long, so these treatments were not repeated.

4.7.3.2 Experiment 2. The Effect of Enzyme Mixtures on Protoplast Yield and Viability.

The results of this experiment are summarized in Table 4.26. It can be seen that treatment one, with 1% w/v cellulase and 0.25% w/v pectinase had a significantly lower yield of protoplasts and protoplast viability. The other enzyme mixtures did not have a significantly different effect on the yield of protoplasts or protoplast viability. Thus treatment three, with 0.5% w/v cellulase and 0.25% w/v pectinase and treatment four with 1.0% w/v cellulase and 0.1% w/v pectolyase were good enzyme mixtures for the release of protoplasts with a 14 to 16 hour incubation.

4.7.3.3 Experiment 3. The Effect of Centrifugation Speed on Protoplast Yield and Viability

There is no statistical difference between the yield of the protoplasts from the various spin speeds, as can be seen in Table 4.27, other than the yield of 100 x g spin, which was larger than the yield of the 60 x g spin. The viability was low in all cases and the various centrifuge speeds had no significant effect on the protoplast viability. Thus the 100 x g centrifugation speed was selected for continued use.

4.7.3.4 Experiment 4. Comparison of the Effect of Media on Protoplast Survival

The average yield of the ten replicates was 7.18×10^5 per gram of leaf tissue with a standard deviation of 2.95×10^5 and the average viability was 78.43% with a standard

Table 4.26 The Effect of Enzyme Mixtures on Protoplast Yield and Viability (Experiment 2).

Treatment #	Enzyme Mixture	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	1.0% cellulase + 0.25% pectinase	9.4 a	61.1 a
2	0.5% cellulase + 0.125% pectinase	21.0 b	80.7 b
3	0.5% cellulase + 0.25% pectinase	27.0 b	79.2 b
4	1.0% cellulase + 0.10% pectolyase	26.6 b	78.8 b
Analysis of variance			
Treatment		*	ns

+ : values based on the mean of 6 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
*,ns: significant at $p < 0.05$ and not significant, respectively.

Table 4.27 The Effect of Centrifugation Speed on Protoplast Yield and Viability.

Treatment #	Centrifugation speed (x gravity)	Protoplast yield (x 10000) /g fr. wt.+	% viability+
1	100	12.3 a	55.3 a
2	60	5.8 b	54.2 a
3	45	7.4 ab	52.4 a
4	30	7.5 ab	60.5 a
Analysis of variance			
Treatment		ns	ns

+ : values based on the mean of 4 replicates.
a,b: difference between means in a column with the same letter is not significant by Duncan's Multiple Range Test at $p < 0.05$.
ns : not significant.

deviation of 11.00%, both of which are low, especially the yield. No change in the protoplasts was observed during two weeks and debris prevented evaluation with Calcofluor White fluorescence. Cell wall formation was not likely to have occurred, since the protoplasts retained their spherical shape. The effect of different media was impossible to deduce from this experiment.

Protoplasts were plated in all of the experiments and what was not destroyed by contamination failed to grow. The occurrence of contamination decreased as each improvement was incorporated into the basic protocol, to finally achieve 90% contamination-free cultures. The protoplasts never lost their characteristic spherical appearance and they swelled with age. The cultures lost their green colour, after about a week and "budding", the production of irregular rounded outgrowths from the protoplasts, was noticeable (see Figure 22).

4.7.4 Preliminary Evaluation of Protoplast Isolation from Cotyledons

The yield from the first experiment was 6.4×10^5 protoplasts per gram of cotyledon tissue and the yield from the second experiment was 8.3×10^5 per gram of cotyledon tissue. The viability was 91.67% for the first experiment and 92.57% for the second experiment. There was very little debris in the culture and the protoplasts kept their green colour for almost two weeks (see Fig. 23).

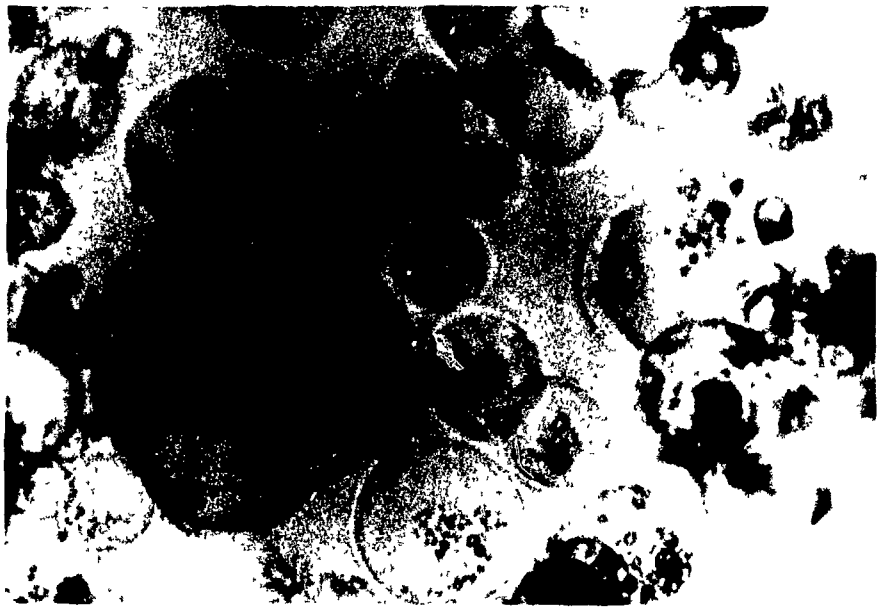

Figure 22. Protoplasts after one week of culture on Kao's medium.

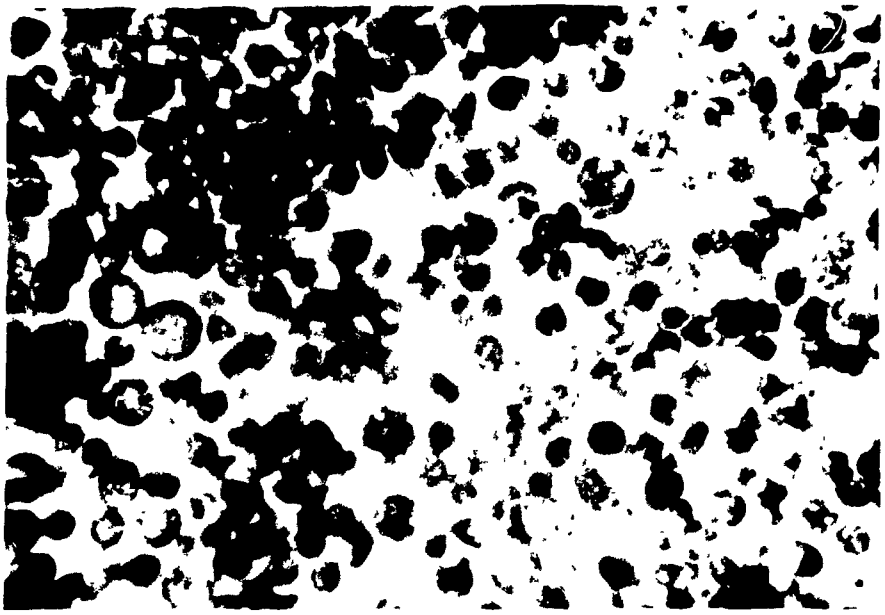

Figure 23. Protoplasts from cotyledons of *I. walleriana* "F_2 economy" mixture.

5. DISCUSSION

5.1 Crosses

The lack of fertility found in the crosses between *I. repens* and *I. walleriana* confirms the observation of Arisumi (1980a) that these two species are incompatible. The failure of the crosses within the species *I. walleriana* was surprising but can be explained by the existence of male sterility in the species. Male infertility ranges from partial to complete and is due to abnormal microsporogenesis (Tara and Namboodiri, 1976). Male fertility can be restored with the appropriate crosses and Leban and Myers (1980a,b) suggested that the phenomenon is due to a complex set of genes, of which, some are environmentally sensitive.

I. walleriana var. *variegata* is a chimeral plant, demonstrated by the formation of completely white or green shoots after a severe pruning (see Fig. 1). Stewart (1978) proposed that the generative cells arise from the L2 layer, which is white in this plant. This factor could affect the viability of both ovules or embryos. Observations of the reproductive apparatus of the plant revealed anthers that did not open and little noticeable pollen. It is unfortunate that the crosses were not successful, since the seeds and embryos were potential explant sources for both callogenesis and protoplast isolation.

5.2 Surface Sterilisation of *I. walleriana*

The semi-succulent nature of plants in the genus *Impatiens* and the presence of a great number of extra floral nectaries (Elias and Gelband, 1977), create an environment

suitable for the proliferation of micro-organisms. Surface sterilisation of both *I. walleriana* and *I. repens* was not successful with simple conventional methods. The surface sterilisation experiments demonstrated a fairly low rate of contamination but when large amounts of tissue were processed for experimental purposes the contamination rates soared. Both Arisumi (1985) and Koenisgberg (1978) observed the problem with contamination, the former losing 12% of his ovule/embryo cultures and the latter being unable to establish the species *I. linearifolia* in culture. Only the surface sterilisation technique modified from Debergh and Maene (1981), described in 3.3.3.3, fulfilled the criteria of efficacy and lack of tissue damage. This method achieved a 70% success rate of clean, healthy cultures (see Table 4.4) by leaving the petioles on the stem cutting to protect the axillary buds during the first exposure to the 10% bleach, but removing them prior to a brief second exposure to the bleach solution. It was not until the stock plants were cultured according to Debergh and Maene (1985), see section 3.1.1, that it was possible to produce about 80% clean cultures. The most important factors of this protocol involve the avoidance of overhead watering, the culture of stock plants in a controlled environment and constant pruning to promote vigorous growth. It was, however, still beneficial to screen for contamination by pre-culture of the explants prior to experimental use. The problem of internal or perhaps better described as inaccessible contamination is a phenomenon observed by many researchers, especially in tropical plants, although not specifically in *Impatiens* (Mathias and Alderson, 1987; Fisse *et al.*, 1987).

5.3 Callogenesis of *I. walleriana*

Callogenesis has been observed to occur in a wide range of species and from many different explants (Evans *et al.*, 1981), so lack of callus formation in *I. walleriana* is unusual. This phenomenon was observed by both Stephens (1985) with seedling explants of *I. walleriana* and by Koenisgberg (1978) with a New Guinea hybrid, although both workers were using hormone levels conducive to adventitious shoot proliferation. They both found copious numbers of roots formed in their cultures, exactly what characterised any attempt at callogenesis in this study. Both the strongest and the weakest auxins, 2,4-D and IAA (Murashige, 1974) were used within and encompassing the range of levels suggested for callus production (Constabel, 1984). Cytokinins, often beneficial to the formation of callus, were included in the experimental media but they only caused inhibition of root growth at early stages of growth. Koenigsberg (1978) reported the highest amount of rooting of internode sections with 10 mg/L kinetin and 0.1 mg/L NAA. Cytokinins are known to be detrimental to root growth in many cases, but not to *Impatiens* and auxins are generally included at low levels to encourage root formation (George and Sherrington, 1984)

The stem sections did not require growth regulators for root formation but the leaf sections generally did not survive without growth regulators, either auxins or cytokinins. At the higher levels of 2,4-D root growth was abnormal, so the response of the tissue sections was definitely being affected by the presence of the plant growth regulators in the culture media. It is possible that the growth regulators, especially the cytokinins were

complexed with the agar and became unavailable to the explant, after a certain period in culture (Bornmann and Vogelmann, 1984). Kinetin could have been causing the endogenous production of auxins, which would have increased root production (Slabnik *et al*, 1986). Also the degradation of plant growth regulators due to heat and light might explain the disappearance of the inhibition of rooting by kinetin.

The seedling stem sections rooted equally as well with or without light, however they did not produce callus. Rooting has been observed to occur without light on pea epicotyls (Nougarede *et al.*, 1987) and under the normal culture conditions of low light (Debergh and Maene, 1981). Tissue sections can be incubated with or without light to induce callus growth (Constabel, 1984).

Bhojwani and Rhazdan (1983) suggested that different levels of sucrose and other carbohydrate sources be tested in the range of 20 to 50%. Various species of plants have been observed to perform better with different carbohydrate sources or concentrations (Chong and Pua, 1985; Hew and Mah, 1989). Since different growth regulators did not induce callus production, sucrose concentrations in the range of 10 to 50% were tested, as well as the molar equivalents of glucose in MS and B5 media. Callus was eventually produced but the induction period was so long that it would not have been a reasonable system for a protoplast donor tissue source. The callus has quite an unhealthy appearance but callus with a similar description, from ovule cultures, was induced to produce shoots (Arisumi, 1985). This callus also took a long period of time to develop. Perhaps the survival and growth of the axillary buds, in this experiment was so

low, due to the harsh nature of the surface sterilisation technique used prior to adapting the method from Debergh and Maene (1981), although Warren-Wilson and co-workers (1986) found that up to 30 minutes in 10% bleach did not interfere with the formation of the abscission layer in *I. walleriana* internode cultures.

5.4 Micropropagation of *I. walleriana*

The three basal media compared for this species are among the media generally recommended for micropropagation and no statistically significant differences were observed in the effects of these media on the growth parameters of the explant. Neither the half strength LS or MS media improved culture growth but they were not inhibitory. These results are the means from the statistical analyses, but fluctuations within the individual experiments, possibly obscured by the high variability among the replicates, suggest that there may actually be an interaction between growth regulator ratios and media concentrations. Koenigsberg (1978) found that half strength LS media improved the growth of two New Guinea Impatiens hybrids. Posa and co-workers (1987) found that *Pogostemon cablin* proliferated well on half strength MS medium.

BAP alone was observed to cause shoot proliferation of *I. walleriana* at a fairly low rate and low shoot height, so different growth regulators and ratios were evaluated. The second micropropagation experiment, section 3.3.5.2., showed that the shoot proliferation rate increased with the increasing cytokinin to auxin ratio, especially with IBA and BAP. Stephens and co-workers (1985) found that NAA and kinetin were effective for the proliferation of several New

Guinea Impatiens, so this growth regulator combination was tested with *I. walleriana*. The highest rate of shoot proliferation per month was with 0.1 mg/L NAA and 30 mg/L kinetin, which is similar to the results with the New Guinea Impatiens. A relatively high rate of kinetin was required for shoot proliferation, but not NAA. Two tenths of a mg per litre NAA was found to be inhibitory to shoot proliferation for *I. walleriana*, whereas 0.1 mg/L was inhibitory for the New Guinea hybrids (Stephens *et al.*, 1985). The rate of shoot proliferation was lower for *I. walleriana* than for the New Guinea Impatiens, probably due to the lower light intensity used for this project (80 $\mu mol/m^2/sec$ versus 27 $mol/m^2/sec$). Kinetin inhibition of shoot lengthening was observed in both experiments. This phenomenon was recorded by Stephens *et al.*(1985) with Impatiens and for *Prunus* by Baraldi *et al* (1988).

The rate of proliferation could still have been improved so the effect of the growth regulators IBA and BAP was evaluated on shoot number, shoot height and total shoot fresh weight. In the first experiment, section 3.3.5.5, shoot multiplication increased with increasing cytokinin levels, because the most effective range of BAP was not used but was subsequently tested in the next trial. Shoot proliferation, in the second experiment, section 3.3.5.6, was high at 5.0 mg/L BAP for the first three levels of IBA but the highest level of IBA (3.0 mg/L) limited proliferation. Inhibition of shoot height was not observed in the first experiment, probably because the evaluation was done after eight weeks of growth. In the second experiment shoot height decreased significantly with the increasing level of BAP, an effect noticed by Han and Stephens (1987).

The curvilinear effect of IBA on mean shoot height is due to the fact that at the lowest level the shoots were taller and at the highest level there was more likely to be a single shoot, which was the original explant, and therefore taller than any new shoots.

Total shoot weight is a reflection of both the shoot number and the shoot height. Single shoots grew significantly without IBA, so there was a high culture weight. The highest level of IBA overcame the inhibition of BAP on shoot height and limited shoot proliferation, so the cultures were taller and therefore weighed more. Koenigsberg (1978) observed this increase of shoot fresh weight, with high levels of kinetin which was probably associated with the increased shoot proliferation rate. He did not include high levels of auxin in his experiment. Rooting was observed on the lowest levels of both BAP and IBA in the two experiments and could have biased the results, since the rooted plantlets grew vigorously. The vigorous growth was probably due to the greater access by the roots, for the plantlets, to nutrients throughout the medium.

Ideally one would like to find an auxin-cytokinin combination that would cause shoot proliferation, with shoots tall enough to move onto the rooting and acclimation stage of tissue culture (de Fossard, 1986). The shoots produced in the previous experiments were usually less than one centimetre tall, if there was active proliferation. The natural auxin, IAA, which is often used for axillary shoot propagation, was included in the last experiment to determine if there was any effect on shoot height (Murashige, 1974). Instead IAA had no effect on any of the

growth parameters other than total culture weight, which was probably due to a slight tendency for rooting at the highest level of IAA. This experiment included levels of BAP that had not previously been tested within the range of 3 to 8 mg/L and it can be concluded that the optimum for shoot proliferation with BAP is 5.0 to 7.5 mg/L (see Table 4.15). This is above the usual range of 1 to 3 mg/L BAP, although concentrations can go as high as 10 mg/L (Zimmerman, 1983). Shoots cultured with 2iP and IAA had a lower rate of multiplication but the propagules were taller, an observation worth pursuing. Han and Stephens (1987) found this combination to be the most effective for one of the New Guinea hybrids that was tested.

The requirement of *Impatiens* for fairly high levels of cytokinins and the lack of response to or inhibition by auxins has been observed in studies on New Guinea species (Stephens *et al.*, 1985; Han and Stephens, 1987) This apparent insensitivity of *Impatiens* to growth regulators is also noticeable in their lack of reaction to the growth retardants ancymidol, chloremquat and SADH (succinic acid-2, 2-dimethyl hydrazide), used on bedding plants (Murray *et al.*, 1986; Pasutti and Weigle, 1980b). Proliferation with the cytokinins, albeit low, is due to axillary shoot propagation. The maximum proliferation observed was three shoots per eight weeks on 0.1 mg/L NAA and 30 mg/L kinetin, well below the expected five to ten shoots in four to eight weeks (Hussey, 1983). If any other form of proliferation was occurring the chimeral nature of *I. walleriana* var. *variegata* would not have been maintained. Maintenance of the genetic integrity is obvious from Figures 13 and 14 and is characteristic of axillary bud propagation (Hussey, 1984).

The fact that *I. walleriana* propagates very easily by cuttings, which root well in water, and the presence of roots throughout all the culture phases, indicated that rooting should not have been a problem. As expected rooting and acclimatization were not problematic, a routine procedure after embryo rescue of *Impatiens* (Arisumi, 1985) which can occur spontaneously in the micropropagation of herbaceous ornamentals (Hughes, 1981).

5.5 Protoplast Isolation

The first experimental efforts to isolate protoplasts with *I. walleriana* var. *variegata* were an indication of the challenge to follow. Raphids are characteristic of the genus *Impatiens* and are found throughout the plant (Esau, 1976). Gibbs (1974) reported mucous cells in almost fifty of the species and the presence of extra-floral nectaries has been observed in equally as many species (Elias and Gelband, 1977). These factors contribute to the consistency of the protoplast suspension and are probably detrimental to protoplasts, especially the raphids, which look like needles capable of piercing protoplasts. No technique evaluated was able to completely eliminate the raphids or the mucous, which were present even in *vitro* grown donor leaves. No reports in the literature have been found that described protoplast isolation from a plant species with raphids and/or mucous. Wolfson and Sears (1989) reported that the culture of *Oenothera* meristems on a low calcium medium significantly decreased the amount of raphids and allowed the isolation of intact chloroplasts, a technique worth investigating.

The cuticle of the *Impatiens* is very waxy and repels liquids, implying that for protoplast isolation, the enzymes would have had to have been quite concentrated and diverse. A long incubation period would have been required and vacuum infiltration of the enzyme solution into the leaves would have been necessary. Preliminary experiments revealed that this was quite to the contrary of the actual situation. The protoplasts were really quite fragile, and were sensitive to Rhozyme, Driselase and high levels of pectinase, none of which digested the mucous. Vacuum infiltration and long incubations were detrimental to the protoplasts and destroyed them in the first experiments.

The protoplasts of *I. walleriana* were stable in solutions containing from 0.4 to 0.6 M mannitol, well within the observed range of osmotic levels for protoplast isolation (Gamborg, 1976). There was no apparent differences in the reaction to any of the commonly used osmotic stabilizers, mannitol, glucose or sorbitol. Thus a 0.5 M combination of mannitol, metabolically inactive, and glucose, metabolically active, was selected for the enzyme mixture and culture media, to allow a gradual decrease of the osmotic level as the glucose was metabolized (Vasil and Vasil, 1985). Dark pretreatment and preplasmolysis of the donor tissue proved beneficial for the yield and viability of the protoplasts, agreeing with the study on eggplant by Saxena and co-workers (1987). The most useful achievement of the preliminary experiments was the effectiveness of the method of separation that involved removal of the intact, but digested leaves from the enzyme for washing. The digested leaves were carefully transferred, with fine forceps, to a small funnel with a nylon mesh filter and

teased apart to release the protoplasts which were then washed through with CPW. This technique enabled the removal of a significant proportion of the raphids and mucous.

The protoplasts did not float in 20% sucrose in CPW, nor did they float on 20% sucrose or 10% Ficoll, due to the viscous nature of the suspension. However, flotation was effective on the higher concentrations of sucrose, Ficoll and the Ficoll/sodium metrizoate mixture (Lymphoprep), since these solutions were dense enough to support the protoplast suspension. The bands at the interface of these solutions were more distinct for the latter treatments but the protoplasts were sensitive to these chemicals. These floatation treatments decreased the amount of debris, somewhat, but also decreased the yield of protoplasts and were not appropriate for inclusion in the protocol. This is contrary to the success that Davey and co-workers (1974) reported with flotation on sucrose. Larkin (1976) found that Lymphoprep was superior to Ficoll for flotation of protoplasts and gave a high yield of viable protoplasts. If the protoplast suspension from *I. walleriana* was less viscous then the results may have been comparable. Slabas and co-workers (1980) observed a similar problem with carrot suspension protoplasts that would not float predictably in sucrose, so they developed a two-phase system for protoplast purification.

Quantification of the protoplast yield and viability enabled more specific evaluation of the isolation parameters. Since contamination was a significant problem, surface sterilisation techniques had to be optimized that were sufficiently effective but non-detrimental to the tissue. Both bleach and ethanol are known to be phytotoxic,

a factor that must be evaluated before incorporation of the procedure into the protocol (Potrykus and Shillito, 1988). The protoplasts of *I. walleriana* were sensitive to ethanol, but not to bleach. The fifteen minute exposure to bleach had the greatest yield even when compared to no surface sterilisation, so possibly the bleach aids in the break down of the waxy cuticle. Even the most intense treatments were not completely effective and contamination affected at least half of the experiments. After the technique of holding the leaves in the bleach was developed and Garamycin included in the solutions, about 90% of the experiments were free of contamination.

Several tests for the appropriate enzyme recipe revealed that 1% w/v cellulase and 0.25% w/v pectinase or w/v 0.1% pectolyase was the most effective for a 12 to 14 hour incubation and 0.5 % w/v cellulase with 0.25% w/v pectinase for a 16 to 18 hour incubation. These mixtures are in the low range of concentrations recommended for protoplast isolation (Gamborg, 1976). Although the yield of protoplasts increased with the incubation time and the initial viability was still high, minimizing the exposure of the protoplasts to the enzymes is recommended. Pectolyase, although effective, is known to decrease the plating efficiency of protoplasts, unless the enzyme is used at very low percentages (Potrykus and Shillito, 1988). The latter recipe is excellent for an overnight incubation at room temperature.

As mentioned above the pretreatment of the donor tissue improved protoplast yield and viability. The best pretreatment or source of tissue is from *in vitro* grown plants, either from seedlings or shoots. Lee and Wetzstein

(1988) found this to be the case with grapes as well. The suspensions have less mucous, fewer raphids and less debris, although there were still crumpled protoplasts and chloroplasts. This source of donor tissue was not included in the protocol because preparation took at least eight weeks, required a greater number of plants for sufficient donor tissue and had specific isolation criteria. Although the yield was higher, the leaves were smaller and the plants could not be reused as tissue sources.

Mature leaves were the poorest donor tissue with a viability of 46% (see table 4.21), but juvenile leaves of any size produced significant yields of protoplasts (up to 2.75×10^4 per gram fresh weight of leaf tissue). Robertson and co-workers (1988) studied the effect of leaf age and showed that young or juvenile leaves yielded protoplasts with a high rate of division. Nylon mesh filters were used to separate the protoplasts from the undigested tissue and larger debris. Ideally a mesh size must be selected that is just larger than the protoplasts. Those of *I. walleriana* ranged from 10 to 35 µm, similar to those of *Brassica juncea* (Chatterjee *et al.*, 1985) but smaller than those of olive (Canas *et al.*, 1987). There were no significant differences between the yields from the various mesh sizes, but there was less debris with both the 44 and 62 µm filters. The 62 µm mesh was selected since the 44 µm mesh was quite close to the protoplast size and other trials had lower yields from that filter. Glass wool was also tested as a filter material, but there was no less debris and it is difficult to ascertain that the same thickness was used for each funnel, adding an unnecessary source of variation.

Comparison of centrifugation speeds was done to find a

speed that pelleted the maximum number of protoplasts, while crushing the fewest. There was little variation among the different spins, largely due to the difficulty of stabilizing the centrifuge at low speeds. The protoplast yield decreased with the centrifugation speeds but the amounts of the debris and raphids were unchanged, so 100 x g spin was considered optimum, which is the speed recommended in most general protocols (Dodds and Roberts, 1982).

Gravitational sedimentation of the protoplast suspension was effective but there is a trade-off between oxygen starvation from standing in solution for too long and the production of uncrushed protoplasts. The viscosity of each suspension varied, so this procedure was unpredictable and time-consuming. Davey and co-workers (1974) found that gravitational sedimentation was a very effective protoplast cleaning method. Another method to prevent crushing of protoplasts in the pellet is to use rounded centrifuge tubes. The pellet formed in these tubes was less cohesive and therefore more difficult to handle, although the yield was good, this treatment was not included in the general protocol.

Quantification of protoplast yield and viability is difficult to achieve in a statistically sound fashion, due the problems with handling several replications of several treatments at the same time. The second approach to quantification of protoplast techniques was designed to process small, identical volumes of leaf tissue separately, so as to include enough replications for statistical significance. However the use of one leaf per replication will cause the loss of the replication if the leaf is not suitable for protoplast isolation. This technique is quite

applicable for media and growth regulator optimization, since the protoplasts from several leaf disks can be pooled and then divided among treatments. The problem of variability of results is a long-known feature that Ochatt and Caso (1986) demonstrated with the observation of up to 50% variability between assays, which is why data from protoplast experiments are usually only expressed with standard deviation or standard error.

Even the highest yield from these experiments, 2.70 x 10^5 per gram fresh weight of leaf tissue was lower than the range of yields from 1.3 to 6.9 x 10^6 per gram fresh weight of leaf tissue produced from *Dimorphotheca* and *Rudbeckia* by Al-Atabee and Power (1987) or yields such as 1.97 to 6.00 x 10^6 per gram of leaf tissue from clover and lotus (Webb *et al.*, 1987).

5.6 Protoplast Viability

Protoplast viability generally varied very little within each experiment, unless there was a significantly deleterious treatment and is comparable to viabilities observed for *Dimorphotheca* and *Rudbeckia* (Al-Atabee and Power, 1987). This parameter is more an indication of the condition of the stock plants and the execution of the general protocol for that specific experiment. If, for example, the plants had been slightly underwatered or the protoplasts were allowed to sit too long in suspension, a lower viability was observed. For instance, experiment three in Table 4.27, had such low viability, that the results of the experiment are questionable. These results could be due to one of the above mentioned problems or any one of numerous other factors. Both the yield and the

viability of experiment four, section 4.7.3.4 were low, probably due to aging plants or the new lights in the growth chamber that increased the light intensity to an unacceptable brilliance for the shade-loving *Impatiens*.

5.7 Protoplast Culture

The lack of successful growth and development in culture can be attributed to many different reasons. The protoplasts had to be handled quite roughly to separate them from the tissue matrix and the mucous. Centrifugation with raphids and debris could have caused piercing and crushing of the protoplasts, although after all this abuse viability of the protoplasts was still reasonably high. The low culture density, with the interference of debris was not propitious for protoplast survival, as well as the fact that the protoplasts all settled at the centre of the media drop with the debris and smothered themselves. Culture contamination was an ongoing problem, that became less significant during the experimental period. Shahin (1985) found that isolation and culture of tomato protoplasts from greenhouse and growth chamber grown plants was impossible due to the amount of debris present and the variable quality of the protoplasts. For successful culture to occur a method of eliminating the debris must be developed.

6. CONCLUSIONS

The crosses performed between *I. walleriana* and *I. repens* and within the former species were unsuccessful. Surface sterilisation of tissue of *I. walleriana* was impossible unless the plants were healthy and growing vigorously. The most successful technique involved a two-phase surface sterilisation procedure in which the petioles protected the axillary buds from the full strength of the exposure to bleach. Pre-experimental screening of the shoot tips on media without growth regulators provided clean, healthy axillary buds for growth regulator trials.

Callogenesis was not observed in *I. walleriana* with any plant growth regulator combination. Instead, prolific root formation occurred on the stem and leaf sections.

The highest observed shoot proliferation rate was from MS media with 3 mg/L BAP and 0.1 mg/L IBA, which occurred in the second experiment, Table 4.10. Shoot proliferation was also high with 30 mg/L kinetin and 0.1 mg/L NAA in MS media, 20 mg/L kinetin, 10 mg/L BAP with any level of IBA that was tested (0 to 1.0 mg/L), 5.0 mg/L BAP with three of the tested levels of IBA (0, 0.03, 0.3 mg/L), the fourth level, 3.0 mg/L was inhibitory, and 6.25 and 7.0 mg/L BAP with either 0, 0.017 or 0.17 mg/L IAA. Other than the second experiment, the tallest proliferating shoots were with the IAA treatments. The highest rate of propagation was 3.0 shoots in eight weeks and 2.7 shoots in 6 weeks for the highest BAP treatments in experiment 7, Table 4.15. This is a very slow rate of proliferation with short shoots.

The protoplast isolation procedure was optimized to a

24 hour dark pretreatment, one hour preplasmolysis in CPW with 0.3 M mannitol and 0.3 M glucose, a short incubation (12 to 14 hours) in 1.0% w/v cellulase Onazuka R10^{R} and 0.25% w/v pectinase Macerase^{R} or a longer incubation in 0.5% w/v cellulase and 0.25% w/v pectinase, in CPW with 0.25 M mannitol and 0.25 M glucose. After incubation the leaves are lifted into a small funnel with 62 µm nylon mesh filter, sitting in a small erlenmyer flask. The protoplasts are washed out of the tissue matrix with a five times the enzyme volume dilution with CPW containing 0.25 M mannitol and 0.25 M glucose and the suspension pelleted at 100 x g for four minutes. The pellet is resuspended in Kao's media amended with 0.1 mg/L BAP, 1.0 mg/L 2,4-D and 1.0 mg/L NAA and the osmotic regulators described above and pelleted once more to complete the washing of the protoplasts. The average yield ranged from 3.3 x 10^{4} to 2.70 x 10^{5} per gram of leaf tissue with viabilities ranging from 16.4% to 93.1% (Fig. 24).

Culture of the leaf mesophyll protoplasts was not successful, due to the debris, raphids and mucous present in the petri dishes, which could not be entirely eliminated. *In vitro* grown donor tissue had a much higher yield of protoplasts, but was not entirely free from debris and raphids. Protoplasts isolated from cotyledons had a high viability and no mucous, raphids or debris were present.

A few more growth regulator trials could be carried out to evaluate the possibilities of micropropagation with 2iP and IAA. The protoplast procedure should be optimized, both with *in vitro* shoots and cotyledons. Parameters for successful protoplast culture must be established.

The fulfilment of the conservative goals is described above, except for the unsuccessful culture of the

protoplasts. The ultimate goals of the project can be attained by fusing cotyledon generated protoplasts of *I. repens* with protoplasts from albino shoots of the chimeral *I. walleriana* grown *in vitro* on a low calcium medium to prevent raphid production (Wolfson and Sears, 1989), without growth regulators, to produce a plant with leaves large enough for protoplast isolation. These shoots would come from stock cultures maintained on MS medium with one of the plant growth regulator combinations described above, that is conducive to shoot proliferation. The heterokaryons would be selected visually and then manually transferred to specialized culture conditions for the growth and regeneration of the hybrids.

An alternative to the use of somatic cell hybridization for the transfer of yellow pigment, is mutagenesis. The preliminary parameters for both chemical and physical mutagens have been established for *I. balsamina* (Bose and Basu, 1967; Klozova, 1962). Spontaneous mutations have been observed in the genus and a dwarf mutant was induced in *I. platypetala* (Simmonds, 1983; Weigle and Butler, 1983). Mutagenesis has been successfully used in vegetatively propagated ornamental crops, such as carnation and chrysanthemum to produce different flower colours (FAO/IAEA, 1989).

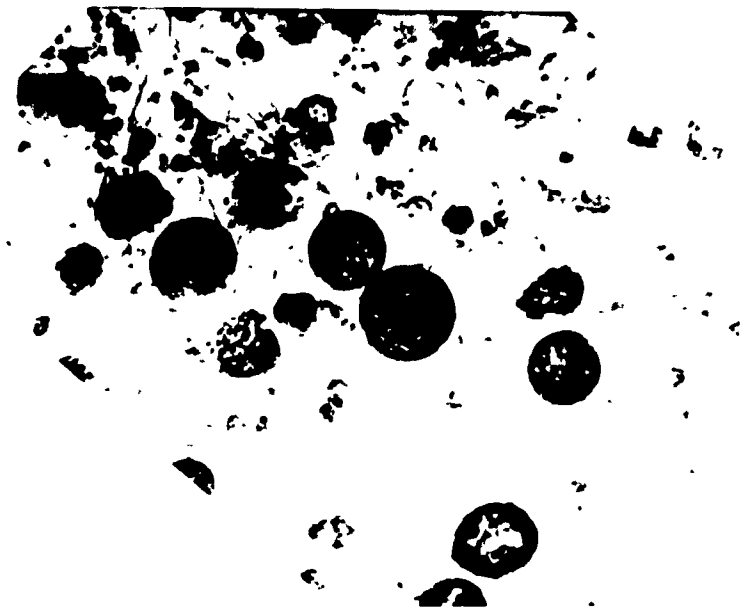

Figure 24. Protoplasts of *Impatiens walleriana* var. *variegata*, isolated with the optimized protocol.

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APPENDIX 1

Impatiens Chromosome Numbers and Native Country

Species	Chromosome Number		Locality	Reference
	n	2n		
I. acamanthera Hook.f.	-	20	Himalayas	5
I. auricoma Baill.	-	16	Africa	5
I. balsamina L.	7	14	India	5
I. campanulata Wt.	10	20	India	5
I. capensis Meerb.	-	20	N. America	5
I. dalzellii Hook.f. et T.	-	16	India	5
I. epiphytica G.M. Schultz	-	16	Africa	1
I. flaccida Arn.	7	14	India	3
I. flaccida Arn. var. *alba*	7	14	India	5
I. gordonii Horne ex Bak.	8	16	Africa	5
I. marianae Rchb.f. ex Hook.f.	-	16	Africa	1
I. mirabilis Hook.f.	17	-	Malaya	3
I. niamniamensis Gilg.	16	32	Africa	5
I. nolitangere L.	10	20	N. Europe	2
I. oncidoides Ridley ex. Hood	-	-	Malaya	
I. pallida Nutt.	-	20	N. Amer.	5
I. platypetala Lindl.	7	14	Java	5
I. platypetala var. *aurantiaca* Steen	-	8	Celebes	1
I. repens Moon	7	14		5
I. scabrida DC.	7	14	Himalayas	3
I. sodenii Warb. et Engl. ex Engl.	8	16	Africa	5
I. tuberifera Humbert.	-	16	Africa	1
I. usambarensis Grey-Wilson	-	16	Africa	5
I. verticillata Wight	-	16	India	4
I. walleriana Hook.f.	8	16	Africa	5

Appendix 1 - continued

Synonyms: *I. biflora* Walt. = *I. capensis*
I. holstii Engl. et Warb. = *I. walleriana*
I. petersiana Gilg. ex Grig. = *I. walleriana*
I. sultanii Hook.f. = *I. walleriana*
I. thomasettii Hook.f. = *I. gordonii*
I. uguenensis Warb. = *I. sodenii*

References: 1) Arisumi, 1980.
2) Jones and Smith, 1966.
3) Chinappa and Gill, 1974.
4) Shimizu, 1984.
5) Zinov'eva-Stahevitch, 1984.

APPENDIX 2

LIST OF MICRO MOLAR EQUIVALENTS

GROWTH REGULATOR	mg/L	uM
AUXINS		
2,4-D	1	4.52
	2	9.05
	4	18.10
	8	36.19
IAA	0.017	0.1
	0.17	1.0
	0.5	2.856
	1.0	5.71
	2.0	11.42
IBA	0.01	0.05
	0.03	0.15
	0.10	0.49
	0.30	1.48
	0.50	2.46
	1.0	4.92
	2.0	9.84
	3.0	14.76
NAA	0.1	0.54
	0.2	1.07
	0.5	2.68
	1.0	5.37
	2.0	10.74
CYTOKININS		
BAP	0.1	0.44
	0.5	2.22
	1.0	4.44
	2.0	8.88
	2.5	11.10
	3.0	13.32
	3.5	15.54
	4.0	17.76

List of Micro Molar Equivalents - cont'd

GROWTH REGULATOR	mg/L	uM
CYTOKININS		
BAP	5.0	22.20
	6.25	27.75
	7.5	33.30
	10	44.41
2IP	1.0	4.92
	8.13	39.99
KINETIN	0.3	1.39
	1.0	4.65
	2.0	9.29
	3.0	13.94
	4.0	18.59
	5.0	23.23
	8.0	37.17
	20	92.93
	30	139.40
ZEATIN	1.0	4.56

GROWTH REGULATOR	1 M (g)	1 uM (mg)
2,4-D	221.04	0.22104
IAA	175.18	0.17518
IBA	203.23	0.20323
NAA	186.20	0.18620
BAP	225.20	0.22520
2IP	203.30	0.20330
KINETIN	215.21	0.21521
ZEATIN	219.20	0.21920

from Bhojwani and Razdan, 1983.

APPENDIX 3

Media Composition - Tissue Culture

Chemical	MS(1) (mg/l)	LS(2) (mg/l)	B5(3) (mg/l)
Macronutrients			
NH_4NO_3	1650	1650	----
KNO_3	1900	1900	2500
$CaCl_2 \cdot H_2O$	440	440	150
KH_2PO_4	170	170	----
$NaH_2PO_4 \cdot H_2O$	----	----	150
$MgSO_4 \cdot 7H_2O$	370	370	250
Micronutrients			
H_3BO_3	6.2	6.2	3.0
$MnSO_4 \cdot H_2O$	15.6	15.6	10
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	2.0
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
KI	0.83	0.83	0.75
FeNaEDTA	43.0	43.0	43.0
Vitamins			
Inositol	100	100	100
Nicotinic acid	0.5	---	1.0
Pyridoxine·HCL	0.5	---	1.0
Thiamine·HCL	0.1	0.4	10.0
Sucrose	30000	30000	20000
T. C. Agar	8000	8000	8000
pH	5.7	5.7	5.5

Appendix 3 - cont'd

References:

1. Murashige and Skoog, 1962.
2. Linsmaier and Skoog, 1965.
3. Gamborg *et al.*, 1968.

APPENDIX 4

Media Composition* - Plant Protoplast Culture

Chemical	Kao's(1)	NT(2) (mg/l)	UM(3)
Macronutrients			
NH_4NO_3	600	825	1.650
KNO_3	1900	950	1.900
$CaCl_2 \cdot 2H_2O$	600	220	0.440
$MgSO_4 \cdot 7H_2O$	300	1233	0.370
KH_2PO_4	170	680	0.170
KCl	300		
FeNaEDTA	43	43	43
Micronutrients			
KI	0.75	0.83	0.83
H_3BO_3	3.00	6.2	6.2
$MnSO_4 \cdot H_2O$	10.00	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	2.00	8.6	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.030	
$CuSO_4 \cdot 7H_2O$			0.025
Vitamins			
Inositol	100	100	100
Thiamine·HCl	10	1	10
Ascorbic acid	1		
Nicotinamide	1		5
Pyridoxine·HCl	1		10
Calcium pantothenate	0.5		
Choline chloride	0.5		
Folic acid	0.2		
Riboflavin	0.1		
p-Aminobenzoic acid	0.01		
Vitamin B_{12}	0.01		
Vitamin A	0.005		
Vitamin D_3	0.005		
Biotin	0.005		

Appendix 4 - cont'd

Chemical	Kao's (1)	NT (2) (mg/ml)	UM (3)
Sugars			
Glucose	68400		
Sucrose	125	10	15000
Fructose	125		
Ribose	125		
Xylose	125		
Mannose	125		
Rhamnose	125		
Cellobiose	125		
Sorbitol	125		
Mannitol	125		
Organic acids			
Sodium pyruvate	5		
Citric acid	10		
Malic acid	10		
Fumaric acid	10		
Miscellaneous			
Vitamin-free casamino acid	125		
Coconut water	10		

* All preparations were filter-sterilized.

References:

1. Kao and Michayluk, 1975.
2. Nagata and Takebe, 1971.
3. Uchimiya and Murashige, 1974.