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TITLE OF THESIS/TITRE DE LA THÈSE Somatic and sexual hybridization of
Nicotiana rustica with Nicotiana tabacum.

UNIVERSITY/UNIVERSITÉ Carleton

DEGREE FOR WHICH THESIS WAS PRESENTED /
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Ph.D.

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE 1980

NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. G. Setterfield and Dr. W.A. Keller

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SOMATIC AND SEXUAL HYBRIDIZATION
OF
NICOTIANA RUSTICA WITH NICOTIANA TABACUM

by

C

GERARD CHRISTOPHER DOUGLAS, B.Agr.Sc.(Hort.)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology
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September, 1980

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He who binds to himself a joy
Does the winged life destroy;
But he who kisses the joy as it flies
Lives in eternity's sun rise.

William Blake
(Eternity)

Progress is the exploration of our own error.
Evolution is a consolidation of what have
always begun as errors.

Jacob Bronowski
(The Origins of Knowledge
and Imagination)

TO

GERARDINE

ABSTRACT

Cell suspension cultures of chlorophyll-deficient mutants of Nicotiana rustica and N. tabacum were used as a source of protoplasts for somatic hybridization. Somatic hybrid cells were selected after genetic complementation for chlorophyll synthesis.

Viable protoplasts were readily isolated from cell suspension cultures of chlorophyll-deficient mutants of each species. Protoplast yield was greater and quality superior when cells were grown on medium containing 2,4-D rather than NAA. Plating efficiencies of 60-80% were achieved in protoplast cultures of both species. Protoplast- and suspension-derived cell colonies formed shoots in the presence of cytokinin with the frequency of shoot formation in the latter being greatest. On shoot-induction medium, suspension-derived cells grown with 2,4-D had a higher morphogenic capacity than those grown with NAA. For both species, morphogenic capacity declined with increasing culture age. The chlorophyll-deficient phenotype was evident in cell and protoplast cultures of both species.

Mutant protoplasts of both species were fused using polyethylene glycol (PEG) and subsequently cultured, yielding cell-colonies. As a result of genetic complementation,

chlorophyll synthesis occurred only in somatic hybrid cells as early as 17 days after fusion. Calcium was essential in the PEG solution for hybrid colony development and the addition of DMSO at optimal concentration of 2% (v/v) increased the frequency of hybrid production.

Somatic hybrid colonies showed enhanced morphogenic capacity with over 65% forming shoots on osmoticum-free medium. Rooting capacity was generally low, varied amongst hybrid lines and appeared to be genetically determined.

N. rustica x N. tabacum sexual hybrids were produced readily by culturing immature seeds 3 days after pollination on medium with 4% sucrose.

Somatic hybrids showed a range of morphological, cytological and biochemical phenotypes whereas sexual hybrids were relatively uniform. Restoration of chlorophyll synthesis was observed in all hybrid plants. A heterosis effect for vigor and percent total alkaloid was observed in most somatic hybrid and in all sexual hybrid plants. Zymograms for esterase, aspartate aminotransferase, and superoxide dismutase showed the presence of parental isozymes in both types of hybrid. New isozymes not occurring in the parents as well as absence of certain parental isozymes were also observed in hybrid zymograms. For aspartate aminotransferase a unique hybrid band was found in 9 out of 10 somatic hybrids but not in any of 10 sexual hybrids.

The morphological and biochemical variability in somatic hybrids and relative uniformity in sexual hybrids appears to be related to chromosome number. The chromosome complement of 15 somatic hybrids ranged from 68-96 amongst lines and from 68-92 within lines. For sexual hybrids the $2n$ complement was 48 in 14 plants, 47 in 1, 49 in 1, and highly variable amongst the cells of each of 2 plants.

Ribulose-1-5-bisphosphate carboxylase/oxygenase of 14 somatic hybrids, 2 sexual hybrids and parental species was isolated by immunoprecipitation and subjected to isoelectric focussing. The nuclear-encoded small subunit of the enzyme showed polypeptide bands derived from both parents in all hybrids. The banding pattern for the chloroplast-encoded large subunit was characteristic of the maternal (*N. rustica*) parent in sexual hybrids. In somatic hybrids, the large subunit bands were characteristic of only one species with 11 expressing the chloroplast genome of *N. rustica* and three expressing that of *N. tabacum*.

All sexual hybrids were self-sterile while two somatic hybrids were self-fertile. All hybrids were fertile when used as female parents in backcrosses.

Somatic hybridization was successful in overcoming both prezygotic and post-zygotic incompatibility barriers which normally isolate these species.

ACKNOWLEDGEMENTS

It has been a privilege to study and to carry out this research under the supervision of Professor George Setterfield and Dr. Wilf Keller. I am sincerely grateful to Dr. Keller for his untiring interest, encouragement and assistance during all stages of this study and for the opportunity to use the excellent facilities of his laboratory and those of the Research Branch of Agriculture Canada. I am thankful to Professor Setterfield for his support and guidance throughout the experimental studies and for his helpful advice, particularly in writing this manuscript.

I thank Dr. Leslie Wetter for the opportunity to work in his laboratory in Saskatoon and for sharing his knowledge of biochemistry. Thanks are due to Dr. Chiharu Nakamura for assistance in cytological analyses and to Dr. Radhey Pandeya for performing the alkaloid analyses. Thanks are also due to Drs. Armstrong, Andrews, Murray, and Fedak and to Miss Craig for their encouragement and interest.

I thank Mrs. Mona Gibson for typing this manuscript with such care and expediency. I also thank Mrs. Nancy Long for her assistance in proof-reading the manuscript and in preparing the photographs.

The financial assistance of the School of Graduate Studies at Carleton University is gratefully acknowledged.

TABLE OF CONTENTS

	PAGE
ABSTRACT	v
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS.	ix
LIST OF TABLES	xii
LIST OF FIGURES.	xv
INTRODUCTION	1
LITERATURE REVIEW.	3
A. The Isolation of Protoplasts; their Properties and Culture.	3
1. Historical perspective.	3
2. Sources of protoplasts.	4
3. Conditions for isolation of protoplasts	7
4. Properties of protoplasts	13
(a) Surface properties and fusion of protoplasts.	13
(b) Metabolic properties of isolated protoplasts.	16
5. Culture of protoplasts.	18
6. Morphogenic potential of protoplast-derived cell cultures	29
B. Somatic Hybridization	32
1. Historical perspective.	32
2. Selection of somatic hybrids.	33
3. Characterization of somatic hybrids	36
(a) Morphological characterization	36
(b) Cytological characterization	38
(c) Biochemical and genetic characterization	40
C. Sexual Hybridization by Culturing Hybrid Embryos In Vitro.	43
1. Historical perspective.	43
2. Culture requirements of embryos	44
3. Culture of <u>Nicotiana</u> embryos <u>in vitro</u>	47

	PAGE
MATERIALS AND METHODS.	49
A. Somatic Hybridization Studies	50
1. Plant material.	50
2. Initiation of callus and suspension cultures.	50
3. Studies on morphogenesis.	50
4. Isolation of protoplasts.	51
5. Culture of protoplasts.	52
6. Fusion of protoplasts and regeneration of hybrid plants.	53
B. Sexual Hybridization Studies.	56
1. Plant material and method of pollination. .	56
2. Culture of sexual hybrid embryos.	57
C. Characterization of Somatic and Sexual Hybrids. .	58
1. Morphological analysis.	58
2. Cytological analysis.	59
3. Analysis of isozymes.	60
(a) Cultures	60
(b) Preparation of extracts.	60
(c) Gel electrophoresis and enzymatic staining	60
4. Analysis of ribulose-1,5 bisphosphate carboxylase/oxygenase (RUBPCase).	61
(a) Plant material.	61
(b) Preparation of RUBPCase.	62
(c) Isoelectric focussing.	62
5. Analysis of alkaloids	63
RESULTS.	64
A. Isolation and Culture of Protoplasts and Regen- eration of Plants from Cell Cultures of Wild- type and Chlorophyll-deficient Mutants.	64
1. Initiation of callus cultures	64
2. Initiation of liquid suspension cultures. .	67
3. Isolation of protoplasts.	67
4. Formation of cell colonies from protoplasts	69
5. Induction of shoots in cell colonies derived from suspension cultures.	75
(a) <u>N. rustica</u>	75
(b) <u>Albino N. tabacum</u>	80
6. Induction of shoots in protoplast-derived cell colonies	82
7. Induction of roots.	82

	PAGE
B. Protoplast Fusion and Selection and Regeneration of Hybrid Plants.	85
1. Observation on the fusion of protoplasts	85
2. Production of hybrid cell colonies	87
3. Induction of shoots in hybrid cell colonies	91
4. Induction of roots in hybrid shoots.	99
C. Morphological, Cytological and Biochemical Analysis of Somatic Hybrids.	115
1. Morphology	115
2. Chromosome determinations.	124
3. Analysis of isozymes	124
4. Analysis of ribulose-1-5 biphosphate carboxylase/oxygenase (RUBPCase) by isoelectric focussing.	131
5. Analysis of alkaloids.	134
D. Sexual Hybridization by <u>In Vitro</u> Culture of Hybrid Embryos	134
1. Development of sexual hybrid seedlings	136
2. Factors affecting the yield and development of hybrid seedlings.	139
E. Morphological, Cytological and Biochemical Analysis of Sexual Hybrids.	146
1. Morphology	146
2. Chromosome determinations.	150
3. Analysis of isozymes	151
4. Analysis of RUBPCase by isoelectric focussing.	156
5. Analysis of alkaloids.	156
DISCUSSION	158
A. Isolation and Culture of Protoplasts and Plant Regeneration in <u>N. rustica</u> and <u>N. tabacum</u>	158
B. Fusion of Protoplasts and Selection and Regeneration of Somatic Hybrid Plants.	168
C. Studies on Sexual Hybridization.	177
D. Comparative Morphological, Cytological, and Biochemical Analysis of Somatic and Sexual Hybrid Plants.	181
CONCLUSIONS.	199
REFERENCES	199
APPENDIX	236

LIST OF TABLES

TABLE		PAGE
1	Genera from which protoplasts derived from leaves were isolated and cultured to form dividing cells (C) and regenerated plants (P).....	5
2	<u>Nicotiana</u> species from which leaf-derived protoplasts were isolated and cultured to form dividing cells.....	8
3	Genera from which protoplasts derived from cell-suspensions were isolated and cultured to form dividing cells (C) and regenerated plants (P).....	9
4	Somatic hybridizations within the genus <u>Nicotiana</u>	37
5	Effect of explant source and auxin type on callus initiation from seedlings of mutant <u>Nicotiana rustica</u> var chlorotica, and mutant and wild-type <u>N. tabacum</u>	65
6	Isolation and response in culture of protoplasts of <u>Nicotiana rustica</u> and <u>N. tabacum</u>	70
7	Effect of different carbohydrates at osmotic concentration 0.35 M on plating efficiency and chlorophyll development in colonies derived from protoplasts of <u>Nicotiana rustica</u> var NRT after 36 days.....	73
8	Frequency of shoot induction in calli of <u>Nicotiana rustica</u> var chlorotica in varying BA/NAA concentrations.....	77
9	Frequency of shoot induction in calli of <u>Nicotiana rustica</u> var NRT in varying BA/NAA concentrations.....	78
10	Effect of time in suspension culture on shoot induction in cell colonies.....	79
11	Effect of cytokinin type and concentration on shoot induction in cell suspension-derived albino <u>Nicotiana tabacum</u> cell colonies.....	81
12	Morphogenic response of cell suspension-derived and protoplast-derived cell colonies of mutant <u>Nicotiana rustica</u> var chlorotica and albino <u>N. tabacum</u> ..	83
13	Induction of roots on protoplast-derived shoots of <u>Nicotiana rustica</u> var chlorotica.....	84

TABLE

PAGE

14	Effect of temperature on formation of <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid cell colonies.....	90
15	Effect of DMSO concentration in PEG fusion solution and the elution medium on formation of <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid colonies.....	92
16	Effect of salts in PEG fusion solution on formation of <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid cell colonies.....	93
17	Effect of basic medium on induction of meristems and/or shoots in <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid calli.....	94
18	Effect of glucose concentration under two hormonal regimes on induction of meristems and/or shoots in <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid calli.....	96
19	Effect of liquid "feeding" medium on subsequent morphogenic response for shoot and/or meristem formation of <u>Nicotiana rustica</u> + <u>N. tabacum</u> hybrid calli.....	98
20	Effect of MS and B ₅ media under different culture conditions on root induction in somatic hybrid shoots..	100
21	Effect of auxin type and concentration on root induction in somatic hybrid shoots.....	104
22	Effect of DMSO on root induction in somatic hybrid shoots.....	105
23	Effect of sequential hormone regimes on root induction in somatic hybrid shoots.....	107
24	Effect of growth inhibitors on root induction in somatic hybrid shoots.....	108
25	Effect of CO ₂ and IBA supplementation on root induction in somatic hybrid shoots.....	109
26	Effect of preparation method of IAA and NAA stocks and their concentration on root formation in somatic hybrid shoots.....	111
27	Effect of boron, IBA, and amino acid supplements on root induction in somatic hybrid shoots.....	112
28	Effect of nicotine and 3-amino pyridine on root induction in somatic hybrid shoots.....	113

TABLE		PAGE
29	Effect of IBA and sucrose concentration on root and secondary shoot induction in somatic hybrid shoots.....	114
30	Breakdown analysis of rooting pattern among individual somatic hybrid lines with combinations of ABA and IBA from Table 24.....	116
31	Vegetative and flower characteristics in <u>Nicotiana rustica</u> + <u>N. tabacum</u> somatic hybrid lines.....	120
32	Chromosome numbers in <u>Nicotiana tabacum</u> + <u>N. rustica</u> somatic hybrids.....	125
33	Alkaloid content of <u>Nicotiana rustica</u> var <u>chlorotica</u> + <u>N. tabacum</u> var <u>WS</u> somatic hybrids, sexual backcross hybrids and the parental varieties.....	135
34	Genotypes of <u>Nicotiana rustica</u> and <u>N. tabacum</u> which have yielded sexual hybrids following crossing and culture of hybrid embryos <u>in vitro</u>	137
35	Effect of culture media on yield and development of <u>Nicotiana rustica</u> var <u>chlorotica</u> x <u>N. tabacum</u> var <u>WS</u> hybrid seedlings.....	140
36	Effect of sucrose concentration on yield and development of <u>Nicotiana rustica</u> var <u>chlorotica</u> x <u>N. tabacum</u> var <u>Delhi 34</u> hybrid seedlings.....	143
37	Effect of time prior to <u>in vitro</u> culture on yield and development of <u>Nicotiana rustica</u> var <u>chlorotica</u> x <u>N. tabacum</u> var <u>WS</u> hybrid seedlings.....	144
38	Effect of culture method on yield and development of <u>Nicotiana rustica</u> var <u>chlorotica</u> x <u>N. tabacum</u> var <u>WS</u> hybrid seedlings.....	145
39	Vegetative and flower characteristics of <u>Nicotiana rustica</u> x <u>N. tabacum</u> sexual hybrids.....	148
40	Alkaloid content of <u>Nicotiana rustica</u> x <u>N. tabacum</u> sexual hybrids and their parental varieties.....	157

LIST OF FIGURES

FIGURE		PAGE
1	Plants of mutant <u>N. rustica</u> var chlorotica and wild-type <u>N. rustica</u> var NRT and F ₁ offspring of reciprocal crosses between these varieties.....	66
2	Seedlings from a heterozygote of <u>N. tabacum</u> var WS showing albino and wild-type segregants.....	66
3	Stem-derived callus of <u>N. rustica</u> var chlorotica on MS medium with 2,4-D or NAA.....	66
4	Stem-derived callus of <u>N. rustica</u> var NRT on MS medium with 2,4-D or NAA.....	66
5	Leaf-derived callus of albino <u>N. tabacum</u> on MS medium with 2,4-D or NAA.....	66
6	Growth characteristics of <u>Nicotiana</u> cell suspension cultures grown with 0.5 mg/L 2,4-D.....	68
7	Effect of cell number on the efficiency of enzyme to release healthy protoplasts.....	68
8	Effect of incubation time on release of healthy protoplasts from <u>Nicotiana</u> cells in enzyme.....	68
9	Freshly-isolated protoplasts of <u>N. rustica</u> var chlorotica derived from cell culture with 2,4-D.....	71
10	Freshly-isolated protoplasts of albino <u>N. tabacum</u> derived from cell culture with 2,4-D.....	71
11	Freshly-isolated protoplasts of <u>N. rustica</u> var NRT derived from cell culture with NAA.....	71
12	Cell wall formation and cell division in <u>N. rustica</u> var NRT culture.....	71
13	High frequency cell division in albino <u>N. tabacum</u> culture.....	71
14	Asymmetric cell division in <u>N. rustica</u> var NRT protoplast-derived culture.....	71

FIGURE		PAGE
15-17	Proembryo-like structures in <u>N. rustica</u> var NRT protoplast-derived cultures.....	71
18	Chlorophyll formation in cell suspension of mutant and wild-type <u>N. rustica</u> grown in MS medium with NAA.....	74
19	Meristem formation on cell suspension-derived callus of mutant <u>N. rustica</u> var chlorotica.....	74
20	Meristem and shoot formation in cell suspension-derived callus of albino <u>N. tabacum</u>	74
21	Shoot formation in cell suspension-derived callus of wild-type <u>N. rustica</u> var NRT.....	74
22	Shoot formation in protoplast-derived callus of mutant <u>N. rustica</u> var chlorotica.....	74
23	High frequency cell colony formation from protoplasts of <u>N. rustica</u> var chlorotica.....	74
24	High frequency cell colony formation from protoplasts of albino <u>N. tabacum</u>	74
25	Freshly isolated protoplast of <u>N. tabacum</u> var chlorotica prior to fusion.....	86
26	Agglutination of protoplasts upon addition of PEG solution.....	86
27	Tight adhesion and plasmolysis of protoplasts in PEG solution.....	86
28	Deplasmolysis of protoplasts upon elution of PEG..	86
29-31	Fusion of protoplasts.....	86
32	Chlorophyll development in cell colonies 5 weeks subsequent to fusion of chlorophyll-deficient <u>N. rustica</u> and <u>N. tabacum</u> protoplasts.....	88
33	Shoot-meristem induction in fusion products.....	88
34	Hybrid shoot differentiation.....	88
35	Comparison of mutant <u>N. rustica</u> , <u>N. tabacum</u> , and somatic hybrid plantlets.....	88
36	Somatic hybrid shoot with abnormal morphology bearing cotyledon-like leaves.....	88

FIGURE		PAGE
37	Somatic hybrid shoot with well developed leaves, shoot axis, and roots.....	88
38	Experimental controls for fusion experiments.....	97
39	Experimental controls for fusion experiments.....	97
40	Effect of glucose at three concentrations on shoot differentiation in hybrid cultures.....	97
41	Proliferation of somatic hybrid meristems and shoots.....	97
42	Root formation by somatic hybrid shoots in petri plates.....	101
43	Effect of IBA and sucrose on callus and secondary shoot formation by somatic hybrid shoots.....	101
44	Somatic hybrid shoots cultured in glass tubes on filter paper bridges.....	101
45	Effect of a range of inhibitors on rooting and secondary shoot formation by somatic hybrid shoots.....	101
46	Multiple shoot formation by somatic hybrid shoot <u>in vitro</u>	102
47-50	Variable shoot morphology and intensity of rooting in somatic hybrid shoots <u>in vitro</u>	102
51-55	Variable shoot and leaf morphologies of somatic hybrid plants in the vegetative phase.....	102
56	Parental species and somatic hybrid of <u>N. rustica</u> + <u>N. tabacum</u> at flowering.....	117
57	Four somatic hybrids at flowering stage exhibiting diverse morphological characteristics.....	117
58	Leaves of parental species and 11 somatic hybrid lines showing diverse morphologies.....	117
59	Somatic hybrid plant bearing two types of flowers...	117
60-61	Flowers of parental species and 8 somatic hybrid lines showing diverse morphologies.....	118
62	Uniform flower morphology of 6 sublines of hybrid line No. 147.....	118

FIGURE		PAGE
63	Variable flower morphology in 2 sublines of hybrid line No. 161.....	118
64	Variable flower morphology in 2 sublines of hybrid line No. 167.....	118
65	Progeny of a self-fertile somatic hybrid at the vegetative stage.....	123
66	Progeny of somatic hybrid (<u>N. rustica</u> + <u>N. tabacum</u>) x <u>N. tabacum</u> cross and somatic hybrid (<u>N. rustica</u> + <u>N. tabacum</u>) x <u>N. rustica</u> cross at the vegetative stage.....	123
67	Esterase isozyme pattern and zymogram for albino <u>N. tabacum</u> , <u>N. rustica</u> var chlorotica, and 10 somatic hybrid lines.....	127
68	Aspartate aminotransferase isozyme pattern and zymogram for albino <u>N. tabacum</u> , <u>N. rustica</u> var chlorotica, and 10 somatic hybrid lines.....	128
69	Superoxide dismutase isozyme pattern and zymogram for albino <u>N. tabacum</u> , <u>N. rustica</u> var chlorotica, and 10 somatic hybrid lines.....	130
70	Analysis of RUBPCase after isoelectric focussing of the carboxymethylated protein of <u>N. rustica</u> and 6 <u>N. rustica</u> + <u>N. tabacum</u> somatic hybrid plants.....	133
71	<u>N. rustica</u> x <u>N. tabacum</u> hybrid embryos, seeds and their germination <u>in vitro</u>	138
72	Abnormal seedlings of <u>N. rustica</u> x <u>N. tabacum</u> during germination <u>in vitro</u>	138
73	Culture and germination of <u>N. rustica</u> x <u>N. tabacum</u> hybrid seeds on placentas <u>in vitro</u>	141
74	Flowers of <u>N. rustica</u> , <u>N. tabacum</u> , and sexual hybrids.....	141
75	Comparison of mutant <u>N. rustica</u> , <u>N. tabacum</u> , and sexual hybrid seedling of <u>N. rustica</u> var chlorotica x <u>N. tabacum</u> var "V".....	141
76	Three sexual hybrids of <u>N. rustica</u> x <u>N. tabacum</u> showing uniform morphology <u>in vitro</u>	141
77	Shoot proliferation in callus of <u>N. rustica</u> var chlorotica and <u>N. rustica</u> var chlorotica x <u>N. tabacum</u> var "V".....	141

FIGURE		PAGE
78	Hybrid <u>N. rustica</u> x <u>N. tabacum</u> plants in the vegetative phase.....	147
79	Parental species and sexual hybrid of <u>N. rustica</u> x <u>N. tabacum</u> at flowering.....	147
80	Flowers of <u>N. rustica</u> , <u>N. tabacum</u> and sexual hybrids.....	149
81	Leaves of <u>N. rustica</u> , <u>N. tabacum</u> and sexual hybrid.....	149
82	Esterase isozyme pattern and zymogram for <u>N. rustica</u> var chlorotica, <u>N. tabacum</u> var "V", and 10 sexual hybrids.....	152
83	Aspartate aminotransferase isozyme pattern and zymogram for <u>N. rustica</u> var chlorotica, <u>N. tabacum</u> var "V", and 10 sexual hybrids.....	154
84	Superoxide dismutase zymogram for <u>N. rustica</u> var chlorotica, <u>N. tabacum</u> var "V", and 10 sexual hybrids.....	155

INTRODUCTION

Interspecific hybridization is an important method of increasing genetic variability to improve existing species. Hybridization by conventional methods is not always possible because complex incompatibility mechanisms have evolved in plants to effectively isolate species. Somatic hybridization has been proposed as a novel method for genetic modification of crop plants. (See Review Vasil 1976). This method involves the isolation, fusion and culture of protoplasts followed by selection of hybrid cell-colonies from the parental types and regeneration of hybrid plants.

Somatic hybrid plants have been obtained in a number of instances (Cocking 1978); success, however, has been limited to a few species combinations and generally yields of hybrid plants have been low. The limited success observed thus far can be attributed to several difficulties including:

- (i) lack of procedures to consistently isolate large numbers of viable protoplasts
- (ii) lack of efficient strategies to aid in the selection of large numbers of hybrid cells
- (iii) difficulties in achieving plant regeneration from hybrid cells

The objectives of this study with respect to interspecific hybridization were three fold. The primary objective was to ascertain if the above mentioned difficulties could be minimized and if procedures for obtaining somatic hybrid cells and plants could be developed through the implementation of a "microbiological" approach as advocated by Melchers (1977). This approach involves

using large numbers of parental protoplasts which show high plating efficiencies when cultured, efficiently fusing protoplasts to give viable hybrid cells and taking advantage of genetic complementation (such as for chlorophyll synthesis) in hybrid cells to distinguish these from parental cells. Two Nicotiana species, N. rustica and N. tabacum were selected for this study since chlorophyll-deficient mutants of both were available. Hybridization of these species was of added interest because they are isolated completely by pre-zygotic incompatibility mechanisms in N. tabacum x N. rustica crosses and incompletely by post-zygotic incompatibility mechanisms in reciprocal crosses. Furthermore, plant breeders are interested in overcoming these isolating mechanisms to assist in the improvement of tobacco.

The secondary objective of this study was to determine if somatic hybridization could overcome both types of incompatibility mechanisms and if post-zygotic incompatibility in N. rustica x N. tabacum crosses could be overcome by culturing embryos in vitro before the onset of incompatible reactions.

The third objective of this study was to ascertain to what extent any hybrids produced by somatic and sexual routes differed with respect to their genetic variability. This would involve the characterization and comparison of any somatic and/or sexual hybrids through morphological, cytological and biochemical analyses. In addition, an evaluation of fertility of hybrids with respect to their potential economic importance was to be undertaken.

LITERATURE REVIEW

Somatic hybridization in higher plants depends essentially on the isolation of protoplasts from somatic cells of prospective parents, the production of hybrid protoplasts through fusion, selection and culture in vitro of protoplast-derived hybrid cells, and finally, regeneration of hybrid plants. Relatively few studies have dealt with the isolation of protoplasts specifically in relation to their post-fusion behavior but many studies have defined the optimal isolation and culture conditions for protoplasts not specifically used for hybridization. Consequently, the first section of this review will deal mainly with previous studies on the isolation and culture of protoplasts not subjected to fusion treatments, with available information pertaining to hybrid protoplasts given in appropriate sections. Subsequent sections deal with studies on fusion of protoplasts, culture and selection of somatic hybrid cells and induction of morphogenesis. Finally, literature pertaining to culture in vitro of sexually-produced hybrid embryos is considered.

A. The Isolation of Protoplasts; their Properties and Culture

1. Historical perspective

The term "protoplast" is attributed to Hanstein and refers to the entity which can be obtained from a cell after the wall has been removed by physical or enzymatic means (Cocking 1972). Protoplasts were first isolated mechanically and stabilized in osmoticum as early as 1892 (Klercker 1892) and subsequently used in fusion studies (Küster 1909). Protoplast isolation by mechanical procedures results in low yields of protoplasts which are also contaminated with cellular

debris. In addition only certain tissues with highly vacuolated cells lend themselves to mechanical isolation procedures.

The use of enzymes for the isolation of protoplasts was first reported by Giaja (1919) who obtained protoplasts by treating yeast cells with gastric juices of the snail, Helix pomatia. Cocking (1960) demonstrated that large yields of debris-free plant protoplasts could be obtained with a fungal enzyme preparation which hydrolyzed wall polysaccharides. Enzyme preparations of fungal origin with potent cellulase, hemicellulase and pectinase activity are now available commercially and isolation of protoplasts by enzymatic means is now the preferred method for most studies except where the enzymes may interfere with the system under investigation (Pilet 1972; Pilet et al. 1972). The demonstration by Nagata and Takebe (1971) that entire plants could be regenerated from isolated protoplasts and that protoplasts could be fused in hybridization experiments (Carlson et al. 1972) has led to the extensive use of protoplasts for studies in plant physiology, cell biology, and genetics.

2. Sources of protoplasts

With the use of hydrolytic enzymes protoplasts can be prepared from tissues of essentially all plant organs including leaves, roots, tubers, petals, fruits, microspores and leguminous root nodules (Wakasa 1973; Vasil 1976). Leaf protoplasts have been used in most studies since they are readily obtainable and their yields are generally high. Leaf protoplasts have been successfully isolated and cultured to form dividing cells from which entire plants could be regenerated for many species (Table 1). The genus Nicotiana has been particularly amenable for studies of protoplast isolation

TABLE 1. Genera from which protoplasts derived from leaves were isolated and cultured to form dividing cells (C) and regenerated plants (R)

<u>Antirrhinum</u>	C	Poirier-Hamon <u>et al.</u> 1974
<u>Asparagus</u>	P	Bui-Dang Ha and MacKenzie 1973
<u>Atropa</u>	P	Lörz and Potrykus 1978
<u>Brassica</u>	P	Kartha <u>et al.</u> 1974; Thomas <u>et al.</u> 1976
<u>Calystegia</u>	P	Harada 1973
<u>Caragana</u>	C	Constabel <u>et al.</u> 1975b
<u>Cucumis</u>	C	Coutts and Wood 1975
<u>Datura</u>	P	Schieder 1975, 1977a, 1978a
<u>Gossypium</u>	C	Khasanov and Butenko 1979
* <u>Glycine</u>	C	Zieg and Outka 1980
<u>Hyoscyamus</u>	P	Wernicke and Thomas 1980
<u>Ipomoea</u>	C	Harada 1973
<u>Lycopersicon</u>	P	Zapata <u>et al.</u> 1977; Muhlbach 1980
<u>Lathyrus</u>	C	Razdan <u>et al.</u> 1980
<u>Manihot</u>	P	Shahin and Shepard 1980
<u>Linum</u>	C	Gamborg and Shyluk 1976
<u>Medicago</u>	P	Kaq and Michayluk 1980
<u>Melilotus</u>	C	Constabel <u>et al.</u> 1975b
<u>Mollugo</u>	C	Chellappan <u>et al.</u> 1980
<u>Nemesia</u>	P	Hess and Leipoldt 1979
<u>Pelargonium</u>	P	Kameya 1975b
<u>Petunia</u>	P	Durand <u>et al.</u> 1979; Frearson <u>et al.</u> 1973; Binding 1974

Continued.....

TABLE 1, continued

<u>Pharbitis</u>	C	Messerschmidt 1974
<u>Phaseolus</u>	C	Pelcher <u>et al.</u> 1974
<u>Pinus</u>	C	David and David 1979
<u>Pisum</u>	C	Constabel <u>et al.</u> 1973; Gamborg <u>et al.</u> 1975
<u>Pseudotsuga</u>	C	Kirby and Cheng 1979
<u>Ranunculus</u>		Dorion <u>et al.</u> 1975
<u>Rosa</u>		Krishnamurthy <u>et al.</u> 1979
<u>Solanum</u>	P	Shepard and Totten 1977; Binding and Nehls 1977; Nehls 1978a; Binding <u>et al.</u> 1978
<u>Vigna</u>	C	Davey <u>et al.</u> 1974

* Protoplasts prepared from pods

and culture. Plants have been regenerated from leaf protoplasts of at least 16 species in this genus (Table 2).

Cell cultures are also a good source of protoplasts as the cells are already free of contaminating microorganisms and adapted to in vitro conditions. Their nutritional and physical regime during growth prior to protoplasting can also be accurately controlled. Protoplasts have been isolated from cell suspensions and cultured successfully in a large number of species (Table 3)

some of which e.g. cereals could not be cultured when isolated from leaves (Potrykus et al. 1976, 1979). Cell cultures are also a useful source of protoplasts from albino or chlorophyll-deficient mutants, since such plants grow very slowly, if at all. Protoplasts isolated from cell cultures of such mutants have been successfully used in somatic hybridization studies (Gleba and Hoffmann 1978; Schieder 1978a; Dudits et al. 1979; Power et al. 1979).

3. Conditions for isolation of protoplasts

For somatic hybridization studies it is essential to obtain high yields of viable protoplasts which possess the capacity for wall synthesis, cell division and subsequent morphogenesis.

Parameters which affect the yield and quality of protoplasts include:

- a) types and concentration of enzymes(s) used
- b) time, temperature, and pH conditions during digestion
- c) nature of the osmoticum and other additives in the enzyme solution
- d) protoplast purification procedure subsequent to isolation and prior to culture.

The type, combination and concentration of enzyme(s) used in the isolation system is usually determined empirically and depends to a large extent on tissue used; for example, with tomato fruit tissue

TABLE 2. Nicotiana species from which leaf-derived protoplasts were isolated and cultured to form dividing cells*

<u>N. tabacum</u>	Nagata and Takebe 1971; Takebe <u>et al.</u> 1971; Nitsch and Ohyama 1971; Bajaj 1972; Raveh and Galun 1975
<u>N. alata</u>	Bourgin and Missonier 1978
<u>N. sylvestris</u>	Nagy and Maliga 1976; Banks and Evans 1976
<u>N. knightiana</u>	Butenko 1979
<u>N. langsdorffii</u> , <u>N. repanda</u> <u>N. nesophila</u> , <u>N. stocktonii</u>	Evans 1979
<u>N. acuminata</u> , <u>N. glauca</u> , <u>N. glutinosa</u> , <u>N. longiflora</u> , <u>N. otophora</u> , <u>N. paniculata</u>	Bourgin <u>et al.</u> 1979; Banks and Evans 1976
<u>N. plumbaginifolia</u> <u>N. suaveolens</u>	Gill <u>et al.</u> 1978
<u>N. rustica</u>	Constabel <u>et al.</u> 1976; Gill <u>et al.</u> 1979

*Note: Entire plants have been regenerated from protoplast derived cell colonies for all species with the exception of N. glutinosa (Bourgin et al. 1979).

TABLE 3. Genera from which protoplasts derived from cell suspensions, were isolated and cultured to form dividing cells (C) and regenerated plants (P)

<u>Acer</u>	C	Bright and Northcote 1974
<u>Ammi</u>	C	Fowke <u>et al.</u> 1974 .
<u>Arabidopsis</u>	P	Xuan and Menczel 1980
<u>Atropa</u>	P	Gosch <u>et al.</u> 1975
<u>Bromus</u>	P	Kao <u>et al.</u> 1973
<u>Browallia</u>	P	Power and Berry 1979
<u>Citrus</u>	C	Vardi <u>et al.</u> 1975
<u>Datura</u>	P	Furner <u>et al.</u> 1978
<u>Daucus</u>	P	Grambow <u>et al.</u> 1972; Dudits <u>et al.</u> 1977
<u>Glycine</u>	C	Kao <u>et al.</u> 1971
<u>Haplopappus</u>	C	Kao <u>et al.</u> 1971
<u>Hordeum</u>	C	Koblitz 1976
<u>Nicotiana</u>	P	Uchimiya and Murashige 1976 Glimelius <u>et al.</u> 1978a; Wallin <u>et al.</u> 1978b; Gamborg <u>et al.</u> 1979
<u>Oryza</u>	C	Tsai <u>et al.</u> 1978; Deka and Sen 1976
<u>Pelargonium</u>	C	Abo El-Nil and Hildebrandt 1976
<u>Pennisetum</u>	P	Vasil and Vasil 1980
<u>Rosa</u>	C	Strauss and Potrykus 1980
<u>Saccharum</u>	C	Maretzki and Nickell 1973; Krishnamurthi 1976
<u>Sorghum</u>	C	Brar <u>et al.</u> 1980
<u>Triticum</u>	C	Dudits and Nemet 1976
<u>Vicia</u>	C	Kao and Michayluk 1975
<u>Zea</u>	C	Potrykus <u>et al.</u> 1979

a pectinase alone is sufficient to yield protoplasts (Gregory and Cocking 1965) whereas leaf tissues and cell cultures generally require a combination of potent cellulases and pectinases. Enzyme efficiency can be optimized by the use of an appropriate cell to enzyme volume ratio and agitation of the cell-enzyme mixture (Takebe et al. 1968; Uchimiya and Murashige 1974). Light may also affect the efficiency of enzymes; low light intensity during enzyme incubation gave a greater yield of protoplasts than either complete darkness or high light intensity (Binding 1974).

Commercial cellulases and pectinases have impurities such as lipases, proteases and phenolics which can affect protoplast viability (Vasil 1976). Specific activity and purity of commercial enzymes may be increased by gel filtration (Constabel 1975). The quality of enzyme preparations has been studied in relation to the fusability of protoplasts and viability of hybrid cells in culture (Kao et al. 1974; Kao 1978).

The effect of incubation time and temperature on yield and quality of protoplasts has been assessed in a number of studies. In order to optimize yield and quality, exposure to enzyme(s) can vary from one and a half hours for thin-walled fruit cells to more than 24 hours for soybean (Gregory and Cocking 1965; Kao 1977). For Nicotiana tabacum cells in suspension culture, there was no increase in yield after two hours of treatment (Uchimiya and Murashige 1974). Enzymes are effective for protoplast release at temperatures ranging from 2–24°C (de la Roche et al. 1977). While exposure time to enzymes may be shortened due to the accelerated release of protoplasts at high temperatures (30–37°C).

such protoplasts often have low viability (Takebe et al. 1968). Temperatures in the range 22-37°C had no significant effect on protoplast yield for suspension-derived cells of N. tabacum (Uchimiya and Murashige 1974). For corn internodes an initial temperature of 12°C for 16 hours followed by 6 hours at 32°C was recommended (Potrykus et al. 1977).

Optimal enzyme pH as determined by protoplast yield usually falls over a broad range. For the enzyme mixture used on cultured tobacco cells the optimum pH ranged from 4.7 to 5.7 (Uchimiya and Murashige 1974). An initial pH of 5.0 yielded fragile Phaseolus protoplasts very rapidly while an initial pH of 7.0 produced stable protoplasts and higher yields but at a slower rate (Pelcher et al. 1974). Low concentrations of MES buffer have been used to stabilize the pH of the isolation system (Kao and Michayluk 1980).

An osmotic agent is an essential component of the enzyme solution during protoplast isolation. Mannitol and/or sorbitol have been most commonly used. For tobacco, mannitol was superior to salts (Takebe et al. 1968) and sucrose was unsuitable (Uchimiya and Murashige 1974). In certain cases the use of inorganic salts resulted in higher yields of superior quality protoplasts (Meyer 1974; Upadhy 1975; Simmonds et al. 1979).

The concentration of osmoticum used during enzymatic isolation procedures is generally in the range 0.2 to 0.7 M. Usually the lowest concentration which permits isolation of protoplasts without excessive rupture is used since physiological stress increases with osmoticum concentration (Premecz et al. 1977). For tissues with

limited intercellular space high osmotic concentrations and vacuum infiltration may facilitate the entry of the enzymes into the tissue (Kirby and Cheng 1979).

The inclusion of agrinine (Altman et al. 1977; Galston et al. 1978) or dextran sulfate (Coutts and Wood 1975) in the enzyme mixture stabilized protoplasts and polyvinylpyrrolidine (Shepard and Totten 1975) increased protoplast yields.

Protoplast preparations must be purified by washing in order to carry out subsequent culture or fusion experiments. Enzymes in concentrations as low as 0.005% inhibit mitotic activity (Landgren 1976; Schilde-Rentschler 1977; Landgren 1978) and debris inhibits fusion (Keller and Melchers 1973). In addition protoplasts must be purified of protoplasmic and vacuolar contents released during isolation. RNase causes protoplast lysis (Ruesink and Thimann 1965; Fisher 1979) and tobacco vacuoles have been shown to have an array of hydrolases which could have deleterious effects on protoplast preparations (Boller and Kende 1979). Protoplast purification usually involves removal of large pieces of undigested tissue by filtration followed by washing in enzyme-free medium. In some cases protoplasts were floated free of debris on Ficoll or sucrose (Eriksson and Jonasson 1969; Schenck and Hildebrandt 1971; Gosch et al. 1975). More commonly protoplasts were sedimented repeatedly by centrifugation in fresh medium (Bui-Dang Ha and MacKenzie 1973; Keller and Melchers 1973; Constabel 1975). Two phase solutions (Kanai and Edwards 1973; Larkin 1976; Slabas et al. 1980) and discontinuous density gradients (Harms and Potrykus 1978b) have also been used. Even with such procedures particulate

matter has been reported to stick to protoplast surfaces and Sepharose beads have been used to facilitate its removal (Keller and Stone 1978). Protoplast viability after isolation and purification may be determined by observing the extent of cyclosis or by the exclusion of Evans blue dye (Kanai and Edwards 1973) or uptake of fluorescein diacetate (Larkin 1976).

4. Properties of protoplasts

(a) Surface properties and fusion of protoplasts

Protoplasts are bound by a plasmamembrane (Withers and Cocking 1972; Fowke et al. 1973; Fowke et al. 1974) which may also have additional surface lipids (Fisher 1979). The plasmamembrane lipid composition is species specific (Yamada et al. 1979) and sterols appear to be present (Fisher 1979). Under certain conditions the plasmamembrane may be discontinuous, facilitating virus uptake (Burgess et al. 1973; Takebe 1975; Kassanis et al. 1977). The presence of binding sites for Concanavalin A (Glimelius et al. 1978c; Williamson 1979; Boss and Ruesink 1979) phytohemagglutinin, and wheat germ agglutinin (Chin and Scott 1979) indicates the presence of glucose, mannose and galactose residues on the membrane surface (Novogrodsky and Katchalski 1971; Burger 1969; Sela et al. 1970). Specific binding sites for fungal toxins have also been located on the plasmamembrane (Strobel and Hess 1974; Strobel and Hapner 1975; Pelcher et al. 1975).

The plasmamembrane of protoplasts carries a net negative charge (Zeta potential) as shown by electrophoresis at physiological pH for eight species (Grout et al. 1973; Nagata and Melchers 1978; Fisher 1979). Phosphate groups are believed to contribute most of this negative charge (Nagata and Melchers 1978). The nature of the

internal electrical charge is controversial. Racusen et al. (1977) reported a positive charge which was dependent on the degree of plasmolysis in leaf protoplasts of six species whereas Briskin and Leonard (1979) observed a negative charge which was independent of plasmolysis in protoplasts of N. glutinosa derived from cell cultures.

The plasmamembrane of the plant protoplast is capable of mediating several important physical processes. Protoplasts are capable of exocytosis of nuclei (Wallin et al. 1978a) and endocytosis of other smaller protoplasts (Ueda et al. 1978), bacteria (Davey and Cocking 1972) and chloroplasts (Bonnett and Eriksson 1974; Potrykus 1973; Uchimiya and Wildman 1979). The plasmamembrane of adjacent cells may be continuous via plasmodesmata and expansion of these channels during enzymatic digestion of the cell walls can lead to fusion of protoplasts (Cocking 1971). Isolated protoplasts aggregate in the presence of agents such as antibodies (Hartmann et al. 1973), Concanavalin A (Glimelius et al. 1974) other lectins (Chin and Scott 1979) and poly-l-lysine (Burge's and Fleming 1974) but do not fuse under these conditions.

A number of procedures have been developed and claimed to induce fusion of protoplasts: sodium nitrate (Power et al. 1970), centrifugation to induce membrane contact (Ito 1973), calcium buffered at high pH (Keller and Melchers 1973), polyethylene glycol (Kao and Michayluk 1974), dextran sulfate (Kameya 1975a, 1979), polyvinyl alcohol (Nagata 1978), charged phospholipids (Nagata et al. 1979), and electrical stimulation (Senda et al. 1979). While there is some evidence for genetic control of fusability (Kao 1978;

Stadler et al. 1980; Roos and Davidson 1980) the fusogen polyethylene glycol has been used for intergeneric (Kao and Michayluk 1974) and even interkingdom (Davey et al. 1978; Ward et al. 1979) fusions.

Various factors affect the efficiency of PEG-induced fusion, including the molecular weight and concentration of PEG, presence of calcium and other ions, source of protoplasts (leaf or cell suspension) and age of donor tissue, enzyme type and concentration used for protoplast isolation, protoplast density and genetic constitution of the protoplasts (Kao and Michayluk 1974; Wallin et al. 1974; Kao 1978; Kameya 1979). The efficiency of PEG-induced protoplast fusion may be enhanced by DMSO (Haydu et al. 1977), elution with calcium ions buffered at high pH (Kao et al. 1974), preparation of the PEG solution in a calcium medium buffered at high pH (Schieder 1978a; Binding and Nehls 1978), or the use of Concanavalin A (Glimelius et al. 1978b). Since protoplasts rapidly lose their ability to fuse within minutes of removal of the enzyme(s) the washing step(s) should be rapid (Ito 1973; Weber et al. 1976; Williamson et al. 1977). Protoplasts which fuse very readily usually have a very low tolerance to the fusion method and are killed easily. The deleterious effects of fusing agents on protoplast survival may be lessened by reducing the enzyme concentration during protoplast preparation, shortening the exposure time to the fusing agent, and selection of appropriate starting materials (Kao 1978). The aim of most fusion experiments is to strike a balance between optimum formation of heterokaryons (particularly dikaryons) and viability (Cocking 1976b).

The mechanism of membrane fusion has been studied intensively and is still not fully understood (Poste and Nicholson 1978). A number of different processes which characterize fusion are mediated by one or more fusogens. For relatively simple synthetic phospholipid vesicles calcium and DMSO can induce a complete or partial crystallization of the outer lipid monolayer (Papahadjopoulos et al. 1977, 1978). These molecular condensations may result in exposure of hydrocarbon chains to water thus creating structural instabilities which would make them more susceptible to fusion (Papahadjopoulos 1978). Fusing agents such as calcium buffered at high pH (Nagata and Melchers 1978), PEG (Maggio et al. 1976) and DMSO (Lucy 1978) decrease the surface potential of membranes, presumably allowing close membrane apposition and fusion (Poste and Allison 1973). PEG-induced fusion occurs as the PEG solution is eluted and protoplasts undergo deplasmolysis (Constabel and Kao 1974; Kao and Michayluk 1974). Fluidity of the protoplast membrane is increased during deplasmolysis (Borochoy and Borochoy 1979), which may contribute to the fusion process. The observation that free movement of membrane proteins (Volsky and Loyter 1978; Wilairat et al. 1978) or their removal (Constabel and Kao 1974; Ward et al. 1979; Kameya 1979) facilitates membrane fusion gives support to the suggestion that initiation of fusion occurs at protein-free areas (Burgess and Fleming 1974; Burgess et al. 1977; Knutton 1979).

(b) Metabolic properties of isolated protoplasts

The metabolic activity of protoplasts can be shown by their uptake and incorporation of protein, RNA, and DNA precursors and other metabolites. The uptake of amino acids and analogues into protoplasts occurs against a concentration gradient at similar

(Robinson and Mayo 1975) or somewhat reduced (Ruesink 1978; Rubinstein and Tattar 1980; Guy et al. 1978) rates as compared to cultured cells. Uptake was stimulated by "aging" (Rubinstein and Tattar 1980; Robinson and Mayo 1975) and lowering the external pH from 7.5 to 5.5 (Rubinstein and Tattar 1980). Similar uptake responses have been shown for sugars (Guy et al. 1978; Guy et al. 1980). The ability to retain metabolites was shown to be lower in protoplasts than in cells while protoplasts had a higher respiration rate (Taylor and Hall 1976). Cells and protoplasts showed a number of similar physiological responses, e. g. death due to fungal toxins (Pelcher et al. 1975; Strobel and Hapner 1975), hydrogen ion excretion induced by auxins (Pilet 1971; Bayer and Sonka 1976; Böttger et al. 1978) or fusicoccin (Rollo et al. 1977) and rapid induction of lipid synthesis by kinetin (Kull and Ultes 1980).

The incorporation of RNA and DNA precursors has been studied in protoplasts such as tobacco which develop into viable cells during culture. The incorporation of radioactive precursors into RNA in tobacco protoplasts was low during the first 12 hours of culture but subsequently a dramatic increase was observed (Paszkowski and Kleczkowski 1979; Premecz et al. 1978). This increase preceded DNA synthesis and cell division (Zelcer and Galun 1976). A lag period was not observed in RNA synthesis in protoplasts of Zea (Wasilewska and Kleczkowski 1974) or Centaurea (Kulikowski and Mascarenhas 1978) protoplasts. In oat and corn protoplasts, which cannot be induced to divide (Euchs and Galston 1976; Wasilewska and Kleczkowski 1974), or tobacco protoplasts

cultured without growth regulators (Zelcer and Galun 1976; Paszkowski and Kleczkowski 1979) uridine incorporation declined sharply. Evidence for faulty processing of hnRNA in such protoplasts relative to cells was also shown (Kulikowski and Mascarenhas 1978). Ribonuclease activity increased rapidly during culture of oat protoplasts, the rise being completely inhibited by arginine (Kaur-Sawhney et al. 1976; Altman et al. 1977). In tobacco, the rise in ribonuclease activity increased with increasing osmotic stress but decreased on incubation with kinetin or cycloheximide (Premecz et al. 1977). Osmotic stress also decreased the uptake and incorporation of RNA and protein precursors in tobacco protoplasts but the synthesis of all RNA species was not affected to the same extent (Premecz et al. 1978). Synthesis of fraction-1-protein in protoplasts stopped almost completely during culture (Fleck et al. 1979).

Leaf protoplasts are capable of photosynthesis and the products which are excreted into the bathing medium are qualitatively similar to those of leaf cells in vivo (Wegmann and Mühlbach 1973). The rates of O_2 exchange and CO_2 fixation by tobacco protoplasts were similar to those of leaf tissue (Morris and Thain 1980) and were affected by osmotic stress (Colman and Mawson 1978).

5. Culture of protoplasts

After the isolation of protoplasts, suitable physical and physiological conditions must be established for wall synthesis, cell division and colony formation. One method of culture involves embedding protoplasts in agar-solidified medium (Nagata and Takebe 1971; Gosch et al. 1975; Upadhy 1975; Shepard and Totten 1977);

the type and concentration of agar used has been shown to be important (Binding 1974). Liquid suspensions of protoplasts have been dispensed in 5-10 mL volumes in culture vessels (Takebe and Nagata 1973; Dorion et al. 1975; Coutts and Wood 1975; Uchimiya and Murashige 1976) or in smaller volumes to form thin layers (Binding 1974; Kao and Michayluk 1975). Protoplasts have been cultured in 0.25- 5.0 μ L droplets in petri plates (Kao et al. 1971, 1973; Gleba 1978b). Inverted drop cultures have also been used (Potrykus et al. 1976). Fabric supports have been shown to be beneficial in some cases (Kirby and Cheng 1979). Agitation of protoplast cultures was shown to be harmful (Kao et al. 1971).

The response of protoplasts during culture is evaluated as the percentage of protoplasts which ultimately undergo synthesis of cell walls and enter cell division, i.e. plating efficiency (Cocking 1972). These responses of protoplasts are determined in part by:

- a) source material and protoplast isolation procedures
- b) density of protoplasts plated
- c) temperature and light conditions during culture
- d) composition of nutrient medium.

The yield, and more importantly the response of leaf protoplasts in culture is variable and dependent on a number of factors involving the source plants. These factors include:

- a) Genotype (Watts et al. 1974; Crowder et al. 1979; Kao and Michayluk 1980; Muhlbach 1980; Izhar and Power 1977)
- b) Physiological condition of the donor including, temperature relative humidity and light regime during growth (Upadhya 1975; Frearson et al. 1973; Shepard and Totten 1977; Hughes et al. 1978; Kao and Michayluk 1980), age of leaves (Gamborg et al. 1975; von Arnold and Eriksson 1976; Watts et al. 1974) and nutritional regime of the donor plant (Cassells and Barlass 1978).
- c) Pretreatments of donor tissue prior to digestion such as holding leaves in darkness or low light intensities (Constabel et al. 1973; Grun and Chu 1978) and incubation in nutrient

solutions (Gatenby and Cocking 1977; Donn 1978; Kirby and Cheng 1979; David and David 1979; Kao and Michayluk 1980), or in solutions containing protein synthesis inhibitors such as cycloheximide (Kaur-Sawhney et al. 1976).

Variability of leaf-derived protoplasts was reported to be reduced by the use of inorganic osmoticum during isolation (Upadhy 1975) and in early stages of protoplast culture (Meyer 1974).

Protoplasts isolated from leaves of shoots which had been cultured and propagated in vitro have been shown to undergo cell wall formation and subsequent cell division with greater consistency than those isolated from greenhouse-grown plants (Binding 1974, 1975; Schieder 1975, 1977a; Binding et al. 1978; Nehls 1978a; Lörz and Potrykus 1978). Axenic shoot culture is successful for many Solanaceous species but attempts to establish Vicia faba and Zea mays shoot cultures in vitro as sources of protoplasts have failed (Binding and Nehls 1978; Potrykus et al. 1976). The response of protoplasts derived from shoots grown in vitro was affected by the composition of the shoot culture medium and light intensity employed (Binding 1975; Durand 1979).

The use of cell cultures for protoplast isolation affords the greatest measure of control over the growth conditions of source cells. The nutritional milieu and environmental conditions of cultured cells can be carefully controlled and optimized. Increasing the auxin level, reducing the sucrose concentration and adding sulphur-containing amino acids or mercapto ethanol to the cell suspension medium prior to enzyme digestion increased protoplast yield from Haplopappus gracilis cells (Wallin et al. 1977).

Ammonium ions were essential for good yields of protoplasts from suspension cultures of alfalfa, soybean and flax while they were not essential for tobacco; nitrogen starvation of wheat and rice

cultures for three days enhanced protoplast yield (Fukunaga and King 1978). With N. tabacum, optimal protoplast yields were obtained from cell cultures in log phase growth (Uchimiya and Murashige 1974; Opatrny et al. 1975). While a number of workers have reported that actively growing cell cultures yield "high quality" protoplasts which are responsive to further culture (Vasil 1976; Eriksson et al. 1978), there has been little systematic work on this aspect of protoplast research. Two recent studies with Rosa 'Paul's Scarlet' (Strauss and Potrykus 1980) and Zea mays (Potrykus et al. 1979) in which protoplasts were isolated from cell suspensions and cultured in up to 27 different media under various conditions have shown that colony formation was largely independent of the culture medium employed but highly dependent on the quality of the culture used for protoplast isolation. In both of these species, isolation of protoplasts from actively dividing donor cultures were essential for success in subsequent protoplast culture.

For somatic hybridization studies, the relative advantages of leaf and cell suspension-derived protoplasts in relation to fusability and viability have to be evaluated. Properties and viability of suspension-derived protoplasts are usually more consistent than those of leaves. In addition, fusability may be manipulated; for example, protoplasts isolated from rapidly-growing cultures can be induced to fuse more readily than those from slow growing cultures and subsequent to fusion the frequency of cell division is higher in hybrid cells in which one of the fusion partners was derived from a rapidly growing culture (Kao 1978). Despite certain advantages of culture-derived protoplasts, leaf protoplasts are often used in hybridization studies because their

chloroplasts provide a visible marker to aid in selection of heterokaryons after protoplast fusion. (Kao and Michayluk 1974; Kao et al. 1974). Additionally, leaf protoplasts are thought to possess inherently higher morphogenic potential than established cell lines. The decline in morphogenic potential of cultured cells with time has been well documented (Murashige and Nakano 1968; Smith and Street 1974, Negrutiu et al. 1978). Leaf cells (Takebe et al. 1968; Evans 1979) are also genetically more uniform than cultured cells (D'Amato 1978) although considerable genetic variation has been found in regenerated plants from leaf protoplasts of potato (Shepard et al. 1980).

The density of protoplasts during culture affects their plating efficiency. This response is species specific (Nagata and Takebe 1971; Binding 1974; Landgren 1976; Grun and Chu 1978; Durand 1979; Kao and Michayluk 1980). For N. tabacum mesophyll protoplasts the optimal density was 2×10^4 mL (Nagata and Takebe 1971). Density dependence is related to nutrition and/or metabolite leaching since single protoplasts in large volumes (4 mL) of rich medium can divide and form cell colonies (Kao and Michayluk 1975).

Environmental conditions during culture affect protoplast response. Protoplasts are usually cultured between 25°C and 27°C. In the case of Lycopersicon esculentum and L. peruvianum a critical influence of temperature was observed in the range of 27 to 29°C (Zapata et al. 1977) and for pea (Landgren 1976) and cotton (Bhojwani et al. 1977) 27°C was optimal. A low light intensity (400 lux) during the first two days followed by higher intensities (3000 lux) was more favorable for colony formation from tobacco leaf protoplasts than continuous culture at low or high intensities.

(Enzmann-Becker 1973). When cultured under continuous light the plating efficiency of tobacco leaf protoplasts was twice as high at 2300 lux than at 700 lux (Nagata and Takebe 1971). Initial periods of culture in low light have also been employed by others (Kao and Michayluk 1974; Durand 1979; Kao and Michayluk 1980). Protoplasts derived from pea roots (Landgren 1976), tomato leaves (Zapata et al. 1977) and lily cell cultures (Simmonds et al. 1979) responded best in darkness whereas light (1-2000 lux) stimulated division of wild-type and albino leaf-derived protoplasts of Petunia hybrida (Binding 1974). High light intensities of 80,000 lux were necessary for maximal response of Nemesia strumosa leaf protoplasts (Hess and Leipoldt 1979).

The media developed for protoplast nutrition are qualitatively similar to cell culture media but generally have a lower concentration of inorganic constituents (Eriksson 1977). Protoplasts do not respond well to high nitrogen levels; high ammonium levels were particularly deleterious to potato leaf protoplasts (Upadhy 1975; Shepard and Totten 1977). The ammonium nitrate requirements of cell suspension-derived protoplasts of Vicia and Bromus were lower than the level used for cell growth (Kao et al. 1973). The form in which ammonia is supplied may also be important; glutamine is beneficial for culturing protoplasts of Ranunculus scleratus (Dorion et al. 1975) asparagus (Bui-Dang Ha and MacKenzie 1973) pea (Gamborg et al. 1975) and Douglas fir (Kirby and Cheng 1979). For Vicia narbonensis a specific requirement for asparagine has been demonstrated and this could not be replaced by glutamine or casein hydrolysate (Donn 1978). Elevated levels of calcium were essential or beneficial for protoplasts of Vicia and Bromus (Kao et al. 1973).

Pisum (von Arnold and Eriksson 1977), Petunia (Binding 1974) and Arabidopsis (Xuan and Menczel 1980). Phosphate supplementation has also been shown to enhance cell division (Kao et al. 1973). The micronutrient requirements of protoplasts have not been studied extensively except for pea where iron and zinc were optimal at 50 and 10 μ M, respectively (von Arnold and Eriksson 1977).

The osmoticum is the most abundant constituent of protoplast culture media and can greatly influence the response of protoplasts during culture. The osmotic potential required to stabilize protoplasts depends on protoplast source; cell-suspension-derived protoplasts usually have a lower osmotic potential than those of leaves. The isotonic value of leaf cells depends on the physiological condition of the plant (Shepard and Totten 1975). The concentration of osmoticum determines the time of initiation and rate of cell division in protoplast cultures (Binding 1974). Mannitol and/or sorbitol are the most commonly used osmotica. While it seems that mannitol is not metabolized by plant cells (Trip et al. 1964) protoplast cultures of N. sylvestris (Nagy and Maliga 1976) and Arabidopsis thaliana (Xuan and Menczel 1980) were sensitive to its presence. This sensitivity may be related to its ability to block enzyme systems such as phenylalanine ammonia-lyase (Kopp et al. 1977). In another study, (Landgren 1976), however, mannitol (with 2% sucrose) could not be replaced by sucrose or glucose (alone) in supporting cell division. Glucose best supported division in carrot (Grambow et al. 1972), Vicia and soybean (Michayluk and Kao 1975), while sucrose was superior for brome grass (Michayluk and Kao 1975). For N. sylvestris (Nagy and Maliga 1976) and pea-root protoplasts (Landgren 1976) sucrose was superior to glucose. Other carbohydrates

such as fructose, ribose, xylose, mannose, rhamnose and cellobiose have been used to support growth of single Vicia protoplasts (Kao and Michayluk 1975). Galactose supplements were reported to inhibit (Uchimiya and Murashige 1976) and stimulate (Khasanov and Butenko 1979) division of protoplast-derived cells of tobacco and cotton, respectively.

Auxins and cytokinins are usually included in protoplast culture media. Auxin was not essential during the early hours of culture of tobacco leaf protoplasts (Meyer and Cooke 1979). In the absence of auxin cell walls formed on tobacco protoplasts derived from cell suspensions but auxin was required for division; NAA was superior to 2,4-D or IAA (Uchimiya and Murashige 1976). For carrot protoplasts auxin was also required for wall synthesis (Asamizu and Nishi 1980). Cytokinin was not required for cell division of protoplasts derived from a cell culture of tobacco (Uchimiya and Murashige 1976) and actually caused sugar cane protoplasts to lyse (Maretzki and Nickell 1973). Tobacco leaf and pea root protoplasts, however, required cytokinin for sustained cell division (Nagata and Takebe 1971; Meyer and Cooke 1979; Potrykus et al. 1979; Landgren 1976). Experiments with tobacco leaf protoplasts in which cytokinin was withheld for various periods of time have shown that the time before onset of division decreased with increasing intervals of cytokinin deprivation suggesting that cytokinin is required to overcome a specific block in the cell cycle (Meyer and Cooke 1979). Of five auxins and three cytokinins tested at various concentrations for support of cell division in tobacco protoplasts, 2,4-D was superior to dicamba, p-chlorophenoxyacetic acid, indolepyruvic acid, indoleacetic acid and indolebutyric acid;

kinetin was superior to zeatin or 2-IP (Harms et al. 1979).

In a more limited survey with Arabidopsis 2,4-D was found to be superior to NAA and benzyladenine superior to kinetin (Xuan and Menczel 1979). Thousands of media variations with auxin and cytokinin have shown that a relatively broad range of hormone concentrations will suffice for tobacco and corn (Harms et al. 1979; Potrykus et al. 1979).

It has been proposed that failure to achieve cell division and colony formation with protoplasts may be due to leakage of essential metabolites from the protoplasts into the medium and/or accumulation of toxic compounds (Kao and Michayluk 1975). Use of large volumes of enriched medium with a variety of carbohydrates, amino acids and coconut water allowed division of single isolated protoplasts (Kao and Michayluk 1975) supporting both proposals. Evidence for the accumulation of inhibitors has been obtained in a study with tobacco; hormones apparently blocked inhibitor production (Meyer and Cooke 1979). Non-dividing x-irradiated protoplasts (Raveh et al. 1973) or viable albino protoplasts (Menczel et al. 1978; Evans 1979) have been used as "feeders" to support division in protoplasts under normally unfavorable conditions. Similarly, the use of conditioned medium taken from precultured cells has been beneficial in certain cases (Kao et al. 1970; Abo El-Nil and Hildebrandt 1976). Medium pH, which can significantly affect responses of protoplasts is usually set at 5.5 to 6.0. In Phaseolus, however, a pH of 7.0 was most suitable (Pelcher et al. 1974). Many essential requirements for culturing protoplasts may still not be fully understood since, for example, with corn over 80,000 variations in culture medium and plant material have failed to achieve sustained division (Potrykus et al. 1976).

Changes in morphology are characteristic of protoplasts in culture. The nucleus moves to a central position and cytoplasmic organelles and vacuolar strands change their distribution. In addition protoplasts increase in volume and change from a spherical to oval shape as a result of cell wall deposition (Kao et al. 1970; Constabel et al. 1973). The manner in which cellulose fibrils are assembled on protoplast surfaces and their relationship to the topology of the plasmamembrane and to each other is not well understood (Herth and Meyer 1977; Willison and Grout 1978; Burgess and Linstead 1979). At the ultrastructural level Ammi and Skimmia protoplasts were rich in endoplasmic reticulum (ER) with associated polysomes and golgi, and the nucleus appeared somewhat lobed (Fowke et al. 1973, 1974; Robenek 1980). Close association between the ER and plasmamembrane, and the similarity in membrane bound particle arrays in the membranes of the two organelles led to the suggestion of a possible interaction between these organelles during wall deposition on Skimmia protoplasts (Robenek and Paveling 1977; Robenek 1980). On the other hand, regular arrays of particles in the fracture faces of carrot and Petunia plasmamembranes were believed to be essentially an artifact of 20% glycerol used in fixation (Davey and Mathias 1979). Wilkinson and Northcote (1980) could not find any clear relationship between the position of regular arrays of intramembrane particles and the occurrence of microfibrils regenerated on cultured potato protoplasts. The initial wall formed on Ammi (Fowke et al. 1974) and tobacco (Takebe and Nagata 1973) protoplasts was composed of loosely-arranged fibrils. Subsequently, the completed wall appeared similar in texture to that of cultured cells (Fowke et al. 1973, 1974). Time course studies with cell-

suspension-derived Vicia protoplasts showed deposition of wall microfibrils after 10-20 minutes of culture (Williamson et al. 1977). In tobacco leaf protoplasts cellulase-sensitive fibrils first appeared after a lag of 7-10 hours of culture and a dense mat of fibrils covered the protoplast surface within two and a half hours from the start of the process (Burgess et al. 1978). In the presence of colchicine, the fibrils were randomly oriented on protoplasts and the cells generated remained spherical (Marchant and Hines 1979). Similar observations have been made concerning cells (Pickett-Heaps 1967) suggesting a functional relationship between microtubules and wall elaboration. The precise nature of this relationship remains to be elucidated.

The chemical composition of the wall initially formed on protoplasts differs from that subsequently produced and considerable amounts of polysaccharides are excreted into the culture medium before wall material is deposited (Takeuchi and Komamine 1978; Asamizu and Nishi 1980). There is also evidence that new walls are rich in hydroxyproline-rich glycoprotein (Tanaka and Uchida 1979). The synthesis of a normal cellulose wall by protoplasts is an important prerequisite for their ability to form cells with a capacity for nuclear and cell division. Nuclear division but not cytokinesis was observed in tobacco protoplasts cultured in the presence of the cell wall inhibitor 2,6-dichlorobenzonitrile (Meyer and Herth 1978) and in salt solutions which caused synthesis of a 'pseudowall' (Meyer 1974). Nuclear divisions were not observed, however, in tobacco protoplasts cultured in the presence of cellulase (Schilde-Rentschler 1977). In corn and oat protoplasts in which neither nuclear division or cytokinesis takes place it has been shown that

the walls formed during culture were weaker than those of viable tobacco protoplasts (Kinnersley et al. 1978).

The nutrient medium which supports the initial few cell divisions of protoplast-derived cells may not be optimal for sustaining continued cell division (Kameya 1975b).

For corn (Potrykus et al. 1976), cotton (Khasanov and Butenko 1979) and pine (David and David 1979) a few divisions in protoplast-derived cells took place but the cell colonies so formed eventually died and could not be successfully transferred to agar-solidified medium. The requirement for two different media, one for inducing the first few cell divisions and the other to sustain division, has been shown for Pelargonium (Kameya 1975b). It is preferable, however, if a single medium is developed to support sustained division (Nagata and Takebe 1971).

6. Morphogenic potential of protoplast-derived cell cultures

In relation to hormonal regimes and conditions necessary for shoot-induction, protoplast-derived cell colonies generally respond similarly to cells derived from plant organs or cell cultures (Nagata and Takebe 1971; Grambow et al. 1972). In N. tabacum, shoots were induced to differentiate in callus by a high cytokinin : auxin ratio (Skoog and Miller 1957; Vas and Hildebrandt 1965; Thorpe 1978) and this relationship holds for protoplast-derived colonies of N. tabacum and other Nicotiana species listed in Table 2. Also for Nicotiana as well as other species, continued growth on a medium with a high auxin : cytokinin ratio favors root formation (Skoog 1971; Murashige 1977; Kohlenbach 1977). For embryogenic cultures such as carrot, protoplast-derived cells differentiate into somatic embryos when auxin is removed from the culture medium (Grambow et al. 1972;

Dudits et al. 1977); this response is similar to that observed for cells of root explants (Reinert 1959). These simple relationships, however, do not hold for many species and the influence of other growth regulators and conditions have been shown to alter the morphogenic response (Street 1979; Szweykowska 1977). With protoplast-derived cells of Nemesia strumosa, for example, shoot formation occurred with a high auxin concentration in the absence of cytokinin (Hess and Leipoldt 1979) and in Brassica napus cytokinin alone could not induce shoot formation but was effective only when supplied along with gibberellic acid (Kantha et al. 1974).

For a number of species, particularly cereals and legumes, it has not been possible to induce de novo shoot formation in callus derived from protoplasts. Recent studies, however, have shown that the formation of somatic embryos may be more readily induced in protoplast-derived cells for certain cereal and legume species than de novo shoot formation in protoplast-derived callus. For alfalfa (Kao and Michayluk 1980) and Pennisetum (Vasil and Vasil 1980) somatic embryos were formed directly from protoplasts without an intervening callus stage. Also for N. tabacum (Lörz et al. 1977) and N. sylvestris (Facciotti and Pilet 1979) somatic embryos and plants developed from protoplasts under certain conditions — a process never observed in callus tissue. These studies and a more recent one with Hyoscyamus muticus and N. tabacum suggest that protoplasts can undergo morphogenesis after a few cell divisions and the failure to regenerate plants from protoplast cultures of certain species may be due to failure in providing inductive conditions during the initial stages of culture (Wernicke and Thomas 1980).

Shoot induction procedures for somatic hybrid cell cultures are basically similar to those outlined above. In somatic hybrids of Nicotiana spp. manipulation of auxin/cytokinin ratio has generally resulted in shoot induction (Table 4). In wide crosses such as soybean + tobacco (Kao 1977) it has not been possible to regenerate hybrid plants. Certain interspecific (Schieder 1978a; Maliga et al. 1977) and intergeneric (Gleba and Hoffmann 1979; Melchers et al. 1978; Krumbiegel and Schieder 1979) cell hybrids, however, have been shown to possess an enhanced morphogenic potential as compared to the parent cultures from which they were derived. The basis for this developmental 'heterosis' is unclear but certainly it increases the possibility of successful production of plants in somatic fusions (Smith and Mastrangelo-Hough 1979).

Adventitious roots are usually induced on shoots of protoplast-derived somatic hybrids through culture on either hormone-free medium or one containing auxin alone. As with de novo shoot induction many factors modulate the stimulatory influence of auxin in inducing adventitious root formation (Murashige 1974). A differential response to root induction between different somatic hybrid shoot lines has been demonstrated for N. glauca + N. langsdorffii (Smith et al. 1976), N. tabacum + N. knightiana (Maliga et al. 1978) and Solanum tuberosum + Lycopersicon esculentum (Melchers et al. 1978) suggesting a strong genetic determination. In certain cases the resistance to root induction necessitated the grafting of hybrid shoots onto scions (Smith et al. 1976; Melchers et al. 1978).

B. Somatic Hybridization

1. Historical perspective

In 1859 de Bary observed that in the life cycle of certain myxomycetes, single cells fused to form multinucleated plasmodia; this study is regarded as the first to show that multinucleate somatic cells could arise through cell fusion (Harris 1970). In more modern times Barski et al. (1960) working with cultured mouse cells which differed both in morphology and karyotype, reported the appearance of hybrid cells in vitro which arose due to spontaneous cell fusion. Subsequently many other intraspecific hybrid lines were produced and characterized (Ephrussi 1972). Cultured cells could be induced to fuse artificially by "Sendai" virus (Okada 1958). This virus was used by Harris and Watkins (1965) to produce the first interspecific somatic hybrid cells in vitro.

The capacity for fusion of higher plant protoplasts was demonstrated by the observation that 'subprotoplasts' formed within plasmolysed cells could fuse upon deplasmolysis (Kuster 1909). Subsequently, isolated protoplasts were shown to fuse when held in close contact for prolonged periods under certain conditions, and using these methods Michel in 1937 is credited with successfully producing interspecific hybrid protoplasts of Beta vulgaris + Allium cepa (Power et al. 1978). Power et al. (1970) reported that sodium nitrate would act as a fusogen and observed fusion of corn and oat protoplasts in the presence of this salt. Carlson et al. (1972) used sodium nitrate to fuse protoplasts of N. langsdorffii and N. glauca resulting in the production of viable hybrid cells from which plants were

subsequently regenerated. Fusion methods were improved first with the use of a calcium solution buffered at high pH (Keller and Melchers 1973) and later with the development of a polyethylene glycol (PEG) treatment (Kao and Michayluk 1974; Wallin et al. 1974). This agent has now largely replaced "Sendai" virus for fusing animal cells (Pontecorvo 1975; McBurney and Strutt 1979). Viable somatic hybrid plant cells have been produced from many intraspecific, interspecific and intergeneric protoplast fusions (Constabel 1976, 1978; Thomas et al. 1979).

2. Selection of somatic hybrids

In the presence of a fusing agent, a mixed population of protoplasts can fuse at random resulting in the formation of heterokaryons at frequencies of up to 30% (Kao 1978). The post-fusion population will consist of a mixture of homokaryons, heterokaryons and unfused protoplasts. Various selection procedures have been developed in order to differentiate the hybrid protoplasts and cells from homofused or unfused protoplasts and cells.

The first somatic hybrid cells (animal) in vitro were detected because they naturally outgrew parental cells (Barski et al. 1960). Later, conditions favoring preferential proliferation of hybrids such as culturing at low temperature were used to enhance the recovery of hybrid cell lines (Ephrussi 1972). For higher plants, by observing the responses of sexual hybrid cells in vitro it was possible to devise media which preferentially favored their growth; such media could also be used to favor growth of hybrid somatic cells (Carlson et al. 1972; Power et al. 1977). However, in most cases sexual hybrids are not available for developing selective conditions and/or hybrid cells do not necessarily have a more

rapid growth than the parents. More positive selection procedures such as the use of complementing mutants are therefore essential.

A hybrid selection system based on genetic complementation was first developed by Littlefield (1964, 1966) using mouse cell lines with deficiencies in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK). Such deficiencies confer resistance to 8-azaguanine (8-AG) and 5-bromodeoxyuridine (BUdr), respectively. Both HGPRT and TK are necessary for nucleotide synthesis from hypoxanthine and thymidine via the salvage pathway but since an alternative de novo pathway of synthesis from sugars and amino acids is possible these mutant lines survive in culture. The de novo pathway may be blocked with aminopterin. Littlefield (1964) selected somatic hybrid cells in a medium with hypoxanthine aminopterin and thymidine (HAT); in this medium, mutant parental cells were selected against due to the presence of aminopterin while hybrid cells complemented for the deficient enzymes and grew. Several other bidirectional selective media were subsequently formulated for mammalian cells (Chan et al. 1978; Krooth et al. 1979).

A HAT selection system is apparently unsuitable for higher plant cells because they possess greater tolerance to drugs such as aminopterin and where the de novo pathway of nucleotide synthesis could be blocked with aminopterin growth was not restored in such cells with hypoxanthine and thymidine supplements (Cocking 1976a). Furthermore, while cells resistant to nucleotide base analogues have been reported (Maliga et al. 1973; Lescure 1973; Bright and Northcote 1975; Horsch and Jones 1978) such cells may also carry undesirable genetic traits. The capacity for genetic complementation in plant cells has, however, been used where

mutant parental cells were available (Cocking 1978) and in the presence of metabolic inhibitors which select against wild-type parental cells (Nehls 1978b). The following complementation-selection systems have been successfully used:

- a) Complementation for chlorophyll synthesis by fusing chlorophyll-deficient or albino mutants of both parental types: (Melchers and Labib 1974; Gleba et al. 1975; Melchers 1977; Schieder 1977b).
- b) Complementation for chlorophyll synthesis by fusing chlorophyll-deficient or albino with wild-type protoplasts coupled with the use of conditions which select against the wild-type parent: (Cocking et al. 1977; Dudits et al. 1977, 1979; Schieder 1978a; Power et al. 1979, 1980; Krumbiegel and Schieder 1979; Evans et al. 1980).
- c) Complementation selection using biochemical mutants (Power et al. 1976, 1977; Glimelius et al. 1978a; Maliga et al. 1977; White and Vasil 1979; Wallin et al. 1978b; Wullems et al. 1980).

Where mutants are not available to allow a complementation-selection procedure individual hybrid protoplasts may be identified during fusion when each parent carries visible markers. This system is particularly suitable where non-pigmented protoplasts derived from cell suspension are fused with pigmented leaf mesophyll or petal protoplasts. Semi-pigmented heterokaryons may be detected by microscopic observation and isolated mechanically. A great number of viable intergeneric hybrids have been produced using this method. (Constabel 1976, 1978; Reinert and Gosch 1976; Kao et al. 1974; Kao 1977; Gleba and Hoffmann 1979; Gleba and Hoffmann 1979) and since heterokaryons could be identified immediately after fusion the development of hybrids in relation to chromosomal composition (Kao 1977; Kao 1978) and ultrastructural cytology (Fowke et al. 1977) could be studied. Where protoplasts do not carry natural cytoplasmic markers, the plasmamembranes and internal membrane systems may be 'tagged' with fluorescent probes (Galbraith and

Galbraith 1979). Inherent differences in the buoyant densities of protoplasts may also be used to obtain a fraction rich in hybrid protoplasts after centrifugation on a sucrose gradient (Harms and Potrykus 1978a).

Finally, somatic hybrids may be selected on the basis of plant morphology after fusion products have been induced to undergo morphogenesis. This system has been used where the selection systems outlined above were not applicable such as in the production of male-sterile hybrids (Gleba 1978a; Belliard et al. 1977; Izhar and Power 1979; Zelcer et al. 1978).

Virtually all the selection procedures outlined above have been used for selecting somatic hybrids within and between members of the genus Nicotiana. Successful somatic hybridizations within this genus which have yielded hybrid plants are listed in Table 4.

3. Characterization of somatic hybrids

Somatic hybrid cells and organisms are characterized in order to verify hybridity; in addition the expression of particular parental genes in different genetic backgrounds may be studied. Somatic hybrids of higher plants have been characterized by morphological, cytological and biochemical criteria at all stages from the completion of protoplast fusion to mature hybrid plants.

a) Morphological characterization

The morphological features of the flowers, leaves, trichomes and roots of somatic hybrids have been studied with reference to the parental types and where possible to sexually produced hybrids. Analysis of interspecific somatic hybrids of Petunia, Datura and Nicotiana have shown that flower corolla length and pigmentation, leaf shape and trichome density, and general plant forms were intermediate with reference to the parental types (Power et al. 1976;

TABLE 4. Somatic hybridizations within the genus Nicotiana

<u>Somatic hybrid</u>	<u>Reference</u>
<u>N. tabacum</u> + <u>N. tabacum</u>	Melchers and Labib 1974 Gleba 1979 Glimelius <u>et al.</u> 1978a Wullems <u>et al.</u> 1980
<u>N. tabacum</u> + <u>N. alata</u>	Nagao 1979
<u>N. tabacum</u> + <u>N. glutinosa</u>	
<u>N. tabacum</u> + <u>N. sylvestris</u>	Melchers 1977 Zelcer <u>et al.</u> 1978
<u>N. tabacum</u> + <u>N. glauca</u>	Evans <u>et al.</u> 1980
<u>N. tabacum</u> + <u>N. knightiana</u>	Maliga <u>et al.</u> 1978
<u>N. tabacum</u> + <u>N. rustica</u>	Nagao 1978
<u>N. sylvestris</u> + <u>N. knightiana</u>	Maliga <u>et al.</u> 1977 Menczel <u>et al.</u> 1978
<u>N. glauca</u> + <u>N. langsdorffii</u>	Carlson <u>et al.</u> 1972 Smith <u>et al.</u> 1976 Chupeau <u>et al.</u> 1978 Wetter and Kao 1976
<u>N. sylvestris</u> + <u>N. sylvestris</u>	White and Vasil 1979

Smith et al. 1976; Schieder 1978a; Power et al. 1979, 1980; Maliga et al. 1977, 1978; Evans et al. 1980). For somatic hybrids of Daucus, leaf and tap-root morphology was intermediate (Dudits et al. 1977). In the genera Nicotiana and Datura interspecific sexual hybrids were intermediate for leaf shape and corolla tube length and pigmentation (Goodspeed 1954; Avery et al. 1959). An exception was the cross between N. glutinosa x N. bigelovii in which the F_1 possessed larger flowers than either parental type (Smith 1974).

b) Cytological characterization

At the cellular level somatic hybrids have been characterized from the time of fusion. Evidence from fluorescence labelling of cellular membranes indicated that a slow mixing of membrane components occurs in hybrid protoplasts (Galbraith and Galbraith 1979). Complete cytoplasmic mixing takes between 3 and 10 hours (Fowke et al. 1975; Gosch and Reinert 1976) and during this mixing the vacuoles fuse (Constabel et al. 1980). Differentiated chloroplasts of hybrid cells degenerate and decrease in number during subsequent cell divisions while the number of leucoplasts appears to increase (Fowke et al. 1977). While the relative contribution of the parent protoplasts to wall formation are not known this fundamental property of plant cells is expressed even in plant + amphibian hybrid cells (Davey et al. 1978).

Most studies of hybrid nuclear cytology deal with intergeneric hybrids because of the relative ease in distinguishing parental chromatin and chromosomes. Studies on such intergeneric hybrids may provide basic information concerning the regulation and expression of nuclear genes in general and also on processes such as chromosome

elimination. The nuclei of hybrid cells have been observed to fuse while in interphase (Constabel et al. 1975a; Kao 1977, 1978) and this process may be mediated through the formation of nuclear bridges (Fowke et al. 1975, 1977). Nuclei have also been observed to undergo fusion during mitosis when all chromosomes align on a common metaphase plate (Constabel et al. 1975a; Kao 1977, 1978; Gosch and Reinert 1976). Fusion during mitosis resulted in a greater frequency of hybrid cells undergoing division than when fusion of interphase nuclei took place (Kao 1978). In some cases complete nuclear fusion did not occur resulting in hybrid cells with a predominance of chromosomes of one or other parent (Binding and Nehls 1978).

The time required for the initial cell division to occur in cultured hybrid cells seems to be related to the requirement of each parent cell. First mitosis in a number of heterokaryocytes occurred at a time which was intermediate to that required for each of the parental types (Constabel et al. 1976). In some cases it is only necessary that one of the parent protoplasts have a capacity for cell division during culture in order for hybrid nuclei to enter mitosis (Kao et al. 1974).

Intimate mixing of chromatin does not necessarily occur in hybrid cells and areas of parental chromatin can be readily distinguished even after several cell divisions (Constabel et al. 1977; Fowke et al. 1977). At the first mitosis chromosomal fragments, ring, and extremely long chromosomes have been observed in intergeneric hybrid cells (Kao 1977; Binding and Nehls 1978). A progressive and generally preferential loss of one set of parental chromosomes followed subsequent cell division (Kao 1977, 1978;

Binding and Nehls 1978) which in certain studies lead to a complete loss of one set of chromosomes (Power et al. 1975; Dudits et al. 1979). In certain other intergeneric hybrids such as Datura + Atropa and Arabidopsis + Brassica, however, extensive chromosome loss did not occur (Krumbiegel and Schieder 1979; Gleba and Hoffmann 1978). For Nicotiana somatic hybrids (Table 4) the chromosomes of each parent species are usually too similar to distinguish cytologically. In such hybrids, however, aneuploidy is common and chromosome complements greater than the sum of the parental types have been reported among all hybrids except those of N. rustica + N. tabacum (Nagao 1978). The chromosomal stability of somatic N. tabacum + N. knightiana hybrids was very low, with losses and mitotic irregularities occurring even within the hybrid plant body while stability of the corresponding sexual hybrid for chromosome number was high (Maliga et al. 1978).

c) Biochemical and genetic characterization

At the subcellular level somatic and sexual hybrids have been characterized by isoenzyme analysis as it provides direct information of nuclear **gene expression**. Where appropriate differences in electrophoretic banding patterns of parental enzymes exist such analyses can provide definitive evidence of hybridity (Davidson 1971; Carlson et al. 1972; Wetter and Kao 1976; Wetter 1977). A number of isozyme systems have proven useful for hybrid verification; peroxidase (Dudits et al. 1977; Power et al. 1975; Carlson et al. 1972; Power et al. 1976; Butenko and Kuchko 1980), esterase (Maliga et al. 1977, 1978; Gleba and Hoffmann 1979; Menczel et al. 1978); phosphodiesterase

(Maliga et al. 1978), alcohol dehydrogenase (Maliga et al. 1977; Wetter 1977; Menczel et al. 1978), glucose-6-phosphate dehydrogenase (Maliga et al. 1977), alanyl-aminopeptidase (Evans et al. 1980) and aspartate aminotransferase (Evans et al. 1980; Wetter 1977; Menczel et al. 1978). In most cases the hybrid banding pattern was not a simple summation of bands present in the parents; losses of one or more parental bands (Wetter and Kao 1976; Wetter 1977) or the appearance of new bands (Power et al. 1976; Menczel et al. 1978; Evans et al. 1980) have been reported. Two studies suggest that even when the entire diploid complement of parental chromosome has been eliminated a few genes may be retained by the hybrid cells. In Petunia + Parthenocissus hybrid cells Petunia-specific peroxidase isozymes were detected although Petunia chromosomes were not present (Power et al. 1975). Similarly in the case of Aegopodium podagraria + Daucus carota hybrids in which Aegopodium chromosomes were eliminated the presence of Aegopodium genes were suggested by the high level of hybrid nuclear RNA which hybridized to Aegopodium DNA immobilized on nitrocellulose (Dudits et al. 1979).

The expression of cytoplasmic as well as nuclear genes may be studied by analysis of the polypeptide banding pattern of ribulose-1-5-bisphosphate carboxylase/oxygenase (RUBPCase) after isoelectric focussing of the carboxymethylated protein. The small subunit of this enzyme is encoded by nuclear genes (Kawashima and Wildman 1972) while the large subunit is encoded by genes in the chloroplast genome (Chan and Wildman 1972; Coen et al. 1977; Gelvin et al. 1977). Analysis of the small subunit in somatic hybrids of N. langsdorffii + N. glauca (Chen et al. 1977), N. glauca + N. tabacum (Evans et al. 1980), N.

rustica + N. tabacum (Iwai et al. 1980) and potato + tomato (Melchers et al. 1978), has shown electrophoretic bands characteristic of both parental species indicating nuclear hybridity. In some cases, however, a differential staining intensity (Melchers et al. 1978) or complete loss of one parental band (Iwai et al. 1980) has been shown in hybrids.

Somatic hybridization results in the production of heterokaryons containing organelles from both parents. Analysis of the chloroplast-encoded large subunit of RUBPCase of mature somatic hybrid plants by its isoelectric focussing pattern (IF) consisting of polypeptide bands indicates that they express the chloroplast genes of only one parent (Chen et al. 1977; Melchers et al. 1978; Iwai et al. 1980). Two studies reported the presence of both parental large subunits in hybrids (Chen et al. 1977; Gleba 1978a). In the former study subsequent experiments showed that when individual cells from leaves were cloned and new plants regenerated, the large subunit isolated from each plant was that of only one parent, indicating the original plant was a chimera (Uchimiya and Wildman 1979). The presence of cytoplasmic genes in hybrids has been studied by analysis of chloroplast or mitochondrial DNA digests after gel electrophoresis (Belliard et al. 1978, 1979). These studies showed that only one of the two parental chloroplast DNAs was present in all hybrid plants and that recombination of chloroplast DNA did not occur (Belliard et al. 1978, 1979). Evidence was presented, however, that recombination of mitochondrial genomes had taken place (Belliard et al. 1979).

Other genetic evidence for hybridity may be inferred from the appearance of new characteristics such as hybrid vigor (Schieder 1978a; Nagao 1978), spontaneous tumour formation (Smith et al. 1976),

restoration of morphogenic potential in hybrid cell cultures (Maliga et al. 1977; Melchers et al. 1978; Gleba and Hoffmann 1979); complementation for normal chlorophyll synthesis (Melchers and Labib 1974; Gleba et al. 1979; Melchers 1977; Schieder 1977b) and simultaneous expression of parental nuclear markers such as drug resistance (White and Vasil 1979; Willems et al. 1980). The segregation of nuclear genes for chlorophyll-deficiency observed in haploid plants derived from the microspores of somatic hybrids has also provided genetic evidence for nuclear hybridity (Schieder 1978b).

C. Sexual Hybridization by Culturing Hybrid Embryos In Vitro

1. Historical perspective

Laibach (1929) was the first to show that hybrid flax embryos which were normally not viable in nature could be 'rescued' when cultured in vitro before the onset of incompatible reactions and grown to seedling plants. Where the incompatibility barriers to sexual hybridization are post-zygotic, culture of hybrid embryos has facilitated the production of intraspecific, interspecific, and intergeneric hybrid plants in over 20 genera (Raghavan 1977). Hännig (1904) first explored the prospect that excised immature embryos might be cultured to seedling plants in vitro on defined medium. Since this study the immature embryos of species in over a hundred genera have been cultured successfully (Narayan ^{aswami} and Norstog 1964).

The entire processes of sexual embryogenesis can be supported in culture as demonstrated by Kanta et al. (1962). In this study poppy ovules were pollinated in vitro and the embryos developed to maturity within their seed structures. In similar experiments

single-celled zygotes and proembryos enclosed either in ovules or ovaries were cultured to maturity in Nicotiana (Dulieu 1966; Rao 1965; Balatkova and Tupy 1972; Zenkteler and Melchers 1978), Zea (Sladky and Havel 1976; Gengenbach 1977), Argemone and Eschscholzia (Kanta and Maheshwari 1963), Petunia (Shivanna 1965), Antirrhinum (Usha 1965), Dianthus (Zenkteler 1965), and Dicranostigma (Rangaswamy and Shivanna 1969). Hybrid zygotes have also been cultured to maturity following in vitro fertilization to yield interspecific and intergeneric hybrid plants (Zenkteler 1967).

2. Culture requirements of embryos

Although the whole process of embryogenesis from a zygote can occur in vitro provided zygotes or proembryos are cultured while still enclosed within their seed structures, several studies have shown that it is not possible to culture very young proembryos when excised from their seeds. Very young embryos often died and those that survived usually germinated at an early stage giving rise to abnormal plants rather than developing to maturity (La Rue 1936; Tukey 1938). Success in culturing excised embryos is determined largely by the extent of embryo development attained prior to excision (Raghavan and Torrey 1963).

Composition of the culture medium significantly affects the growth of embryos in vitro. When medium was supplemented with B vitamins or coconut milk, young, heart-stage embryos of Nicotiana and Datura were successfully cultured (van Overbeek et al. 1942). Coconut milk was subsequently shown to promote the development of embryos of a number of other species (Narayanaswami and Horstog 1964; Raghavan 1966). Endosperm extracts of other species such as barley have been used (Ziebur and Brink 1951) and

excised embryos have also been cultured directly on excised endosperm tissue (Kruse 1974). Supplementation of media with casein hydrolysate has also been found to be beneficial for young embryos in a number of studies (Raghavan 1966).

Culture media were much improved following the analysis of the chemical constituents of endosperm. Analysis of Capsella endosperm tissue showed that the culture medium should have a supply of reduced nitrogen (preferably glutamine) with a high osmolarity value and acid pH (Rijven 1952). For seven different species the osmolarity of endosperm liquid was high at a time when embryos were immature and declined as they matured and were about to germinate (Ryczkowski 1960). This study provided a rationale for the observation that immature embryos of Datura required 8% sucrose for normal development while nearly mature embryos grew well with 0.1 to 1.0% sucrose (Paris et al. 1953; Rietsma et al. 1953). Similar observations have been made for cultured embryos of barley (Norstøg and Smith 1963), sunflower (Kuo and Wang 1965), flax (Pretova 1974) and pea (Stafford and Davies 1979). For many species sucrose has been shown to be superior to other sugars (Raghavan 1966; Burghardtova and Tupy 1980). Studies by Raghavan and Torrey (1963) questioned the need for a high level of osmoticum for young Capsella embryos. These workers showed that while very young globular embryos could be grown with 12-18% sucrose, the sucrose level could be dropped to 2% provided the medium was supplemented with IAA, kinetin and adenine.

Immature embryos in culture have highly specific nitrogen requirements. Analysis of cotton and bean endosperm indicated the presence of high malate levels as well as higher levels of ammonium

than that supplied in normal culture media (Mauney et al. 1967; Smith 1973). Sodium malate was toxic to cultured cotton embryos whereas ammonium malate enhanced their growth and survival frequency (Mauney et al. 1967); embryos cultured with ammonium malate did not require a high level of osmoticum. Similar studies with cultures of immature barley embryos reported low (2%) sucrose level supported growth if the ammonium level was optimized (Norstog 1979). This requirement of young embryos for ammonium ions seems to result from their inability to metabolize nitrate. Studies with immature orchid embryos showed they lacked nitrate reductase and could only metabolize ammonium. Furthermore, even when nitrate reductase appeared nitrate was not utilized in the presence of ammonium (Raghavan and Torrey 1964b).

In a number of embryo culture experiments certain growth regulators have been shown to have positive effects in very small quantities. IAA when used in low concentrations (0.05 mg/L) was reported to enhance the development of embryos of several species (La Rue 1936). For Capsella a number of different auxins at very low concentration (0.0001-0.001 mg/L) promoted the growth of the primary root, hypocotyl and cotyledons and induced the initiation of embryonic leaves in excised embryos of a range of ages (Raghavan and Torrey 1964a). Gibberellic acid promoted elongation of the root and hypocotyl of older Capsella embryos but for young embryos inhibited initiation of a shoot apex (Raghavan and Torrey 1964a). For immature Hordeum distichum embryos, gibberellic acid promoted normal embryo development and prevented development of callus (Granatek and Cockerline 1978). In this study gibberellic acid promoted utilization of stored starch which effectively raised

the cellular osmolarity and allowed normal embryo development to occur. Exogenous cytokinins are generally disruptive to normal embryogenic processes. In Capsella (0.0001 to 1.0 mg/L) kinetin inhibited growth of the embryonic root and induced precocious expansion of embryonic leaves (Raghavan and Torrey 1964a).

Combinations of kinetin and IAA plus adenine sulphate, however, permitted development of very young globular embryos (Raghavan and Torrey 1963). Absciscic acid (ABA) is generally regarded as a growth inhibitor and has been used in media for culturing embryos. Umbeck and Norstog (1979) reported that while absciscic acid inhibited growth of the scutellums and coleoptiles of excised barley embryos it favored development of normal embryos at low levels (0.01-0.05 mg/L). This 'regularizing' of development by ABA was further enhanced in the presence of ammonium. Norstog (1979) proposes that ABA may be inhibiting onset of the germinative phase in immature embryos and at the same time permitting normal embryogenesis.

3. Culture of *Nicotiana* embryos in vitro

Studies of embryo culture in the genus Nicotiana have involved either culturing entire ovaries containing immature seed or culturing immature seeds which were removed from the ovary. For N. rustica entire unpollinated pistils were excised and placed on culture medium; one day later they were pollinated and subsequently normal seeds developed and germinated (Rao 1965). A progression from globular to heart-shaped embryos was observed from the sixth to twelfth day after pollination and after the fifth week seeds began to germinate in situ (Rao 1965). Similar studies were carried out

with N. tabacum and in addition to culturing immature seeds within the ovary, fertilized ovules were excised 4-5 days after pollination and cultured directly on medium (Dulieu 1966). This study showed that embryos required B-vitamins and sucrose at an optimal level of 4%; casein hydrolysate, kinetin or NAA supplied alone or in combination, however, were toxic. Similar studies by Balatkova and Tupy (1972) on the other hand, reported that casein hydrolysate favored the development of N. tabacum embryos.

In vitro culture techniques have also been utilized to facilitate interspecific sexual hybridization in Nicotiana. Fertilized ovules of N. repanda x N. tabacum, N. stocktonii x N. tabacum and N. nesophila x N. tabacum were successfully cultured in vitro after extensive attempts to achieve sexual hybrids in planta had failed (Reed and Collins 1978). This study showed that ovules from greenhouse crosses were more productive than those from field crosses and that ovules cultured within two weeks of pollination were most viable. While hybrid plants of N. stocktonii x N. tabacum and N. nesophila x N. tabacum survived to maturity N. repanda x N. tabacum hybrids did not survive transition to the greenhouse (Reed and Collins 1978).

MATERIALS AND METHODS

A. Somatic Hybridization Studies1. Plant material

Seeds of Nicotiana rustica L. ($2n=48$) and Nicotiana tabacum L. ($2n=48$) were obtained from Dr. R. Pandeya, Delhi Research Station, Agriculture Canada, Delhi, Ontario and Dr. D. U. Genetel, North Carolina State University, Raleigh, NC, respectively. Strains of N. rustica consisted of a wild-type N. rustica var NRT and a chlorophyll-deficient mutant var chlorotica. Reciprocal crosses between these genotypes established that the chlorotica phenotype is due to a recessive nuclear mutation (Pandeya, personal communication). Seeds of N. tabacum were derived from heterozygous plants of a white seedling (WS) strain which carried a recessive nuclear mutation for lethal albinism.

Plants of both N. rustica genotypes and wild-type N. tabacum were grown in a controlled chamber under a light intensity of 3000 ft-c, with a 16 h (25°C) light and an 8 h (20°C) dark cycle. Callus cultures were initiated from internodal stem sections of plants approximately 30-35 cm high or from seedlings. Cultures of N. tabacum were also initiated from seedlings. Seeds from the WS heterozygote of N. tabacum were germinated on agar-solidified hormone-free B_5 medium (Gamborg et al. 1968; Appendix, Table 1) at 25°C under low light intensity (100 ft-c). The first true seedling leaves and hypocotyls of albino and wild-type plants were excised and used for callus initiation. Seeds and explants were surface sterilized in 7% w/v filtered calcium hypochlorite for

20 min followed by three rinses of sterile demineralized water.

2. Initiation of callus and suspension cultures

To initiate callus cultures, explants were placed on solid (0.8% Difco agar) medium of Murashige and Skoog (1962) with 2% sucrose modified by the addition of 40 mg/L Sequestrene Fe 330 (Geigy) and the vitamins of B₅ medium (above), hereafter referred to as MS medium (Appendix, Table 2). The medium was supplemented with either 1 mg/L naphthaleneacetic acid (NAA) or 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The method for preparing all growth regulator stocks is given in Appendix, Table 6. The pH was adjusted to 5.8 (0.1 M KOH) and the medium autoclaved (18 min at 1.1 kg/cm² pressure).

Cell suspension cultures were obtained from the primary callus cultures by transferring approximately 4 g of callus into 125 mL Ehrlenmeyer flasks containing 30-35 mL of liquid MS medium supplemented with 2,4-D or NAA. The pH of the liquid medium was adjusted to 5.0 prior to autoclaving. Suspension cultures were maintained on a gyrotory shaker at 160 rpm at 25°C in continuous light (140 ft-c). Initially, suspensions were subcultured every 5-7 days but as vigor increased this interval was reduced to 60-84 h.

3. Studies on morphogenesis

Most of the studies on in vitro morphogenesis were carried out using cell suspension cultures. Cell colonies were collected by filtration on 125 µm nylon mesh and washed with 60 mL of hormone-free MS medium. In initial tests, colonies 2-5 mm in diameter were transferred to MS medium containing 2.0 mg/L

kinetin and 0.1 mg/L NAA (Kasperbauer and Collins 1972). In subsequent tests the effect of a range of benzyladenine/auxin ratios on shoot induction was studied. The colonies were cultured on agar-solidified media at 25°C (160 ft-c) and analyzed after 8 weeks with respect to frequency of meristem and/or shoot induction. In some experiments on shoot induction protoplast-derived colonies were used. In these cases the colonies were transferred directly to shoot-inducing media without washing. For root initiation, differentiated shoots (3-6 mm) arising from callus (derived from cell suspensions and protoplasts) were excised and transferred to agar-solidified MS or B₅ medium.

4. Isolation of protoplasts

For isolation of protoplasts cells were filtered from suspension using 125 μ M nylon mesh and approximately 2 g (fr. wt.) cells were placed in 10-mL of enzyme solution in 100 x 15 mm Falcon petri plates, sealed with parafilm and incubated on a reciprocating shaker (26 strokes/min) at 25°C. The enzymes used were Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo), Rhozyme HP 150 (Rohm and Haas, Philadelphia, PA 19105) and Onozuka R-10 (Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan). Individual 10% (w/v) stock solutions of each enzyme were prepared in 0.35 M mannitol and centrifuged on a bench top centrifuge for 12 min at 1500 xg to remove inert material. From these stocks a solution containing 1% Driselase, 1% Rhozyme and 0.1% Onozuka R-10 in 0.35 M mannitol was prepared and pH adjusted to 5.8 with 0.1 M KOH prior to filter sterilization using Nalgene disposable units (0.2 μ M membrane). Following incubation, protoplasts were separated

from undigested tissues by successive passage through 125 and 44 μ M nylon mesh and yields per 2 g cells determined by haemocytometer counts.

5.. Culture of protoplasts

For subsequent culture the protoplasts were collected by centrifugation (100 x g, 3 min) and washed three times in the medium described by Nagata and Takebe (1971), containing 40 mg/L Sequestrene Fe 330 and mannitol or glucose at 0.35 M, hereafter referred to as NT medium (Appendix, Table 3). When used for culturing protoplasts this medium was filter-sterilized. Where other carbohydrates were tested for their effect on chlorophyll formation in protoplast-derived cell colonies the protoplasts were washed three times in NT medium with 0.35 M mannitol and cultured in medium containing alternative carbohydrate osmotica at 0.35 M. In studies on embryogenesis from protoplast-derived cells NT medium was supplemented with 1.1 mg/L p-chlorophenoxyacetic acid and 5.4 mg/L kinetin (Lörz *et al.* 1977). For culture the protoplasts were suspended at a density of $2-8 \times 10^4$ /mL and plated in 100-200 μ L drops in 15 x 60 mm Falcon petri dishes (10-14 drops per dish), sealed with parafilm and incubated at 25°C (22 ft-c) except where otherwise indicated. In each experiment a minimum of 6 plates were incubated. Every second day observations were made on a minimum of 6 drops per treatment. Plating efficiency was taken as the maximum percentage of protoplasts which eventually formed cell walls and underwent cell division.

6. Fusion of protoplasts and regeneration of hybrid plants

Protoplasts were prepared from cell colonies of N. rustica var chlorotica and albino N. tabacum maintained for 18 and 4 months, respectively, in suspension culture on MS medium with 0.5 mg/L 2,4-D. The protoplasts were pelleted by centrifugation (100 xg, 3 min) and resuspended in protoplast culture medium (NT) at densities of $1.5-2.0 \times 10^5/\text{mL}$ (N. rustica) and $1.0-1.5 \times 10^5/\text{mL}$ (N. tabacum). The total time for this wash was less than 10 min to minimize possible wall regeneration. Aliquots of each protoplast suspension (3.5-4 mL) were dispensed into a 10 x 1.5 cm disposable glass tube and centrifuged at 100 xg. Most of the supernatant was decanted and the protoplast mixture was gently resuspended in the remaining medium (~0.2 mL). Four mL of polyethylene glycol (PEG) solution, with or without dimethyl sulfoxide (DMSO), was gently added along the wall of the tube and the protoplasts gently dispersed. The basic PEG solution was that of Kao and Michayluk (1974) using 50% w/v PEG 1540 (Baker Chemicals); method of preparation and components are given in Appendix, Table 7. The concentration of calcium and phosphate in the PEG solution was varied in one experiment. When DMSO was used it was filter-sterilized and mixed with PEG solution immediately prior to use. Protoplasts were incubated 20 min in PEG alone and 3 min in PEG/DMSO. Following incubation, the PEG solution was eluted using either NT protoplast culture medium or calcium-glycine buffer at pH 10.5 (Keller and Melchers 1973); method of preparation and components are given in Appendix, Table 8. The elution procedure of Power et al. (1976) was followed. Aliquots of 0.5, 1.0, 2.0, and 5.0 mL of eluting

solution were sequentially added to the protoplast suspension at 5 min intervals. Additions were made slowly with a Pasteur pipette along the wall of the tube and mixing was achieved by gentle agitation. Following the last addition protoplasts were sedimented by centrifugation, the supernatant aspirated and replaced by 7-8 mL of NT medium and the suspension allowed to stand for 1-2 h. During this interval protoplasts were examined microscopically. Finally, the protoplasts were sedimented, resuspended in 6-8 mL of NT medium, plated in 100-200 μ L drops in 15 x 60 mm Falcon petri plates and incubated at 25°C. Up to 12 tubes of protoplasts could be handled per experiment.

For the first 10-15 days the drop cultures were incubated at a light intensity of 22 ft-c. Subsequently they were transferred to 150 ft-c to induce chlorophyll synthesis in any potential hybrid colonies. The frequency of presumed hybrid colonies, i.e. colonies distinctly greener than either parent, was scored 3-4 weeks post-fusion. Except where mentioned, culture drops containing these hybrid colonies were supplied approximately 50 μ L of NT medium containing either 0.35 M or 0.175 M glucose and after a further 1-2 weeks individual hybrid colonies were transferred to solid shoot-induction medium, with and without osmotic stabilizer. The basic medium was NT medium with benzyladenine (BA) at 1.0-2.0 mg/L and a range of NAA concentrations from 0.05-1.0 mg/L ~~were~~ added. Hybrid calli were maintained for about 8 weeks on shoot-induction medium at 25°C (light intensity 120-150 ft-c) and scored for frequency of shoots.

Individual hybrid calli which produced shoots were selected at random from each fusion experiment and assigned a line number.

Over 250 lines resulting from 12 separate fusion experiments were used in studies on shoot and root morphogenesis. These lines were maintained as stock cultures on NT medium without NAA. For root induction, shoots were excised from these cultures and planted on MS or B₅ media containing a variety of growth regulators. The following growth regulators were used in various experiments on root induction: indoleacetic acid (IAA), indolebutyric acid (IBA), phenylacetic acid (PAA), abscisic acid (ABA), gibberellic acid (GA)^{*}, 7-azaindole (AZA), 8-azaguanine (8-AZAG), cycocel (CYC). ABA, GA, 8-AZAG and CYC were filter-sterilized and added to the medium after autoclaving; other hormones were autoclaved with the medium. Other treatments including supplementation of medium with boron, 3-aminopyridine or nicotine are described in detail in the Results section B.4. Stocks of all growth regulators were solubilized as indicated in Appendix, Table 6 and prepared at a concentration of 1.0 mg/mL. Where CO₂ was supplied (Canadian Oxygen Ltd., Ottawa, Canada) material was cultured in a small incubator which was sealed after introducing CO₂; concentrations of CO₂ were measured using an infra red analyzer (Beckman Instruments, Canada Ltd.). Shoots were cultured in pyrex 25 x 15 mm and 38 x 200 mm tubes, 4 fl. oz. glass food jars and 15 x 60 mm Falcon petri dishes under different conditions of lighting and temperature. The frequency of root formation was scored after about 8 weeks of culture. Rooted shoots were planted in greenhouse soil (1:1:1, loam : peat : sand) in 125 mm pots covered with a small bell jar and placed in a mist chamber. The bell jars were removed after one week and after a further week the plants were transferred to a greenhouse.

^{*} Gibberellic acid (GA) used throughout was GA₃.

B. Sexual Hybridization Studies

1. Plant material and method of pollination

Since preliminary crosses using N. tabacum as the female parent failed, the detailed experiments carried out in this study involved N. rustica as the maternal parent. The source of N. rustica var. chlorotica and N. rustica var. NRT are given above (Section A. 1). For N. tabacum three genotypes were used as pollen sources: (i) Wild-type N. tabacum var. WS either homozygous or heterozygous for a recessive albino mutation (obtained from Dr. D. U. Gerstel); (ii) wild-type N. tabacum var. Delhi 34 (obtained from Dr. R. Pandeya); (iii) a light sensitive "virescent" (V) mutant of N. tabacum (obtained from Professor G. Melchers, Max Planck Institute for Biology, Tübingen, W. Germany). Except for the "V" mutant of N. tabacum all plants were germinated and grown under standard greenhouse conditions and fertilized with 20:20:20 (N:P:K) liquid fertilizer once weekly. Seeds of the "V" mutant were germinated on agar-solidified B₅ medium and after 4-6 weeks seedlings were transferred to Jiffy-7 peat pellets and placed in a mist chamber for about 2 weeks. They were then potted and grown to flowering in a greenhouse under shaded conditions.

Flowering N. rustica plants of various ages were used in sexual hybridization studies. Flowers which were not used and mature seed capsules were removed from experimental plants. Flowers in which the corolla had fully extended from the sepals but which did not show signs of corolla widening at the distal end were judged to be at a stage where anther dehiscence would occur within 24 h. These flowers were emasculated and artificially pollinated 24 h later. Pollen from N. tabacum flowers was obtained

by collecting freshly ruptured anthers with attached pollen in a glass petri plate. Pollen was applied to N. rustica stigmas which showed a glistening surface by rubbing N. tabacum anthers over the stigmatic surface.

2. Culture of sexual hybrid embryos

Hybrid embryos were allowed to develop on the maternal N. rustica plants in the ovary for 3 days except where indicated. After 3 days the ovaries with peduncle attached were excised. The corolla and sepals were removed and the ovaries surface-sterilized by a 1 min dip in 70% ethyl alcohol followed by 20 min in 7% w/v filtered calcium hypochlorite and 3 x 5 min rinses in sterile demineralized water. The ovary walls were then removed and placentas bearing immature hybrid seeds (ovules) were excised and cultured. Alternatively, hybrid seeds were removed from the placentas and cultured independently. The basal medium was agar-solidified B₅ (Appendix, Table 1) containing 4% sucrose, except where indicated. The formulations for alternative media used in one experiment (i.e. MS, Kao, Lörz) are given in Appendix, Tables 2, 4 and 5, respectively. Lighting was continuous and intensity during culture was 22-50 ft-c for the first 3 weeks followed by an increase to 150 ft-c. Temperature of culture was 25°C constant. The number of hybrid seedlings germinating and their morphology was scored after a minimum of 5 weeks of in vitro culture. Seedlings with one, three or fused cotyledons, or those lacking cotyledons or roots were scored as abnormal. After normal seedlings had developed the first set of true leaves they were transferred to 25 x 15 mm glass pyrex tubes containing B₅ medium with 2% sucrose and cultured for 4 weeks. After 4 weeks they were

then potted and maintained for 10 days in a mist chamber before transfer to the greenhouse.

C. Characterization of Somatic and Sexual Hybrids

1. Morphological analysis

As described above each somatic hybrid cell colony which produced shoots was assigned a line number. Where more than one rooted plant was obtained from a given line each plant was designated as a subline (by a letter a, b...) of the original hybrid line. Individual sexual hybrid plants which arose from hybrid seeds in vitro were numbered. Plants of parental species and somatic and sexual hybrids were grown in a greenhouse and supplied weekly with 20:20:20 (N:P:K) liquid fertilizer. Albino N. tabacum mutants could not be grown to flowering. Measurement of a number of traits was made on individual somatic hybrid plants; mean values of similar measurements were calculated from data obtained from four plants of each parental species. Plant height was measured from soil level to the base of the terminal bud and leaf number was taken as the total number of leaves produced in the course of flowering. Leaf areas were means obtained from a sample of the lower five (N. tabacum and somatic hybrids) or lower three (N. rustica) consecutive leaves in the middle third region of each plant. Leaf length determinations did not include the petiole. Relative leaf areas were obtained by the tracing and weighing method. For comparative photography the highest leaf subtending a vegetative bud was selected. The number of floral branches was based on the number bearing functional flowers and also included the terminal flower bud. Corolla width was measured as the greatest diameter of the open flower and

corolla length as the distance from the corolla face to the sepal tips. At least five flowers were measured for each floral trait.

The morphological characteristics of sexual hybrids for plant height, floral characteristics, leaf number and dimensions were determined as outlined above for somatic hybrids. To compare sexual hybrids with the parental species, four plants of *N. rustica* var *chlorotica* were analyzed but because plants of the "V" mutant of *N. tabacum* were very slow growing only one flowering plant was available for analysis.

Pollen viability of sexual and somatic hybrids was ascertained by spreading pollen on agar-solidified B₅ medium with 4% sucrose and scoring the frequency of germination of at least 300 grains/flower after 24 h incubation at 25°C under a light intensity of 50 ft-c. Selfing and backcrossing of somatic and sexual hybrids were performed using newly-opened flowers and applying appropriate pollen liberally to receptive stigmas. To ascertain if seeds were formed in various crosses, the seed capsules were collected when they naturally abscised. Capsules were opened and individual seeds examined under a dissecting microscope.

2. Cytological analysis

To determine the chromosome number of both somatic and sexual hybrids, cells of root tips and vegetative buds were analyzed. Root tips and vegetative buds of actively-growing plants in the vegetative phase were excised, pretreated in demineralized water for 24 h at 0°C, fixed in Carnoy's solution (ratio by volume = 6 ethanol : 3 chloroform : 1 acetic acid), stained in Snow's solution (Snow 1963) and squashed in 45% acetic acid. At least five clear metaphase plates from each preparation were counted.

3. Analysis of isozymes

- a) Cultures. From a total of 250 somatic hybrid lines which produced shoots 10 were selected at random for analysis. With the sexual hybrids 10 seedlings showing normal morphology were used. Hybrid shoots were maintained 4 weeks at 25°C, 300 ft-c. on solid B₅ medium prior to transfer to the Prairie Regional Laboratory, National Research Council in Saskatoon where analyses were performed. Upon arrival at Saskatoon the shoot cultures were kept for 7 days at 25°C 93 ft-c, and then processed. For comparative purposes plantlets of the wild-type and mutants of both parental species grown in vitro were also analyzed.
- b) Preparation of extracts. Shoots were rinsed and ground with a PT 10-35 Polytron homogenizer (Brinkman Instruments, Canada Ltd.), with a ratio of extraction medium to tissue fresh weight of 1 mL-1 g. The extraction medium contained 1M sucrose and 0.056M 2-mercapto-ethanol in 0.2M Tris HCl buffer (pH 8.5). The homogenate was centrifuged at 30,000 xg at 5°C for 1 h and the pellet discarded. Protein content of the supernatant was determined by a modified Lowry method (Wetter 1977).
- c) Gel electrophoresis and enzymatic staining. Polyacrylamide gels at pH 8.9 were employed according to the method of Davis (1964). The gels were cast in 5 mm x 90 mm glass tubes. A stacking gel (Wetter 1977) was used to concentrate proteins entering the running gel. Fifty to 100 µg of protein sample (containing 25% sucrose and a trace of bromophenol blue) was layered on the gel and migrated into the stack. Electrophoresis was carried out with a constant power of 10 watts per 12 tubes and terminated when the bromophenol

blue band was about 0.5 cm from the end of the tube. Aspartate aminotransferase isozymes were detected using aspartic acid as substrate in the reaction aspartic acid + α ketoglutarate $\rightleftharpoons$ oxaloacetate + glutamic acid (Wetter 1977). Oxaloacetate gives a brown color reaction in the presence of fast violet; staining solution given in Appendix, page 247. For esterase staining the method of Scandalios (1969) modified by using 2% 1-naphthyl acetate was followed and gels were incubated at room temperature. Superoxide dismutase bands were visualized according to the method described by Edwards *et al.* (1978) in which the gels were stained in a 0.1 M Tris HCl buffer containing 0.1 mg tetrazolium and 0.05 mg phenazine methosulphate per mL. The gels were then exposed to a fluorescent lamp until the background was dark blue. To test for cyanide insensitivity 1 mM NaCN was added to the staining solution (del Rio *et al.* 1978).

4. Analysis of ribulose-1-5 bisphosphate carboxylase/oxygenase (RUBPCase)

(a) Plant material. Fourteen somatic hybrid and two sexual hybrid plants were selected at random for analysis. For comparison, plants of the parental species, *N. rustica* var NRT and chlorotica and wild-type and virescent "V" *N. tabacum* were used. Albino *N. tabacum* plants die at the seedling stage and were not analyzed. All somatic hybrid and parental plants were grown in the greenhouse. Sexual hybrids were cultured in vitro to a height of approximately 10 cms in 15 x 20 mm tubes at a light intensity of 150 ft-c. Leaves from the somatic hybrids and parental plants, and entire sexual hybrid plantlets were used for the analyses (performed in Saskatoon).

(b) Preparation of RUBPCase. Approximately 5 g deribbed leaves were ground in a glass tissue grinder for 3 min in 1.3 mL of Gray's (see Appendix, Table 9) extraction buffer (Gray and Wildman 1976) containing 0.2 g Dowex-1 (x8) acetate (200-400 mesh). The homogenate was centrifuged at 48,000 xg for 30 min and the supernatant retained. RUBPCase was isolated according to a modification of the method of Uchimiya et al. (1979). The supernatant (approx. 1 mL) was mixed with 0.3 mL of anti-RUBPCase rabbit serum and incubated for 1 h at 40°C followed by overnight storage in a refrigerator. Anti-RUBPCase rabbit serum was prepared using as an antigen crystalline RUBPCase isolated from N. tabacum leaves by the method of Gray and Wildman (1976). After incubation the antibody-RUBPCase complex was centrifuged and washed twice with a borate-saline buffer containing 0.0025 M borax, 0.04 M boric acid and 0.12 M NaCl, and adjusted to pH 7.3. The washed precipitate was then solubilized, carboxymethylated and passed through a Sephadex G-25 column to absorb excess iodoacetate as described by Uchimiya et al. (1979).

(c) Isoelectric focussing. The isoelectric focussing was carried out on a slab gel prepared according to the method of Kung et al. (1974). In this investigation 40% Ampholine, pH range 4-6 (LKB Instruments) was employed. The following modifications were used: 0.01 M H_3PO_4 replaced H_2SO_4 in the bottom reservoir and a pH gradient in the gel from pH 4.0 (bottom) to 6.0 (top) was established at 2.5 watts at 2°C for 30 min. Approximately 40 µg of sample was added to each well and electrofocussing carried out at 2°C for 1 h at 1 watt followed by 2 h at 2.5 watts. The gel was stained for protein in 0.04% Coomassie Brilliant Blue G250.

in 3.5% perchloric acid for 1 h (Reisner et al. 1975) and destained overnight in a solution containing (ratio by volume) 10 ethylacetate : 78 water : 5 acetic acid : 7 ethanol (Vesterberg et al. 1977).

5. Analysis of alkaloids

Plants of the parental species, somatic, sexual and backcross hybrids were grown in a greenhouse and supplied weekly with 20:20:20 (N:P:K) liquid fertilizer. For alkaloid analysis of parental species and sexual hybrids, leaves were selected from plants which were just starting to flower. Leaf material of the somatic hybrids was taken from flowering shoots of plants approximately one year old; by cutting back old plants a continuous supply of fresh shoots was produced. One week prior to analysis the top third of all plants was removed to allow alkaloids to accumulate in the leaves. The four topmost leaves from each decapitated plant were harvested on the same day, bulked and analyzed. Total alkaloids were extracted using sodium hydroxide, steam distilled and collected in sulphuric acid as outlined by Griffith 1957. Percent total alkaloids calculated as nicotine in the extracts was determined after ultraviolet spectrophotometry at 236, 259, and 282 mμ from the relationship:

$$\begin{array}{l} \text{\% total alkaloids} \\ \text{\% calculated as nicotine} \end{array} = \frac{D_{259} - \left[\frac{D_{236} + D_{282}}{2} \right] \times 0.78}{\text{gram sample}}$$

Where D_{236} , D_{259} , D_{282} are the optical densities (D) observed at corresponding wavelength [mμ (Griffith 1957)].

RESULTS

A. Isolation and Culture of Protoplasts and Regeneration of Plants from Cell Cultures of Wild-type and Chlorophyll-deficient Mutants1. Initiation of callus cultures

Callus was initiated equally well from hypocotyl, stem or leaf explants of N. rustica seedlings and from stem or leaf explants of greenhouse-grown plants using MS medium with either NAA or 2,4-D (Table 5). The original explants from mutant N. rustica var chlorotica plants always bleached while explants from wild-type N. rustica var NRT plants remained dark green. Calli derived from mutant explants were white and friable on either hormone (Fig. 3), whereas those from wild-type plants remained green on NAA but after about a year in culture on 2,4-D turned light green (Fig. 4). The phenotypic differences between the two N. rustica strains in vivo (Fig. 1) were therefore maintained in vitro although modulated by the hormone regime. In addition to callus, roots or small root-like structures formed on the NAA and 2,4-D media, respectively (Figs. 3 and 4).

Callus from albino seedlings of N. tabacum (Fig. 2) was readily obtained from leaf or hypocotyl explants (Fig. 5) and 2,4-D was more effective in promoting its growth than NAA (Table 5). Callus also proliferated readily from stem and leaf explants taken from wild-type N. tabacum plants grown in a greenhouse. Wild-type calli were initially light green with both auxins but bleached over a period of several months. Callus from albino explants was initially friable and very light green on NAA and brown on 2,4-D (Fig. 5).

TABLE 5. Effect of explant source and auxin type on callus initiation from seedlings of mutant *Nicotiana rustica* var chlorotica, and mutant and wild-type *N. tabacum*

Species	Genotype	Explant	Auxin, n. callus- induction medium	No. explants tested	No. explants producing callus	Callus development*
<i>N. rustica</i> var chlorotica	mutant	hypocotyl	2,4-D NAA	11	11	+++
		stem	2,4-D NAA	11	11	+++
		leaf	2,4-D NAA	8	8	+++
<i>N. tabacum</i>	mutant	hypocotyl	2,4-D NAA	12	12	+++
		cotyledon	2,4-D NAA	12	12	+++
		leaf	2,4-D NAA	11	11	++
		hypocotyl	2,4-D NAA	11	9	+
		leaf	2,4-D NAA	12	5	+
<i>N. tabacum</i>	wild-type	hypocotyl	2,4-D NAA	12	3	+
		leaf	2,4-D NAA	12	12	+++
		hypocotyl	2,4-D NAA	12	10	++
		petiole	2,4-D NAA	9	9	++
		leaf	2,4-D NAA	9	9	++
<i>N. tabacum</i>	wild-type	hypocotyl	2,4-D NAA	12	12	++
		leaf	2,4-D NAA	12	11	+
		leaf	2,4-D NAA	11	11	+++
<i>N. tabacum</i>	wild-type	hypocotyl	2,4-D NAA	12	12	++
		leaf	2,4-D NAA	12	12	++

Note: Basic medium throughout was MS with 2,4-D and NAA used at 0.5 mg/L and 1.0 mg/L, respectively.

*Callus diameter obtained for the majority of explants in each treatment after approx. 8 wk culture:
(+) = 1-3 mm (++) = 3-5 mm (+++) = 5-10 mm (++++ = 10-15 mm.

FIGURE 1. Characteristics of mutant and wild-type N. rustica plants. Note chlorophyll-deficiency in stem (arrow) of mutant N. rustica var chlorotica (third from left) and dark green stem of wild-type N. rustica var NRT (second from left). Reciprocal hybrids of these varieties are wild-type (first from left and fourth from left).

FIGURE 2. Seedlings from a heterozygote of N. tabacum var WS showing albino and wild-type segregants.

FIGURE 3. Stem-derived calli of N. rustica var chlorotica on MS medium with NAA (top) or 2,4-D (bottom). Calli are chlorophyll-free and have produced root-like structures (arrows).

FIGURE 4. Stem-derived calli of N. rustica var NRT on MS medium with NAA (top) or 2,4-D (bottom). Chlorophyll and root-like structures with NAA.

FIGURE 5. Leaf-derived calli of albino N. tabacum var WS medium with NAA (top) or 2,4-D (bottom); arrows indicate leaf explant.

8

2. Initiation of liquid suspension cultures

Following transfer into liquid medium the callus of both species remained in large clumps and grew slowly during the first four weeks. To obtain fast growing cultures consisting of small cell clusters suitable for protoplast isolation it was essential to subculture as often as possible. Initially subculture was at 5-7 days; after approximately 12 weeks the suspension cultures had increased their growth rate so as to require subculture every 2-3 days and readily yielded protoplasts upon digestion with enzymes. Cultures of both species grew at approximately the same rate with either NAA or 2,4-D (Fig. 6).

After 6 months suspension cultures of N. rustica var chlorotica were white on either hormone while var NRT cultures were green on NAA and white on 2,4-D (Fig. 18). Cultures of albino and wild-type N. tabacum were initially brown. After 6 months, the albino cultures were white on either hormone while the wild-type cultures were pale green on 2,4-D and darker green on NAA.

3. Isolation of protoplasts

Preliminary experiments were carried out to optimize the production of healthy protoplasts using 10 mL of a standard enzyme mixture. The best protoplast preparations were obtained using cells 72 h after subculture, i.e. cultures in log-phase growth (Figs. 9 and 10). The optimal fresh weight of cells in 10 mL of enzyme was 2.0 g (Fig. 7). Protoplasts began to be released after 1 h for N. rustica and 3 h for N. tabacum and the optimal number of good quality protoplasts from both species was obtained after 5-6 h of incubation in enzyme (Fig. 8).

FIG. 6 GROWTH CHARACTERISTICS OF *NICOTIANA GLAUCOPOLYLLA* CELL SUSPENSION CULTURES GROWN WITH 0.5 mg/L 2,4-D. STARTING INOCULUM 2.0 gms.

FIG. 7 EFFECT OF CELL NUMBER (gms fr. wt. cells) ON THE EFFICIENCY OF ENZYME (1% DRISE-LASE, 1% RHIZOZYME, 0.1% ONOZUKA RIO-10 ML CONSTANT VOLUME) TO RELEASE HEALTHY PROTOPLASTS.

FIG. 8 EFFECT OF INCUBATION TIME ON RELEASE OF HEALTHY PROTOPLASTS FROM *NICOTIANA* CELLS IN ENZYME.

Yields of protoplasts from a number of experiments with both species are summarized in Table 6. Since there was substantial unavoidable variation between experiments, ranges of values rather than means are given. Both wild-type and mutant N. rustica cultures yielded substantially more protoplasts than N. tabacum. Cells of N. rustica grown on 2,4-D gave up to 50% more protoplasts than those grown on NAA. Protoplasts derived from all 2,4-D-grown cells appeared healthier cytologically, had fewer starch grains and were less fragile than those derived from cells grown on NAA (cf. Figs. 9 and 10 with 11). Mutant and wild-type N. tabacum cultures grown with NAA gave low yields of poor quality protoplasts, consequently data are presented only for the cultures grown on 2,4-D. Albino N. tabacum cultures released more protoplasts than the wild-type N. tabacum (Table 6).

4. Formation of cell colonies from protoplasts

Within 36 h of culture most protoplasts lost their spherical shape indicating synthesis of cell walls. Cell division was subsequently observed (Figs. 12 and 13). The times for initial and maximum frequency of division of protoplast-derived cells is shown in Table 6. N. rustica cells (NRT and chlorotica) derived from cultures originally grown on NAA entered division more rapidly than cells from 2,4-D cultures. N. tabacum cultures from cells grown on 2,4-D divided at about the same rate as N. rustica cultures derived from cells grown on NAA.

Plating efficiency (i.e. percent of original protoplasts eventually dividing) was influenced by a number of culture parameters. Efficiencies ranging from 60-80% were obtained for all cultures

TABLE 6. Isolation and response in culture of protoplasts of *Nicotiana rustica* and *N. tabacum*

Species	Genotype	Auxin in cell culture medium	No. of isolation experiments	Protoplast yield (x.10 ⁶)	Time of initial division (days)	Time of maximum division (days)
<i>N. rustica</i>	wild-type	2,4-D	4	1.6 - 4.5	9 - 16	17 - 20
		NAA	3	1.0 - 2.4	2 - 5	8 - 10
	mutant	2,4-D	6	2.0 - 4.5	9 - 16	17 - 20
		NAA	4	0.7 - 2.1	2 - 5	8 - 10
<i>N. tabacum</i>	wild-type	2,4-D	3	0.04 - 0.2	3 - 5	8 - 10
	mutant	2,4-D	6	0.6 - 1.6	3 - 5	8 - 10

Note: Concentration of 2,4-D and NAA was 0.5 mg/L and 1.0 mg/L, respectively. Protoplast yields are based on 2.0 g fr. wt. starting cells and represent the maximum ranges observed in different experiments. Time of initial and maximum division are also given as ranges.

FIGURE 9. Freshly-isolated protoplasts of N. rustica var chlorotica derived from cell culture with 2,4-D (x 750).

FIGURE 10. Freshly-isolated protoplasts of albino N. tabacum derived from cell culture with 2,4-D (x 750).

FIGURE 11. Freshly-isolated protoplasts of N. rustica var NRT derived from cell culture with NAA. Note relatively poor cytoplasmic strand network and starch grains (x 750).

FIGURE 12. Cell wall formation and cell division in N. rustica var NRT culture (x 1400).

FIGURE 13. High frequency cell division in albino N. tabacum culture (x 150).

FIGURE 14. Asymmetric cell division in N. rustica var NRT protoplast-derived culture (x 450).

FIGURES 15-17 Proembryo-like structures in N. rustica var NRT protoplast-derived cultures. Note asymmetry of the structures and dense cytoplasm of cells (x 300).

when protoplasts were plated at a range of densities; the optimal density was $5 \times 10^4/\text{mL}$. Protoplasts were routinely cultured at low light intensities (22 ft-c) since light intensities of 150 ft-c were generally found to be detrimental if the protoplast preparation was of low quality, e.g. containing noticeable debris. No differences were observed in plating efficiencies between protoplasts cultured in high or low light intensities when the preparations appeared of good quality microscopically. Culturing at 30°C decreased the time for initial cell division and subsequent colony formation, but overall plating efficiency did not change. While the plating efficiencies with both mannitol and glucose were similar, where glucose was used the colonies better survived subsequent transition to media lacking an osmotic stabilizer.

The effect of a number of carbohydrates supplied as osmotica (0.35 M) to protoplast-derived colonies obtained from cells of *N. rustica* var. NRT is presented in Table 7. All the compounds except galactose supported cell division equally well (plating efficiency 45-65%). Throughout the experiment protoplasts in galactose showed protoplasmic streaming but most remained spherical, apparently failing to form cell walls, and were unable to divide. Colonies cultured in raffinose, maltose and sucrose adhered to each other and floated in the culture drops while colonies in the other carbohydrates settled on to the culture dish. Young colonies grown in the presence of different carbohydrates expressed a wide range of chlorophyll development with glucose being most effective in induction of greening (Table 7).

Four weeks after initiation of cell division when a large number of cell colonies had developed (Figs. 23 and 24) the

TABLE 7. Effect of different carbohydrates at osmotic concentration 0.35 M on plating efficiency and chlorophyll development in colonies derived from protoplasts of Nicotiana rustica var NRT after 36 days

Osmoticum	Mean plating efficiency (%)	Relative greening
Glucose	65	+++++
Maltose	65	++++
Sucrose	65	++++
Mannitol	65	+++
Sorbitol	65	+++
Fructose	45	+++
Raffinose	50	++
Galactose	No division	—

Note: Protoplasts were derived from a cell suspension grown with NAA. All protoplasts cultured in NT medium with 1% sucrose.

FIGURE 18. Cell suspension of N. rustica grown in MS medium with NAA; left, chlorophyll-free N. rustica var chlorotica; right, the chlorophyll-containing wild-type N. rustica var NRT.

FIGURE 19. Meristem formation on cell-suspension-derived callus of mutant N. rustica var chlorotica. Note chlorophyll development only in meristems; non-induced callus on the right is white.

FIGURE 20. Meristem and shoot formation in cell-suspension-derived callus of albino N. tabacum. Note the appearance of chlorophyll in some tissues (arrow).

FIGURE 21. Shoot formation in cell-suspension-derived callus of wild-type N. rustica var NRT.

FIGURE 22. Shoot formation in protoplast-derived callus of mutant N. rustica var chlorotica. Note leaves are light green in comparison to the wild-type (Fig. 21).

FIGURE 23. High frequency cell colony formation from protoplasts of N. rustica var chlorotica.

FIGURE 24. High frequency cell colony formation from protoplasts of albino N. tabacum.

2

culture drops were supplied with approximately 50 μ L of NT medium containing 0.15 M glucose. After another week the colonies were transferred to a variety of solid media lacking an osmotic stabilizer. Protoplast-derived colonies of chlorotica were white (Fig. 23) while colonies from the wild-type N. rustica were bright green when glucose was used as the initial osmoticum. Colonies derived from NAA cell suspensions of N. rustica var NRT became green earlier and were brighter in color than those derived from 2,4-D suspensions. Protoplast-derived colonies of albino N. tabacum were slightly brown (Fig. 24) while those of the wild-type were pale green.

Occasionally proembryo-like structures were observed in protoplast-derived cultures of N. rustica var NRT in NT medium (Figs. 15-17). The structures were found only in cultures derived from cells grown on 2,4-D and their frequency increased if the protoplast culture medium contained kinetin and p-chlorophenoxy-acetic acid (Lörz et al. 1977). These structures were typified by their polarized nature and thin-walled cytoplasmic cells which divided very rapidly. After 4-5 weeks in culture the cells became vacuolated and the structures lost their organized appearance.

5. Induction of shoots in cell colonies derived from suspension cultures

a) N. rustica. Morphogenic responses could be elicited in cell colonies of the chlorotica mutant of N. rustica with benzyladenine (BA) only whereas N. rustica var NRT responded to both benzyladenine and kinetin (2.0 mg/L) with 0.1 mg/L NAA. On inductive medium chlorotica cell colonies remained white in color with light

chlorophyll synthesis occurring only in localized areas where meristems differentiated (Fig. 19). Cell colonies of wild-type N. rustica turned green on shoot-induction medium and meristems were less readily detectable. Small differentiated shoots of the chlorotica mutant were distinctly lighter in color than those of the wild-type variety (cf. Figs. 21 and 22).

When the response of chlorotica cell colonies to benzyladenine and NAA was studied further (Table 8) the highest frequency of shoots was obtained with 1-2 mg/L BA in the presence of 1.0 mg/L NAA and the colonies derived from 2,4-D-grown cultures gave a consistently better morphogenic response than those from NAA. Shoot organogenesis did not occur in the absence of BA while BA induced a low frequency of shoots in the absence of NAA. A similar test of NAA and BA levels on suspension-derived cell colonies from N. rustica var NRT gave essentially the same results (Table 9).

The highest frequency of shoot induction was obtained with 2,4-D-derived cell colonies on medium containing 1.0 mg/L NAA and 1-4 mg/L BA. With NAA-derived NRT cell colonies the optimum NAA concentration (0.01-0.1 mg/L) was lower than with chlorotica-derived colonies (1.0 mg/L; Table 8). Morphogenesis in the absence of NAA occurred only in one case, i.e. NAA-derived cell colonies supplied with 4 mg/L BA.

Over a large number of experiments it was consistently observed that cell colonies taken from newly-initiated suspensions had the greatest morphogenic capacity. Cell colonies of N. rustica subcultured for prolonged periods failed to respond to media which were inductive for new cultures (Table 10).

TABLE 8. Frequency of shoot induction in calli of *Nicotiana rustica* var *chlorotica* in varying BA/NAA concentrations

Auxin conc. mg/L in shoot induction medium	Auxin in suspension medium of source cell cultures	BA conc. mg/L in shoot induction medium			
		0	1.0	2.0	4.0
0	2,4-D NAA	0 0	3 (1) 0	10 (6) 3 (1)	6 (4) 0
0.01	2,4-D NAA	0 0	3 (7) 0	17 (6) 3 (5)	13 (6) 0
0.1	2,4-D NAA	0 0	7 (4) 3 (8)	7 (11) 0	10 (4) 0
1.0	2,4-D NAA	0 0	64 (5) 31 (6)	77 (8) 20 (4)	37 (6) 13 (6)

Note: Unbracketed figures give percent individual calli showing shoots and/or meristems (based on 30 calli per treatment). Mean number of shoots per callus given by figures in brackets. Cell colonies were maintained seven weeks in suspension culture on MS medium with 2,4-D (0.5 mg/L) or NAA (1.0 mg/L) as indicated in the second column and then transferred to shoot-induction medium with varying BA and NAA concentrations for six weeks at 25°C under 160 ft.c. and scored.

TABLE 9. Frequency of shoot induction in calli of *Nicotiana rustica* var NRT in varying BA/NAA concentrations

Auxin conc. mg/L in shoot induction medium	Auxin in suspension medium of source cell cultures	BA conc. mg/L in shoot induction medium			
		0	1.0	2.0	4.0
0	2,4-D	0	0	0	7
	NAA	0	0	0	0
0.01	2,4-D	0	0	10	20
	NAA	0	37	14	0
0.1	2,4-D	0	10	23	23
	NAA	15	19	23	23
1.0	2,4-D	0	50	60	50
	NAA	3	3	10	4

Note: Figures give percent individual calli showing shoots and/or meristems (based on 10 calli/test and 22-30 calli/test for cell cultures grown with 2,4-D and NAA, respectively). Cell colonies were maintained 72 weeks in suspension culture on MS medium with 2,4-D (0.5 mg/L) or NAA (1.0 mg/L) as indicated in the second column and then transferred to shoot-induction medium with varying BA and NAA concentrations for 8 weeks at 25°C under 160 ft.c. and scored

TABLE 10. Effect of time in suspension culture on shoot induction in cell colonies

Species	Cytokinin	Age of suspension culture (wks)	No. of calli tested	No. of calli with shoots and/or meristems	Frequency of calli responding (%)
<u>N. rustica</u> var NRT	Kinetin	4	18	18	100
		16	12	10	83
		32	6	3	50
		70	20	0	0
<u>N. tabacum</u> albino	Kinetin	5	30	23	77
		16	21	21	100
		22	18	5	28
		24	18	2	11
		49	27	0	0
<u>N. tabacum</u> albino	Benzyladenine	11	16	8	50
		14	16	4	25
		15	20	5	25
		20	18	0	0
		28	35	0	0
		41	31	0	0

Note: Cell colonies were taken from suspension cultures grown with 0.5 mg/L 2,4-D. Basic medium for shoot induction was MS throughout with 2.0 mg/L cytokinin and 0.1 mg/L NAA.

b) Albino N. tabacum. Since conditions for induction of differentiation in wild-type tobacco callus are well established (Skoog and Miller 1957; Vasil and Hildebrandt 1965; Thorpe 1978) studies on N. tabacum morphogenesis were restricted to the albino mutant. Kinetin and benzyladenine were tested at a range of concentrations in combination with 0.1 mg/L NAA for shoot induction (Table 11). The optimal concentration of cytokinin was 2.0 mg/L. At this concentration there were no substantial differences in the effect of kinetin or benzyladenine on the frequency of responding calli. Kinetin, however, allowed a more normal development of shoots and leaves whereas with benzyladenine mostly buds were produced. In responsive cell colonies both elongated shoots and small meristematic buds formed (Fig. 20). Shoots developed to about 1.5 cm in length and showed small green patches on their leaves (Fig. 20); in this respect they resembled the slightly-variegated seedlings used to initiate the original callus.

As with N. rustica the morphogenic response of albino N. tabacum cell colonies declined with time in culture. Cell colonies were grown in suspension for various periods ranging from 5-49 weeks and transferred to solid MS medium containing 2.0 mg/L of either kinetin or benzyladenine with 0.1 mg/L NAA (Table 10). Cell colonies taken from old cultures could not be induced to undergo shoot differentiation although they grew at about the same rate as younger responsive calli. While young responsive calli produced shoots, older responsive calli tended to produce buds only which did not develop further. Increasing the concentration of cytokinin and NAA by up to five-fold over the optimal level (Table 11) failed to elicit a morphogenic response in old cultures.

TABLE 11. Effect of cytokinin type and concentration on shoot induction in cell suspension-derived albino *Nicotiana tabacum* cell colonies

Cytokinin	Cytokinin conc. mg/L	No. of calli tested	No. calli with shoots and/or meristems	Frequency of calli responding (%)
Kinetin	0	32	0	0
	1.0	27	8	30
	2.0	36	18	50
	4.0	27	12	44
	8.0	32	7	22
Benzyladenine	0	30	0	0
	1.0	33	14	43
	2.0	24	11	46
	4.0	33	11	33
	8.0	33	11	33

Note: Basic medium throughout was MS with 0.1 mg/L NAA and cytokinin as indicated in second column. Cell colonies were taken from an 8-wk-old suspension grown with 0.5 mg/L 2,4-D.

6. Induction of shoots in protoplast-derived cell colonies

Protoplast-derived cell colonies of both N. rustica and N. tabacum had a lower morphogenic capacity than those taken directly from suspension cultures. The morphogenic response of 24-week-old cell colonies which were obtained from protoplasts or directly from cell cultures are compared in Table 12. For N. rustica var chlorotica none of the protoplast-derived cell colonies responded while 37% of the cell colonies taken from suspension culture produced shoots and/or meristems. In subsequent experiments, however, shoots were induced at a low frequency (5-24%) using benzyladenine at 2.0 mg/L and NAA at 0.1 mg/L; the capacity for shoot differentiation was observed to be greater in protoplast-derived calli of newly established cell suspensions than in calli from older cultures. Protoplast-derived calli obtained from cells originally grown on 2,4-D exhibited a greater morphogenic response than those from NAA; in this respect they were similar to cell colonies taken directly from suspension culture.

As with N. rustica, protoplast-derived cell colonies of albino N. tabacum had a lower morphogenic potential than those taken directly from suspension cultures (Table 12). For calli which could not be induced to form shoots on the initial shoot-inducing medium, increasing the cytokinin concentration in subsequent transfers from two to five times above the optimal level failed to elicit a morphogenic response in these calli.

7. Induction of roots

Using MS or B₅ medium lacking hormones, roots were induced on excised shoots of N. rustica var chlorotica (Table 13). The frequency of rooting was 40-50% for chlorotica but only 0-13% for NRT.

TABLE 12. Morphogenic response of cell suspension-derived and protoplast-derived cell colonies of mutant Nicotiana rustica var chlorotica and albino N. tabacum

Species	Origin of cell colonies	Frequency of shoot and/or meristem induction
<u>N. rustica</u> var		
chlorotica	cell suspension	37
	protoplasts	0
<u>N. tabacum</u> albino		
	cell suspension	93
	protoplasts	13

Note: Basic medium throughout was MS containing 2.0 mg/L BA + 1.0 mg/L NAA (N. rustica var chlorotica) or 2.0 mg/L kinetin + 0.1 mg/L NAA (N. tabacum). Protoplasts and cell suspension-derived cell colonies were cultured in vitro for a total of 24 wks (N. rustica) and 16 wks (N. tabacum) prior to transfer to shoot-induction medium. Frequency of shoot and/or meristem induction based on 30 calli tested for each species.

TABLE 13. Induction of roots on protoplast-derived shoots of
Nicotiana rustica var chlorotica

Medium	Hormones	No. of shoots tested	No. of shoots with roots	% rooted
B ₅ - full strength	0	23	11	48
B ₅ - half "	0	36	14	39
MS - half "	0	36	18	50
MS - full "	IBA 2.0 mg/L Kinetin 0.03 mg/L	39	17	44

This frequency was not improved by the addition of the root-inducing auxin indolebutyric acid at 2.0 mg/L. In cases where rooting did not occur many secondary shoots grew on the initial explants on rooting medium. Subculturing these shoots resulted in further shoot proliferation allowing for the production of large numbers of cloned shoots. For root induction in N. tabacum, shoots were excised and transferred to MS and B₅ media lacking auxins. While the wild-type rooted readily (85%) the albino shoots showed no root formation.

B. Protoplast Fusion and Selection and Regeneration of Hybrid Plants

1. Observations on the fusion of protoplasts

Clean preparations of healthy protoplasts were used for fusion (Fig. 25). It was not possible to distinguish protoplasts of N. rustica from those of N. tabacum in the light microscope (Figs. 9 and 10). Within 30 seconds of adding PEG protoplasts began to agglutinate and became more opaque in the light microscope indicating changes in the cytoplasm (Fig. 26). A clear line indicated the boundary of adjoining protoplasts (Fig. 26). Subsequently, groups of protoplasts amassed together and became very dehydrated as indicated by a loss of turgidity (Fig. 27). Protoplasts in these groups lost their network of cytoplasmic strands and boundaries with adjoining protoplasts were difficult to distinguish (Fig. 27). Upon elution of PEG with (NT) culture medium or medium buffered at high pH (Keller and Melchers 1973) the protoplasts became deplasmolysed within 30 min and their boundaries became more discernible (Fig. 28). During this process of deplasmolysis protoplast fusion and mixing of cytoplasms occurred (Fig. 31). Adjacent protoplasts which were tightly agglutinated in PEG did

FIGURE 25. Freshly isolated protoplast of N. rustica var chlorotica prior to fusion.

FIGURE 26. Agglutination of protoplasts upon addition of PEG solution.

FIGURE 27. Tight adhesion and plasmolysis of protoplasts in PEG, and loss of cytoplasmic-strand network.

FIGURE 28. Deplasmolysis of protoplasts upon elution of PEG with NT medium.

FIGURE 29. Tightly adhering pair of protoplasts after PEG elution and single protoplast with normal network of cytoplasmic strands.

FIGURE 30. Protoplasts sticking together after elution of PEG is complete.

FIGURE 31. Two fusing protoplasts and initiation of cytoplasmic mixing.

not always fuse but remained attached for a period of time (Figs. 29 and 30) during which they slowly rehydrated and regained their spherical shape and normal cytoplasmic-strand network (Fig. 29).

2. Production of hybrid cell colonies

Three to five days after fusion most protoplasts lost their spherical shape indicating wall deposition, and the resulting cells began to divide. Most cells capable of division divided within 10-20 days. Since the microscopic appearance of the protoplasts of either species was similar no attempt was made to identify or follow the development of potential hybrid cells.

Seven to ten days after the cultures were moved from 22 ft-c to 150 ft-c, i.e. 17-30 days post-fusion numerous cell colonies were visible macroscopically. The majority of these calli were white presumably arising from unfused or homofused parental cells. Interspersed in the albino colonies, however, were substantial numbers of bright green colonies presumably arising from heterofusions (Fig. 32). The green calli grew somewhat faster than the albino types and were either loose or dense in texture (Fig. 32).

In control protoplast fusions using N. rustica var chlorotica the protoplasts survived well and gave rise only to cell colonies which were white or cream-colored (Fig. 38). Most protoplasts died in the control fusions using N. tabacum alone; those that survived gave rise to white cell colonies only which eventually became light brown in color (Fig. 38). When protoplasts of both parent species were cultured together without prior fusion only white or light brown calli formed (Fig. 38). When green colonies from heterofusions were co-cultured with white calli derived from

FIGURE 32. Chlorophyll formation in cell colonies 5 wks subsequent to fusion of chlorophyll-deficient N. rustica and N. tabacum protoplasts.

FIGURE 33. Shoot meristem induction in fusion products on NT medium with 1.0 mg/L BA. Parental cultures remain chlorophyll free.

FIGURE 34. Hybrid shoot differentiation. NT medium + 1.0, mg/L BA.

FIGURE 35. Comparison of mutant N. tabacum (left) N. rustica (right) and somatic hybrid (centre) plantlets.

FIGURE 36. Somatic hybrid shoot (recalcitrant to root induction) with abnormal morphology bearing cotyledon-like leaves.

FIGURE 37. Somatic hybrid shoot with well-developed leaves, shoot axis, and roots.

homofused protoplasts of the parent species chlorophyll synthesis was not induced in the parental calli indicating that hybrid calli could not cross-feed the parental types (Fig. 39). The results of these control experiments indicated that the green colonies formed in heterofusion conditions resulted from genetic complementation for chlorophyll synthesis in somatic hybrid cells formed between the chlorophyll-deficient parent species. A fusion experiment performed one year later, using the original cultures as sources of protoplasts, has demonstrated that the cultures retained their competence for complementation.

The number of green colonies produced using a constant number of parental protoplasts for fusion offers a simple rapid assay for successful heterofusion. Using this assay the effect of a number of variations of the PEG treatment were examined in an attempt to establish optimum fusion conditions for these species. Since the possible combinations of factors which might affect fusion are extremely numerous no attempt was made to be completely systematic. Rather, the PEG solution developed by Kao and Michayluk (1974) with and without DMSO (Haydu *et al.* 1977) was tested in a variety of situations and successful modifications identified.

Table 14 shows the effect of temperature of two fusion solutions, i.e. PEG + 5% DMSO, on the production of hybrid colonies. Considering the variation between replicate experiments this study gave little evidence of consistent temperature effects, except that PEG + DMSO was noticeably less effective in promoting fusion at 18°C than at higher temperatures. Subsequent fusion experiments were carried out at 27°C.

TABLE 14. Effect of temperature on formation of Nicotiana rustica
+ N. tabacum hybrid cell colonies

Fusion temperature (°C)	Fusion method			
	PEG alone (20 min)		PEG + 5% DMSO (3 min)	
	Expt. I	Expt. II	Expt. I	Expt. II
18 - 19	190	123	71	63
25 - 27	154	166* 143*	270	175* 113*
37 - 38	312	125	272	146

Note: Data represent total numbers of green colonies per fusion experiment using constant volumes of protoplasts. PEG treatment was 50% w/v PEG 1540 followed by elution with NT medium.

*Replicate fusion experiments

The effect of a range of concentrations of DMSO in the PEG solution is summarized in Table 15. Again there was some uncontrollable variation between experiments, but low concentrations of DMSO (2-5%) in the PEG solution were superior to PEG alone when elution was carried out with NT medium. Elution with high pH-calcium solution gave erratic results and at best was no more effective than NT elution.

The results of an experiment varying calcium and phosphate ions in the PEG fusion solution are shown in Table 16. Within the combinations tested the various levels of phosphate had little effect. Calcium concentration, however, was important. Complete death occurred with 0 and 105 mM CaCl_2 while 1.05-10.5 mM gave a good yield of hybrid colonies.

3. Induction of shoots in hybrid cell colonies

To achieve shoot induction, hybrid cell colonies were transferred to media containing a high ratio of cytokinin to auxin. A comparison of MS and NT media each supplemented with 2.0 mg/L benzyladenine and 0.1 mg/L NAA revealed that NT was superior for shoot organogenesis (Table 17). On NT shoot-induction medium the hybrid calli remained bright green in color while the parental calli were white or light brown (Fig. 33). Numerous shoot meristems differentiated on the surface and interior of responsive hybrid calli and these later developed into shoots with dark green leaves (Figs. 34 and 35). Out of approximately 250 different shoot lines, no evidence of sectoring for chlorophyll-deficiency was observed in leaves of the hybrid shoots in vitro. Not all lines had normal morphology. Many lines bore cotyledon-like leaves the bases of which were fused with the 'stem'. These shoots had no distinct

TABLE 15. Effect of DMSO concentration in PEG fusion solution and the elution medium on formation of *Nicotiana rustica* + *N. tabacum* hybrid colonies

DMSO v/v (%)	Eluting solution	Expt. I	Expt. II
0	NT medium	154	123
2	NT "	223	267
4	NT "	—	171
5	NT "	179	—
8	NT "	0	129
16	NT "	D	D
0	high pH-Ca ²⁺	D	131
4	high pH-Ca ²⁺	D	—

Note: Data represent the total numbers of green colonies per fusion experiment using constant volumes of protoplasts. DMSO was added to 50% w/v PEG 1540. Incubation time prior to elution was 3 min. D-no survival of protoplasts. The high pH-Ca²⁺ medium is that of Keller and Melchers (1973).

TABLE 16. Effect of salts in PEG fusion solution on formation of Nicotiana rustica + N. tabacum hybrid cell colonies

Salts		Total no. of hybrid colonies
KH_2PO_4 (mM)	CaCl_2 (mM)	
0	10.5	90
0.7	10.5	116
1.4	0	0
1.4	1.05	141
1.4	10.5	149
1.4	105.0	0

Note: Data represent total number of green colonies per fusion experiment using constant volumes of protoplasts.

The fusion solution was 50% w/v PEG 1540 plus 5% v/v DMSO for 3 min. Elution with NT medium. D- No survival of protoplasts.

TABLE 17. Effect of basic medium on induction of meristems and/or shoots in Nicotiana rustica + N. tabacum hybrid calli

Basic medium	Sucrose conc. (%)	No. calli tested	No. calli with meristems and/or shoots	Frequency of induction (%)
MS	2	24	3	13
NT	1	30	14	47

Note: Basic media each contained benzyladenine at 2.0 mg/L and NAA at 0.1 mg/L with 0.175 M glucose.

internodes and could not be induced to form roots (Fig. 36).

Using NT medium, the effect of two hormonal regimes and various glucose concentrations in the shoot-induction medium were assayed (Table 18). Continued culture of protoplast-derived hybrid colonies in levels of osmotica necessary for protoplast stabilization (0.35 M) inhibited the development of shoots irrespective of the hormones used. The optimal frequency of shoot induction was obtained where glucose was omitted and benzyladenine was used at 1.0 mg/L (Table 18). The presence of glucose even at 0.175 M depressed the frequency of hybrid calli which produced meristems, and those meristems formed with glucose usually failed to develop further. Glucose also depressed chlorophyll synthesis and the growth of hybrid and parental type cell colonies (Fig. 40). In the absence of glucose the hybrid cultures turned green and produced well-developed shoots. The effect on shoot induction of NAA (0, 0.05, 0.1, 0.2 mg/L) in the absence of glucose was tested (data not shown). NAA at 0.05–0.2 mg/L slightly enhanced the frequency of shoot differentiation from 53–67% and at 0.05 mg/L was routinely used thereafter in combination with BA (1.0 mg/L):

Hybrid cell colonies were supplied supplementary liquid medium about 5–6 weeks after fusion to support their continued growth. The type of medium and its hormone composition were tested for capacity to support subsequent shoot induction (Table 19). NT medium was superior to MS but neither the ratio or absolute amount of auxin and cytokinin seemed to affect the ability of hybrid calli to form shoots when subsequently transferred to agar-solidified shoot-inducing medium (Table 19).

Hybrid calli continued to differentiate shoots **profusely** upon subculturing with 1.0 mg/L BA without glucose (Fig. 41). In this

TABLE 18. Effect of glucose concentration under two hormonal regimes on induction of meristems and/or shoots in Nicotiana rustica + N. tabacum hybrid calli

Hormonal regime mg/L	Glucose concentration (M)	No. of calli tested	No. of calli with meristems and/or shoots	Frequency of induction (%)
BA 1.0	0	20	12	60
	0.175	26	6	23
	0.35	16	2	13
BA 2.0 + 0.1 NAA	0.175	30	14	47
	0.35	15	3	20

Note: Basic medium throughout was NT containing 1% sucrose

FIGURE 38. Controls for fusion experiments: colonies derived from homofused protoplasts of mutant N. rustica (extreme right) and N. tabacum (extreme left); products of heterofusion (upper-most center dish); colonies derived from a mixture of protoplasts from mutant N. rustica and N. tabacum (lower-most center dish). All colonies on NT medium with 1.0 mg/L BA and 1% sucrose.

FIGURE 39. Controls for fusion experiment: callus from homofused protoplasts of mutant N. rustica (lower left) and N. tabacum (lower right). Green hybrid callus (lower center). Co-culture of hybrid callus with mutant N. rustica (upper right) and with N. tabacum (upper left). All colonies on NT medium with 1.0 mg/L BA + 3.0 mg/L NAA and 1% sucrose.

FIGURE 40. Effect of glucose on growth, chlorophyll formation and shoot induction on products of heterofusion. Glucose-free medium (left), 0.175 M glucose (center) and 0.35 M glucose (right); cell colonies on NT medium with 1.0 mg/L BA + 1% sucrose.

FIGURE 41. Proliferation of shoots and meristems in hybrid calli subcultured for 5 months on glucose-free NT medium with 1.0 mg/L BA + 1% sucrose.

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TABLE 19. Effect of liquid "feeding" medium on subsequent morphogenic response for shoot and/or meristem formation of *Nicotiana rustica* + *N. tabacum* hybrid calli

Salts and vitamins	Feeding medium		No. of calli tested	No. of calli with meristems and/or shoots	Frequency of induction %
	Sucrose %	Hormones mg/L			
MS	2	BA 0.5 NAA 1.5	142	81	57
MS	1	BA 0.5 NAA 1.5	86	43	50
NT	1	BA 1.0 NAA 3.0	61	41	67
NT	1	BA 0.5 NAA 1.5	68	46	68
NT	1	BA 1.0 NAA 0.05	59	39	66

Note: Six weeks after fusion, culture plates were supplied liquid "feeding" medium with 0.175 M of glucose (approx. 2.0 mL/plate). After a further three weeks of culture cell colonies were transferred to the optimal shoot-inducing medium, i.e. NT basic medium with 1% sucrose containing 1.0 mg/L BA and 0.05 mg/L NAA and the morphogenic response scored four weeks later.

way lines could be maintained in vitro for prolonged periods.

After a year of subculturing, however, these hybrid calli produced mainly meristems and shoots did not develop with the same vigor as on newly cultured calli.

4. Induction of roots on hybrid shoots

The results of a number of experiments in which hybrid shoots were cultured on MS or B₅ media under a variety of conditions designed to induce rooting are summarized in Table 20. Although the frequency of rooting was very low, MS medium was superior to B₅, particularly with continuous white light. Rooting frequency was erratic ranging from 0% in one experiment to 23% in another. The highest frequency obtained with hormone-free MS medium (23%) was used as a standard against which the effectiveness of other treatments was evaluated. At the end of the period on root-induction medium most shoots had developed several secondary shoots indicating a lack of apical dominance in the original explant. Cultured shoots also produced callus at their bases. The axes of shoots which formed roots were generally more developed than unresponsive shoots. Roots tended to be few in number, usually two or three, and tended to be brittle.

Altered lighting such as red light (Letouze and Beauchesne 1969) and temperature shocks [4°C for 2-8 days (Negrutiu and Jacobs 1978a)] failed to enhance root formation (Table 20), although these treatments promote rooting in other species. Two weeks etiolation (Delargy and Wright 1978) also failed to stimulate rooting. Further rooting experiments were carried out with MS medium and continuous white light. Shoots were also cultured in pyrex tubes or jars.

As compared to petri plates (Fig. 42) tall vessels favored development of the shoot axis and leaves (Figs. 43-50) but did not

TABLE 20. Effect of MS and B₅ media under different culture conditions on root induction in somatic hybrid shoots

medium	Culture variables	Total no. expts.	No. shoots tested per expt. (range)	% forming roots
MS	W	5	12-30	0-23
	P	1	10	0
	R	1	8	0
	C	2	6-11	9
B ₅	W	2	12	0
	P	1	12	0
	P	1	20	0
B ₅ 1/2 strength	W	2	96-96	0

W - White fluorescent light continuous (ft-c)

P - Photoperiod 12/12 white

R - Red light continuous

C - Cold treatment (5°C) for 2, 4, 6, 8 days prior to 25°C culturing

FIGURE 42. Root formation (arrows) in somatic hybrid shoots of line #148. Note proliferation of callus and shoot buds and poor development of shoot axes. MS medium with 1% sucrose, IBA 2.0 mg/L and kinetin 0.03 mg/L according to Tran Than Van (1977).

FIGURE 43. Effect of sucrose and IBA on somatic hybrid shoots. Sucrose concentration 0.1% (top row), 2% bottom row. IBA concentration for top and bottom rows, (L-R) 0.1 mg/L; 0.4 mg/L; 0.8 mg/L. Note proliferation of callus with 2% sucrose.

FIGURE 44. Culture of somatic hybrid shoots on filter paper bridges. Note proliferation of callus and secondary shoots in some cultures.

FIGURE 45. Effect of growth inhibitors ABA, AZA, **CYC**, and ~~8~~-AZAG used alone and in combination on shoots of somatic hybrid line #6. Note reduction in secondary shoot formation with certain combinations. Legend is given in Appendix, Table 10.

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FIGURE 46. Multiple shoot formation from a single shoot of a non-rooting somatic hybrid in vitro.

FIGURES 47-50. Variable shoot morphology and intensity of rooting in different lines of somatic hybrids in vitro.

FIGURES 51-55. Variable shoot and leaf morphology in different lines of somatic hybrids in the vegetative phase. Intensity of apical dominance is variable; plant in Fig. 54 has two equally vigorous main shoots.

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61

prevent proliferation of secondary shoots (Fig. 46). In addition to culturing shoots on agar, hybrid shoots were also cultured on filter paper bridges as illustrated in Fig. 44. This method was reported to stimulate root formation in shoot meristems of N. rustica (Walkey and Woolfitt 1968) and was tested with a range of NAA concentrations (Table 21). The tall vessels used tended to favor shoot elongation but frequency of rooting was not enhanced.

A range of auxins and other growth regulators were tested at various concentrations to stimulate the development of normal shoot and root formation. The results with four auxins used at a range of concentrations are summarized in Table 21. None of these increased frequency of root formation over that found with MS alone (Table 20). Auxins induced prolific callus formation at the base of the hybrid shoots. In cases where root induction occurred (Fig. 42) it was not clear whether roots arose from the callus or the shoots. A high auxin concentration (0.2%) was reported to induce root formation of somatic hybrid shoots of N. rustica + N. tabacum by Nagao (1978) but this treatment did not affect rooting in this study (data not shown). To possibly enhance entry of auxins into the base of stems, hybrid shoots were cultured in media with DMSO at 0.5 and 5% (Table 22). Root formation was not improved by these treatments and all shoots were killed with 5% DMSO.

In some studies on root formation the stimulatory effect of auxin and gibberellic acid (GA) was dependent on the time at which they were supplied during culture (Gaspar et al. 1977).

TABLE 21. Effect of auxin type and concentration on root induction in somatic hybrid shoots

Auxin type	Conc. (mg/L)	Sample size/treatment	% of shoots forming roots
IBA	2, 10, 20	8-18	0-7
IAA	1.75, 17.5	32-40	3
NAA	1.86, 18.6,	24-39	0-24
	*1, *3, *10, *30	11-12	0
PAA	1.0	20	0

Note: Basic medium throughout was MS containing 2% sucrose except where indicated by *.

*Denotes half-strength Hoaglands solution (Hoagland and Arnon 1939) with shoots cultured on filter paper bridges rather than agar.

TABLE 22. Effect of DMSO on root induction in somatic hybrid shoots

DMSO conc. (v/v)	No. of shoots tested	No. of shoots forming roots	% of shoots forming roots
0	63	2	3
0.5%	30	2	7
5.0%	36	0	0

Note: Basic medium throughout was MS with the hormone and sucrose addition of Tran Thanh Van (1977); sucrose 1%; IBA, 2.0 mg/L; Kinetin, 0.03 mg/L.

The results of an experiment in which shoots were precultured with IBA, GA or IBA + GA, followed by transfer to media with different combinations of these was tested (Table 23). Under these conditions gibberellic acid did not promote root formation.

In an effort to inhibit secondary shoot formation and favor rhizogenesis, two growth inhibitors 8-azaguanine (8-AZAG) and abscisic acid (ABA) were tested (Table 24). With these inhibitors 32% of cultured shoots formed roots and secondary shoot formation was reduced slightly. High concentrations (10.0 mg/L) of 8-AZAG and ABA in combination with IBA inhibited shoot elongation. Since 32% of ~~the~~ shoots tested in this experiment formed roots with 0.1 mg/L ABA in combination with 4.0 mg/L IBA a second experiment was carried out in which the inhibitors ABA (2.5 mg/L), 7-azaindole [AZA (2.5 mg/L)], 8-AZAG (5.0 mg/L) and cycocel [CYC (1.6 g/L)] were tested alone and in all possible combinations. All inhibitors when used alone or in combination tended to reduce the number of secondary shoots formed by lines unresponsive to rooting (Fig. 45) but did not change the overall morphology of these shoots. The frequency of rooting did not exceed 3%.

Ethylene and ethanol have been shown to inhibit adventitious root formation in tomato and Phaseolus, respectively (Coleman et al. 1980; Middleton et al. 1978a). Since carbon dioxide is a known inhibitor of ethylene action (Burg and Burg 1965) hybrid shoots were cultured in the presence of high levels of CO₂ (Table 25). The presence of CO₂ when supplied with or without auxin did not stimulate root formation. Root formation in Phaseolus was inhibited by ethanol at concentrations as low as 0.02 ml/L.

TABLE 23. Effect of sequential hormone regimes on root induction in somatic hybrid shoots

Preculture hormones (2d)	Culture hormones (35d)	No. of shoots tested	No. of shoots forming roots	% of shoots forming roots
0	0	26	0	0
0	IBA	23	0	0
0	GA	23	0	0
0	IBA + GA	23	1	4
IBA	IBA	28	0	0
IBA	IBA + GA	31	0	0
IBA + GA	IBA + GA	23	0	0
IBA + GA	IBA	23	0	0
GA	IBA	29	0	0

Note: Basic medium throughout was MS containing 2% sucrose, IBA conc. 2.0 mg/L; GA conc. 3.5 mg/L ($10^{-5}M$). A minimum of 5 hybrid lines were assayed per treatment.

TABLE 24. Effect of growth inhibitors on root induction
in somatic hybrid shoots

Growth inhibitor	Inhibitor concentration (mg/L)	IBA concentration (mg/L)	No. of shoots forming roots	% of shoots forming roots
8-Azaguanine	0	0	1	3
	0	2	4	13
	0.1	2	6	20
	1.0	0	5	17
	1.0	2	2	7
	10.0	2	1	3
Absciscic Acid	0	0	7	23
	0	4	2	7
	0.1	4	11	32
	1.0	0	3	10
	1.0	4	8	27
	10.0	4	1	3

Note: 30 shoots were tested for each treatment. Basic medium throughout was MS.

TABLE 25. Effect of CO₂ and IBA supplementation on root induction in somatic hybrid shoots

CO ₂ concentration (ppm)	IBA concentration (mg/L)	No. of shoots tested	No. of shoots forming roots	% of shoots forming roots
* C	0	48	0	0
2200	0	42	0	0
C	2.0	39	1	2
2200	2.0	47	1	2

Note: Basic medium throughout was MS containing 2% sucrose. All shoots were cultured in darkness. A minimum of 11 hybrid lines were assayed per treatment.

*C = 'control', represents 350 ppm CO₂, i.e., atmospheric concentrations.

(Middleton et al. 1978a); since auxin stock solutions (100 mg/L) used throughout these experiments were solubilized with ethanol (~ 3 ml/L) the effect of an alternative solubilizing agent was tested with IAA and NAA. However, the mode of preparing stocks of IAA or NAA did not appear to affect the rooting frequency of somatic hybrid shoots (Table 26).

Since commonly used growth regulators such as auxins did not enhance rooting frequency above that obtained with hormone-free medium, a number of other stimulatory agents which were reported to enhance rooting in other species were tested. MS medium supplemented with boron, which was essential for root initiation in Phaseolus aureus (Middleton et al. 1978b), and amino acids was tested (Table 27). Increasing boron by up to 20-fold or supplying amino acids alone or in combination with IBA did not enhance rooting frequency. Other supplements such as nicotine (Peters et al. 1974) and 3-amino pyridine (Phillips and Collins 1979; Evans 1979) were reported to induce rhizogenesis in Nicotiana species but including these compounds at 25-100 mg/L did not increase frequency of rooting in hybrid shoots (Table 28). For sugar cane a high concentration of sucrose alone was sufficient to induce roots on shoots cultured in vitro (Maretzki and Hiraki 1980). In the present study increasing concentrations of sucrose (2%, 4%, 8%, 16%, 20%) were tested but were not stimulatory. Lowering the sucrose concentration from 2%-0.1% reduced the production of secondary shoots substantially (Table 29) and also reduced the proliferation of callus at the base of cultured shoots (Fig. 43). Rooting frequency, however, was not enhanced (Table 29).

TABLE 26. Effect of preparation method of IAA and NAA stocks and their concentration on root formation in somatic hybrid shoots

* Mode of preparation of hormone stocks					
Hormone tested	concentration (mg/L)	Abs. ethanol		KOH (2M)	
		No. shoots tested	% of shoots forming roots	No. shoots tested	% of shoots forming roots
IAA	1.75	40	3	34	12
	17.5	32	3	41	0
NAA	1.86	24	4	26	4
	18.6	39	0	45	7

Note: Basic medium throughout was MS containing 2% sucrose. A minimum of 9 hybrid lines were assayed per treatment.

* To make hormone stocks IAA and NAA were initially solubilized in 2.5 mL (2M) KOH, or 5.0 mL absolute ethanol prior to bringing to final volume of 1 L conc. (10 mL stk. soln. = 1.0 mg hormone).

TABLE 27. Effect of boron, IBA, and amino acid supplements on root induction in somatic hybrid shoots

Medium tested	No. of shoots tested	No. shoots forming roots	% shoots forming roots
H ₂ O	30	0	0
H ₂ O + IBA	30	0	0
MS	30	1	3
MS + IBA	30	1	3
MS + Boron (X10)	30	1	3
MS + Boron (X10) + IBA	30	1	3
MS + Boron (X20)	30	1	3
MS + Boron (X20) + IBA	30	0	0
MS + a.a.	25	0	0
MS + IBA + a.a.	25	1	4

Note: MS contains 6.3 mg/L boric acid; Figures in brackets represent degree of boric acid supplementation above this level. IBA was supplied at 2.0 mg/L where indicated. Amino acids (a.a.) were supplied as casein hydrolysate at 1.0 g/L.

TABLE 28. Effect of nicotine and 3-amino pyridine on root induction in somatic hybrid shoots

MS medium supplement	Conc. of supplement (mg/L)	No. of shoots tested	No. of shoots forming roots	% of shoots forming roots
Nicotine	0	12	0	0
	25	9	2	22
	50	8	1	13
	100	11	0	0
3-amino pyridine	25	11	0	0
	50	9	0	0
	100	13	1	8

Note: Basic medium throughout was MS containing 2% sucrose. A minimum of 5 hybrid lines were assayed per treatment.

TABLE 29. Effect of IBA and sucrose concentration on root and secondary shoot induction in somatic hybrid shoots

IBA (mg/L)	0.1%			Sucrose concentration			2%	
	No. shoots tested	% of shoots forming roots	*Mean No. 2° shoots/shoot	No. shoots tested	% of shoots forming roots	*Mean No. 2° shoots/shoot	% of shoots forming roots	*Mean No. 2° shoots/shoot
0.1	35	3	2	41	0	9		
0.4	36	0	2	39	0	6		
0.8	36	0	3	41	0	8		

Note: Basic medium throughout was MS containing 2% sucrose. A minimum of 10 hybrid lines were assayed for rooting ability per treatment.

*Denotes the mean number of secondary shoots which developed from the original shoot explant during the course of the experiment. Secondary shoot formation data is for line No. 1 (3 shoots/treatment) - a line with cotyledon-like leaves which never rooted.

During the course of these experiments it became apparent that the capacity for rooting varied among the individual hybrid lines which were originally isolated at the green colony stage. A breakdown analysis of part of the experiment recorded in Table 24 is given in Table 30. From this table it is clear that lines which did not have an inherent capacity for rooting, i.e. lines 171, 244, 37, 253, 183 failed to produce roots on all media tested while line 34, for example, which was 'competent' rooted on all media.

Hybrid lines which did form roots varied with respect to the number of roots formed and exhibited highly variable shoot and leaf morphologies in vitro among different lines (Figs. 46-50). Shoots which rooted in petri plates were usually given one subculture in a glass vessel prior to their transition to the greenhouse. Eighty to 90% of the shoots which had rooted in vitro survived the transition to the greenhouse.

C. Morphological, Cytological and Biochemical Analysis of Somatic Hybrids

1. Morphology

The majority of hybrid plants transferred to the greenhouse grew vigorously. Differences in shoot and leaf morphology observed between lines in vitro (Figs. 47-50) were maintained in the greenhouse (Figs. 51-55). Unlike the parents most hybrid lines expressed only weak apical dominance and produced many lateral vegetative and flowering shoots (Figs. 51 and 57). While most of these shoots arose from axillary buds a few developed at the apical region giving rise to plants with two equally vigorous apices (Fig. 54).

With the exception of line 155 (Figs. 57 and 61), young stems, leaves and sepals of the hybrids were dark green, resembling

TABLE 30. Breakdown analysis of rooting pattern among individual somatic hybrid lines with combinations of ABA and IBA from Table 24

Shoot lines tested	Hormone regime ABA mg/L/IBA mg/L					
	0/0	0/4	0.1/4	1.0/0	1.0/4	10/4
34	3 (1)	3 (2)	6 (5)	3 (2)	6 (6)	6 (1)
143	3 (3)	3 (0)	3 (3)	2 (1)	-	-
165	2 (2)	-	2 (1)	-	3 (0)	-
245	3 (0)	3 (0)	2 (2)	3 (0)	3 (0)	3 (0)
31	-	-	1 (1)	-	-	-
16	2 (1)	-	3 (0)	3 (0)	2 (1)	3 (0)
171	3 (0)	6 (0)	3 (0)	5 (0)	3 (0)	6 (0)
244	5 (0)	6 (0)	3 (0)	5 (0)	-	3 (0)
253	3 (0)	5 (0)	4 (0)	3 (0)	5 (0)	3 (0)
183	6 (0)	-	-	3 (0)	5 (0)	3 (0)
37	-	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)

Note: Basal medium throughout was MS. Unbracketed figures represent the number of shoots of a given line tested with the hormone regime indicated; bracketed figures represent the number of shoots forming roots in each test.

FIGURE 56. Somatic hybrid line 158 (center) exhibiting hybrid vigor with parental types N. rustica var chlorotica (left) and wild-type N. tabacum right.

FIGURE 57. Four somatic hybrids exhibiting diverse morphological characteristics (left to right) lines 141, 8, 159, 155.

FIGURE 58. Leaves of 11 somatic hybrid plant lines flanked by parental types N. rustica var chlorotica (extreme left), and wild-type N. tabacum (extreme right); hybrid lines (top row L-R) 15, 139, 158, 141, 69, 155; bottom row (L-R) 161, 147, 163, 167, 159.

FIGURE 59. Somatic hybrid line 147 bearing two types of flowers wide-throated (small arrow) and narrow-throated (large arrow) corollas.

FIGURES 60 and 61. Flowers of 8 somatic hybrid lines face and side view with parental types N. rustica var chlorotica (extreme left) and wild-type N. tabacum (extreme right) hybrid lines (top L-R) 165, 164, 159, (middle L-R) 146, 143, 64; bottom (L-R) 155, 8.

FIGURE 62. Uniform flower morphology in 6 sublines of line No. 147.

FIGURE 63. Variable flower morphology in 2 sublines of line No. 161.

FIGURE 64. Variable flower morphology in 2 sublines of line No. 167.

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wild-type N. rustica rather than the parent N. rustica var chlorotica or wild-type N. tabacum (Figs. 56, 58 and 61). Small light green islands were observed in the leaves of a few lines.

The analysis of vegetative and floral characteristics of 17 lines is summarized in Table 31. One-third of the lines were taller than either parent (Fig. 56) while the remainder were intermediate in height. All hybrids produced more leaves than the parents and most leaves had a length/width ratio equal to or greater than N. tabacum. Similar to N. tabacum the leaves of most lines had short winged petioles but none bore stipules (Fig. 58). The leaves of two lines (147 and 161) were distinctly petiolated (Fig. 58), a trait present in N. rustica. The mean leaf area among the hybrid lines tended to be close to or somewhat less than that obtained for wild-type N. rustica. Six lines bore very small leaves, while only three produced leaves approaching those of N. tabacum in surface area (Table 31 and Fig. 58). Shortly after flowering, most of the hybrids developed symptoms resembling magnesium-deficiency in their leaves (Fig. 58). This was not observed in the parental species and could not be corrected by additional magnesium applications.

Flower morphology was intermediate with respect to the parent species for corolla length, width and pigmentation and for sepal length (Table 31 and Figs. 60 and 61). Corollas of all the flowers had some pink anthocyanin typical of N. tabacum although the degree of pigmentation varied among the lines, e.g. line No. 159 was dark pink while line No. 155 was only faintly pink (Fig. 60). Where a number of sublines were grown some variation between plants was found in vegetative and flower morphology (see Table 31,

TABLE 31. Vegetative and flower characteristics in *Nicotiana rustica* + *N. tabacum* somatic hybrid lines

	Height (cm)	No. of leaves/ plant	Leaf index $\frac{\text{length}}{\text{width}}$	Leaf area cm ²	No. of flower branches	Corolla		Sepal length
						length	width	
<i>N. tabacum</i>	99	16.5	1.5	164.1	7	3.3	3.2	2.1
<i>N. rustica</i> var <i>chlorotica</i>	66.8	10.7	1.2	163.1	10.5	0.7	1.7	1.0
<i>N. rustica</i> var NRT	46.8	11.7	1.2	88.4	14.5	0.9	1.3	0.9
Hybrid Lines								
164	144.0	53	1.9	68.1	8	1.0	1.2	1.0
153	137.5	33	1.7	183.1	11	1.2	2.6	1.6
8	133.0	47	1.5	24.1	-	1.0	2.7	1.6
158	114.0	25	2.1	99.2	4	-	-	1.6
139	98.0	27	1.9	95.6	11	1.3	2.0	1.2
150	97.0	25	2.6	40.9	6	1.0	2.7	1.6
64	95.0	22	1.5	112.9	5	1.4	2.0	1.2
143	94.4	22	1.6	72.1	5	-	2.0	1.1
163	93.5	30	1.5	112.9	4	1.2	2.1	1.0

... Continued next page

TABLE 31. Continued

Hybrid Lines	Height (cm)	No. of leaves/ plant	Leaf index $\frac{\text{length}}{\text{width}}$	Leaf area cm ²	No. of flower branches	Corolla		Sepal length
						length	width	
161a	92	24	2.2	48.7	6	1.3	2.3	1.1
b	121	26	1.3	149.9	13	1.5	3.0	1.5
159	89.5	29	2.0	83.8	8	1.3	2.3	1.8
165	84.0	18	1.9	81.8	6	1.4	1.9	1.3
69	83.5	25	1.6	44.8	9	1.3	2.3	1.4
167	77.5	30	1.5	60.4	4	0.9	1.8	1.0
147a	77	24	1.4	91.5	7	1.5	2.8	1.4
b	81	42	2.3	81.8	7	1.5	2.7	1.5
c	84	39	1.4	157.8	11	1.7	2.8	1.4
d	91.5	41	1.4	130.5	8	1.7	3.0	1.5
141	58.5	20	1.5	74.0	11	2.3	1.8	1.0
146	56.5	22	2.2	23.2	4	1.6	-	1.5

Note: Hybrid lines are denoted by numbers and sublines by letters.

Lines 147 and 161). The flowers of a number of sublines of No. 147 appeared quite similar (Fig. 62) except for one plant which was clearly chimeric and bore two kinds of leaves and flowers (Fig. 59). Flowers of sublines of Nos. 161 and 167 were, however, strikingly different (Figs. 63 and 64). The number of floral branches produced by one-third of the lines was less than either parent while the remainder of lines tended to resemble either N. rustica or N. tabacum for this character. Flowers of all hybrid lines bore pistils and stamens (Fig. 61). In some cases the flowers contained a distorted pistil and more than five stamens, one of which was frequently fused to the corolla.

The anthers of many somatic hybrid lines produced pollen whose viability was assayed by germination on B₅ medium with 4% sucrose. Under these conditions 88% of the N. rustica var chlorotica and 94% of the wild-type N. tabacum var WS pollen germinated. Pollen from somatic hybrid lines 141, 147, 153, 159, 161, 163, and 164 germinated with frequencies of 8%, 0%, 0.3%, 0%, 0.3%, 0.2%, and 0.1%, respectively. At least ten seed capsules from each of the somatic hybrid lines listed in Table 31 were examined for the presence of mature seeds following self-pollination. While capsules of a few lines contained seeds of normal size most seeds were hollow and not viable. From two lines (139 and 64) six viable seeds were obtained and were germinated in vitro. One resultant plant was transferred to the greenhouse and grown to flowering (Fig. 65); it was self-sterile and leaf morphology of this plant was intermediate with respect to N. rustica and N. tabacum. In an effort to enhance self-fertility of somatic hybrids, one plant from each of lines 147, 163, and 159 which were sterile under greenhouse conditions were

FIGURE 65. Progeny from a self-fertile-somatic hybrid line 64.

FIGURE 66. Backcross progeny of somatic hybrids (SH) with parental species. Top row (left) SH line 139 x N. tabacum var WS. Top row (right) SH line 139 x N. rustica var NRT. Middle and bottom rows, four siblings from a SH line 143 x N. tabacum var WS cross.

66

65

grown at 35°C and 5°C under a 16 h photoperiod as recommended by Kostoff (1943). While these plants produced from 4-14 flowers during five weeks of culture under these conditions self-fertility was not enhanced and no viable seeds were obtained.

Fertility of somatic hybrids was greater when the hybrids were used as maternal parents in backcrosses to the parent species. A total of 23 somatic hybrid pistils of five different lines were pollinated with pollen from N. tabacum var WS to yield 18 seeds which germinated. For backcrosses with N. rustica, a total of 17 somatic hybrid pistils of eight different lines were pollinated to yield seven seeds which germinated. There was great morphological variation amongst sibs of the backcross progeny (Fig. 66) all of which were self-sterile when self-pollinated.

2. Chromosome determinations

The cytological analysis of 15 hybrid lines is summarized in Table 32. It was not possible to identify the individual chromosomes of each species. Considerable variation in chromosome number was found among the different lines ($2n = 68$ to 96) and between sublines ($2n = 68$ to 92 in line 34). With the exception of line 139, all the hybrids examined had less than the amphidiploid complement of 96 chromosomes (N. tabacum $2n = 48$; N. rustica $2n = 48$). There were no gross irregularities in chromosome numbers among the cells of a given root tip although in a few cases disconstrictional chromosomes, chromosome fragmentation and stickiness were observed. There were no obvious morphological characters that could be correlated with chromosome complement.

3. Analysis of isozymes

Comparative enzyme analyses of the wild-type and chlorophyll-

TABLE 32. Chromosome numbers in Nicotiana tabacum + N. rustica
somatic hybrids

Line number	Sub-lines	2n, respectively
34	a, b, c, d	83, 92, 68, 83
164	a, b	76, 84
147	a, b	87, 86
158	a, b; c	85, 86, 86
143	a, b	90, 93
139	a, b	96, 95
144	a	92
167	a, b	68, 86
159	a	89
141	a, b	91, 94
165	a, b	91, 94
143	a, b	90, 84
146	a	84
161	a	83
152	a	87

-deficient forms of both parental species showed no differences in electrophoretic band patterns for all enzymes studied (data not shown). Therefore, the electrophoretic band patterns of hybrids were compared to those of the parental mutant types of both species. Analysis of esterase, aspartate aminotransferase, and superoxide dismutase isozyme patterns provided evidence of combined genotypes in the hybrid plants. A number of other enzymes were assayed i.e. alanyl aminopeptidase, phosphorylase, alcohol dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase, acid phosphatase, and peroxidase but the isozyme patterns of the parents were not sufficiently distinct to reveal mixed enzyme composition in the hybrid plants.

A number of esterase isozymes were readily detectable in the parents and hybrids (Fig. 67). Considerable complexity was found in the Rf region 0.22 to 0.40. Electrophoretic bands of Rf 0.22-0.35 which were present in N. tabacum appeared in some hybrids only (particularly hybrid 3 and 7) while the band at Rf 0.4 was exclusive in the hybrids only. Bands at Rf 0.53 and 0.58 were contributed by N. tabacum and N. rustica, respectively and both were present in all hybrids at high concentration. While there was a trace of the Rf 0.53 band in N. rustica, the high concentration (typical of N. tabacum) present in the hybrids suggests this band was contributed by N. tabacum. At least two bands appeared in the Rf 0.70 region in all hybrids; these bands were present singly in either parent. In some instances (hybrids 3 and 4) three bands appeared in this region.

The isozymes for aspartate aminotransferase separated into three zones on the gels (Fig. 68). In the Rf 0.1 zone each

FIGURE 67. Esterase isozyme pattern and zymogram obtained for albino N. tabacum (gel 1), N. rustica var chlorotica (gel 2) and 10 somatic hybrid lines (gels 3-10).

FIGURE 67

FIGURE 68. Aspartate aminotransferase isozyme pattern and zymogram obtained for albino N. tabacum (gel 1), N. rustica var chlorotica (gel 2) and 10 somatic hybrid lines (gels 3-10).

FIGURE 68

parent had two bands, one of which was common to both species.

The hybrids had all three bands. The middle band at Rf 0.11 was more intense in the hybrids indicating an additive effect. In the Rf 0.28 zone, both the Rf 0.26 band of N. rustica and the Rf 0.29 band of N. tabacum were present in the hybrids. N. rustica also contributed a band at Rf 0.29 but this band was considerably more intense in the hybrids indicating an additive effect. A hybrid band at Rf 0.28 not present in either parent appeared in nine out of ten hybrid lines. To rule out the possibility that this band was an anomaly due to preparation procedures, three separate electrophoretic runs at different protein concentrations and times were undertaken in which equal portions of N. rustica and N. tabacum protein extracts were incubated prior to electrophoresis. The hybrid band appeared consistently only in extracts taken from hybrid tissue.

The electrophoretic pattern for superoxide dismutase is summarized in Fig. 69. Superoxide dismutase catalyses the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ and occurs in all aerobic organisms as the primary defense mechanism against oxygen radicals (Fridovich 1974). The enzyme may contain one of several metals (Cu, Zn, Fe, Mn) as prosthetic groups. Those containing Cu or Zn are inhibited by cyanide (CN) which prevents their visualization on the gels, while the Mn and Fe enzymes are CN-insensitive (del Rio et al. 1978). The isozyme pattern developed in the absence of CN is shown in Fig. 69. Bands which appeared in the uppermost part of the gels (Rf 0.0 to 0.2) were peculiar to the hybrids and never appeared in either parent species. These bands were all CN-sensitive. The Rf 0.35 zone consisted of a triplet of bands in the hybrids at

FIGURE 69. Superoxide dismutase isozyme pattern and zymogram obtained for albino N. tabacum (gel 1) N. rustica var chlorotica (gel 2) and 10 somatic hybrid lines (gels 3-10); *denotes CN-sensitive bands. (Hybrid No. 6 and 10 were not assayed for CN-sensitivity).

FIGURE 69

least two of which were derived from N. rustica. One band of this triplet was also common to a triplet derived from N. tabacum but the two other N. tabacum components were absent in the hybrids. These triplets were insensitive to CN in both parents and hybrids. In the Rf 0.50 zone two CN-insensitive bands which indicate hybridization appeared in nine hybrids. One band, Rf 0.53 was contributed by N. tabacum and the other, Rf 0.50, by N. rustica. The Rf 0.50 band also occurs in N. tabacum but was CN-sensitive while all hybrids showed CN-insensitive (hybrid 6 and 10 were not assayed) bands at this position. Another CN-insensitive band was found at Rf 0.46 in all hybrids assayed, with the exception of hybrid 7, and this band was not present in either parent.

4. Analysis of ribulose-1-5 bisphosphate carboxylase/oxygenase (RUBPCase) by isoelectric focussing

In agreement with Uchimiya et al. (1979) the isoelectric focussing (IF) pattern in a pH gradient of 5.0 to 4.0 gave the same polypeptide composition for RUBPCase purified by ~~immune~~-precipitation and by crystallization (data not shown). Furthermore, no differences were found between the IF patterns of chlorophyll-deficient N. rustica var chlorotica and wild-type N. rustica var NRT. These observations for N. rustica and those of Chen et al. (1975) in which the IF pattern for ten different N. tabacum cultivars including two chlorophyll-deficient mutants was constant indicated the validity of comparing the IF patterns of wild-type N. tabacum and mutant N. rustica with those of the somatic hybrids.

A representative analysis of RUBPCase after isoelectric

focussing for both parental species and six somatic hybrids is shown in Fig. 70. The small subunit (SS) polypeptide composition of both N. rustica and N. tabacum was found to consist of two heavy and one light band. In both species the two most acidic bands had the same isoelectric point but the more basic band of N. rustica had a slightly higher isoelectric point than that of N. tabacum. All 14 somatic hybrids (six of which are illustrated in Fig. 70) contained two bands in this region, i.e. four bands total for the SS. This result indicates the presence and expression of nuclear genes from both parental species.

The large subunits (LS) of N. tabacum and N. rustica were found to consist of three prominent IF bands. The isoelectric point of one N. tabacum band was slightly higher than that of N. rustica allowing resolution of large subunit components in somatic hybrids. The large subunit protein distribution pattern in lanes 4, 5 and 6 (Fig. 70) is similar to N. tabacum while that of lanes 7, 8 and 9 is similar to N. rustica. In addition to these, illustrated in Fig. 70, analysis of a further eight somatic hybrid plants revealed a large subunit IF pattern in each which was typical of N. rustica. Isoelectric focussing of an RUBPCase enzyme mixture from both species gave four prominent bands in the large subunit zone (lane 2). None of the 14 somatic hybrid plants analyzed had the large subunit protein of both species.

Of 14 somatic hybrids analyzed here morphological characteristics of 10 and chromosome complement of eight are described above. Apart from hybrid No. 8 which bore small leaves (Fig. 57) and unusual flowers (Figs. 60 and 61) there were no

FIGURE 70. Distribution pattern of polypeptides of ribulose-1-5 bisphosphate carboxylase/oxygenase (RUBPCase) after isoelectric focussing in a pH gradient 4.0-6.0.

N. tabacum var WS (lane 1); mixture of equal amounts of protein from N. tabacum var WS and N. rustica var chlorotica (lane 2); N. rustica var chlorotica (lane 3); somatic hybrid lines 64a, 158b, 141a, 205a (lanes 4-9, respectively).

SS = Small subunit

LS = Large subunit

FIGURE 70

obvious morphological characteristics which distinguished plants expressing the chloroplast genes of N. tabacum from those of N. rustica.

5. Analysis of alkaloids

The alkaloid content of ten somatic and two backcross hybrids and their parental varieties is summarized in Table 33. The alkaloid content of N. rustica was more than twice that of N. tabacum. Alkaloid content varied considerably between the 11 somatic hybrid lines analyzed. Five plants produced levels of alkaloids which were more than double the parental values. Of the remaining five plants one showed levels lower than either parent, two were similar to N. tabacum and three to N. rustica. Three backcross hybrid plants (somatic hybrid x N. tabacum var WS) showed an alkaloid content which was almost double that of N. tabacum but less than that obtained for N. rustica.

D. Sexual Hybridization by In Vitro Culture of Hybrid Embryos

Attempts to obtain sexual hybrids of these species using N. tabacum as females failed. Flowers of 21 such crosses abscised completely within two weeks. In reciprocal crosses a total of 82 N. rustica var chlorotica flowers were emasculated and pollinated with N. tabacum pollen taken from three different varieties. Seed capsules swelled and appeared to develop normally for the first five days but later appeared sunken. After about 28 days all capsules were harvested and examined for the presence of seed. Many seeds appeared to have developed but all seeds were hollow when dissected and none of these germinated. To obtain sexual hybrid plants of N. rustica x N. tabacum, immature embryos still

TABLE 33. Alkaloid content of Nicotiana rustica var chlorotica + N. tabacum var WS somatic hybrids, sexual backcross hybrids and the parental varieties

Genotype	*% Total alkaloid
<u>N. rustica</u> var chlorotica	0.54
<u>N. tabacum</u> var WS	0.21
Somatic hybrid 165	1.95
" " 8	1.87
" " 64	1.47
" " 163	1.41
" " 139	1.24
" " 167	0.47
" " 164	0.44
" " 158	0.35
" " 163	0.29
" " 141	0.18
" " 134 x WS	0.51
" " "	0.35
" " 139 x WS	0.32

* Expressed on a dry weight basis. Values are means obtained from four leaves of each plant.

contained within their seed structures were removed from ovaries three days after cross pollination and cultured in vitro. Seeds produced in this way contained hybrid embryos and such seeds readily germinated. Sexual hybrids involving two varieties of N. rustica and three of N. tabacum (Table 34) were readily obtained by this procedure.

1. Development of sexual hybrid seedlings

Hybrid seeds from cross pollinations were white in color when initially excised from the ovary three days after pollination. After two weeks in vitro these turned brown and developed a distinct pattern on the seed coat. By three weeks, hybrid seeds attained a size intermediate between seeds of the parent species (Fig. 71) and began to germinate. After five weeks, most seeds born on placental tissue germinated (Fig. 73), first with the emergence of the radicle followed by the cotyledons (Fig. 71). Not all seedlings which germinated were normal; seedlings developed with none, one, two unequal, three or fused trumpet-shaped cotyledons (Fig. 72). Germination in these embryos was also often abnormal with the cotyledons emerging first and in some cases no radicle developing (Fig. 72). Subsequent development of seedlings with morphological abnormalities was not studied. Dissection showed that all seeds which reached full size by the end of the culture period but which failed to germinate contained a torpedo-shaped embryo (Fig. 71). Twenty-five such embryos were cultured on B₅ medium with 0.5 mg/L kinetin and 0.25 mg/L 2,4-D and 2% sucrose. Four weeks after initiating these cultures 15 embryos produced green calli with many shoot primordia (Fig. 77) while the remaining 10 died. IAA at 1.0 mg/L could not replace 2,4-D. These primordia

TABLE 34. Genotypes of Nicotiana rustica and N. tabacum which have yielded sexual hybrids following crossing and culture of hybrid embryos in vitro

<u>N. rustica</u> ♀				<u>N. tabacum</u> ♂			
<u>N. rustica</u> var chlorotica				x <u>N. tabacum</u> var "V"			
"	"	"	"	"	"	"	Delhi 34
"	"	"	"	"	"	"	WS
"	"	"	NRT	"	"	"	Delhi 34
"	"	"	"	"	"	"	WS

$$\left(\begin{array}{c} \text{N. rustica var chlorotica} \\ \times \\ \text{N. rustica var NRT} \end{array} \right) F_1 \times \text{N. tabacum var WS}$$

$$\left(\begin{array}{c} \text{N. rustica var chlorotica} \\ \times \\ \text{N. rustica var NRT} \end{array} \right) F_1 \times \text{N. tabacum var Delhi 34}$$

FIGURE 71. N. rustica var chlorotica x N. tabacum var WS
hybrid seeds and their germination in vitro.
Mature hybrid seed on its edge dissected to
show embryo (a). Hybrid seed on its side
(beneath "c") is intermediate in size with
respect to seed from N. rustica var chlorotica
(d) and N. tabacum var WS (b).
c = torpedo-shaped hybrid embryo dissected
from seed.

FIGURE 72. Abnormal seedlings of N. rustica var chlorotica
x N. tabacum var WS during germination in vitro.

developed into shoots which readily rooted when transferred to B₅ medium with 2% sucrose.

In control experiments unfertilized N. rustica ovules were cultured but did not germinate indicating that parthenogenic plant development from ovular tissue or cells of the embryo-sac was rare or impossible (Fig. 73). Evidence for the hybrid nature of seedlings was suggested by their dark green color when compared to seedlings of the chlorophyll-deficient parental species (Fig. 75) and also by the synthesis of chlorophyll in callus derived from isolated hybrid embryos (Fig. 77). The development of chlorophyll indicated that genetic complementation for normal chlorophyll synthesis had taken place in the hybrid seedlings. This complementation occurred equally well with hybrid seedlings produced from a number of crosses involving N. rustica var chlorotica listed in Table 34.

2. Factors affecting the yield and development of hybrid seedlings

Using seeds on cultured placentas a number of studies were carried out. A study of the effect of four basic culture media on yield and development of hybrid seedlings revealed that highest yields (39 seedlings/placenta) occurred with Lörz medium (Table 35). Each placenta of N. rustica produces approximately 230 seeds when self-fertilization occurs; a yield of 39 hybrid seedlings therefore represents only a fraction of the potential number obtainable. While Lörz medium gave the greatest number of hybrid seedlings most seedlings were morphologically abnormal (Table 35). There was little difference in yield between B₅ or MS media but B₅ supported the development of the greatest number of normal embryos and was therefore used in subsequent experiments.

TABLE 35. Effect of culture media on yield and development of Nicotiana rustica
var chlorotica x N. tabacum var WS hybrid seedlings

Culture media	No. of placentas cultured	Mean no. seeds germinating/placenta		
		Normal	Abnormal	Ratio normal/abnormal
B ₅	18	20	12	1 : 0.6
MS	17	15	14	1 : 0.9
Lörz	17	15	24	1 : 1.6
Kao	20	9	10	1 : 1.1

Note: Placentas bearing immature hybrid seeds were cultured in vitro 3 days after pollination on media indicated; each medium contained 4% sucrose. Nutrient composition of B₅, MS, Lörz and Kao media are given in Appendix Tables 1, 2, 4, 5, respectively.

- FIGURE 73. Germination of N. rustica var chlorotica x N. tabacum var Delhi 34 seedlings in situ on cultured placentas after 6 wks of culture. Note when placentas of unpollinated N. rustica flowers were cultured (lower left and upper right of picture) placentas did not develop and seedlings were not obtained.
- FIGURE 74. Face and side view of flowers of N. tabacum var WS (upper left) and var Delhi 34 (upper right); N. rustica var chlorotica (upper center). Flowers of N. rustica var chlorotica x N. tabacum var WS (lower left) and N. rustica var chlorotica x N. tabacum var Delhi 34 (lower right). Note enhanced development of anthocyanin in hybrids of N. rustica var chlorotica x N. tabacum var WS.
- FIGURE 75. Comparison of seedlings of chlorophyll-deficient N. rustica var chlorotica (left) and N. tabacum var "V" (right) with sexual hybrid (center). Note restoration of normal chlorophyll content and vigor in hybrid.
- FIGURE 76. Three sexual hybrid plantlets of N. rustica var chlorotica x N. tabacum var "V" showing uniform morphology and profuse rooting in vitro.
- FIGURE 77. Callus and meristem formation from cultured embryo of N. rustica var chlorotica x N. tabacum var "V" (top) and from hypocotyl explant of N. rustica var chlorotica (bottom). Note chlorophyll development only in hybrid tissue. B5 medium containing 0.5 mg/L kinetin + 0.25 mg/L 2,4-D.

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B₅ medium with various concentrations of sucrose was tested. A concentration of 4% was optimal supporting both the development of the greatest number of hybrid embryos and the greatest frequency of normal seedlings (Table 36).

Supplements of malt extract at 0.1, 0.2, and 0.4% did not affect the ratio of normal : abnormal seedlings obtained but the mean number of hybrid seedlings obtained per placenta was reduced slightly in the presence of 0.1% malt extract.

The effect of allowing hybrid embryos to develop on the plant for various periods prior to culture in vitro is shown in Table 37. Immature hybrid seeds were very loosely attached to the placental tissue three days after pollination. Immature hybrid seeds cultured earlier than three days after pollination did not usually develop to maturity. Those cultured three days after pollination yielded half as many hybrid seedlings as those cultured after 4, 5, or 7 days but the greatest number of normal seedlings per cultured placenta was obtained by culturing immature seeds three days after pollination. The ratio of normal : abnormal seedlings was 1:0.8 when immature seeds were cultured three days after pollination and 1:8.0 when culture was delayed for seven days.

The effect of culturing immature hybrid seeds directly in contact with agar-solidified culture medium rather than attached to placental tissue is shown in Table 38. Yield of hybrid seedlings was increased by culturing immature seeds directly on the culture medium. The ratio of normal : abnormal seedlings obtained by both methods was similar.

TABLE 301 Effect of sucrose concentration on yield and development of *Nicotiana rustica*
var chlorotica x *N. tabacum* var Delhi 34 hybrid seedlings

Sucrose conc. % w/v	No. of placentas cultured	Mean no. seeds germinating/placenta		
		Normal	Abnormal	Ratio normal/abnormal
2	6	0	0.5	0.5
4	7	11	2	13 1 : 0.2
6	7	5	2	7 1 : 0.4
8	7	2	3	5 1 : 1.5

Note: Basic medium throughout was B₅. Placentas bearing immature hybrid seeds were cultured in vitro 3 days after pollination.

TABLE 37. Effect of time prior to in vitro culture on yield and development of Nicotiana rustica var chlorotica x N. tabacum var WS hybrid seedlings

Mean No. hybrid seeds germinating/placenta				
Time post pollination prior to <u>in vitro</u> culture (days)	No. of placentas cultured	Normal	Abnormal	Total
1	10	0	0	0
2	10	4	0	0
3	8	14	12	26
4	10	10	40	50
5	10	10	48	58
7	10	7	55	62
10	5	2	14	16
				Ratio normal/abnormal
				1 : 0.8
				1 : 4.0
				1 : 5
				1 : 8
				1 : 7

Note: Basic medium throughout was B₅ with 4% sucrose. Placentas bearing immature hybrid seeds were excised at various intervals after pollination as indicated and cultured in vitro.

TABLE 38. Effect of culture method on yield and development of Nicotiana rustica var chlorotica x N. tabacum var WS hybrid seedlings

Culture method	Mean No. hybrid seeds germinating/placenta			Ratio normal/abnormal
	Normal	Abnormal	Total	
Immature seeds cultured directly on medium	24	20	44	1 : 0.8
Immature seeds cultured attached to placentas	14	12	26	1 : 0.8

Note: Basic medium throughout was B5 with 4% sucrose. For each culture method the immature hybrid seeds of eight placentas were cultured three days after pollination.

Using the alternative method of culturing immature hybrid seeds directly in contact with the culture medium the effects of sucrose alternatives such as mannitol, fructose, glucose and glucose + fructose were tested for their ability to support development of hybrid seedlings (data not shown). All carbohydrates were supplied at an equivalent molarity of 4% sucrose (0.12 M). No seedlings were obtained with mannitol. Equal numbers of seedlings were obtained with sucrose, fructose, and glucose but a greater frequency of abnormal seedlings were obtained in the presence of glucose. This adverse effect of glucose was lessened when glucose was supplied in combination with fructose.

E. Morphological, Cytological and Biochemical Analysis of

Sexual Hybrids

1. Morphology

At least twenty individual sexual hybrid plants were grown to flowering. The morphology of leaves, stems and roots of these sexual hybrids (Figs. 74, 76, 78 and 80) was extremely uniform by comparison with that of somatic hybrids (Figs. 46-55, 56-61). All plants grew vigorously in vitro and in the greenhouse, produced many roots (Fig. 76) and expressed strong apical dominance (Figs. 76 and 79). After several weeks of flowering, however, growth of axillary buds occurred as in N. rustica (Fig. 79). All plants had dark green stems, leaves and sepals resembling wild-type N. rustica and N. tabacum rather than the chlorophyll-deficient parent species (Figs. 74, 75 and 81)..

The analysis of vegetative and floral characteristics of four sexual hybrid plants is summarized in Table 39. No plants appeared phenotypically chimeric and little inter-plant variation was observed.

FIGURE 78. Progeny of N. rustica var chlorotica crossed with

- (i) N. tabacum var WS (top)
- (ii) N. tabacum var "V" (middle)
- (iii) N. tabacum var Delhi 34 (bottom)

Note uniformity in morphology

FIGURE 79. Parental species N. rustica var chlorotica

(right), N. tabacum var WS (left) and hybrids
of N. rustica var chlorotica x N. tabacum var
WS (center).

79

78

TABLE 39. Vegetative and flower characteristics of *Nicotiana rustica* x *N. tabacum* sexual hybrids

	Height (cm)	No. of leaves/ plant	Leaf index $\frac{\text{length}}{\text{width}}$	No. of flower branches	Corolla $\frac{\text{length}}{\text{width}}$	Sepal length
<i>N. rustica</i> var chlorotica	67	11	1.2	11	0.7	1.7
<i>N. tabacum</i> var "V"	126	34	4.4	8	2.3	2.8
Sexual hybrids						
Plant No. 1	158	15	1.5	15	1.6	2.6
" 2	150	16	1.5	15	1.6	2.6
" 3	148	11	1.7	15	1.6	2.6
" 4	142	17	1.6	15	1.6	2.6

FIGURE 80. Face and side view of flowers of sexual hybrids of N. rustica var chlorotica crossed with (i) N. tabacum var Delhi 34 (two specimens upper left) and (ii) N. tabacum var "V" (two specimens upper right) and parental types N. tabacum var Delhi 34 (lower left) and N. rustica var chlorotica (lower right). Note uniformity in morphology among hybrids of different genotype and among siblings.

FIGURE 81. Leaves of chlorophyll-deficient N. tabacum var "V" (left) and N. rustica var chlorotica (right). Leaf of sexual hybrid of N. rustica var chlorotica x N. tabacum var "V" (center).

All hybrids were taller than either parent at flowering (Fig. 79) and produced an intermediate number of leaves. The leaf length/width ratio of all hybrids was closer to N. rustica than N. tabacum (Fig. 81). Leaves of all plants were distinctly petiolated resembling N. rustica; like N. tabacum a slender margin of leaf tissue extended along the petioles to the stem. All sexual hybrids produced a greater number of flower branches than the parents. Flower morphology was intermediate with respect to the parent species for corolla length, width, and pigmentation and for sepal length (Table 39 and Figs. 74 and 80). Flowers of N. tabacum var WS were more heavily pigmented with anthocyanin than those of var Delhi 34, (Fig. 74). N. rustica var chlorotica x N. tabacum var WS hybrids were more heavily pigmented than hybrids produced from var Delhi 34 (Fig. 74). Apart from these differences in pigmentation there was no extreme variation in flower morphology among the sexual hybrids of the type found in somatic hybrids (Figs. 60 and 61). The flowers of sexual hybrids contained normal pistils and five stamens per flower.

Pollen viability tests revealed that all sexual hybrids were male sterile. Seventeen sexual hybrids were backcrossed with N. tabacum and from these crosses four seeds were obtained which germinated. In 12 backcrosses using N. rustica pollen no viable seeds were obtained.

2. Chromosome determinations

The chromosome numbers of 18 sexual hybrid plants were analyzed. Fourteen plants showed a chromosome complement of 48, one showed 47 and one 49; the two remaining plants contained variable numbers of chromosomes among different cells of the same root tip. One such

chimera had 48 chromosomes in some cells while other cells had 66 plus a fragment chromosome. For the other chimera chromosome numbers varied from 41 to 86 among cells.

3. Analysis of isozymes

While a number of N. tabacum and N. rustica varieties were used in the sexual crosses (Table 34) the sexual hybrids chosen for isozyme analysis were N. rustica var chlorotica x N. tabacum var "V".

As for the somatic hybrids the electrophoretic pattern of esterase isozymes proved most useful for the analysis of sexual hybrids. The esterase patterns obtained for the virescent "V" mutant of N. tabacum was somewhat different than that of the albino segregants of the white seedling (WS) strain of N. tabacum used in the somatic hybridizations (cf. Fig. 67 with 82). The isozyme pattern for N. rustica var chlorotica was essentially the same in the electrophoretic runs of both the sexual and somatic hybridization analyses.

All of the hybrids analyzed gave the same electrophoretic pattern for esterases; this pattern showed isozymes derived from both parents (Fig. 82). All hybrids showed bands contributed by N. tabacum at Rf 0.38 and at Rf 0.45. A band contributed by N. rustica at Rf 0.51 and a band at Rf 0.57 from N. tabacum appeared in all ten hybrids. The triplet of bands in the Rf 0.7 to 0.75 zone in the hybrids also provided evidence of hybridity. The uppermost doublet, Rf 0.7 and 0.73, were derived from N. tabacum and the lowermost band, Rf 0.75 from N. rustica. All hybrids lacked the light Rf 0.48 band of N. rustica but showed the entire

FIGURE 82. Esterase isozyme pattern and zymogram
for N. tabacum var "V" (gel 1),
N. rustica var chlorotica (gel 2) and
10 sexual hybrids (gels 3-10).

Figure 82

complement of N. tabacum bands.

The separation of aspartate aminotransferase bands obtained during the analysis of sexual hybrids was not as good as that obtained for the somatic hybrids (cf. Fig. 83 with 68). As a result the aspartate aminotransferase system did not provide unequivocal evidence for hybridity. A band at Rf 0.33 appeared strongly in N. tabacum but weaker in N. rustica. All hybrids showed this band strongly, suggesting the expression of N. tabacum genes alone or a co-expression of genes from N. tabacum and N. rustica. The expression of N. rustica genes in the hybrids was indicated by the presence of electrophoretic bands at Rf 0.39 and 0.42 which were clearly derived from N. rustica. Both N. rustica and N. tabacum showed a band at Rf 0.45 but this band did not appear in the hybrids. Similarly, the hybrids did not show the Rf 0.29 band of N. rustica. Unlike the somatic hybrids in which nine out of ten plants expressed a unique isozyme (Fig. 68) none of the sexual hybrids showed such a band.

The zymogram pattern for superoxide dismutase developed in the absence of cyanide is shown in Fig. 84. This zymogram for the hybrids is representative of a sample of ten sexual hybrid plants analyzed. A triplet of electrophoretic bands at Rf 0.32, 0.34, and 0.36 was found for N. tabacum and Rf 0.38, 0.40, and 0.42 for N. rustica. All hybrids showed the triplet derived from N. rustica but not from N. tabacum. All sexual hybrids showed a band at Rf 0.54 which was derived from N. rustica. Evidence for the expression of N. tabacum genes was indicated by the presence of a single band at Rf 0.57 in all hybrids which was present in

3 3

OF / DE

FIGURE 83. Aspartate aminotransferase isozyme
pattern and zymogram for N. tabacum
var "V" (gel 1), N. rustica var chlorotica
(gel 2) and 10 sexual hybrids (gels 3-10).

Figure 83

FIGURE 84. Superoxide dismutase zymogram for N. tabacum
var "V" (1) N. rustica var chlorotica (2)
and a sexual hybrid (3). Note the
zymogram in "3" is representative of a
sample of 10 sexual hybrids each of which
displayed an identical pattern.
Zymogram was obtained for gels stained in
the absence of cyanide.

FIGURE 84

N. tabacum but not N. rustica. All hybrids also showed a band at Rf 0.61 which was common to both N. rustica and N. tabacum. The pattern of isozymes developed in the presence of cyanide was poorly resolved and could not be interpreted.

4. Analysis of RUBPCase by isoelectric focussing

The polypeptide composition of RUBPCase of two N. rustica var chlorotica x N. tabacum var "V" sexual hybrids was analyzed after isoelectric focussing (figure not shown). The small subunit polypeptide composition of these hybrids contained four bands indicating the presence and expression of nuclear genes from both parental species.

The large subunit polypeptide distribution pattern was similar to N. rustica for both sexual hybrids demonstrating the presence and expression of chloroplast encoded genes of the maternal parent in sexual hybrids.

5. Analysis of alkaloids

The alkaloid content of ten sexual hybrid plants obtained from crosses of two varieties of N. rustica with two of N. tabacum is summarized in Table 40. Alkaloid content of N. rustica var chlorotica was highest but that of commercial tobacco N. tabacum var Delhi 34 was slightly higher than wild-type N. rustica. All ten sexual hybrids produced much higher levels of alkaloids than the parents. Highest levels (of up to four times the parental values) were obtained where high yielding N. tabacum var Delhi 34 was used in the cross.

TABLE 40. Alkaloid content of Nicotiana rustica x N. tabacum sexual hybrids and their parental varieties

Genotype	* % Total alkaloid
<u>N. rustica</u> var chlorotica	0.54
<u>N. rustica</u> var NRT	0.44
<u>N. tabacum</u> var Delhi 34	0.50
<u>N. tabacum</u> var WS	0.21
<u>N. rustica</u> var chlorotica	
x <u>N. tabacum</u> var Delhi 34	2.18
" "	1.75
<u>N. rustica</u> var chlorotica	
x <u>N. tabacum</u> var WS	1.24
" "	0.93
<u>N. rustica</u> var NRT	
x <u>N. tabacum</u> var Delhi 34	2.52
" "	1.94
" "	1.89
" "	1.69
<u>N. rustica</u> var NRT	
x <u>N. tabacum</u> var WS	1.72
" "	1.10

* Expressed on a dry weight basis. Values are means obtained from four leaves of each plant.

DISCUSSION

A. Isolation and Culture of Protoplasts and Plant Regeneration in N. rustica and N. tabacum

Wild-type and mutant genotypes of N. rustica and N. tabacum can be manipulated in vitro to produce callus, and cell suspension cultures which are morphogenically competent, and from which high yields of viable protoplasts can be obtained. Other studies established that callus could be readily initiated from explants of wild-type N. rustica (Hunt and Loomis 1976) and N. tabacum (Skoog and Tsui 1948). In the present study all explants of N. rustica produced more callus than those of N. tabacum when cultured on the same medium (Table 5). In agreement with Venketeswaran and Mahlberg (1962) explants of albino N. tabacum produced more friable callus than those of the wild type. Leaf explants were most suitable for callus initiation in both species. The source of explant for callus initiation is often critical (Yeoman 1973; Murashige 1977), particularly with cereal species (Dale and Deambrogio 1979). In both Nicotiana species the auxin 2,4-D was superior to NAA for callus initiation; use of 2,4-D is often recommended for callus initiation (Gamborg 1975).

For reproducible somatic hybridization it is essential to produce substantial numbers of parental protoplasts with consistent membrane properties and a high inherent capacity for cell division. Leaf protoplasts of both N. rustica and N. tabacum have been

previously isolated and cultured (Nagata and Takebe 1971; Constabel et al. 1976; Gill et al. 1979; Landgren and Bonnett 1979).

However, plating efficiency was generally low and variable.

Furthermore, the capacity of leaf protoplasts to undergo fusion (Kao 1978; Kameya 1979) and subsequent cell division (Watts et al. 1974) was strongly influenced by leaf age and growth regime of donor plants. Because of these difficulties in working with leaf protoplasts and because albino seedlings of N. tabacum are lethal in vivo and grow very slowly in vitro, cell suspension cultures were established and maintained under constant conditions as a source of protoplasts. Initially these suspension cultures grew slowly and protoplast preparations derived from them contained much cellular debris. With frequent subculturing for about 12 weeks, however, the growth rate of the cultures increased and both the yield and quality of resultant protoplasts greatly improved. This improved yield and quality was maintained in cultures up to one year old. Recent studies with corn (Potrykus et al. 1979) and rose (Strauss and Potrykus 1980) cell suspensions also reported that protoplasts were produced most readily from cells in the exponential phase and such protoplasts synthesized cell walls and underwent division with the greatest frequency. Protoplasts obtained from rapidly-growing cells are also most suitable for somatic hybridization studies since they can be induced to fuse more readily than those from older cultures and heterokaryocytes formed undergo cell division with a greater frequency than heterokaryocytes having one fusion partner derived from a slow-growing cell culture (Kao 1978).

Satisfactory yields of good quality protoplasts were consistently obtained from cell cultures of both species (Table 6). Substantial yields of viable protoplasts have been previously obtained from cultured cells of wild-type (Uchimiya and Murashige 1974) and albino (Su/Su) N. tabacum (Gamborg et al. 1979) and from leaf protoplasts of N. rustica (Gill et al. 1979). A considerable difference in yield of protoplasts was found between species with N. rustica outyielding N. tabacum (Table 6). Using cells from comparable suspension media there was little difference in the yield of protoplasts from wild-type and mutant N. rustica genotypes but with N. tabacum the albino culture outyielded the wild-type by up to 15-fold (Table 6). These results strongly suggest that plant genotype influences yield in agreement with previous studies (Watts et al. 1974; Izhar and Power 1977). Greatest yields of clean stable protoplasts of both species were obtained from cells after 5.5 h of enzyme treatment (Fig. 8). This is in contrast to a study with wild-type N. tabacum by Uchimiya and Murashige (1974) in which optimal yields were reported after 2-3 h under different conditions. Also, for N. rustica leaf protoplasts, Gill et al. (1979) reported that a 12 h incubation was necessary for yields similar to those of this study and the protoplasts which they eventually obtained were extremely fragile.

The type of auxin in the culture medium of the cell suspensions prior to protoplast isolation was also found to affect yield, quality, and subsequent time of cell division in cultures. 2,4-D enhanced the yield of protoplasts over NAA by 2- to 3-fold in N. rustica (Table 6). This effect has been previously noted with Haplopappus gracilis (Wallin et al. 1977). In addition, protoplasts from 2,4-D

cultures were more stable in handling and appeared morphologically superior with a well-developed cytoplasmic network and fewer starch grains (Figs. 9, 10, and 11). On the other hand, the onset of cell division in these protoplast-derived cultures was delayed up to 10 days over comparable cultures from NAA-grown cells (Table 6). This effect was not observed in N. tabacum. On the basis of the superior quality of protoplasts and the greater morphogenic potential of 2,4-D-grown cells (discussed below) it was decided to use cultures grown with 2,4-D for somatic hybridization studies.

High plating efficiencies in the 60-80% range were consistently obtained in protoplast-derived cells of both species in NT culture medium. This is in contrast to other studies which reported low plating efficiencies of 15% for leaf protoplasts of N. rustica (Constabel et al. 1976; Gill et al. 1979) and 20% for albino N. tabacum (Landgren and Bonnett 1979).

The development of pro-embryo-like structures from N. rustica protoplasts was observed in the presence of NT medium containing p-chlorophenoxyacetic acid and kinetin (Results, Section A 4). These structures were only observed when the original cell cultures were grown in the presence of 2,4-D. In previous studies somatic embryos have been obtained directly from protoplasts of N. tabacum (Lörz et al. 1977), N. sylvestris (Facciotti and Pilet 1979) and alfalfa (Kao and Michayluk 1980) and these subsequently developed into plants. However, in this study it was not possible to culture the pro-embryos to maturity. Pro-embryo-like bodies transferred to the medium recommended by Lörz et al. (1977) deteriorated possibly because of osmotic shock. The ability to obtain embryos from somatic

hybrid protoplasts after fusion would be advantageous since separate procedures for root and shoot induction in hybrid cells would be obviated.

The phenotypic differences in the genetic variants of both species were observable in vitro though they were modulated by the culture medium employed. While leaves, stems and petioles of N. rustica var chlorotica plants were pale green; callus, cell suspension cultures and protoplast-derived colonies appeared completely chlorophyll-free (Figs. 1, 3, 18, 23). The absence of chlorophyll in vitro in this mutant of N. rustica makes it a potentially valuable parent for somatic hybridization studies where complementation for chlorophyll synthesis is used as the selection system. Cultures of this mutant only became green in localized areas of callus undergoing shoot differentiation. Wild-type N. rustica var NRT plants were much darker green than the mutant; cell colonies and induced shoots from this genotype were also greener. The albino mutation of N. tabacum was leaky. Seedlings germinated in vitro, callus cultures grown on NAA-containing medium and shoots regenerated from suspension-derived cell colonies produced chlorophyll in some cells. Chlorophyll-containing albino leaf cells with varying degrees of thylakoid organization in plastids have been previously observed in this mutant (Susalla and Mahlberg 1975). Cell suspension cultures and protoplast-derived cell colonies from the mutant were, however, chlorophyll-free (Figs. 18 and 24). It appears therefore that while the mutants of both N. rustica and N. tabacum have some capacity to form chlorophyll, this capacity is depressed in undifferentiated cultured cells. A close relationship between the extent of cell and chloroplast

differentiation has been previously observed in cells of many other species (Granick 1955). Mutants of both species were stable in vitro since in the course of many experiments no evidence for reversion to the wild-type was found.

The auxin 2,4-D depressed chlorophyll synthesis in callus and suspension cultures. This effect was most striking with wild-type N. rustica var NRT suspension cultures which were bright green when NAA was used but chlorophyll-free in 2,4-D. Bergmann (1967) and Yamada et al. (1978) have shown that 2,4-D depresses RUBP carboxylase and chlorophyll synthesis in cultured N. tabacum cells while NAA enhances these systems. With NRT the amount of chlorophyll produced in protoplast-derived cell colonies from 2,4-D-grown suspensions was diminished and the onset of chlorophyll synthesis delayed relative to cultures derived from NAA-grown suspensions. Since the protoplast culture medium did not contain 2,4-D, there was likely a carry-over effect from cell suspension to protoplast-derived colonies.

Chlorophyll synthesis in cell cultures is also affected by the type and amount of ~~carbohydrate~~ carbohydrate employed (El-Hinnawy 1974; Dalton and Street 1977) and for different plant materials a given sugar e.g. sucrose has been shown to either promote (Bornman and Fanshawe 1976) or depress (Pamplin and Chapman 1975) chlorophyll development. In the present study the carbohydrates glucose, maltose, sucrose, mannitol, sorbitol, fructose and raffinose used as osmotica at 0.35 M along with 1% sucrose all allowed cell colony formation from protoplasts (Table 7). Glucose was most effective in promoting chlorophyll synthesis in protoplast-derived cell colonies and was therefore used in cultures following protoplast fusion to enhance greening of somatic hybrid colonies. The carbohydrates tested apparently have a direct

effect on chlorophyll synthesis since the energy demands of these cells should have been met by the included sucrose. The direct inhibitory effect of sucrose on chlorophyll synthesis and the promotive effects of glucose on chlorophyll content of carrot tissues has been demonstrated by Pamplin and Chapman (1975).

The absence of cell division when galactose was used as osmoticum may have been due to its inhibition of wall synthesis. Wall regeneration in tobacco leaf protoplasts is a prerequisite for subsequent cell division (Schilde-Rentschler 1977). Other studies have shown that galactose can inhibit cellulose synthesis, wall deposition and growth in higher plants (Baker and Ray 1965; Winter et al. 1970). More recently de Kock et al. (1979) have shown that as little as 0.1% galactose impairs cellulose synthesis in axenic cultures of Lemna gibba while promoting senescence. Galactose also inhibited respiration in spinach tissue cultures (Dalton and Street 1977). These studies indicate that the inhibitory action of this agent is at a fundamental level of cell metabolism.

In both species conditions were established for shoot induction in cell colonies derived from suspension cultures. The morphogenic response depended on concentration of cytokinin in the shoot-induction medium, type of auxin in the culture medium prior to induction and age of culture prior to induction (Tables 8, 9, 10, and 11). At 2 mg/L, kinetin, was effective for N. rustica var NRT concurring with Gill et al. (1979) but not for var chlorotica and it was superior to BA in ensuring development of shoots in albino N. tabacum. For N. rustica var chlorotica, BA was most effective when the cytokinin : auxin ratio was 2, giving shoots on 77% of treated calli (Table 8).

Phosphate ions were not important for fusion in the present study (Table 16). For chick erythrocyte fusion, however, phosphate ions in combination with calcium were found to be essential; calcium-phosphate induced an aggregation of membrane-associated proteins exposing protein-free sites in the membranes (Majumdar and Baker 1980). One hypothesis for the mechanism of membrane fusion is that fusion is initiated at such protein-free sites (Volsky and Loyter 1978; Knutton 1979). Observations on the fusion of plant protoplasts are in keeping with this hypothesis; localized areas of contact between apposing plasmamembranes (Fowke et al. 1977; Burgess et al. 1977; Burgess and Fleming 1974) have been observed and enhanced fusion frequencies occurred when surface proteins were removed (Constabel and Kao 1974; Ward et al. 1979).

The enhancement of PEG-induced fusion by DMSO, observed in this study (Table 15) concurs with previous studies (Haydu et al. 1977; Norwood et al. 1976) but unlike these studies, 2-5% DMSO rather than 10-15% DMSO was optimal. Different responses obtained here may be due to the species used since it is known that plasmamembranes of different species have differing phospholipid compositions (Yamada et al. 1979) which in turn affects the fluidity of protoplast membranes and their capacity for fusion (Senda et al. 1980). These studies provide a rationale for observations that fusability and tolerance to fusing agents are species specific (Kao 1978). One way in which PEG and DMSO contribute to processes of fusion is by decreasing the surface potential of the plasmamembrane (Maggio et al. 1976; Lucy 1978), presumably allowing close membrane apposition which is a prerequisite for fusion (Poste and Allison 1979). PEG and DMSO

carrot (Smith and Street 1974) and Arabidopsis thaliana (Negrutiu et al. 1978). The reason for such losses in morphogenic competence is not clear but it may be related to modifications of the genome such as change in chromosome number or size, or to epigenetic factors (D'Amato 1978). Aneuploidy or polyploidy, which is often characteristic of long-term cell cultures and parallels the decline in morphogenic competence (Murashige and Nakano 1968; Smith and Street 1974; Negrutiu et al. 1978) does not necessarily preclude morphogenic competence (Sacristan and Melchers 1969; Smith and Street 1974). Regenerants from cell cultures tend to have approximately similar chromosome numbers and these values do not necessarily reflect the ploidy of the cell cultures from which they were derived (D'Amato 1978). While the ploidy level of the cell cultures in the present study was not determined, a study with albino (Su/Su) N. tabacum has shown that cell cultures retained diploidy and morphogenic capacity for over two years (Gamborg et al. 1979). In relation to the present study the decline in morphogenic potential was regarded as undesirable since it could have led to difficulties in regeneration of somatic hybrid plants from hybrid protoplasts derived from these cells.

Shoots could be induced at low frequencies in Protoplast-derived cell-colonies of both species. Morphogenic response was variable with frequencies ranging from 0-24% in different experiments for N. rustica var chlorotica and 0-13% for albino N. tabacum. These differences may be related to different ages of the source cell cultures as discussed above or (as discussed below) to the timing of transfer to shoot-inducing medium. In all the experiments, however, it was consistently observed that induction of shoots from

protoplast-derived colonies was less successful than from cell colonies obtained directly from cell cultures. In one experiment with N. rustica, 37% of cell suspension-derived colonies formed shoots while protoplast-derived colonies cultured for the same period failed to respond (Table 12). Similarly with albino N. tabacum, the frequency of shoot induction in protoplast-derived cells was 13% while that of cell suspension-derived cells was 93%. This phenomenon has not been studied closely heretofore and the reason for reduced morphogenic response in protoplast-derived cells is not clear. It is possible that the protoplast culture medium had improperly conditioned the cells for subsequent shoot induction since studies by Negrutiu and Jacobs (1978b) have shown the importance of a proper sequence of media for organogenesis. Alternatively, as suggested by Wernicke and Thomas (1980), medium for culturing protoplasts may preferentially favor proliferation of non-morphogenic cells. The time of transfer to shoot-inducing conditions may therefore be critical. Transfer of protoplast-derived cells at an early stage to medium supporting shoot induction allowed prolific colony development and shoot formation in protoplast-derived colonies of N. tabacum and Hyoscyamus muticus (Wernicke and Thomas 1980).

The frequency of root induction in shoots of mutant N. rustica was higher than in the wild-type; formation of adventitious roots was not enhanced by auxin. For N. tabacum, roots were readily initiated on wild-type shoots but not on albinos. Roots were readily formed by albino (Su/Su) shoots of N. tabacum (Gamborg et al. 1979) but not by shoots of albino N. sylvestris (Szilagyi and Nagy 1977). Differences in rooting ability of shoots derived from protoplasts

have been previously observed among different species of Nicotiana (Banks and Evans 1976).

B. Fusion of Protoplasts and Selection and Regeneration of Somatic Hybrid Plants

The green cell colonies which appeared after fusing N. rustica and N. tabacum protoplasts were apparently the result of non-allelic genetic complementation for chlorophyll synthesis in hybrid cells. Green colonies failed to appear in control experiments ruling out the possibility of cross-feeding and/or greening due to homofusion (Figs. 38 and 39).

Since protoplast-derived colonies of wild-type N. rustica readily synthesized chlorophyll and appeared deep green while those of N. tabacum did not, it is likely that the N. rustica genome was responsible for chlorophyll synthesis in the hybrid cells. If this suggestion is correct, the early intense greening characteristic of N. rustica var chlorotica upon complementation may be useful for selection of hybrids from fusions of this mutant with a wide range of species.

The hybrid green colonies were easily detectable within three weeks following fusion. This is in contrast to other studies using complementation selection where hybrid colonies were only recognized after at least one subculture on solid medium (Melchers and Labib 1974; Schieder 1977b; Melchers 1977; Glimelius et al. 1978a). Other selection systems in which only one parent was albino or chlorophyll-deficient (Cocking et al. 1977; Dudits et al. 1977; Schieder 1978a; Maliga et al. 1978; Power et al. 1979; Dudits et al. 1979) or where biochemical mutants were used (Maliga et al. 1977, Willems et al. 1980;

White and Vasil 1979) required even greater post-fusion intervals for the manifestation of characteristics that indicate hybridity. Early selection of hybrid cell colonies is particularly desirable in combinations involving unrelated species since a prolonged culture period subsequent to fusion often leads to chromosome elimination (Kao 1977). Where selection is based on genetic complementation, rapid chromosome elimination may lead to failure of complementation and in addition the resulting chromosomal imbalance could hinder subsequent induction of morphogenesis (Murashige and Nakano 1968).

When protoplasts of either species were cultured separately, they underwent division asynchronously, the onset of cell division differing by up to 11 days (Table 6). While the onset of cell division could not be determined in the hybrids it was apparently not delayed since hybrid cell colonies appeared as soon as parental types.

The hybrid cell colonies had a higher growth rate than either parental types. Although this might be partly due to an advantage conferred by the capability for photosynthesis with resultant synthesis of important metabolites it probably involves a general heterosis effect depending on other factors since it has persisted in many of the mature hybrid plants. A similar heterosis effect was observed by Schieder (1978a) in interspecific Datura somatic hybrids. This enhanced growth rate of hybrid cells has been suggested as a method to aid in the selection of somatic hybrid cells produced from combinations in which heterosis in the progeny could be anticipated (Smith and Mastrangelo-Hough 1979).

Polyethylene glycol (PEG) was effective for inducing fusion of N. rustica and N. tabacum protoplasts. The appearance of large

numbers of hybrid cell-colonies subsequent to heterofusion is attributed to the use of large numbers of parental protoplasts (approximately 10^6 per fusion treatment), the effectiveness of PEG for inducing fusion without excessive damage and the high capacity for wall and subsequent colony formation in parental protoplasts. Polyethylene glycol was originally developed for fusing plant protoplasts (Kao and Michayluk 1974; Wallin *et al.* 1974) and is now also used extensively for fusing animal cells (Pontecorvo 1975) or fusing plant protoplasts with animal cells (Ward *et al.* 1979). Severe plasmolysis with loss of spherical shape and disappearance of cytoplasmic strands, as was observed in this study are typical responses to this fusogen (Kao and Michayluk 1974; Constabel and Kao 1974). These changes indicate that protoplasts undergo rapid dehydration as a result of the PEG treatment (Wallin *et al.* 1974). In agreement with previous studies (Kao and Michayluk 1974; Constabel and Kao 1974), fusion of protoplasts occurred during dilution of PEG and not all agglutinated protoplasts underwent fusion (Figs. 29-31). Recent studies with myoblasts have shown that membrane fluidity varies over cell surfaces (Herman and Fernandez 1978). If similar properties were demonstrated for protoplasts it could be hypothesized that protoplasts would fuse only where surfaces with high membrane fluidity came in contact. This could also account for the large numbers which aggregate but never fuse.

To assess the efficiency of PEG-induced fusion under a variety of conditions the number of green colonies produced per fusion experiment was used as an assay. Since this assay was carried out approximately 3-4 weeks after fusion, it is therefore a function of the frequency

of heterofusion and the capacity of heterokaryocytes to form hybrid cell colonies. Uncontrollable variation in the frequency of hybrid colony formation occurred between experiments, presumably because a number of unknown subtle factors affect the fusion process. Similar variation has been found in other studies (Ward et al. 1979). For any given experiment, however, it was possible to generally assess the effectiveness of individual factors in modulating fusion and subsequent survival.

Temperature was not critical in PEG-induced fusion over a 18–35°C range except at 18°C in the presence of DMSO (Table 14). A similar observation was made in inter-kingdom fusions over a 26–37°C range (Ward et al. 1979). On the other hand PEG-induced fusion of Rauwolfia serpentina protoplasts (Senda et al. 1980), calcium-induced fusion of phospholipid vesicles (Breisblatt and Ohki 1976) and fusion of leaf protoplasts of N. tabacum induced by calcium buffered at high pH (Keller and Melchers 1973) were all reported to be temperature dependant. It is possible in the present study that higher temperatures resulted in higher frequencies of heterokaryons but that less of these survived to form cell colonies.

In agreement with Kao and Michayluk (1974) it was found that calcium ions were essential for good fusion frequencies (Table 16). The calcium requirement was fulfilled by 1.05 mM CaCl_2 and a 10-fold increase did not enhance fusion concurring with Ward et al. (1979). Calcium may contribute to fusion of protoplasts by altering the fluidity of membrane phospholipids (Boss and Mott 1979). The role of calcium-induced phase changes is complex and the precise mechanism of calcium-induced fusion is not well understood (Papahadjopoulos et al. 1977).

Phosphate ions were not important for fusion in the present study (Table 16). For chick erythrocyte fusion, however, phosphate ions in combination with calcium were found to be essential; calcium-phosphate induced an aggregation of membrane-associated proteins exposing protein-free sites in the membranes (Majumdar and Baker 1980). One hypothesis for the mechanism of membrane fusion is that fusion is initiated at such protein-free sites (Volsky and Loyter 1978; Knutson 1979). Observations on the fusion of plant protoplasts are in keeping with this hypothesis; localized areas of contact between apposing plasmamembranes (Fowke et al. 1977; Burgess et al. 1977; Burgess and Fleming 1974) have been observed and enhanced fusion frequencies occurred when surface proteins were removed (Constabel and Kao 1974; Ward et al. 1979).

The enhancement of PEG-induced fusion by DMSO, observed in this study (Table 15) concurs with previous studies (Haydu et al. 1977; Norwood et al. 1976) but unlike these studies, 2-5% DMSO rather than 10-15% DMSO was optimal. Different responses obtained here may be due to the species used since it is known that plasmamembranes of different species have differing phospholipid compositions (Yamada et al. 1979) which in turn affects the fluidity of protoplast membranes and their capacity for fusion (Senda et al. 1980). These studies provide a rationale for observations that fusability and tolerance to fusing agents are species specific (Kao 1978). One way in which PEG and DMSO contribute to processes of fusion is by decreasing the surface potential of the plasmamembrane (Maggio et al. 1976; Lucy 1978), presumably allowing close membrane apposition which is a prerequisite for fusion (Poste and Allison 1979). PEG and DMSO

also alter the biophysical properties of membranes with DMSO inducing phase changes in membrane lipids (Papahadjopoulos 1978) and PEG increasing protoplast membrane fluidity as a result of deplasmolysis (Borochoy and Borochoy 1979) which occurs when PEG is eluted. Phase changes induced by DMSO occur at a higher temperature in synthetic membranes (Lyman et al. 1976) and may explain the depressed fusion index observed with DMSO at 18°C.

Hybrid colonies were readily induced to undergo shoot morphogenesis. Shoot induction in somatic hybrid colonies of N. rustica + N. tabacum has also been reported previously by Nagao (1978). With NT medium 47% of tested hybrid colonies formed shoots while with MS only 13% responded (Table 17). Since hybrid cells were cultured on NT medium from the protoplast stage the poorer response on MS medium may have been due to a shock imposed on the cells upon transfer; alternatively, the composition of NT medium particularly its lower sucrose content, may have made it superior to MS. MS and NT media with equal sucrose concentrations were also used to 'feed' small hybrid cell colonies prior to transfer to solid NT medium for shoot induction. When used to 'feed' colonies NT medium was superior to MS for subsequent morphogenesis (Table 19). Surprisingly, the hormonal regime of the 'feeding' medium did not appear to affect subsequent shoot formation in hybrid calli. On NT shoot-induction medium, the optimal morphogenic response was obtained with supplements of 1.0 mg/L BA and 0.05-0.2 mg/L NAA and 1% sucrose in the absence of glucose. Media with high osmotic potentials had a deleterious effect on shoot morphogenesis. Dilution of the initial iso-osmotic level of glucose was essential for shoot morphogenesis since continued

culture on 0.175 or 0.35 M glucose was inhibitory (Table 18). Studies by Brown *et al.* (1979) have shown that 3% sucrose was optimal for shoot formation in tobacco callus and that part of the sucrose was acting as an osmoticum; potentials generated by other osmotica with values greater than the equivalent of 3% sucrose inhibited shoot organogenesis.

Somatic hybrid cell-colonies had a high capacity for shoot morphogenesis (68%, Table 19). This enhanced response appears to be in part at least a direct consequence of the hybrid condition since the parent suspension cultures from which protoplasts were obtained for fusion experiments were already quite old (*N. rustica*, 18 months; *N. tabacum*, 4 months) and had low morphogenic capacity as discussed above (Table 10). Maliga *et al.* (1977) and Gleba and Hoffmann (1979) have also reported that hybrid cells had an enhanced capacity for differentiation. The basis for this developmental 'heterosis' is unclear but it certainly increases the possibilities for the production of hybrid plants by somatic hybridization. It has been suggested by Smith and Mastrangelo-Hough (1979) that selection of parental *Nicotiana* species from appropriate sections of the genus known to exhibit heterosis upon sexual crossing may facilitate the selection of hybrid colonies after somatic fusions. It might be possible to select hybrid colonies solely on the basis of morphogenic potency without a requirement for other markers.

The frequency of root induction in hybrid shoots could not be consistently increased above 23%, which was obtained on MS medium with 2% sucrose under standard culture conditions. A wide variety

of treatments (Table 20) such as red light, temperature shocks or etiolation were unsuccessful although in other species they were stimulatory (Letouze and Beauchesne 1969; Negrutiu and Jacobs 1978a; Delargy and Wright 1978). Supplementing the culture medium with boron (Table 27) did not enhance rooting although it was stimulatory for bean (Middleton et al. 1978b). Nicotine and 3-amino pyridine were successfully used to induce rooting in N. rustica (Peters et al. 1974) and four other Nicotiana species (Evans 1979) respectively, but were not stimulatory for hybrids in the present study (Table 28). Auxins are usually employed alone (Murashige 1974) or followed by gibberellic acid (Gaspar et al. 1977) to induce rooting in stem cuttings and shoots propagated in vitro. With shoots of N. rustica + N. tabacum a positive stimulatory influence on rooting by the auxins IAA, IBA, NAA, or PAA or sequential regimes of IBA with gibberellic acid as recommended by Gaspar et al. (1977) could not be demonstrated (Tables 21, 23, 26 and 29). Furthermore, supplements of ABA and other inhibitors such as cycocel were ineffective although significantly stimulatory for other species (Basu et al. 1970; Chin et al. 1969; Sadhu 1979). Also, employing procedures to minimize the presence of potential inhibitory compounds such as ethanol (Middleton et al. 1978a) and ethylene (Coleman et al. 1980) in cultures did not effect increased rooting (Tables 25 and 26).

The inability to form roots may be linked to the developmental state of the shoots since most of the incompetent shoots had a more juvenile morphology and lacked apical dominance while competent shoots developed well-defined leaves and stems and were apically

dominant. While proliferation of secondary shoots could be lessened by lowering the sucrose concentration of the culture medium this did not ensure resumption of complete apical dominance. Shoots with abnormal morphology which were also unresponsive to normal root-inducing procedures have been reported for protoplast-derived shoots of N. otophora x N. tabacum (Banks and Evans 1976).

Analysis of a typical rooting experiment showed that some lines of hybrid shoots formed roots irrespective of the treatments employed while others remained unresponsive (Table 30). This suggests that capacity for rooting may be genetically determined in these somatic hybrids. Evidence from isozyme, morphological and cytological analysis of hybrid plants support the hypothesis that in different hybrids different sets of genes were expressed thereby permitting a range of developmental responses. This hypothesis is supported by other studies in which a differential response to root induction between lines was demonstrated for somatic hybrids of N. tabacum + N. knightiana (Maliga et al. 1978), N. glauca + N. langsdorffii (Smith et al. 1976), Solanum tuberosum + Lycopersicon esculentum (Melchers et al. 1978). Furthermore, shoots unresponsive to root induction which were derived from protoplasts of N. tabacum x N. otophora displayed abnormal morphologies lacked one particular chromosome (Banks and Evans 1976). Loss of certain chromosomes in somatic hybrids of this and other studies may be responsible for the inability to form adventitious roots.

C. Studies on Sexual Hybridization

In agreement with previous studies (Kostoff 1930; East 1935) attempts to produce N. tabacum x N. rustica hybrids failed. Kostoff (1930) reported that pollen tubes of N. rustica germinated and grew in the styles of N. tabacum but they never reached the ovary. Sexual hybrids of N. rustica x N. tabacum, however, were readily obtained when immature seeds were cultured 3 days after pollination. Complementation for normal chlorophyll synthesis occurred in all sexual hybrids when chlorophyll-deficient mutants of one or both parental species were hybridized. Complementation for normal chlorophyll synthesis in intraspecific hybrids with the "V" mutant of N. tabacum has been previously reported (Melchers and Labib 1974). Using chlorophyll-deficient mutants in sexual hybridization followed by embryo culture offers the advantage that products of self- and cross-fertilization can be readily distinguished at an early stage.

Cytological events characterizing the incompatibility reactions which partially isolate N. rustica from N. tabacum have been studied by Brink and Cooper (1941). Their observations included: retarded growth of endosperm, abnormal nucellar proliferation and lack of differentiation in integumentary cells. These authors hypothesized that all these abnormal developmental processes, in particular hyperplasia of the nucellus, effectively interrupted the flow of nutrients from the integuments to the embryo via the endosperm thereby inducing embryo abortion. This hypothesis is supported by the present experiments which showed that immature hybrid seeds could develop to maturity when cultured in vitro and furthermore,

yield of hybrid seedlings was enhanced when seeds were cultured directly on the medium rather than attached to placental tissue (Table 38).

While many mature seeds were obtained after five weeks of culture a proportion of these did not germinate. These seeds may have gone into a naturally quiescent state imposed by the presence of endosperm as was shown in *Ilex* (Hu et al. 1979) and *Cercis* (Profumo et al. 1979). When embryos were excised and cultured with 2,4-D and kinetin, however, 60% produced callus and shoot meristems indicating that many seeds were viable.

The greatest yield of hybrid seedlings obtained per placenta in these studies (Table 37), was estimated to represent only a quarter of the potential obtainable. Where self-fertilized ovules of *N. tabacum* were cultured in vitro similar reductions in potential yield were reported (Dulieu 1966; Zenkteler and Melchers 1978). Since embryo development for this hybrid is not synchronized among the population of a given ovary (Brink and Cooper 1941) those that survived probably represent the fraction which could best respond to culturing in vitro.

Of the four basic culture media tested for supporting growth and development of *N. rustica* x *N. tabacum* hybrid embryos Lörz's medium gave the greatest yield of seedlings but over half of these were abnormal in morphology (Table 35); this medium has been previously used to support continued growth of protoplast-derived embryos of *N. tabacum* (Lörz et al. 1977). For the other media tested, B₅ and MS media are commonly used to culture plant cells and Kao's highly enriched medium has been used for culturing

somatic hybrid cells of soybean + N. glauca (Kao 1977). All four media are characterized by considerable differences in the content of macronutrients and other components (see Appendix, Tables 1, 2, 4, and 5) and only further experimentation will identify the most important nutrient(s) for hybrid embryo culture.

The concentration of sucrose in the culture medium affected the yield and frequency of normal seedlings obtained. The greatest yield and lowest frequency of abnormal seedlings was obtained with 4% sucrose (Table 36). Where immature seeds of N. rustica (Rao 1965) and N. tabacum (Dulieu 1966) were cultured sucrose at 4% was also reported optimal. The relatively low yields of N. repanda x N. tabacum, N. stocktonii x N. tabacum and N. nesophila x N. tabacum sexual hybrids obtained by Reed and Collins (1978) may be due to an inadequate level (2%) of supplied sucrose. High sucrose concentrations (8%) increased the frequency of seedlings with abnormal morphology (Table 36); this was also reported for N. tabacum (Dulieu 1966). This detrimental effect of high sucrose concentrations was probably due to its effect in increasing the osmotic potential of the medium (Narayanaswami and Norstog 1964). While sucrose was optimal at 4% in the present study the seedling yield could probably be improved if sucrose was supplied at gradually decreasing concentrations over time. For a number of species, embryos have been shown to require high sucrose concentrations during the initial phases of growth and low concentrations during the latter phases (Paris et al. 1953; Kuo and Wang 1965; Pretova 1974; Nakajima and Wakizuka 1977). A gradually decreasing concentration of supplied sucrose and salts

can be achieved by combining two media of different composition, so that by diffusion through the agar matrix a gradient in concentration is gradually established over time (Monnier 1978).

Both yield and development of hybrid seedlings were affected by the time elapsed between pollination and in vitro culture (Table 37). Immature seeds cultured earlier than 3 days after pollination usually failed to develop and germinate. Self-fertilization of N. rustica ovules occurs 1 day after pollination (Cooper and Brink 1940; Sehgal and Gifford 1979) and the appearance of the occasional hybrid seedling from 2-day cultures (Table 37) suggests fertilization was complete in the hybrids by two days after pollination. Most 2-day-old zygotes perished in this study probably because sucrose supplied at 4% was too low for these young embryos; studies with Petunia hybrida ovules cultured 2 days after pollination showed a requirement for at least 7% sucrose whereas ovules cultured 4 days after pollination developed satisfactorily with 4% sucrose (Nakajima and Wakizuka 1977). Immature seeds cultured 3 days after pollination gave the greatest frequency of seedlings with normal morphology but total yield was only half that obtained by delaying culture for 4-7 days after pollination (Table 37). Supplying nutrients to embryos 3 days after pollination may have coincided with the onset of critical processes in embryogenesis and ensured normal development in that fraction of embryos surviving the culture regime employed. High yields of abnormal seedlings obtained from immature seeds cultured 4-7 days after pollination may be a consequence of greater survival of embryos in such seeds because they are larger than those in 3-day-old seeds (Brink and Cooper 1941). Since incompatibility

reactions are also more advanced with increasing time after pollination (Brink and Cooper 1941), embryos may have incurred more damage in vivo, and this may have adversely affected their subsequent development in vitro. The yield of hybrid seedlings sharply dropped when culturing was delayed for 10 days after pollination (Table 37). By this time the incompatibility reactions may have intensified such that supplied nutrients may have failed to reach most embryos due to hyperplasia of the nucellus or collapse of the endosperm (Brink and Cooper 1941). Extensive histological studies on the development of embryos in vitro are required for a better understanding of the results obtained in this study.

D. Comparative Morphological, Cytological, and Biochemical Analysis of Somatic and Sexual Hybrid Plants

Leaf and flower morphology of both somatic and sexual hybrid plants of N. rustica with N. tabacum was generally intermediate with respect to the parental species (Figs. 58, 60, 61, 74, 80, and 81). Intermediate morphological traits were previously reported for somatic (Nagao 1978) and sexual hybrids (Christoff 1928; Chobanova 1977) of these species and is characteristic of many interspecific Nicotiana hybrids produced by somatic (Smith et al. 1976; Maliga et al. 1977, 1978; Nagao 1978, 1979; Evans et al. 1979) and sexual (Kostoff 1943; Goodspeed 1954) hybridization.

Morphological variation in leaf and floral traits amongst individual somatic hybrid plants was extensive (Figs. 57, 58, and 60). In a number of instances this variation extended to sublines within the hybrid lines (Figs. 63 and 64). This morphological

variation between and within lines is most likely related to variation in chromosome number (Table 32). Considerable variation between Nicotiana somatic hybrid plants was also observed and associated with variable numbers of chromosomes in other studies (Smith et al. 1976; Maliga et al. 1978) while their corresponding F_1 or amphiploid sexual hybrids were relatively uniform. Although Nagao (1978) reported variable chromosome numbers in N. rustica + N. tabacum hybrids he did not report extensive morphological variation. Three factors may account for this apparent discrepancy. Nagao (1978) produced fewer somatic hybrid plants, used leaf-derived protoplasts and used different varieties of parental species than the present study. Uniformity in morphology coupled with chromosomal instability was previously reported in backcross hybrids of (N. rustica x N. tabacum) x N. rustica (Murty and Swaminathan 1957).

N. rustica x N. tabacum sexual hybrids were uniform for leaf and flower characteristics (Fig. 78) and this uniformity was associated with constant chromosome numbers (Results, E 2). Where F_1 sexual hybrids of N. rustica x N. tabacum have been produced previously (East 1928; Christoff 1928; Kostoff 1943; Roman 1968; Chobanova 1977) extensive variable morphology was not reported, although two segregates for flower morphology were observed in allotetraploid N. rustica x N. tabacum (Kostoff 1943). Sexual Nicotiana hybrids were also uniform in other studies in which they were compared to their corresponding somatic hybrids (Smith et al. 1976; Maliga et al. 1978).

Most somatic and sexual hybrids were intra-plant stable for chromosome number. Two out of 18 sexual hybrids, however, were

chimeric for chromosome number though phenotypically they appeared normal. One somatic hybrid plant which was phenotypically chimeric bearing two kinds of leaves and flowers (Fig. 59) may have had variable chromosome numbers among different cells of the plant body. Intra-plant variability in chromosome number has been previously observed in N. tabacum + N. knightiana (Maliga et al. 1978) and N. suaveolens x N. glutinosa (Gupta and Gupta 1973) hybrids. Chromosomal instability in many other Nicotiana hybrids has been studied and in a few cases causal factors elucidated (Smith 1968).

A number of factors peculiar to somatic hybridization may have contributed to chromosomal instability in somatic hybrids in the present study. Chromosome elimination may have occurred as a consequence of combining two alien cytoplasms after the fusion of protoplasts. Chromosome elimination is a common occurrence especially in intergeneric hybrid cells (Kao 1977; Binding and Nehls 1978). A study with cultured cells of N. tabacum, N. sylvestris, and reciprocal hybrids showed chromosome stability was relatively high in N. tabacum and low in N. sylvestris cultures; hybrids with N. tabacum cytoplasm were stable for chromosome number whereas those with N. sylvestris cytoplasm were unstable (Nizeki et al. 1978). The variability in chromosome number in somatic hybrid plants may have its origin in the parental protoplasts which were obtained from cell cultures; cells grown in suspension are frequently characterized by chromosomal instability (D'Amato 1978).

Surprisingly, somatic hybrid plants with a hyperploid chromosome complement, i.e. a number greater than the sum of the parental species ($2n = 96$) were not obtained. This observation agrees with a study by Nagao (1978) on similar hybrids. Hyperaneuploids are a common outcome of somatic hybridization experiments (Smith *et al.* 1976; Schieder 1977b; Maliga *et al.* 1978; Melchers and Sacristan 1977) presumably due to fusions involving more than two protoplasts or fusion of polyploid protoplasts followed by chromosome elimination. In the present study hyperploid cells probably comprised a proportion of fusion products but these may have been non-viable or were selected against during protoplast culture and/or subsequent shoot induction procedures. Hyperaneuploid sexual hybrids of *N. rustica* x *N. tabacum* ($2n = 116$) have, however, been identified (Kostoff 1943, pp. 758). The recovery of mostly hyperaneuploids in somatic hybrids of *N. langsdorffii* + *N. glauca* (Smith *et al.* 1976; Chapeau *et al.* 1978) and aneuploids in the present study also supports the hypothesis of Smith and Mastrangelo-Hough (1979) that cells with specific chromosomal compositions may be more successful than other genotypes in yielding viable and totipotent cultures.

The heterosis effects observed in *N. rustica* with *N. tabacum* hybrids of this study have been previously reported in allodiploid (Chobanova 1977) and allotetraploid (Kostoff 1943) sexual hybrids and in somatic hybrids (Nagao 1978) of these species. Contrary to the findings of Nagao (1978) however, the present study revealed that hybrid vigor for plant height was restricted to one-third of the somatic hybrid lines and all lines produced more leaves than either parent in the course of flowering. The vigor observed

in sexual and somatic hybrids is due in part to the interaction of the species genomes. Hybrid vigor in somatic hybrids can not be attributed to polyploidy per se since autotetraploid N. rustica and N. tabacum plants are small and stunted in comparison to respective diploids (Smith 1939). For sexual hybrids, hybrid vigor can not be attributed to an initial advantage in embryo size since hybrid embryos have been shown to be smaller than those of either parent (Kostoff 1938).

In agreement with previous studies sexual hybrids of N. rustica x N. tabacum were completely self-sterile (Kostoff 1943, pp. 758; Chobanova 1977). Somatic hybrids and allotetraploid sexual hybrids would be expected to show some self-fertility since pairing of homologous chromosomes in meiosis would be likely. Although Kostoff (1943) reported the production of allotetraploid N. rustica x N. tabacum plants, self-fertility in these was not referred to. The somatic hybrids were largely infertile but six progeny from two selfed-lines have been obtained. In some lines seed development was initiated but did not continue successfully. Self-sterility of somatic hybrids may therefore be due to post-zygotic incompatibility of the type encountered in the production of F_1 sexual hybrids. The low seed set of somatic hybrids produced in this study is in contrast to that of Nagao (1978) in which high pollen fertility and seed set was reported. This difference from observations of the present study (see Result Section C.1) may have been due to the genotypes and/or growth conditions employed. The influence of genotype and environment on pairing efficiency and fertility of other interspecific Nicotiana hybrids has been demonstrated (Williams 1975; Williams and Pandey 1975).

Backcross progeny were obtained when both somatic and sexual hybrids were used as the female in crosses with each parental species except from sexual hybrids crossed with N. rustica. Failure to obtain backcross progeny in this case may be due to the low number of trials carried out. Other studies have shown that backcrosses to either parent can be achieved and that successive backcrosses to either parent gave some partially-fertile segregants with varying chromosome numbers and a variety of phenotypes (Eghis 1933; Kostoff 1943). Successive backcrossing of sexual-hybrids has been successful in transferring genes from N. rustica to N. tabacum (Legg and Mann 1961) and in the transfer of the mammoth gene of N. tabacum to N. rustica (Smith 1950).

The presence of isozymes in somatic and sexual hybrids which were clearly derived from parental species provided convincing evidence that products of somatic and sexual hybridization were true hybrids (Figs. 67-69 and 82-84). Furthermore, lightly staining aspartate aminotransferase bands with identical mobilities in the parental species often appeared heavily stained in both somatic and sexual hybrids suggesting the co-expression of genes of parental origin (Figs 68 and 83). The success of isozyme analysis for evidence of sexual (Smith et al. 1970) and somatic (Carlson et al. 1972; Power et al. 1975; Evans et al. 1980) hybridization has demonstrated the usefulness of this technique.

While isozymes of esterase, aspartate aminotransferase and superoxide dismutase showed the presence of electrophoretic bands of parental origin in both types of hybrid, in no case was the pattern simply the summation of those in the parental species.

In the case of esterase, certain parental bands were absent in both somatic and sexual hybrids. In addition, isozymes of esterase and superoxide dismutase which were clearly absent in parental species appeared in somatic hybrids. The absence of certain parental bands (Smith et al. 1970; Wetter and Kao 1976) and the presence of new bands (Szilagy and Nagy 1978) in hybrids have been previously reported. The appearance of new isozymes in hybrids may represent new molecular species produced as a result of altered post-transcriptional modification or may represent the derepression of genes present in the ancestral genomes of either N. rustica or N. tabacum. The latter phenomena has been demonstrated for esterase isozymes in tobacco hybrids by Szilagy and Nagy (1978). Similarly, the loss of N. tabacum bands in both somatic and sexual hybrids. (Figs. 69 and 83) may be due to gene repression in the hybrids. Alternatively, loss of isozymes in somatic hybrids may be due to the loss of specific chromosome(s) with the coinciding loss of genes for specific isozymes.

With each enzyme studied, the complement of bands among each of ten somatic hybrids analyzed usually differed while all ten sexual hybrid lines showed an identical complement of bands. This difference was presumably a consequence of variable chromosome numbers among somatic hybrid plants and constant numbers in sexual hybrids.

For the enzyme aspartate aminotransferase, a hybrid band appeared in 9 out of 10 somatic hybrids but not in any of 10 sexual hybrids analyzed. This isozyme probably represents a heteropolymer between subunits coded by N. tabacum (Rf 0.29) and

N. rustica (Rf 0.26) since it had an intermediate mobility (Rf 0.28) in hybrids. Such hybrid bands have been previously demonstrated in sexual hybrids of maize (Scandalios et al. 1975) and in somatic hybrids of N. tabacum and N. glauca (Evans et al. 1980). For alcohol dehydrogenase and catalase, unique hybrid bands have been shown to exhibit improved physiochemical properties over the less efficient parental forms (Scandalios 1977). The consistent appearance of this hybrid band and failure to demonstrate its presence in mixtures of parental proteins which were preincubated prior to electrophoresis indicates that the hybrid molecule is synthesized and assembled in a concerted fashion in hybrid cells only. Regulation of genes governing synthesis of various aspartate aminotransferase isozymes is poorly understood. The significance of hybrid bands in 9 out of 10 somatic but not in sexual hybrids is not understood at present. Although different varieties of parental species were used for somatic and sexual hybridization, genotypic effects can probably be discounted since one of the ten somatic hybrids did not show a hybrid band. It is possible that such unique bands were due to the presence of a greater number of chromosomes in somatic hybrids and that in the one somatic hybrid lacking this unique band a certain chromosome(s) conferring the ability to synthesize such enzymes was lost.

Apart from providing evidence for nuclear hybridity superoxide dismutase was also interesting from the point of view of nuclear/cytoplasmic gene interaction. Cytosol isozymes have Cu or Zn as prosthetic groups and are inhibited by cyanide while isozymes in mitochondria have Mn or Fe and are not CN-sensitive. This observation

applies to a diverse range of organisms (Fridovich 1974). If it applies to Nicotiana species the CN-insensitive triplet bands of the Rf 0.30 zone, and the Rf 0.53 band contributed by N. rustica and N. tabacum, respectively, in somatic hybrids suggest the presence of mitochondrial-type proteins from both species in hybrids (Fig. 69). Since, however, studies on this enzyme in yeast (Weisiger and Fridovich 1973) and man (Creagan et al. 1973; Shows 1977) demonstrate that the mitochondrial enzyme is coded by nuclear genes the presence of both proteins cannot be taken as evidence that mitochondria derived from both parents are present in the hybrids of this study. While isozymes of the sexual hybrid were analyzed in the presence of cyanide, results were inconclusive with respect to bands of interest to this question. In other studies involving DNA hybridization, it has been shown that recombination of mitochondrial genomes can occur in hybrid cytoplasms of Nicotiana species (Belliard et al. 1979).

The expression of cytoplasmic genes was studied by analysis of RUBPCase after isoelectric focussing (IF). The IF pattern of RUBPCase provided sufficiently distinctive phenotypic markers of both the chloroplast and nuclear genomes of N. rustica and N. tabacum to allow the products of protoplast fusion and sexual crosses between these species to be analyzed (Fig. 70). The relative positions of the polypeptides from the large and small subunits of both species were consistent with studies on these species carried out by Wildman et al. (1975). The pattern of small subunit bands demonstrated the presence of nuclear markers from both parental species and reconfirms evidence for hybridity obtained by isozymes

(Fig. 70). Previous studies on sexual (Kawashima and Wildman 1972) and somatic (Chen et al. 1977; Melchers et al. 1978; Evans et al. 1980; Iwai et al. 1980) hybrids have shown that similar analysis of this enzyme may be used to demonstrate nuclear hybridity. By contrast with these previous studies, hybrids expressing a differential staining intensity of certain nuclear bands as reported for somatic hybrids by Melchers et al. (1978) or the complete loss of a band characteristic of N. rustica as reported by Iwai et al. (1980) were not observed in the present study. Since N. rustica + N. tabacum somatic hybrids show a great variation in chromosome number (Table 35) absence of some nuclear gene products is not unexpected.

The pattern of large subunit bands in somatic hybrids showed the expression of either the chloroplast genome of N. rustica or N. tabacum. No evidence for the simultaneous expression of both types of chloroplast genes was obtained. This indicates that the mixture of two plastid types originally present after protoplast fusion subsequently undergoes segregation leaving only one type in differentiated tissues. Lack of expression of chloroplast-encoded markers has been shown to parallel the loss of chloroplast DNA (Belliard et al. 1979; Belliard and Pelletier 1978; Aviv et al. 1980). Plastid sorting is not unexpected and has been previously shown in sexual crosses in which biparental inheritance of plastids is possible (Sager 1975; Tilney-Bassett 1975) and in previous reports on intraspecific and interspecific somatic hybrids (Chen et al. 1977; Melchers et al. 1978; Belliard and Pelletier 1978; Aviv et al. 1980; Iwai et al. 1980).

The molecular mechanism(s) involved in plastid segregation are not well understood. This process seems to be an active one since it has been shown by Chen et al. (1977) that it is not due to random losses occurring with successive cell divisions. It appears that the role of the nucleus is secondary to the interaction between populations of plastids since this and other studies (Chen et al. 1977; Melchers et al. 1978; Belliard and Pelletier 1978; Aviv et al. 1980; Iwai et al. 1980) have shown that different plants with a common nucleus may play host to different organelle genomes. Studies of the molecular mechanism by which one type of chloroplast predominates in Chlamydomonas have shown that the DNA of chloroplasts destined for elimination is modified and digested (Sager 1975). In somatic hybrids, the total number of chloroplasts per hybrid cell would be expected to increase relative to that found in parent cells since chloroplast number (Butterfass 1973) and chloroplast DNA content per cell (Whiteway and Lee 1977) has been shown to increase upon autopolyploidization. In somatic hybrids it is possible that those chloroplasts which can replicate most efficiently will predominate in hybrid cells.

In the present study 11 plants expressed the chloroplast genes of N. rustica while 3 expressed those of N. tabacum. Other studies have shown an approximately 50/50 ratio of plants expressing alternate chloroplast genes (Chen et al. 1977; Iwai et al. 1980). Unlike these previous studies, in the present experiments neither N. rustica nor N. tabacum protoplasts carried differentiated plastids at the time of fusion and nuclear complementation was required to activate the chloroplast genomes. Therefore once

nuclear complementation occurred in somatic fusion products it is reasonable to suggest that there was an equal opportunity for the plastids of either species to increase in number and undergo differentiation. As previously noted, the early appearance and intensity of chlorophyll development in the hybrid cell colonies subsequent to protoplast fusion was characteristic of wild-type N. rustica rather than N. tabacum suggesting an early phenotypic "dominance" of N. rustica chloroplasts over those of N. tabacum. As a result, a preponderance of cell colonies likely to be already expressing and containing greater numbers of N. rustica chloroplast genomes may have been selected for plant regeneration. This does not rule out the possibility that both plastid types were differentiated at the selection stage and the greening due to N. tabacum chloroplasts was masked by those of N. rustica with sorting out taking place during subsequent differentiation. It is also possible that chlorophyll development in N. tabacum plastids may have been enhanced due to the presence of N. rustica nuclear genes and so account for the appearance of three somatic hybrids expressing the chloroplast genome of N. tabacum. Alternatively, these three plants may have arisen from the fusion of two protoplasts of N. tabacum with one of N. rustica thereby establishing an initial preponderance of N. tabacum plastids. If this was the case, however, it would appear that massive chromosome elimination must have taken place since, in agreement with Nagao (1978), no somatic hybrids with a chromosome complement greater than the sum of the parental species were found (Table 32). Isoelectric focussing analysis of RUBPCase in conjunction with chromosome counts of the

hybrid cell colonies shortly after fusion and during differentiation might resolve some of these problems.

The pattern of large subunit IF bands in two sexual hybrids demonstrated the expression of the chloroplast genome of the maternal parent (N. rustica) used in sexual crosses. This is not unexpected since maternal inheritance in many interspecific Nicotiana hybrids has been proven unequivocally (Wildman et al. 1975).

While attempts were made to produce reciprocal sexual hybrids using N. tabacum as female, these failed (as discussed above). As in other studies (East and Hayes 1912; Kostoff 1943; Chobanova 1977) sexual hybridization of these species was achieved only by using N. rustica as the female parent. The use of N. tabacum as female parent in hybridization with N. rustica would be desirable in programs to improve N. tabacum since the problem of N. rustica cytoplasm would be avoided. Somatic hybridization has, however, facilitated the production of unique hybrids expressing N. tabacum chloroplast genes. Such hybrids with N. tabacum cytoplasmic genes may be valuable in breeding programs aimed at the introgression of N. rustica genes into plants which have a predominantly N. tabacum background.

The content of alkaloids in both somatic and sexual hybrids was ascertained. In N. rustica and N. tabacum the predominant alkaloid is nicotine (Smith 1968). Nicotine is synthesized mostly in roots, consequently the concentration in leaves is a function of rate and amount of synthesis in roots, followed by the rate of translocation and finally, by the storage capacity of the leaves (Smith 1968). In agreement with previous studies (Legg and Mann

1961), N. rustica was shown to have a higher percent total alkaloid than N. tabacum. Apart from one somatic hybrid line, all plants derived from somatic and sexual hybridizations yielded higher percent total alkaloid than respective parental genotypes (Tables 33 and 40). Studies with N. tabacum have shown that inheritance of percent total alkaloid is primarily controlled by two genes; the additive effects of polygenes, however, were also important (Legg et al. 1969). Enhanced percent total alkaloid in both somatic and sexual hybrids may be a consequence of the presence of N. rustica genes plus a general heterosis effect due to the interaction of polygenes. A greater variation in percent total alkaloid was found among somatic hybrids (0.18-1.95% total alkaloid) than sexual hybrids (0.93-2.52% total alkaloid). The reason for this is not clear but may be due to variability in chromosome number between somatic hybrid lines or the expression of different alleles amongst plants. Evidence for the expression of different alleles was obtained from an analysis of isozymes. In progeny of somatic hybrids backcrossed to N. tabacum, percent total alkaloid was higher than in N. tabacum but lower than the somatic hybrid parent value. In the third backcross generation of N. rustica x N. tabacum with N. tabacum, Legg and Mann (1961) observed that no lines contained less, most were equivalent and a few were greater for percent total alkaloid than the parental N. tabacum value. Somatic and sexual hybrids produced in the present study are currently being evaluated in breeding programs to improve commercial tobacco.

CONCLUSIONS

Suspension cultures of chlorophyll-deficient N. rustica and N. tabacum were a good source of cells from which large numbers of viable protoplasts could be routinely isolated. These protoplasts had high plating efficiencies in culture and were morphogenically competent for shoot formation.

The morphogenic capacity of protoplast-derived cells was lower than cells which did not go through the protoplasting step. Cells of newly-initiated cultures had a greater morphogenic capacity than cells from established cultures.

The chlorophyll-deficient phenotype of each species in vivo was expressed in vitro as complete absence of chlorophyll in cultured cells; this characteristic was a prerequisite for identifying parental cells from hybrid cells subsequent to fusing N. rustica protoplasts with those of N. tabacum.

Protoplasts of these mutants were readily fused using polyethylene glycol (PEG). The efficiency of PEG-induced fusion, was dependent on the presence of calcium but was independent of temperature. The production of somatic hybrid cells was enhanced when PEG was used in conjunction with DMSO.

Capacity for chlorophyll synthesis was restored in somatic hybrid cells as a result of genetic complementation. Chlorophyll-synthesizing cells appeared only in products of heterofusions. Green colonies appeared in large numbers within 17 days of heterofusion and the "early greening" trait was attributed to the mutant

N. rustica genome which was corrected by the presence of genes from N. tabacum. This "early greening" trait of N. rustica makes it a valuable mutant for somatic hybridization with other species. The ability to consistently produce hybrid cell colonies which were detectable shortly after fusion allowed their frequency of appearance to be used as an assay to optimize the fusion procedure.

Somatic hybrid cells grew faster than those of the parental species and this hybrid vigor persisted in mature plants. Shoot morphogenesis was readily induced in somatic hybrid cells and these cells showed an enhanced capacity for shoot differentiation with respect to the parental species. Hybrid shoots showed little response to root-inducing compounds such as IBA or to other treatments. Only shoots with particular genotypes could be induced to form roots.

Sexual incompatibility in N. rustica x N. tabacum crosses could be overcome by culturing immature seeds in vitro but it was not possible to obtain reciprocal hybrids.

Genetic variability in somatic hybrids as ascertained from morphological, cytological and biochemical analyses was greater than in sexual hybrids. The hybrid nature of plants produced by somatic and sexual hybridization was evident from morphological characteristics. Plants produced by somatic hybridization, however, were highly variable for a number of morphological traits whereas those of the sexual hybrids were uniform.

The chromosome number of somatic hybrids varied between different lines and also among members of the same line. Sexual hybrids showed relatively constant numbers of chromosomes.

Isozyme analysis was useful for verifying hybridity and providing information concerning the expression of parental genes in somatic and sexual hybrids. The variability in pattern of electrophoretic bands among somatic hybrids and relative uniformity among sexual hybrids may be a consequence of variable chromosome numbers in the former and constant numbers in the latter hybrids.

Analysis of RUBPCase permitted the evaluation of the expression of nuclear and cytoplasmic genes in somatic and sexual hybrids. Evidence for nuclear hybridity in somatic and sexual hybrids was obtained by the presence of markers from both parental species. Chloroplast sorting occurred in somatic hybrids as demonstrated by the expression of chloroplast encoded markers from only one of the parental species. The sorting mechanism, was not undirectional, however, as some plants expressed the chloroplast-encoded genes of N. rustica while others expressed those of N. tabacum. The presence of somatic hybrids expressing cytoplasmic genes of N. tabacum demonstrated that the pre-zygotic incompatibility mechanism which leads to failure in N. tabacum x N. rustica sexual crosses could be overcome by somatic hybridization. Maternal inheritance of N. rustica chloroplast encoded genes was verified in N. rustica x N. tabacum hybrids.

The alkaloid content of both somatic and sexual hybrids was greater than the parental species and the range found amongst somatic hybrids was greater than amongst sexual hybrids.

Somatic hybrids exhibited a greater degree of self-fertility than sexual hybrids, however, both types of hybrid were fertile in backcrosses to parental species. Somatic and sexual hybrids

produced by in vitro procedures have potential to serve as bridges for transferring genetic information from N. rustica to N. tabacum.

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APPENDIX I

TABLE 1. Nutrient composition of B₅ medium

Component		Concentration
Vitamins	Nicotinic acid	1.0 mg/L
	Thiamine . HCl	10.0 mg/L
	Pyridoxine . HCl	100.0 mg/L
	Myoinositol	10.0 mg/L
Micronutrients	MnSO ₄ ·H ₂ O	10.0 mg/L
	H ₃ BO ₃	3.0 mg/L
	ZnSO ₄ ·7H ₂ O	2.0 mg/L
	Na ₂ MoO ₄ ·2H ₂ O	0.25 mg/L
	CuSO ₄ ·5H ₂ O	0.025 mg/L
	CoCl ₂ ·6H ₂ O	0.025 mg/L
	KI	0.83 mg/L
Macronutrients	NaH ₂ PO ₄ ·H ₂ O	150 mg/L
	KNO ₃	2500 mg/L
	(NH ₄) ₂ SO ₄	134 mg/L
	MgSO ₄ ·7H ₂ O	250 mg/L
	CaCl ₂ ·2H ₂ O	750 mg/L
	Iron (Sequestrene - Fe330 Geigy)	40 mg/L
Sucrose		20000 mg/L

TABLE 2. Nutrient composition of MS medium

Component	Concentration
Vitamins	As in Table 1
Micronutrients	
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3 mg/L
H_3BO_3	6.2 mg/L
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6 mg/L
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25 mg/L
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025 mg/L
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025 mg/L
KI	0.83 mg/L
Macronutrients	
NH_4NO_3	1650.0 mg/L
KNO_3	1900.0 mg/L
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440.0 mg/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.0 mg/L
KH_2PO_4	170.0 mg/L
Iron (Sequestrene-Fe 330-Geigy)	40.0 mg/L
Sucrose	20000.0 mg/L

TABLE 3. Nutrient composition of NT medium

Component		Concentration
Vitamins	Meso-inositol	100.0 mg/L
	Thiamine - HCl	1.0 mg/L
Micronutrients		As in Table 2
Macronutrients	NH_4NO_3	825.0 mg/L
	KNO_3	950.0 mg/L
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	220.0 mg/L
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1233.0 mg/L
	KH_2PO_4	680.0 mg/L
Sucrose		10000.0 mg/L
Hormones	1-Naphthalene acetic acid	3.0 mg/L
	6-Benzylaminopurine	1.0 mg/L
* Osmoticum	Mannitol or glucose	0.35 M

TABLE 4. Nutrient composition of Kao's medium

Component		Concentration
Vitamins (mg/L)	Myo-inositol	100.0
	Nicotinamide	1.0
	Pyridoxine . HCl	1.0
	Thiamine . HCl	10.0
	D-Calcium pantothenate	0.5
	Folic acid	0.2
	p-aminobenzoic acid	0.01
	Biotin	0.005
	Choline chloride	0.5
	Riboflavin	0.1
	Ascorbic acid	1.0
	Vitamin A	0.005
	Vitamin D ₃	0.005
	Vitamin B ₁₂	0.01
Micronutrients		As in Table 1
Macronutrients (mg/L)	NH ₄ NO ₃	600.0
	KNO ₃	1900.0
	CaCl ₂ . 2H ₂ O	600.0
	MgSO ₄ . 7H ₂ O	300.0
	KH ₂ PO ₄	170.0
	KCl	300.0
	Iron (Sequestrene-Fe 330 -Geigy)	28.0

continued...

TABLE 4 continued

Component		Concentration
Sugars (mg/L)	Sucrose	125.0
	Fructose	125.0
	Ribose	125.0
	Xylose	125.0
	Mannose	125.0
	Rhamnose	125.0
	Cellobiose	125.0
	Sorbitol	125.0
	Mannitol	125.0

Organic acids (mg/L) (Adjusted to pH 5.5 with NH_4OH)

Sodium pyruvate	5.0
Citric acid	10.0
Malic acid	10.0
Fumaric acid	10.0

Other supplements: Vitamin-free casamino acid	125.0 mg/L
Coconut water (from mature fruits: heated to 60°C for 30 min and filtered)	10.0 mL

TABLE 5 Nutrient composition of Lörz medium

Component		Concentration
Vitamins (mg/L)	Myo-inositol	100.0
	Pyridoxine . HCl	0.1
	Nicotinic acid	0.5
	Thiamine . HCl	0.1
Micronutrients (mg/L)	MnSO ₄ . H ₂ O	4.7
	H ₃ BO ₃	1.5
	ZnSO ₄ . 7H ₂ O	2.7
	KI	0.75
Macronutrients (mg/L)	NaH ₂ PO ₄ . H ₂ O	218.0
	NaNO ₃	202.0
	(NH ₄) ₂ SO ₄	790.0
	MgSO ₄ . 7H ₂ O	1738.0
	CaNO ₃ . 4H ₂ O	288.0
	KCl	910.0
	Iron (Sequestrene-Fe-330-Geigy)	28.0
	Glycine	3.0

TABLE 6. Method for preparing growth-regulator stocks

Growth regulator	Solubilizing agent and amount
2,4-Dichlorophenoxyacetic acid ¹	
Naphthaleneacetic acid ²	
7-Azaindole ¹	
Indolebutyric acid ¹	
p-Chlorophenoxyacetic acid ¹	Absolute alcohol ~ 3.0 mL
Indoleacetic acid ¹	
Phenylacetic acid ³	
Kinetin ¹	
Benzyladenine ¹	Hot HCl minimal amount
8-Azaguanine ¹	
Gibberellic acid ¹	
Absciscic acid ¹	Absolute ethanol ~ 3.0 mL
Nicotine ²	H ₂ O
3-Aminopyridine ¹	H ₂ O
"Cycocel" ⁴	Used as commercial product 11.8% a.i.

¹ "Sigma" Chemical Company, St. Louis, MO, U.S.A.

² Baker Chemical Company, Phillipsburg, NJ, U.S.A.

³ Aldrich Corp., Santa Cruz, U.S.A.

⁴ American Cyanamid Company, Princeton, NJ, U.S.A.

All stock solutions were prepared so that 10 mL of stock solution = 1.0 mg/L growth regulator.

TABLE 7. Preparation method and composition of medium used for fusion of protoplasts

	Amount/100 mL	
Glucose	1.98 g (0.1 M)	Solution A
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.15 g (0.0105 M)	
$\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.009 g (0.0007 M)	

To 50 mL of solution A 25 g of PEG 1540 (Baker Chemicals) was added and pH adjusted to 5.5 prior to autoclaving.

TABLE 8. Preparation method and composition of medium used for elution of PEG

Solution A (100 mL)CaCl₂ · 2 H₂O 0.7351 g (0.05 M)

Mannitol 3.6434 g (0.2 M)

NaOH 0.2 g (0.05 M)

Solution B (100 mL)

CaCl₂ · 2H₂O 0.7351 g (0.05 M)

Mannitol 3.6434 g (0.2 M)

Glycine 0.3752 g (0.05 M)

Solutions A and B were made up separately. The solution for eluting PEG was made by mixing equal volumes of A and B; to obtain pH of 10.5 a slight excess of solution A is required.

TABLE 9. Composition of Gray's buffer

0.05 M Tris

0.10 M NaCl

0.001 M KCN

0.01 M Sodium metabisulfite

0.1% Beef serum albumin

pH adjusted to 7.8 with HCl

TABLE 10. Legend for Figure 45

Location in Figure 45	* Growth inhibitor(s) tested on shoots in Figure 45 (from left to right)				
	Top row	CYC	CYC + ABA	CYC + AZA	CYC + 8-AZAG
Second row		CYC + ABA + 8-AZAG + AZA	CYC + ABA + AZA	CYC + ABA + 8-AZAG	CYC + ABA + 8-AZAG
Third row		ABA + AZA + 8-AZAG	ABA	ABA + AZA + 8-AZAG	ABA + 8-AZAG
Bottom row		AZA	AZA + 8-AZAG	8-AZAG	**

* CYC = Cycocel (1.6 g/L)
 ABA = Abscissic acid (2.5 mg/L)
 AZA = 7-Azaindole (2.5 mg/L)
 8-AZAG = 8-Azaguanine (5.0 mg/L)

** = Inhibitor-free MS medium

Staining solution and procedures for detecting aspartate amino- transferase isozymes on polyacrylamide gels

Solution "A"

Fast violet 68 mg dissolved in 9.0 mL sodium phosphate buffer
(0.2 M) pH 7.5 (prepared immediately prior to use)

Solution "B"

	Concentration of stock solutions	Amount of stock solutions in solution "B"
Sodium phosphate buffer pH 7.5	0.2 M	24.6 mL
Pyridoxal phosphate	5 mg/10 mL	0.9 mL
Bovine serum albumin	3 g/100 mL	1.8 mL
L-aspartic acid pH 7.5 (adjusted with KOH)	0.2 M	7.6 mL
α -ketoglutarate pH 7.5 (adjusted with KOH)	0.1 M	2.2 mL
Polyvinylpyrrolidone	—	1.116 g

Procedure

Mix solution "A" and solution "B" immediately prior to
staining gels.

END

1 6 1 2 8 1

FIN