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PLANT REGENERATION FROM
STEM CORTEX PROTOPLASTS

OF

BRASSICA JUNCEA

by

② ANNE SPROULE, B.Sc.

A thesis submitted to the Faculty
of Graduate Studies and Research
in partial fulfillment of the
requirements for the degree of
Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

April, 1987.

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ABSTRACT

Stem cortex protoplasts of Brassica juncea cv Zem and Domo were isolated by digesting epidermal strips in 1% Macerozyme R-10 and 0.5% Cellulase R.S.. Numerous culturing media were tested, with the optimal being a modified S₁ (Schenck and Robbelen) containing 0.12mM MgSO₄ and 6mM CaCl₂. Agarose (0.4%) was also beneficial for cultured protoplast viability.

A requirement for NAA (naphthalene acetic acid) was noted for callus survival on induction medium. Modified S₁ medium was as good as, or superior to, MS induction medium for callus survival and shoot organogenesis. Numerous experiments altering the growth regulators in the induction medium failed to increase the shoot induction frequency above the 1% frequency found with 3mg/l BA (6-benzyl-aminopurine) and 1mg/l NAA.

Regenerated plants from protoplast derived calli flowered, and the majority set seed, but only 20% of the plants examined cytologically exhibited a normal chromosome number of 36 (n=18).

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TABLE OF CONTENTS

	PAGE
ABSTRACT	.iii
ACKNOWLEDGEMENTS	.iv
TABLE OF CONTENTS	.v
LIST OF TABLES	.ix
LIST OF FIGURES	.xi
LIST OF APPENDIX TABLES	.xiii
LIST OF ABBREVIATIONS	.xiv
INTRODUCTION	1
LITERATURE REVIEW	4
A. Development of protoplast technology	
1. Introduction	4
2. Sources	5
3. Tissue pretreatment and protoplast	
isolation procedures	6
4. Protoplast-culture	8
5. Plant regeneration	.11
6. Plant genetic engineering through the use	
of protoplasts	.13
B. <u>Brassica</u> protoplast technology	
1. Sources of protoplasts	.16
2. Protoplast isolation	.20
3. Culture	.22

4. Regeneration	.25
5. The use of <u>Brassica</u> protoplasts in genetic engineering	.27
MATERIALS AND METHODS.	.31
General preparations	.31
Experimental protocol	.32
RESULTS	.40
1. Genotypic differences in protoplast yield and viability	.40
2. Comparison of stems and axillary branches as protoplast sources	.40
3. Protoplast-derived plants as protoplast sources	.43
4. Effect of pretreatment and enzyme treatments on protoplast yield and viability	.43
5. Assessment of leaf mesophyll tissue as a protoplast source	.44
6. Effect of tissue density on protoplast yield . .	.44
7. Effect of sucrose wash on protoplast yield and viability	.47
8. Effect of culture conditions on protoplast survival	.51

9. Effect of basal medium on viability and plating efficiency	.52
10. Effect of altering S_1 media constituents on protoplast viability and plating efficiency	
A. Ammonium levels	.59
B. Calcium and magnesium levels	.59
11. Assessment of embedding in agarose beads	.66
Plant regeneration	
12. A broad survey of growth regulators	.68
13. Effect of alternative or additional growth regulators	.68
14. Comparison of B5 and MS as regeneration media. . .	.79
15. Comparison of gelling agents in regeneration media	.79
16. Effect of sucrose concentration on callus survival	.79
17. Effect of altering KNO_3 and NH_4NO_3 ratios in MS media on callus survival and shoot induction	.81
18. Effect of temperature shock on shoot stimulation	.85
19. Effect of half strength MS media on callus survival and organogenesis	.85
20. Effect of removal of liquid medium from protoplast cultures on subsequent regeneration	.88
21. Effect of modified S_1 as a regeneration medium	.88
22. Production of plants from regenerated shoots . . .	.90

23. Cytological studies of the regenerated plants . . .	92
DISCUSSION	96
CONCLUSIONS	107
REFERENCES	109
APPENDIX	122

LIST OF TABLES

TABLE	PAGE
A. Examples of enzyme mixtures employed in the isolation of <u>Brassica</u> protoplasts	21
B. Examples of <u>Brassica</u> protoplast culture	26
1. Effect of genotype on protoplast viability in <u>B.juncea</u>	41
2. Comparison of stems and axillary branches of <u>B.juncea</u> Zem as protoplast sources . .	42
3. Effect of strip density on yield of <u>B.juncea</u> protoplasts	48
4. Effect of agarose underlayers on viability of <u>B.juncea</u> cv Domo protoplast cultures . . .	53
5. Effect of added Ficoll or agarose to culture media cell division in <u>B.juncea</u> cv Domo protoplast cultures .	54
6. Effect of agarose sublayer volume on cell division of <u>B.juncea</u> cv Domo protoplast cultures	55
7. Effect of altered ammonium levels on plating efficiency of <u>B.juncea</u> cv Domo protoplast cultures	60
8. Effect of altered magnesium and calcium levels in S ₁ on protoplast viability in <u>B.juncea</u> . . .	61
9. Effect of altered magnesium and calcium levels in S ₁ on protoplast viability in <u>B.juncea</u> . . .	62
10. Effect of embedding protoplasts in agarose beads on viability and plating efficiency in <u>B.juncea</u> cv Domo protoplast cultures . . .	67
11. Effect of BA and NAA concentrations on organogenesis in protoplast-derived calli of <u>B.juncea</u> cv Zem	71

12.	Effect of GA_3 and NAA on callus survival in protoplast-derived calli of <u>B.juncea</u> cv Domo	73
13.	Effect of kinetin, kinetin riboside, and GA_3 on callus survival and shoot regeneration in protoplast-derived calli of <u>B.juncea</u> cv Domo	74
14.	Effect of thidiazuron on survival of protoplast-derived calli from <u>B.juncea</u> cv Domo	76
15.	Effect of sucrose concentrations on callus survival of protoplast-derived calli of <u>B.juncea</u> cv Domo	80
16.	Effect of altered $KNO_3 : NH_4NO_3$ ratios on callus survival and shoot induction in protoplast derived-calli of <u>B.juncea</u> cv Domo	82
17.	Effect of high temperature ($35^\circ C$) treatment on survival of protoplast-derived calli of <u>B.juncea</u> cv Domo	86
18.	Effect of 1/2 strength MS media on callus survival and organogenesis in protoplast-derived calli of <u>B.juncea</u> cv Domo	87
19.	Effect of modified S_1 on shoot regeneration in protoplast-derived calli of <u>B.juncea</u> cv Domo	89
20.	Effect of modified S_1 , tested at pH 5.0 and 5.8, on callus survival and shoot induction in protoplast-derived calli of <u>B.juncea</u> cv Domo	91
21.	Morphological and cytological observations of <u>B.juncea</u> plants derived from protoplast-derived calli	93

LIST OF FIGURES

FIGURE

PAGE

- A Cytogenetic relationship of the Brassicaceae. . . 1
- 1 Cross section of Brassica juncea
main stem showing approximate depth of cut . . .46
- 2 Freshly isolated protoplasts of
Brassica juncea from stem cortical layers. .46
- 3 Freshly isolated mesophyll protoplasts of
Brassica juncea from in vitro grown
leaves46
- 4 Overloaded enzyme solution, 27 strips
(approximately 0.55g) in 10 ml solution . . .50
- 5 Optimally loaded enzyme solution, 18 strips
(approximately 0.35g) in 10 ml solution . . .50
- 6 First cell division in B.juncea cv
Zem stem cortex protoplast culture grown in
S₁ medium58
- 7 First cell division in B.juncea cv
Zem stem cortex protoplast culture grown in
Pell B medium58
- 8 Colony formation from B.juncea cv Domo
protoplasts after approximately one
month in S₁58
- 9 Protoplast-derived B.juncea cv Domo
cells after culture in X medium for 9 days . .65
- 10 Protoplast-derived B.juncea cv Domo
cells after culture in Y medium for 9 days . .65
- 11 Protoplast-derived B.juncea cv Domo
cells after culture in Z medium for 9 days . .65
- 12 Protoplast-derived B.juncea cv Domo
cells after culture in S₁ medium for 9
days65

plant regeneration are general prerequisite methodologies for employing biotechnology in crop improvement.

Protoplasts have the potential for numerous genetic manipulations, for example, fusion with other species, incorporation of cytoplasts or organelles, injection with desirable genes, and retrieval of useful mutants.

The objective of the present study was to develop a dependable, repeatable protocol for isolating B. juncea protoplasts and regenerating plants from them. The stem cortex protoplast system developed by Klimaszewska and Keller (1987) for B. napus was primarily evaluated in this study. Modifications in protoplast isolation, culture and plant regeneration protocols were evaluated in order to develop an efficient system. The availability of such technology could lend itself to novel approaches for B. juncea improvement, either as an oilseed or a condiment crop.

LIST OF APPENDIX TABLES

TABLE	PAGE
1- MS Medium	122
2- S ₁ , Pell B, Lichter's Media	123
3- Kao's Medium	124
4- Chatterjee's BG Culture Medium	126
5- Pelletier C Medium	127
6- B5 Medium	128

LIST OF ABBREVIATIONS

ABA	abscisic acid
BA	6-benzyl-aminopurine
FDA	fluorescein diacetate
GA ₃	gibberellic acid
NAA	1-naphthalene acetic acid
2,4-D	2,4-dichlorophenoxy acetic acid
PEG	polyethylene glycol
No Damp tm	2.5% oxine benzoate (fungicide)
Jiffy 7 tm	peat pellet (approx. 6cm high X 4cm diameter) used as a planting medium for small plants or seeds

INTRODUCTION

Brassica juncea is an important world crop. In some countries, such as India, it is grown primarily as an oilseed (Nagamani et al. 1981). In Canada, it is commonly known as brown or oriental mustard and its production is for condiment purposes. B. juncea, along with B. hirta (yellow mustard), seed production exceeds 80,000 tons annually, much of which is exported. The Canadian B. juncea acreage represents 90% of the world's production (Collins, 1986). Although drought resistant and suitable for growth in the drier areas of Canada, it is not cultivated as an oil crop because of its high levels of potentially toxic glucosinolates in the meal, making it unfit for animal consumption.

Canola is the generic name given to Canadian grown Brassica oilcrop species with less than 5% erucic acid in its oil, and less than 30 μ M glucosinolate/g in the meal. The first Canola, B. Napus var. Tower, was licensed in 1974. Today, with double low cultivars in both B. napus and B. campestris, Canola comprises 100% of the rapeseed oil crop in Canada (Kondra, 1985). With oil levels of approximately 38% of dry weight, compared to approximately 41% in B. napus (Downey, 1985), B. juncea has potential as a

third Canola species. Not only does B. juncea have acceptable oil quality and high seed yield, but it is drought tolerant, and resistant to pod shatter and blackleg (Leptosphaeria maculans) (Tewari, 1985).

B. juncea also matures earlier than the Canola Westar, and has a higher seed yield (Downey, 1985).

B. juncea would greatly increase the 4 million ha. on which Canola can be grown in Canada (Kondra, 1985). However, this development has not occurred because of the high glucosinolate levels which limit the cultivation of B. juncea in North America to a condiment crop. Efforts to produce low glucosinolate and low erucic acid have been undertaken. Nagamani et al. (1981) treated seeds with gamma rays, but obtained plants with higher erucic acid content. Kirk and Oram (1981) isolated two lines of erucic acid free B. juncea by screening populations from parents already low in erucic acid. However, the development of stable low glucosinolate lines has not yet been reported.

It is quite possible that conventional breeding methods will lead to the development of Canola type B. juncea. However, the possibility of achieving such an objective may be increased if other strategies, i.e. biotechnology, are also employed. Protoplast culture and

plant regeneration are general prerequisite methodologies for employing biotechnology in crop improvement.

Protoplasts have the potential for numerous genetic manipulations, for example, fusion with other species, incorporation of cytoplasts or organelles, injection with desirable genes, and retrieval of useful mutants.

The objective of the present study was to develop a dependable, repeatable protocol for isolating B. juncea protoplasts and regenerating plants from them. The stem cortex protoplast system developed by Klimaszewska and Keller (1987) for B. napus was primarily evaluated in this study. Modifications in protoplast isolation, culture and plant regeneration protocols were evaluated in order to develop an efficient system. The availability of such technology could lend itself to novel approaches for B. juncea improvement, either as an oilseed or a condiment crop.

LITERATURE REVIEW

A. Development of protoplast technology

1. Introduction

A plant protoplast can be defined as the membrane bound entity found within the cell walls of plant tissue. The isolation of protoplasts was reported as early as 1892, when Klercker mechanically isolated small numbers of protoplasts by slicing open the cell walls in thin slices of plant tissue (see Butenko, 1979). In 1960, Cocking produced large yields of protoplasts by enzymatically digesting the cell walls of root tissue. This opened the door to a new field of plant study. Protoplasts were initially used for ultrastructural and physiological studies, such as the investigation of membrane transport, and the isolation of active organelles. (Butenko, 1979). Although wall regeneration around protoplasts had been observed by Townsend in 1887 (see Burgess, 1983), the division of cells regenerated from protoplasts was not observed until 1970, when it was reported by Kao et al. In 1971, Takebe et al reported the regeneration of tobacco plants from protoplast cultures. This demonstration of the totipotency of protoplasts generated great interest,

as their potential for practical applications was greatly enhanced.

2.Sources

Within ten years of the first report of plant regeneration, plants from over 60 species had been regenerated from protoplasts (Binding, 1982). Protoplasts have been isolated from many plant tissues, with leaf mesophyll being the most common (Davey, 1983). Leaves from such diverse sources as milkweed (Singh, 1984), wheat (Chin and Scott, 1979), poplar (Russell and McCown, 1986), alfalfa (Kao and Michayluk, 1980) and tomato (Tal and Watts, 1979) have been used to isolate protoplasts. Other tissue sources for protoplasts include roots (Xu et al. 1981), cotyledons, hypocotyls (Glimelius and Ottosson, 1983), flower petals (Flick and Evans, 1983), pollen tetrads (Bhojwani and Cocking, 1972), and stem cortical tissue (Klimaszewska and Keller, 1987). Cell suspensions, dividing plant cells maintained in liquid media, are another source for protoplasts (Gleddie et al. 1986).

Although leaves are a convenient source for large yields of protoplasts, the ability of the culture to undergo sustained division is highly dependant on growth

conditions of the donor material (Binding, 1982).

Controlled growth chambers are considered a suitable environment for donor plant growth, with greenhouse conditions a less productive alternative (Davey, 1983).

Sterilization procedures, necessary to eliminate competitive bacteria and fungi, can adversely affect protoplast isolation, and so another alternative to greenhouse grown leaves is axial shoot cultures maintained on sterile media (Russell and McCown, 1986). Once established, these cultures can be maintained indefinitely through clonal transfer of shoot meristems, thus providing a well controlled source of experimental tissue (Schenck and Robbelen, 1982). In vitro culture also lends itself to control of the donor environment, shown to affect the yield and viability of the protoplasts (Davey, 1983).

3. Tissue pretreatment and protoplast isolation procedures

Donor tissue is often pretreated in a plasmolysing medium to aid in the consistent release of protoplasts and improve viability. The plasmolysed protoplast has sealed plasmodesmata which help the cell survive by limiting loss of cytoplasm and intake of the digestion solution (Davey, 1983). Plasmolysing media can be complex, as the modified Kao's medium used by Van Slogteren et al: (1980), or a simple sugar solution (Rose, 1980). Calcium,

in the form of CaCl_2 , is often added to help maintain membrane integrity (Grun and Chu, 1978; Rose, 1980). Growth regulators have also been added; Shahin (1985) reported that the addition of 2,4-D and BA to the pretreatment medium was essential for sustained division in tomato protoplasts. Maintaining excised tissue in low light or darkness before isolation can also aid in a more consistent release of protoplasts (Dijak et al. 1986).

The aseptically grown or sterilized tissue is subjected to a combination of enzymes in order to break down the walls and release the protoplasts. The enzymes act on the structural components of the cell wall, but do not digest the cell membrane. Various hemicellulases, cellulases and pectinases have been employed (Davey, 1983). Earlier studies often employed a two step isolation procedure, using a pectinase to release the cells, then using a cellulase to digest the walls (Nagata and Takebe, 1970). Currently, enzymes are generally used as a mixture rather than in sequential use and always in the presence of an osmoticum which protects the naked protoplasts from lysis. The enzyme mixture suitable for one species may not be suitable for another, and so enzyme regimes must be empirically determined for each protoplast source (Davey, 1983).

4. Protoplast culture

Isolated protoplasts are cultured in a suitable medium to initiate division and sustain growth.

Protoplasts have basic nutritional requirements similar to those of whole plants, with some additional components required that are normally synthesized by the plant. For example, thiamine is required for growth, as is an auxin source (Gamborg, 1984). An osmoticum is an important addition to any protoplast medium, as the protoplasts normally depend on the cell wall to protect them from swelling and bursting. The osmotic potential of the osmoticum ideally balances the water potential of the interior of the protoplast, thus protecting the membrane (Evans and Bravo, 1983). Mannitol is the most frequently used osmoticum in protoplast media (Evans and Bravo, 1983), but there have been several reports of alternative sugars or sugar alcohols. For example, Xu et al. (1981) and Maeda et al. (1983) both reported increased survival of protoplasts using glucose as an osmoticum, and Van Slogteren et al. (1980) reported sucrose to be an optimal osmoticum. Glucose and sucrose can be metabolized by the plant cells; this allows for an advantageous reduction of the osmotic potential in the medium during the growth of the cultured protoplasts since the need for

an osmoticum in the medium decreases after the protoplasts have regenerated cell walls. This regeneration occurs rapidly in viable cells, generally between one to 24 hours after culturing (Burgess 1983).

A basic protoplast culture medium consists of inorganic nutrients, carbon source(s), vitamins, growth regulators, and possibly some organic supplements. Not only is the presence of the nutrients important, but also the actual and relative concentrations (Gamborg, 1984). For example, Von Arnold and Eriksson (1977) reported that although a low calcium concentration could be partially compensated by a higher magnesium supply, they found a high (12mM) calcium concentration was superior for protoplast survival and division.

Viability, cell division, and plating efficiency are terms often used in the description of protoplast cultures. Cyclosis signifies active metabolism, and is commonly used as an indicator of protoplast viability (Larkin, 1979). Fluorescein diacetate and other fluorochromes are also employed in observing protoplast and cell viability (Bornman and Bornman, 1985). Cell division is noted when one can observe distinct wall formation across the cell, resulting in cleavage of the

cell. Plating efficiency has had several interpretations, for example, Xu et al (1981) count the number of cells which have divided after 7 days, but the generally accepted definition refers to the percentage of protoplasts which undergo multiple divisions to reach the microcalli stage (Shahin, 1985).

A high plating efficiency increases the possibility of retrieving any individual protoplast to the plant stage. Since genetic manipulations often depend on a unique event within thousands of protoplasts, considerable work has been done in an attempt to optimize the conditions for viability, division and plating efficiency. A filter paper base soaked in nutrient medium (Oelck et al. 1982), renewal of the medium (Hayashi and Nakajima, 1984), and feeding of protoplasts with slightly altered media with reduced osmotic potential after a few days in culture (Pelletier et al. 1983) have all been shown to be beneficial for some systems. Nurse cultures, which are established cell cultures grown in a layer below the freshly isolated protoplasts, are also used to improve viability (Yarrow et al, 1986). Takeuchi and Komamine (1982) reported an agar underlayer to be beneficial to protoplast culture. Numerous workers have further observed increased benefits with purified agar or agarose

(Shillito et al, 1983a&b, Maeda et al. 1983, Linsefors and Brodelius, 1985, and Adams and Townsend, 1983).

5.Plant regeneration

The objective of most protoplast studies is to achieve plant regeneration. Generally, the division of the protoplast-derived cells results in the formation of multicellular colonies which develop into macroscopic calli. Plant regeneration may be achieved through an organogenic or embryogenic pathway. The organogenic route initially involves the induction of shoot bud differentiation. The shoots can be excised and generally induced to form roots (e.g. Muhlbach, 1980). Alternatively, cells within the callus can be induced to undergo embryogenesis (Vardi et al. 1975). In some instances, even the initial cell divisions are highly organized and embryogenesis proceeds directly without an intervening callus stage (Dijak et al. 1986).

Generally, a change in growth regulator type or concentration is necessary for plant regeneration. Although auxin has been shown to stimulate cell division, cytokinin has been associated with shoot induction and so regeneration media for organogenesis tend to have a high cytokinin to auxin ratio (Gamborg, 1984). The specific

medium for plant regeneration must be developed for each species, and can involve testing numerous combinations and concentrations of different cytokinins and auxins (Kantha, 1975). For example, zeatin has been reported as superior to BA as a cytokinin source for many species (Engler and Grogan, 1982/83, Singh, 1984, and Shahin, 1985). The addition of other growth regulators, for example, gibberellic acid (GA_3), can stimulate shoot organogenesis in some plant species (Bidney *et al.* 1983). Other changes in culture conditions may also be necessary to achieve plant regeneration; Grun and Chu (1978) reported the stimulation of light was necessary for plant regeneration from Solanum.

Embryogenesis is not triggered by the same conditions that stimulate organogenesis. In alfalfa, a medium free of growth regulators is gradually added to the culture (Kao and Michayluk, 1980). Protoplast-derived calli of ~~eggplant~~ underwent embryogenesis only if they had been in contact with low levels of 2,4-D (Gleddie *et al.*, 1986). Physical factors can also play a role; low voltage electrical fields have been reported to stimulate embryogenesis in Medicago sativa (Dijak *et al.* 1986).

6. Plant genetic engineering through the use of protoplasts

Although protoplasts are useful for structural and physiological studies, their greatest potential lies in their ability to provide an isolated cell system for genetic manipulation. Protoplasts can be induced to fuse to form heterokaryons from which hybrid cells, and ultimately hybrid plants, can be obtained. Such hybrid plants are referred to as somatic hybrids. Reliable methods for achieving protoplast fusion have been developed with polyethylene glycol treatment (Chupeau et al, 1978) and electrofusion (Bates and Hasenkamp, 1985) being the most commonly used approaches. A number of selection systems for hybrid cells have also been developed, such as manual selection, selective media, and genetic complementation (Keller et al, 1982). Numerous intraspecific and interspecific hybrids have been regenerated and characterized. Most of these hybrids have involved combinations of species within the following genera: Nicotiana, Datura, Petunia, Solanum, and Brassica (Harms, 1983).

Protoplast fusion has also been utilized to achieve a limited transfer of nuclear genetic information. Somers et

al (1986) fused irradiated barley protoplasts with protoplasts of a nitrate reductase mutant of tobacco and recovered tobacco plants containing the nitrate reductase structural gene of barley.

Because cytoplasm of parental lines are combined, fusion provides a novel mechanism for transferring cell organelles. The nuclei of one population can be chemically or physically inactivated to ensure that only cytoplasmic components are transferred by the fusion. Numerous examples of cytoplasmic organelle transfer of "cybridization" are available. For example, Fluhr et al (1983) demonstrated the transfer of chloroplasts in Nicotiana species, and Medgysey et al (1985) reported transfer of chloroplast and mitochondrial traits in Nicotiana. Spheroplasts from Agrobacterium and E.coli have also been directly fused with protoplasts (Shillito and Steinbiss, 1983).

Protoplasts can also be used to advantage in mutant isolation, whether selecting for naturally occurring somaclonal variations found in culture, for example, chlorophyll deficiency, and reduced fertility (Orton, 1984), or inducing new mutations with the use of mutagens, for example, streptomycin resistance, or auxotrophy. Haploid plants can be used as the source of protoplasts

for mutagenesis, allowing the detection of recessive mutations (Bourgin, 1983). The isolated mutants are potentially superior sources for transformation experiments, if the mutation is easily selected for in vitro.

Gene transfer into plant protoplasts is another potentially useful approach for plant improvement. Several methods for introduction of genes into protoplasts have been developed. Agrobacterium, DNA-loaded liposomes, Ti plasmids, and cauliflower mosaic virus (CaMV) have been used to carry genetic material into the protoplast. (Manzara and Lurquin, 1983, Shillito and Steinbiss, 1983). Cocultivation with Agrobacterium, or infection with CaMV allows for incorporation, as does using a fusogen with liposomes, or naked DNA (Manzara and Lurquin, 1983). Microinjection can deliver genes directly into the nucleus of a protoplast (Miki et al. 1987). However, for any or all of the above genetic manipulations to come to fruition, it should be emphasized that efficient technologies for culturing protoplasts and regenerating plants from the cultures must be developed.

B. Brassica protoplast technology

1. Sources of protoplasts

The genus Brassica comprises several economically important species including the diploid species B.oleracea, B.campestris, and B.nigra and the amphiploid hybrids B.napus, B.junceae and B.carinata. The close species interrelationship was originally described by U (1935) (see Figure A). Protoplasts have been successfully isolated from all six species (Glimelius, 1984, Choung et al, 1987).

Leaves from in vitro grown young plants and shoot cultures are the most commonly reported source of Brassica protoplasts (eg., Schubert and Matzke, 1985, Li and Kohlenbach, 1982, and Newell et al., 1984). In vitro grown shoots can be maintained under precisely controlled environmental conditions; it is also possible to clonally propagate lines which have been shown to yield protoplasts which can be successfully cultured (Schenck and Robbelen, 1982). Leaf tissues are an excellent source for producing large yields of protoplasts from a single plant, but have the disadvantage of being very susceptible to growth conditions, and, in Brassica, are relatively fragile in culture. Sundberg and Glimelius (1986) used this latter

FIGURE A
Cytogenetic relationship of the Brassicaceae*

*- adapted from U, 1935

characteristic as a means of eliminating one parental line in somatic hybridization; they reported a drastic decrease of vitality in mesophyll protoplasts within 24 hours. Thin epidermal/cortical cell layers derived from stems (Klimaszewska and Keller, 1987) are another alternative protoplast source. These cortex protoplasts have the advantages of mesophyll protoplasts, large yields from one plant source and ease of preparation, without the extreme fragility.

Other tissues include cotyledons (Alexander and Dubert, 1983), hypocotyls (Glimelius and Ottosson, 1983), and roots (Xu et al., 1982) as alternative protoplast sources. These alternatives have the advantage of not requiring extensive growth facilities, and the seed can be sterilized prior to germination, thus avoiding damage to the tissue to be protoplasted. However, large numbers of seedlings are required, and the protoplasts derived from them may not be genetically uniform. Suspension cultures have also been used as a protoplast source (Klimaszewska and Keller, 1986).

The physiological condition of the donor tissue plays a major role in protoplast viability and capacity to undergo sustained division. Bidney et al. (1983), indicated that the growth of the source material under

defined conditions was vital for isolation of viable cabbage (B.oleracea) mesophyll protoplasts. Hoffman et al. (1979) tested greenhouse versus in vitro grown plants and found of four species tested - B.napus, B.oleracea, B.nigra, and B.campestris - only B.napus produced colonies from protoplasts derived from leaves grown in the greenhouse. All four species produced colonies from in vitro grown plants. By contrast, Fu et al. (1985) were able to regenerate plants from B.oleracea mesophyll protoplasts obtained from greenhouse grown leaves.

Growth chamber grown plants have the advantage of developing under environmentally controlled conditions. It is therefore possible to study environmental effects and thus optimize growth conditions. Schubert and Matzke (1985) studied proton release in phytohormone treated B.napus protoplasts and observed differences between growth chamber and field grown plants, illustrating physiological differences between plants grown under different conditions. Controlling, and thus being able to define, growth conditions helps to standardize experimental tissue as far as possible in a biological system. Growing plants have also been conditioned for better protoplast viability. For example, Schubert and

Matzke (1985) fed B.napus with nutrient solutions, and Bidney et al. (1983) fed B.oleracea weekly with MS salts.

Pretreatment of the tissues prior to culture has often been employed in experiments with Brassica. The pretreatment serves to condition the tissue towards response, or to help improve consistency in the results. For example, Klimaszewska and Keller (1987) reported that protoplasts isolated from fresh tissue reached the cell colony stage in only 66% of the experiments, compared with 100% of the experiments utilizing tissue pretreated in MS salts for 24 hours. Pretreatment of tissue in various osmotica has been beneficial in a number of cases, with pretreatment times varying from a few hours to several days. Gatenby and Cocking (1977) used a seven day pretreatment of leaf explants on a solidified medium; more commonly the tissue is treated in a liquid medium for a few hours (Spangenberg et al. 1986), to a day (Bidney et al. 1983).

2. Protoplast isolation

Isolation procedures for Brassica protoplasts have been variable, with a number of different pectinases, cellulases, and hemicellulases of various grades of purity being employed (Table A). The enzymes are normally used

TABLE A

Examples of enzyme mixtures employed in the isolation of Brassica protoplasts

Enzyme, concentration and source	Reference
1% Cellulase R-10 Onozuka	Klimaszewska and Keller 1987
1% Macerozyme R-10 Onozuka	
4% Meicelase Meiji Seika Kaisha Ltd.	
0.5% Driselase Kyowa Hakko Kogyo Co.	Gatenby & Cocking 1977
0.4% Macerozyme Kinki Yakult Manuf.	
1% Pectinase Serva	Chatterjee <u>et al.</u> 1985
1% Cellulase R-10 Onozuka	
0.02% Pectolyase Seishin Pharm. Co. Ltd.	
1% Rhozyme Rohm and Hass Ltd.	Alexander & Dubert 1983
2% Cellulase Yakult Biochemicals Ltd.	

at concentrations of less than 5%, and are usually dissolved in a carbohydrate osmoticum. Salts such as CaCl_2 have been added as membrane stabilizers, and numerous other components have been added to the enzyme solution in attempts to increase protoplast viability (eg., Gatenby and Cocking, 1977, and Pelletier et al., 1983).

Once digested, the isolated protoplasts are washed free of the enzyme solution before being cultured. Again, methodologies have been varied. Protoplasts have been pelleted (Chuong et al., 1985), suspended on density gradients of percoll (Alexander and Dubert, 1983) or sucrose and mannitol (Schubert and Matze, 1985) or floated upon a 0.5M sucrose solution (Klimaszewska and Keller, 1987). There has rarely been a detailed discussion on the various merits of the different procedures, although some report enrichment of viable protoplasts using floatation cleaning methods (Alexander and Dubert, 1985).

3. Culture

Many researchers use a modification of B_5 medium for culturing Brassica protoplasts (Sundberg and Glimelius, 1986, Pelletier et al. 1983). Although Spangenberg et al (1986) reported microculture of single protoplasts of

B.napus using modified Kao's medium, protoplasts are normally cultured at high densities ($0.5-2.5 \times 10^5 \text{ ml}^{-1}$) (Alexander and Dubert, 1983, Kohlenbach et al. 1982).

Much work has been done in an attempt to optimize the conditions for growth, division, and eventual regeneration to plants. Renewal of the medium (Pisan et al., 1983), dilution of the medium (Fu et al. 1985), and feeding of protoplasts with slightly altered media (Pelletier et al. 1983) after a few days in culture have all been shown to be beneficial for some systems. On the other hand, Chuong et al. (1985) demonstrated that using high sucrose (13%) and Ficoll (Pharmacia Fine Chemicals, Sweden) in the culture medium caused the protoplasts to float and enabled a one step culturing procedure for Brassica protoplasts without any feeding or dilution being necessary.

In 1982, Takeuchi et al. reported an agar underlayer to be beneficial to Vinca rosea protoplast culture. Shillito et al. (1983a) tested numerous agars and agarose in culture with a wide range of species, including Brassica protoplasts, and found the purified agarose (agar with the ionic agaropectin removed) to be far superior to agar. Shillito et al. (1983b) and Pisan et al. (1983) reported embedding protoplasts in agarose-solidified media to be beneficial, providing the agarose medium containing

the embedded protoplasts is cut into segments and floated in liquid medium. Shillito emphasized the importance of transferring B.rapa protoplasts into the liquid medium as soon as the agarose media has set around the protoplasts.

The production of brown pigments (assumed to be phenolics) by cells in culture is a major problem in Brassica protoplast culture. The cells release the 'phenolics' into the medium, often at high concentrations that rapidly kill the culture. Hoffman et al. (1979) found it necessary to screen for low phenolic producers by growing shoots in culture and selecting only those shoots which did not turn the agar brown. Although some have tried different tissues sources (e.g. Xu et al. (1982) used roots) to decrease production, replacing or diluting the culture media have been the most common approaches. Even with roots as a source, Xu found it essential to add fresh medium for continued growth. Pisan et al. (1983) and Spangenberg et al. (1986) replaced the medium every 3 -4 days. Kohlenbach et al. (1982) centrifuged isolated stem embryo protoplasts after 3-5 days in culture and resuspended the pellet in fresh medium, and Hoffman et al. (1979) washed the growing Brassica cultures up to two times a week. On occasion, the phenolic problem has been used to advantage; Schenck and Robblen (1982) used the

ability of B.napus to better resist the particles as a selection system in fusion experiments.

4.Regeneration

There have been numerous reports of plant regeneration in Brassica protoplast culture, through both organogenesis and embryogenesis (Table B). Embryogenesis is the rarer phenomenon, generally arising from in vitro sources. Klimaszewska and Keller (1986) recovered somatic embryos from protoplasts derived from a embryogenic suspension culture of B. nigra. Kohlenbach et al. (1982) reported embryogenesis from protoplasts of B.napus; these protoplasts were derived from a stem embryo culture system which originally arose from a single event out of 98 different protoplast isolation experiments. Embryo formation occurred at the base of the hypocotyl of the regenerating plant, and these embryos formed more embryos in a similar manner. Li and Kohlenbach (1982) utilized mesophyll protoplasts and recovered somatic embryos; but the leaves were obtained from plants which had been regenerated from the stem embryo system. There is some evidence that plants arising from tissue culture may be more amenable to further tissue culturing; Robertson and Earle (1986) reported that plants

TABLE B

Examples of Brassica protoplast culture

Species	protoplast source	regeneration observed	reference(s)
napus	root	shoot	Xu <u>et al.</u> 1982
	hypocotyl	shoot	Glimelius 84
		shoot	Chuong <u>et al.</u> 85
		-----	Spangenberg <u>et al.</u> 85,86
		-----	Alexander <u>et al.</u> 83
	cotyledon	-----	
	mesophyll	shoot	Newell <u>et al.</u> 84
	stem embryo	embryo	Kohlenbach <u>et al.</u> 82
	stem embryo's mesophyll	embryo	Li and Kohlenbach 82
	stem cortex	shoot	Klimaszewska & Keller 87
oleracea	hypocotyl	shoot	Glimelius 84
		shoot	Robertson and Earle 86
		shoot	Fu <u>et al.</u> 85
		shoot	Bidney <u>et al.</u> 83
	root	-----	Gatenby and Cocking 83
		shoots	Xu <u>et al.</u> 82
campestris (rapa)	mesophyll	-----	Shillito <u>et al.</u> 83
		-----	Paszkowski <u>et al.</u> 83,86
		-----	Pisan <u>et al.</u> 83
		-----	Kumar <u>et al.</u> 81
	hypocotyl	-----	Glimelius 84
	root	-----	Xu <u>et al.</u> 82
nigra	suspension	embryo	Klimaszewska & Keller 86
	hypocotyl	-----	Glimelius 84
alba (hirta)	root	-----	Xu <u>et al.</u> 82
carinata	hypocotyl	shoot	Chuong <u>et al.</u> 87
juncea	mesophyll	-----	Aggarwal <u>et al.</u> 83
		shoot	Chatterjee <u>et al.</u> 85
		embryo	"
	hypocotyl	-----	Glimelius 84
	cotyledon	shoot	Wenbin <u>et al.</u> 86

regenerated from hypocotyl explants were vastly superior to seed grown plants for protoplast culture.

The Murashige and Skoog (MS) medium is commonly used with a variety of growth regulators when regenerating shoots from either explants or protoplast derived calli, Benzyladenine (BA) and naphthalene acetic acid (NAA) are frequently used, although other cytokinins and auxins have also been surveyed. Klimaszewska and Keller (1987) found zeatin improved regeneration in B.napus protoplast culture. GA₃ has been reported helpful in regenerating shoots from cabbage protoplasts (Bidney et al. 1983, and Fu et al. 1985), although Xu et al. (1982) regenerated cabbage using only BA, and NAA or IAA.

5. The use of Brassica protoplasts in genetic engineering

The successful regeneration of plants from Brassica tissue culture has encouraged researchers to attempt genetic manipulation of the protoplasts in culture. As early as 1974, Kartha et al. demonstrated fusion between B.napus and Glycine max protoplasts followed by limited division of hybrid cells. There have been several reports of interspecific somatic hybridization with Brassica, with various selection schemes. Toriyama et al. (1987a) used a double mutant Sinapis turgida as a universal hybridizer.

for Brassica. The mutant was unable to grow on medium with nitrate as the sole nitrogen source, and was resistant to 5-methyltryptophan; thus the somatic hybrids could be easily selected for, being both resistant to 5-methyltryptophan and able to grow on nitrate media. Curiously, Toriyama et al., (1987b) also reported 100% hybrid regeneration from fusions between B.oleracea and Moricandia arvensis although no selection was performed, and suggested hybrid vigour as the cause. Hoffman and Adachi (1981) have shown their "Arabidobrassica" (a fusion product of Arabidopsis thaliana and B. campestris) to be asymmetric intergenic hybrids. Klimaszewska (unpublished) has fused Diplotaxis harra to B.napus, and used differences in calli morphology as a selection system.

One of the more interesting possibilities of somatic hybridization in Brassica is the resynthesis of the amphiploids. Because of their origins, with isolated crossings between the two diploid parents, the genetic diversity of B.napus, for example, is very low. Fusing the protoplasts of the parent species has been suggested as a way to resynthesize the amphiploid and increase its genetic pool (Hoffman et al. 1979). Schenck and Robbelen, (1982), Glimelius et al. (1986), and Sundberg and Glimelius, (1986) have fused protoplasts of B.oleracea and

B.campestris to resynthesize B.napus. Selection systems included visual determinations with micromanipulation to isolate the fusion products (Sundberg and Glimelius, 1986), increased vigour of the hybrids (Schenck and Robbelen, 1982), and combined fluorescence of the hybrids allowing separation using flow cytometry (Glimelius et al. 1986).

A number of studies have focused on using protoplast fusion to transfer and/or combine cytoplasmic traits. Pelletier et al. (1983) have used PEG fusion to combine useful cytoplasmic traits of B.napus, B.campestris and Raphanus sativus. Yarrow et al (1986) have also reported the production of B.napus "Cybrids", combining mitochondrial-associated male sterility with plastid associated herbicide tolerance. Spangenberg and Schweiger (1986) have employed electrofusion for organelle and karyoplast transfer in B.napus protoplasts.

Brassica protoplasts may serve as recipient cells for the transfer of foreign genetic information. The cauliflower mosaic virus (CaMV), which infects several Brassica species, has been studied for its potential as a vector. Paszkowski et al. (1983, 1986) developed a system to transform turnip protoplasts with CaMV and demonstrated that genetically engineered CaMV was integrated and

proliferated with the dividing protoplasts. Brassica protoplasts are presently being utilized as recipients for microinjection of organelles and nuclear genes in B.napus (D.Simmonds, personal communication).

MATERIALS AND METHODS

General Preparations

Stock 7% (w/v) calcium hypochlorite was prepared in demineralized water then filtered through a Whatman #1 filter. A working solution of approximately 2% was made with the addition of two parts demineralized water to one part 7% calcium hypochlorite.

Stock solutions of 100mg/l of the various growth regulators were prepared. Naphthalene acetic acid (NAA), gibberellic acid (GA_3), and 2,4-dichlorophenoxy acetic acid (2,4-D) were dissolved in absolute alcohol (10 mg auxin powder to 2-5 ml ethanol), then brought up to 100 ml with distilled water. Zeatin, kinetin, and 6-benzyl-aminopurine (BA) were dissolved in 1N HCl (10 mg cytokinin powder to 5-10 ml HCl), then brought to volume with distilled water. An additional cytokinin, thidiazuron (N-phenyl-N'+1,2,3-thiadiazol-5'yl urea, Nor-Am Chemical Company) was dissolved in hot 6N HCl before being brought to volume with water. Stock solutions were stored at 2°C until use.

Distilled water was sterilized in large volumes (5 l) by autoclaving (15psi) for 30 minutes. Glassware was

rinsed in distilled water, wrapped in foil and autoclaved for 20-30 minutes. Protoplast culture media were filter sterilized (Nalgene 115 ml, 0.2 μ m disposable filter); regeneration media and root induction media were autoclaved for 17 minutes. Agarose underlayer plates for protoplast culture were prepared by mixing double strength filtered culture media with autoclaved double strength agarose (0.8% w/v) in distilled water ; then the mixed media were pipetted into sterile 60 x 15 mm petri dishes.

Experimental Protocol

The development of an efficient protoplast culture technology necessitates altering many variables within the protocol. Therefore, the basic system will be outlined here, and variations will be discussed in the Results section.

Seeds of Brassica juncea cv Domo and Zem, obtained from Dr.R.K.Downey, Research Station, Agriculture Canada, Saskatoon, were sown in 12 cm pots containing soil:peat:sand mixture (1:2:1), thinned to one seedling per pot, and grown in a growth chamber with day:night temperature of 20:15°C, 16 hour photoperiod and light intensity of 400 μ Em⁻²sec⁻¹ derived from cool white fluorescent tubes (type F96/T12/VHO) and incandescent

(45W) bulbs producing 84% and 16% of the intensity, respectively. Relative humidity was maintained at 70%. The plants were watered daily with tap water and fed a 20:20:20 (N:P:K) liquid nutrient once weekly.

Approximately twenty days after sowing, at the onset of stem elongation, the primary inflorescence was removed to encourage bolting and axillary shoot development. Within five to seven days the axillary shoots reached a length of 15 to 20 cm and the internodal explants were collected at this stage. The first three to four terminal sections (non-pithy internodes), harvested just prior to flowering, were cut into 5 cm lengths.

Explants were placed in small jars and surface sterilized with a brief rinse (10 to 15 seconds) with 70% ethanol followed by a 20 minute immersion in 2% calcium hypochlorite and four rinses, each of five minutes duration, in sterilized distilled water. Longitudinal strips comprising the epidermis and several layers of cortical tissue were aseptically isolated within a laminar flow unit. In order to do this a Beaver 75 micro-sharp blade (Waltham, MA, USA) was used to cut through the epidermis and subepidermal tissue. The epidermal strip was then isolated by either slicing horizontally along the internode or by grasping the small

flap cut by the blade with forceps and gently pulling the strip from the internodal section. The strips were transferred to 100x15mm petri dishes (with strips from up to five internodes per dish) filled with 10 ml of pretreatment medium. The pretreatment medium was composed of the salts of Murashige and Skoog medium (1962) as modified by Douglas et al (1981) (MS; Appendix, Table 1), pH 5.8, and sterilized by autoclaving.

The strips were pretreated in darkness at 2°C for 24 hours. The pretreatment medium was removed, and the tissue density was adjusted to approximately 0.5 g per petri dish (0.5 g.f.wt. of tissue is roughly equivalent to the isolated strips from 2 to 3 of the 5cm internodal sections). An enzyme solution containing 1% (w/v) Macerozyme R-10 and 0.5% (w/v) Cellulase Onozuka R-S (both from Kinki Yakult Mfg Co Ltd, Nishinomiya, Japan) and 0.4M mannitol in distilled water, pH 5.8, adjusted with 0.1N KOH, was sterilized through a 0.2µm Nalgene™ disposable filter; 10 ml of this solution was added to each petri dish. The dishes were sealed with parafilm, and incubated without agitation for 18 hours darkness, at 25°C.

The digested tissue was filtered through 88 and 44 µm nylon mesh filters (B&SH Thompson Co, Town of Mount Royal,

Quebec) to remove large pieces of debris. The filtrate was transferred to 100X15 mm test tubes and centrifuged for 6 min at 100g ; the supernatant was discarded and the pelleted protoplasts were resuspended in 6 ml of culture media. The protoplasts were spun down and resuspended in fresh media two more times to thoroughly remove the enzyme solution.

Various culture media were tested; they were utilized under the following names: "Lichter's" (Lichter, 1982), "Pell B" (Pelletier et al., 1983), "Kao's" (Kao, 1977), "Chatterjee" (Chatterjee et al., 1985), and "S₁" (Schenck & Robbelen, 1982). S₁ and modified S₁ were predominately used (see Appendix, Tables 2-4). The protoplasts were counted under a haemocytometer prior to the final centrifugation and resuspended at densities approximating 10⁵/ ml. Generally, 60 x 15 mm plates were prepared with a 2ml underlayer of the culture media solidified with 0.4% agarose (Seaplaque, Mandel Scientific) and a 1.5 ml aliquot of the protoplast suspension was plated over the agarose medium. Variations of this culture method included plating the protoplasts in liquid media directly into the plastic petri dishes, and embedding the protoplasts in agarose solidified media. The protoplasts were embedded by mixing a protoplast

suspension in double strength medium with an equal volume of melted 0.8% agarose. The agarose-protoplast mixture was either spread over an agarose underlayer, or pipetted out in small drops, or beads, (approximately 0.1ml/bead) directly onto the petri dish bottom. The dishes were sealed with parafilm and incubated at 25°C, in darkness for the first seven to ten days, and in continuous fluorescent light (approximately $70 \mu\text{m}^{-2}\text{sec}^{-1}$) thereafter.

The protoplast cultures were observed at 1, 4 and 7 days after plating. The cultures were monitored by observing cyclosis and/or treating samples with fluorescein diacetate (FDA, Sigma) stock of 5mg/ml acetone mixed 1:100 (v/v) with culture media: the stain and culture sample were mixed 1:1 and the viability assessed under a Zeiss IM35 inverted microscope with HBO 5W high pressure mercury source exciter, exciter filter BG35 and barrier filter 48 77 10 -9904. Thereafter visual assessments were made, with plating efficiencies equal to the percentage of originally viable protoplasts surviving to visible colony stage.

Ten to fourteen days after isolation, the dividing protoplasts had grown to visible microcolonies and the cultures were fed with 0.5-1ml of Pell C (Pelletier et al, 1983) (Appendix, Table 5). When the agarose

underlayer was covered with microcolonies (in 3 to 4 weeks), the culture was subdivided onto a fresh petri dish with an agarose solidified media underlayer. The transfer was made by pipetting up any unabsorbed liquid media and loose colonies suspended within it from the original dish and spreading the suspension over fresh plates. Thus, the original dish was left relatively free of liquid media, with the new petri dish containing the transferred colonies and a small amount of liquid.

Approximately 6 to 8 weeks after isolation, microcolonies 1 to 1.5 mm in size were picked up with forceps and transferred to regeneration media. MS medium with 1.5% sucrose and solidified with 0.6% Difco Bacto agar or 0.4% agarose was used as the basal regeneration medium. Numerous combinations and concentrations of BA, NAA, GA₃, kinetin, and zeatin were assessed for their effect on shoot regeneration. Except for experiments on variation of light and temperature conditions, the colonies were maintained under continuous fluorescent light (approximately $70 \mu\text{E m}^{-2}\text{sec}^{-1}$) and 25°C. Callus survival was scored after four weeks, a surviving callus often exhibited growth, but the criteria used was the absence of brown, necrotic tissue. Essentially, a

callus was scored as surviving if it was not obviously dead over more than 50% of its surface.

Within four weeks of colony transfer, the majority of calli with morphogenic capacity had produced roots or shoots. The shoots were excised and placed on B5 media (Gamborg et al, 1968), lacking growth regulators, with 2% sucrose and solidified with 0.8% agar. The shoots were maintained at 20°C with an 8 hour photoperiod (440 ft/candles) to prevent premature flowering. The shoots rooted easily, and could be transferred to Jiffy 7TM peat pellets treated with 1ml/l No Damp (2.5% Oxine Benzoate from Plant Products Co.Ltd, Ontario) within a month. After 2-3 weeks in a mist chamber, the established plantlets were potted in the original soil mixture and transferred to the greenhouse or growth chamber.

Shoots regenerated from protoplast-derived calli were used as an in vitro mesophyll tissue source. The shoots were maintained in sterilized jars on B₅ medium, with 2% sucrose and 0.8% agar, in a growth chamber at 20°C with an 8 hour photoperiod (440 ft/candles). The shoots, or axial buds from the shoots, were transferred to fresh media every four weeks.

Cytogenetic studies were carried out on some of the regenerants. Flower buds were fixed in a Carnoy solution (six parts of absolute ethanol, three parts of chloroform, and one part glacial acetic acid, by volume) and stored at 4°C. Anthers from the fixed buds were dissected out, and individually crushed in a drop of acetocarmine. Observations were made using phase contrast illumination.

RESULTS

1. Genotypic differences in protoplast yield and viability

Preliminary experiments were carried out using the low erucic acid line Zem. Although it was possible to isolate protoplasts from internodal strips, less than 20% of the protoplast preparations survived past the first week in culture. When Domo, a licensed oriental mustard cultivar, was used as the source material, there was an increase in the number of plants which yielded viable protoplasts, and a increase (to over 60%) in the number of plants which yielded protoplasts which survived through the first week of culture (Table 1). The oriental cultivar Blaze was tested twice, and also yielded viable protoplast cultures which underwent cell division. Subsequent experiments, unless noted otherwise, utilized Domo as the donor source.

2. Comparison of stems and axillary branches as protoplast sources

Axillary branches of Zem were studied as an alternative source for protoplasts, and were found to be at least as suitable as the main stem from the same test plant (Table 2).

TABLE 1. Effect of genotype on protoplast viability
in B. juncea

Cultivar	Zem	Domó
no. plants tested*	41	71
% of plants yielding viable protoplasts	68	80
% plants yielding protoplasts which underwent cell division after 7 days	15	63

* compilation of numerous culture treatments, all in S₁

TABLE 2. Comparison of stems and axillary branches of B. juncea Zem as protoplast sources

	Total no. of plants tested*	% of plants producing viable protoplasts
main	39	54
axillary	23	65

* compilation of numerous culture treatments

3. Protoplast-derived plants as protoplast sources

Regenerated plants were established in soil and maintained in growth chamber and greenhouse. When they were used as a source for donor tissue, both the greenhouse and the growth chamber plants yielded viable protoplasts which underwent sustained division at least as frequently as seed grown plants. Notably, the clones from the one Zem regenerate yielded protoplasts which ultimately produced another regenerate.

4. Effect of pretreatment and enzyme treatments on protoplast yield and viability

Numerous pretreatments of the excised strips and variations of the enzyme solution were tested for improvement in protoplast yield and viability. Some isolated strips were digested without pretreatment, and in other tests, the MS pretreatment salt solution was supplemented with growth regulators. Neither alteration had any effect on yield or initial viability.

The normally used enzyme solution consisting of enzymes, mannitol, and distilled water was buffered with 600 mg/l MES (2-(N-Morpholino)ethane sulphonic acid) and supplied with macronutrients: 27.2mg/l KH_2PO_4 , 100mg/l KNO_3 , 148mg/l CaCl_2 , and 240mg/l MgSO_4 . The enzyme

solution was replaced with fresh enzyme solution after one hour of incubation. The strips were agitated at 15 strokes/min on a reciprocating shaker. None of the above treatments had any discernible effect on the yield or initial viability when assessed microscopically with the aid of a haemocytometer.

5. Assessment of leaf mesophyll tissue as a protoplast source

Leaves from plants grown in growth chambers failed to produce protoplasts. However, leaves from in vitro protoplast-derived regenerants yielded protoplasts, but their viability was consistently inferior to stem cortical protoplasts (Figures 2 & 3) and died within a few weeks when tested in S_1 , Pell B, or Kao's media (see Appendix, Table 2&3). One preparation of mesophyll protoplasts reached the colony stage when cultured in X medium (see section 9); however, no shoot regeneration occurred in the protoplast-derived calli.

6. Effect of tissue density on protoplast yield

Initial tests with Zem to determine optimal tissue density for digesting epidermal strips in 10 ml. of enzyme solution indicated that viable protoplasts could be isolated from levels as high as 45 strips, with each strip

FIGURE 1 Cross section of Brassica juncea
main stem showing approximate depth of cut
(arrow). Slide courtesy K.Klimaszewska.

FIGURE 2 Freshly isolated protoplasts of
Brassica juncea from stem cortical layers.
Magnification: 150X

FIGURE 3 Freshly isolated mesophyll protoplasts of
Brassica juncea from in vitro grown
leaves.
Magnification: 120X

1

2

3

averaging 4 cm x 0.3 cm and 5-7 cell layers in thickness (Figure 1) (up to 1 g/10ml). However, Domo was much more sensitive to overloading, with successful isolation occurring only if the density was kept below 0.5g/10ml. (Table 3). Routinely, Domo protoplasts were isolated at a density of approximately 12 strips (0.25g f.wt.) /10 ml enzyme solution.

When the strip density was too high, the majority of the protoplasts browned and shrivelled in the enzyme solution, and many failed to release from the donor tissue (Figures 4 & 5). The possibility of this effect resulting from enzyme toxicity, rather than a density overloading, was tested by maintaining a standard experimental plate (with approximately 0.25g f.wt. tissue in 10 mls enzyme solution), containing viable protoplasts, at room temperature before washing. Protoplasts remained viable for up to four days in the presence of enzyme, as indicated by their ability to undergo cell division and colony formation.

7. Effect of sucrose wash on protoplast yield and viability

As an alternative to washing by pelleting, the protoplasts were resuspended after the initial

TABLE 3. Effect of strip density on yield of B.juncea protoplasts.

approx. # strips 10 mls enzyme	Zem	Domo
10-20	+++	+++
25-30	+++	+
35-45	++	D

+ - relative yield of viable protoplasts
D = all protoplasts were dead

FIGURE 4 Overloaded enzyme solution, 27 strips
 (approximately 0.55g) in 10 ml solution.
 Note oval shapes indicative of
 incomplete wall digestion
 Magnification: 85X

FIGURE 5 Optimally loaded enzyme solution,
 18 strips (approximately 0.35g) in 10 ml
 solution. Note spherical protoplasts
 illustrating fully digested cell walls.
 Magnification: 85X

4

5

centrifugation in 6 ml of 0.6M sucrose and a thin layer (approximately 0.5ml) of SCM (0.4M sorbitol, 10mM calcium chloride, and 5mM MES; pH 5.8) was layered on top. Further centrifuging, at 100 xg for 10 minutes, divided the protoplasts into three zones, a pellet containing protoplasts and debris, a second zone of protoplasts dispersed throughout the sucrose solution, and a layer of protoplasts forming a band at the interface between the sucrose and the SCM layers. The top band of protoplasts was removed and further washed with protoplast culture medium before plating. Generally, less than 25% of the viable protoplasts were collected in this way; the majority were found in the pellet and dispersed throughout the sucrose solution. Subsequent observations on controlled comparisons with the same plants showed no improvement in the viability of the floated protoplasts over protoplasts washed in the regular manner (i.e. pelleted three times, twice in the protoplast media).

8. Effect of culture conditions on protoplast survival

Protoplast suspensions were generally plated into petri plates containing protoplast media solidified with 0.4% agarose. Although protoplasts could occasionally survive and divide without this underlayer, controlled tests using protoplasts from the same isolation showed the

superiority of an underlayer culturing system (Table 4). Protoplasts were also embedded in a thin layer of 0.4% agarose solidified medium above the agarose underlayer. In another test, 3% Ficoll was added to the liquid media to test its effect on protoplast survival. Both variations had little effect on viability after a week in culture (Table 5). Embedded protoplasts tended to have a higher plating efficiency in some experiments, but this did not consistently occur. An embedded culture had the disadvantage of not being easily diluted, leading to overcrowding problems when a large percentage of the protoplasts underwent cell division (Figures 13 & 14).

Several volumes of the agarose underlayer were tested to determine the optimal level to support protoplast culture, but no significant difference was noted (Table 6). Thereafter, the minimum volume tested, 2 ml, was used routinely in order to minimize agarose consumption.

9. Effect of basal medium on viability and plating efficiency

When Pell B medium (Appendix, Table #2) was tested as an alternative to S_1 , up to a four fold increase in protoplast viability was observed. After one week, protoplasts cultured in Pell B had viabilities up to 100%.

TABLE 4. Effect of agarose underlayers on viability of B.juncea cv Domo protoplast cultures

Expt	% viable protoplasts after 7 days	
	Underlayer *	Liquid layer**
1	25	1
2	19	2.5
3	1.5	0.8
4	1.6	0.8
5	6	0
<hr/>		
Avg	10	1

* 5 ml of S₁ solidified with 0.4% agarose in a 60x15 petrie dish, overlaid with 1.5ml protoplast suspension

** 3 ml of protoplast suspension plated directly into petri dish

- significant difference at P 5% (Student's t-Test)

TABLE 5. Effect of added Ficoll or agarose to culture media cell division in B. juncea cv Domo protoplast cultures

	Expt 1		Expt 2	
	% viable	% dividing	% viable	% dividing*
S ₁	10	38	5	70
+Ficoll **	10	33	4.5	100
+agarose***	14	22	4	100

* % of viable protoplasts which underwent cell division after 7 days of culture

** 3% Ficoll added to S₁

*** protoplasts in overlay embedded in S₁ medium with 0.4% agarose

TABLE 6. Effect of agarose sublayer volume on cell division of B. juncea cv Domo protoplast cultures

underlayer volume (ml)	% viable		% of viable protoplasts which underwent division	
	Expt 1	Expt 2	Expt 1	Expt 2
5	15		6	
4	14		9	
3	16	16	7	10
2	15°	17	9	10

Expt 1 - average of 6 protoplast isolations

Expt 2 - average of 11 protoplast isolations

no significant difference at $P > 30\%$ (Student's t-Test)

with an average of 90% over 4 trials compared to an average of 20% viable protoplasts in S_1 . However, the protoplasts cultured in Pell B did not divide to form tight compact colonies, but rather formed loose mats of budding single cells, which rarely developed into multicellular colonies. Protoplasts in S_1 , although exhibiting very low initial survival, divided without budding, and occasionally went on to form dense, spreading colonies which could be transferred to regeneration media (see Figures 6, 7, & 8). Protoplasts in both Lichter's (Appendix, Table 2) and Kao's medium (Appendix, Table 3) responded similarly to those in Pell B, except that Kao's medium also greatly stimulated the formation of brown particles. Chatterjee's medium (Appendix, Table 4) caused such rapid browning that only a few preliminary tests were run before it was rejected as a suitable protoplast medium.

In summary, although Lichter's and Pell B media both supported high viability in B. juncea protoplast cultures, neither stimulated the type of colony formation found in S_1 . As colony formation is more important than protoplast survival, in relation to plant regeneration, S_1 was chosen as the base medium with which to carry out further experiments.

FIGURE 6 First cell division in B.juncea cv
Zem stem cortex protoplast culture grown in
S₁ medium.
Magnification: 200X

FIGURE 7 First cell division in B.juncea cv
Zem stem cortex protoplast culture grown in
Pell B medium.
Magnification: 250X

FIGURE 8 Colony formation from B.juncea cv Domo
protoplasts after approximately one
month in S₁.
Magnification: 150X

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10. Effect of altering S₁ media constituents on protoplast viability and plating efficiency

A. Ammonium level

In an effort to increase initial viability, S₁ was modified to more closely resemble Lichter or Pell B medium. The amino acid content of Lichter's medium was added to S₁, but no improvement was observed. The ammonium levels were changed to duplicate those in Lichter's or Pell B. Little difference in early viability was observed, but ammonium free S₁ had a greatly reduced plating efficiency (Table 7).

B. Calcium and magnesium level

When the calcium and magnesium levels were altered in S₁, dramatic differences were noted. Initial experiments showed that any reduction of magnesium, independent of the calcium level, was of significant benefit to early protoplast survival, and higher levels of calcium also showed an improvement (Table 8). Further experiments also reflected this trend (Table 9), but morphological differences were also noted. 0.12mM of magnesium coupled with 6mM calcium produced extremely viable, loosely dividing colonies. 0.12mM of magnesium with 1.2mM calcium sustained high viability for the first week, but the

TABLE 7. Effect of altered ammonium levels on plating efficiency of *B. juncea* cv Domo protoplast cultures

% of protoplasts viable after 7 days				
mg/l NH_4NO_3	800 (=S ₁)	134	0 +30mg/l glutathione	0*
Expt				
1	48	44	30	35
2	13	30	43	40
3	11	21	39	28
plating efficiency (%) (avg. of 3 Expts)	1	2	1	0.02

* basal S₁ medium was utilized; another source of nitrogen was KNO_3 (2000mg/l)

no significant difference in initial viability $P > 10\%$
(Student's t-Test)

TABLE 8. Effect of altered magnesium and calcium levels in S_1 on protoplast viability in B. juncea

% viability after one week in culture *				
	S_1	A	B	C
Mg (mM)	7.6	1.22	0.5	0.8
Ca (mM)	1.36	2.04	2.1	13.6
Expt				
1	21	67	72	83
2	15	62	75	80
3	17	64	77	80

Avg	18	64	75	81

* 3 Expts with the same protoplast source

Ca = $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in S_1 , A, and C

Ca = $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ in B

Mg = $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for all media

significant differences between all formulations $P < 5\%$
(Student's t-Test)

TABLE 9. Effect of altered magnesium and calcium levels in S_1 on protoplast viability in B. juncea

Expt	<u>% viable protoplasts after 7 days</u>						
	1	2	3	4	5	6	7
Medium *							
Mg:Ca ratio/conc.							
X (1:50) 0.12mM:6mM	70	73	73	73	76	69	68
Y (1:10) 0.12mM:1.2mM	58	66	-	-	-	-	63
Z (1:1 med) 1.2mM	58	37	12	-	20	14	15
Z' (1:1 high) 6mM	-	-	42	-	-	-	-
V (50:1) 6mM:0.12mM	16	19	-	-	-	-	-
W (10:1) 1.2mM:0.12mM	24	5	-	-	-	-	-
S_1 (5.6:1) 7.6mM:1.36mM	-	-	-	-	13	16	dead
1:1 w. $CaNO_3$ 1.2mM	-	-	-	22	-	-	-

* S_1 with altered $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$

- medium not used in expt

protoplasts remained spherical, with a tendency for the chloroplasts to collect on one side. Little or no division was seen. When the magnesium level was increased to 1.2mM, with calcium at 1.2mM, the viability dropped. But the few surviving protoplasts divided to form compact, rounded microcolonies as found in dividing S_1 preparations, although the magnesium levels are still well below those in S_1 , 1.2mM $MgSO_4 \cdot 7H_2O$ as compared to 7.6mM (see Figures 9-12).

Of particular interest was the consistent survival of the protoplasts in medium X - 0.12mM $MgSO_4 \cdot 7H_2O$:6mM $CaCl_2 \cdot 2H_2O$ - compared to other media. Protoplast isolations of B. juncea normally vary greatly from one experiment to the next, most probably due to genetic variation and uncontrollable growth variation between different plants. However, short term viability in medium X was not only very high, in the 70% range, but also very consistent. Although medium X did not support the formation of compact colonies, it was still possible to obtain colonies of a size that could be transferred.. These colonies were not well defined, due in part to the spreading character of the colonies, and partly because of the large numbers regenerated. It proved impossible to accurately determine plating efficiencies in these plates,

FIGURE 9 Protoplast-derived B. juncea cv Domo
cells after culture in X medium for 9 days.
Magnification: 100X

FIGURE 10 Protoplast-derived B. juncea cv Domo
cells after culture in Y medium for 9 days.
Magnification: 100X

FIGURE 11 Protoplast-derived B. juncea cv Domo
cells after culture in Z medium for 9 days.
Magnification: 100X

FIGURE 12 Protoplast-derived B. juncea cv Domo
cells after culture in S₁ medium for 9
days.
Magnification: 100X

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even if the protoplasts were isolated in small beads. However, estimations of plating efficiencies in the X medium indicated ten times more colony production than in medium Z, when cultured in liquid medium. Colonies were rarely regenerated in the other media tested. Subsequent experiments always included X as one of the media evaluated.

11. Assessment of embedding in agarose beads

In some experiments, protoplasts were suspended in warm media (approx. 35°C) containing 0.4% agarose, plated out in small (0.1ml) drops and allowed to solidify. With this system, the addition of liquid medium was necessary, but the timing was critical. If the liquid medium was added immediately after the agarose solidified, or even 24 hours later, the protoplasts died. If 1-1.5ml of Pell C medium (Appendix, Table 5) was added 7-10 days after plating (shortly after the first signs of sustained growth), agarose droplets proved superior to protoplasts in liquid on an agarose underlayer for colony survival (Table 10).

TABLE 10. Effect of embedding protoplasts in agarose beads on viability and plating efficiency in B. juncea cv. Domo protoplast cultures

Medium *	%viable after 5 days	plating efficiency (%)	
		liquid**	bead***
W	28	0	0
X	67	0.4	1-2
Y	68	0	0.3
Z	39	0	0.8
S ₁	28	0	0.005

* media as in TABLE 9

** control - 1.5 ml protoplast suspension over 2 ml agarose underlayer, average of 2 plates

*** average of 4 plates

PLANT REGENERATION

12. A broad survey of growth regulators

If the developing colonies were left in the original plate, they never grew more than 2mm in diameter and they eventually died (within 3 months after isolation).

Therefore, developing colonies were transferred when they reached 1 mm or more in diameter. Initially, MS medium was used as the induction medium and tested with numerous

combinations of the growth regulators BA and NAA. Only two combinations (2.2mg/l BA:0.10mg/l NAA, and 2.2BA:0.09NAA) showed any shoot regeneration, and then at a frequency of approximately 1 per 100 calli. As the cytokinin BA decreased, the tendency for root organogenesis increased. Low levels of both growth regulators resulted in decreased survival (Table 11).

Since an accidental preparation of 2.5 mg/l BA and 1.35 mg/l NAA produced several shoots, 3mg/l BA and 1mg/l NAA were eventually chosen as a standard growth regulator regime for further experiments in which other variables were assessed.

13. Effect of alternative or additional growth regulators

A requirement for NAA in the induction media was noted when testing the addition of gibberellic acid (GA_3),

FIGURE 13 Colony formation from protoplasts of B. juncea cv Domo cultured in an S₁ liquid overlayer above an agarose solidified media underlayer. (after approximately 6 wks)
Magnification: 1X

FIGURE 14 Colony formation from B. juncea cv Domo protoplasts embedded in S₁ agarose solidified media. (after approx. 6 wks)
Magnification: 1X

FIGURE 15 B. juncea cv Domo protoplast-derived callus exhibiting root formation.
Magnification: 1.2X

FIGURE 16 B. juncea cv Domo protoplast-derived calli with one callus exhibiting shooting.
Magnification: 1.2X

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TABLE 11. Effect of BA and NAA concentrations on organogenesis in protoplast-derived calli of B. juncea cv Zem

% survival of plated calli after one month * (% surviving calli producing roots)						
NAA mg/l	1.9	0.9	0.19	0.09	0.019	
BA mg/l						
2.2 ***	50 (2)	55 (2)	47 (2.4)**	33(3)**	26	
1.1 *****	74 (2.7)	70	64	66 (3)	46	
0.22 *****	82 (22)	76 (18)	70	64	36	
0.11 *****	82 (24)	74 (22)	70 (5.7)	64 (3)	30	
0.022 ***	28 (4)	27 (36)	15 (50)	17 (6)	6	

* MS induction medium- 4% sucrose and 0.8% agar

** - one callus produced a shoot

*** - duplicated plates, totalling 93 calli

***** - duplicated plates, totalling 50 calli

but NAA levels of 10 mg/l were shown to be inhibitory to survival when combined with 3 mg/l BA (Table 12). This NAA requirement was confirmed with when tested with BA; calli transferred to 3mg/l BA in the absence of NAA had a 5% survival rate compared to a 100% survival in the control plates. No shoot induction improvement was noted at levels up to 0.4mg/l GA_3 (Table 12). Further tests showed an inhibitory effect of GA_3 on callus survival at levels of 10 mg/l using freshly transferred calli (Table 13), although older calli, transferred from control induction plates, survived on levels up to 40 mg/l (see Figure 17).

Kinetin and kinetin riboside were tested as alternatives to BA, with kinetin showing no improvement in survival or shoot regeneration. Kinetin riboside was significantly inferior for callus survival at levels at and above 5 mg/l (Table 13). Zeatin showed no improvement in survival or shoot regeneration, when tested as an alternative to BA, and also confirmed the NAA requirement. Survival without NAA was less than 5%, compared to over 50% of the calli placed on media with 3mg/l zeatin and 1mg/l NAA.

Calli established on control media (MS, 3BA:1NAA) (with calli as old as 15 months, but with the majority

TABLE 12. Effect of GA_3 and NAA on callus survival in protoplast-derived calli of B. juncea cv Domo

plate (10calli/plate)	callus survival after 1 month (no./10)					total/50
	I	II	III	IV	V	
control	10	10	7	8	10	45
3BA:0.1mg/1 GA_3	0	0	0	0	0	0
3BA:0.2mg/1 GA_3	0	0	0	0	0	0
3BA:0.4mg/1 GA_3	0	0	0	0	0	0
control + 0.2mg/1 GA_3	9	9	2	7	7	34
control + 0.2mg/1 GA_3	8	9	8	7	10	42
3BA:5mg/1 NAA	10	8	7	3	6	34
3BA:10mg/1 NAA	0	0	0	3	3	6

Control = 3mg/l BA + 1 mg/l NAA

3BA = 3mg/l BA

lack of NAA and 10mg/l NAA significantly different from control (Student's t-Test)

TABLE 13. Effect of kinetin, kinetin riboside, and GA₃ on callus survival and shoot regeneration in protoplast-derived calli of B. juncea cv Domo

medium*	surviving calli (total/60)	calli producing roots (total/60)	calli producing shoots (total/60)
control	41	0	1
0.05K	42	9	0
0.5K	40	2	0
5K	46	1	0
15K	35	0	0
0.05KR	28	1	0
0.5KR	25	0	0
5KR	15	0	0
15KR	5	0	0
control &			
0.02GA	43	0	0
0.2GA	39	1	0
2GA	31	0	0
10GA	21	0	0

* MS media, 1.5% sucrose, 0.4% agarose, with 1mg/l NAA.

other growth regulators: Control = 3mg/l BA

K = mg/l Kinetin

KR = mg/l Kinetin Riboside

GA = mg/l GA₃

kinetin riboside significantly different from control at levels at and above 5mg/l (Student's t-Test)

having been approximately one month on control medium, were transferred to media containing varying concentrations of thidiazuron. Neither autoclaved, or filter sterilized media showed any improvement in shoot induction, although high levels - 5mg/l - stimulated healthy green growth (Table 14). Only one shoot was observed, on a 0.02mg/l filter sterilized plate, shooting from an abnormally green and friable callus. This callus was easily maintained in culture; it was over a year old at the time of transfer to thidiazuron, and was the same callus which responded to the abscisic acid treatment (see below).

Absciscic acid (ABA) was tested, in conjunction with GA_3 , with or without a kinetin pretreatment. The calli were transferred to 0.05mg/l ABA, 1mg/l GA_3 , with some calli previously cultured on 3mg/l kinetin, 1mg/l NAA for one week. One callus responded to this treatment; an abnormally dark green, highly friable callus was stimulated to produce abnormal shoots which did not produce roots and eventually died (see Figure 18). In comparison, shoots from calli grown on 3BA:1NAA rooted readily when transferred to B_5 rooting medium (see Figure 19).

TABLE 14. Effect of thidiazuron on survival
of protoplast-derived calli from B. juncea
cv Domo

Survival after one month (#/95 calli plated)						
mg/l thidiazuron	0**	0.01	0.02	0.1	1.0	5.0
Media*						
Autoclaved	90	91	88	93	86	94
Filtered		87	89	82	91	94

* - MS media, 1.5% sucrose, 1 mg/l NAA, 0.4% agarose with
added thidiazuron autoclaved before use, or filter
sterilized

** - Control medium: 3 mg/l BA

no significant difference (Student's t-Test)

FIGURE 17 Established calli of B.juncea
transferred to induction plates with GA₃
concentrations from 2 to 40 mg/l, and
showing no difference in survival or shoot
induction.
Magnification: 0.3X .

FIGURE 18 Abnormal shoots of B.juncea regenerated
from highly friable, dark green callus.
Magnification; 1X

FIGURE 19 Shoot regenerated from B.juncea .
protoplast-derived callus rooted on B₅
media. A flower bud is discernible.
Magnification :1.3X

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14. Comparison of B₅ and MS as regeneration media

B₅ was tested as an alternative to MS as a shoot regeneration medium. There was no significant difference in callus survival, but the calli on the B₅ media browned and died sooner than those on MS, with 44% of the calli on MS remaining green and viable after four weeks, compared to only 17% of the calli on B₅. MS was chosen as the standard medium for all further experiments.

15. Comparison of gelling agents in regeneration medium

Agar (0.8%) and agarose (0.4%) were compared with similar results; both promoted callus survival with no significant effect on shoot organogenesis. It was noted that the colonies on agar solidified media were not as green and friable as those grown on agarose.

16. Effect of sucrose concentration on callus survival

The original sucrose concentration of 4% in the regeneration medium was found to be significantly inferior for callus survival compared to lower concentrations (Table 15). There was no shoot induction at any concentration of sucrose during this test. Thereafter, 1.5% sucrose was used in all regeneration experiments. When the sucrose was removed completely from the induction

TABLE 15. Effect of sucrose concentrations on callus survival of protoplast-derived calli of B.juncea cv Domo

	<u>% survival after 3 weeks (140 plated calli)</u>			
% sucrose	4	2	1	0.5
% survival	50	70	72	79

4% sucrose significantly different (Student's t-Test)

medium, in an attempt to shock the calli and induce regeneration, the calli survived up to a month, with no visible growth, but no shoots were induced when the calli were transferred to fresh media containing sucrose.

17. Effect of altered KNO_3 and NH_4NO_3 ratios in MS media on callus survival and shoot induction

Normally, shoot induction with 3BA:1NAA did not exceed 1% under any conditions, and when shoots were induced, they were restricted to one shoot per callus (Figure 16). A rooting callus (Figure 15) frequently produced more than one root. However, one experiment following normal procedures resulted in a much higher regeneration frequency - approximately 3% of the calli produced shoots - and one callus, when divided, produced numerous shoots (Figure 20). The calli from this experiment were used in a test studying altered KNO_3 and NH_4NO_3 ratios; no significant difference (Student's T-test) in calli survival or shoot induction was noted, save for a significantly reduced response when only KNO_3 was used in the medium (Table 16).

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MicroD

TABLE 16. Effect of altered KNO_3 : NH_4NO_3 ratios on callus survival and shoot induction in protoplast derived-calli of B. juncea cv Domo

medium	observations after 3 weeks	
	% viable	no. plates with calli producing shoots (no./10 plates)
$\text{KNO}_3/\text{NH}_4\text{NO}_3$ ratio concentration		
60mM NH_4NO_3	60	4
1:16 3.5mM:56.5mM	78	1
1:4 12mM:48mM	65	4
1:2 20mM:40mM	76	3
1:1 30mM:30mM	78	4
2:1 40mM:20mM	75	4
4:1 48mM:12mM	86	2
16:1 56.5mM:3.5mM	83	2
60mM KNO_3	33	0

* % viable - % of calli exhibiting growth (100 calli plated)

FIGURE 20 Prolific protoplast-derived callus of B. juncea subdivided, with the subdivisions exhibiting shooting.
Magnification: 1.4X

FIGURE 21 Regenerated B. juncea protoplast-derived shoot established in a Jiffy 7 peat pellet before transfer to soil.
Magnification: 0.5X

FIGURE 22 Regenerated B. juncea plants established in soil and flowering.
Magnification: 0.1X

FIGURE 23 Seed set in a regenerated B. juncea plant.
Magnification: 0.5X

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18. Effect of temperature shock on shoot stimulation

Young calli were transferred to induction medium and placed in 35°C conditions for several days. Thereafter they were placed in 25°C conditions and monitored for one month. The temperature shock had no effect on survival and failed to stimulate shoot formation (Table 17).

19. Effect of half strength MS medium on callus survival and organogenesis

When MS regeneration medium was diluted in half, no improvement in survival or shooting occurred, but the incidence of rooting increased significantly. In order to test whether the effect was due to lowered growth regulator levels, a further test was carried out, comparing half strength with normal growth regulator levels. However, the variation in calli survival and rhizogenesis between plates in half strength medium with normal growth regulator concentration was too high to statistically show increased root production. A final experiment with a high frequency of surviving calli showed significant increases in root production in both the half strength media formulations when compared to MS medium (Table 18).

TABLE 17. Effect of high temperature (35°C) treatment on survival of protoplast-derived calli of B. juncea cv Domo

calli surviving after one month (10 calli/ expt)				
days at 35°C Expt	<u>0</u>	<u>2</u>	<u>5</u>	<u>7</u>
1	6	4	4	4
2	10	7	3	8
3	6	10	9	7
4	10	5	10	10
5	10	10	10	10
6	10	7	10	9
<hr/>				
average	8.7	7.2	7.5	8.0

no significant difference $P > 30\%$ (Student's t-Test)

TABLE 18. Effect of 1/2 strength MS media on callus survival and organogenesis in protoplast-derived calli of B. juncea cv Domo

	% survival and (% of calli with roots)		
	Control	1/2 MS 3:1	1/2(MS) 3:1
Expt 1 (100 calli)	84(8)	98(26)	90(30)
Expt 2 (100 calli)	100(7)	100(30)	100(49)

* survival - number of calli exhibiting growth after one month

Control - MS media, 3mg/l BA and 1mg/l NAA

1/2 (MS 3:1) - Control diluted by 50%

1/2(MS) 3:1 - MS diluted by 50%, 3mg/l BA and 1mg/l NAA

Student t-Test: Expt 1 marginally significant difference in root production 10% > P > 5%

Expt 2 significant difference in root production P < 0.1%

20. Effect of removal of liquid medium from protoplast
- cultures on subsequent regeneration

It was noted that growing microcolonies survived well and progressed rapidly at the edges of the petrie dishes, above the liquid overlayer. Routinely, some plates were 'dried' by drawing off the liquid with a pasteur pipette after the microcolonies were easily visible to the eye. When compared to colonies from untreated plates, the 'dried' colonies showed no improvement in calli survival but did exhibit increased root organogenesis.

21. Effect of modified S₁ as a regeneration medium

S₁ was used as an alternative to MS as a regeneration medium, using the high calcium levels found to be advantageous in protoplast culture. With 6mM calcium and no magnesium in the medium, the calli failed to survive. When 6mM calcium and 2.4 mM magnesium were added to the medium, survival increased to control levels and shoot and root organogenesis appeared enhanced - although not significantly using Student's T-test (Table 19). With only two shoots on 140 calli, the difference between two and zero is considered to be caused by chance rather than a real difference between the populations.

TABLE 19. Effect of modified S_1 on shoot regeneration
in protoplast-derived calli of B. juncea cv Domo

% callus survival after 1 month *			
Medium **	Control	X'-Mg	X'
% survival	76	0.07	79
# shoots	0	0	2
# roots	0	0	2

* - 14 duplicated plates, with 10 calli per plate

** - Control- MS media, 3mg/l BA and 1mg/l NAA

X'-Mg - S_1 using 6 mM $CaCl_2$; 3mg/l BA and 1mg/l NAA

X' - S_1 using 6 mM $CaCl_2$ with 2.4mM $MgSO_4$;
3mg/l BA and 1mg/l NAA

no significant difference in Control and X' $P > 70\%$ (Student's
t-Test)

The S_1 medium with 6mM $CaCl_2$ and 2.4mM $MgSO_4$ (X') precipitated in the autoclave, and failed to gel solidly. A comparison was made with media which had been filter sterilized, with no significant difference in survival or shoot regeneration. The precipitation caused a lowering of the pH from 5.8 to approximately 5.0, so another test was run with filtered induction media at pH 5.0 and 5.8. A significant improvement was seen in calli survival using modified S_1 as compared to the MS control, but no difference was noted between the two pH's (Table 20).

22. Production of plants from regenerated shoots

Regenerated shoots were excised from the calli and transferred to B_5 medium to induce rooting. Some shoots were lost at this stage. Shoots with very abnormal leaves, large, non-lobed leaflets, did not survive this step unless the first new leaves formed were of a more normal nature. If the shoot began to flower before rooting, chances of rooting were greatly reduced. This problem was eliminated by maintaining the excised shoots in growth chambers under an eight hour photoperiod. Under these conditions, shoots maintained in culture rarely flowered, and never until roots had formed (see Figure 19).

TABLE 20. Effect of modified S_1 , tested at pH 5.0 and 5.8, on callus survival and shoot induction in protoplast-derived calli of B. juncea cv Domo

Expt	callus survival after 1 month (# survive/ 10 calli)		
	Control	X' pH 5.8	X' pH 5.0
1	7	8	9
2	5	7	8
3	6	10	10
4	7	10; shoot	8
Totals	25/40	35/40	35/40

Control- MS media, 3mg/l BA and 1mg/l NAA

X' - S_1 using 6 mM $CaCl_2$ with 2.4mM $MgSO_4$;
3mg/l BA and 1mg/l NAA

Control significantly different from other media $P < 5\%$
(Student's t-Test)

Rooted shoots were transferred to Jiffy 7 peat pellets and maintained in a mist chamber until well established (Figure 21). Initial problems of damping off were eliminated with the application of No Damp™. The plants that were transferred to pots and maintained in growth chamber or greenhouse flowered, (Figure 22), and set seed (Figure 23). All regenerants tested produced pollen, and when selfed, generally set seed. Some seed tended to germinate within the pod, but whether this was due to abnormalities in the regenerants, or to growth conditions was not determined.

23. Cytological studies of the regenerated plants

Cytological studies carried out on the regenerated plants showed some plants to have normal diploid chromosome numbers (Figure 24), and some with lower than normal numbers (Figure 25). Plants with higher than normal were also seen (Table 21). The single regenerate from Zom experiments, was cytologically normal. When a clone of the regenerate was protoplasted, another shoot was obtained, but its chromosome number tested at greater than 36. Other regenerates from Domo experiments also protoplasted well; for example, "7S5" produced "14JY9", both with abnormal chromosome numbers (Table 21).

Table 21. Morphological and cytological observations of B. juncea plants derived from protoplast-derived calli

Regenerant*	flowers	seed set	chromosome number (2n)**
Zem 13	Y	Y	36
16D19	Y	-	-
84D19A	Y	-	-
12D19	Y	Y	-
7JY19	abnormal	-	-
42D19	Y	poor	-
25D19A	Y	Y	G
16D19	Y	poor	56
36024	Y	Y	-
25D19	Y	poor	G
41D19A	Y	Y	-
55D19	Y	Y	-
14029	Y	poor	-
3MY13	Y	poor	-
5024	Y	poor	-
7S5	Y	Y	24
2D5	Y	Y	36
32024	Y	-	54
14JY9 (fr. 7S5)	Y	Y	30
705	Y	Y	-
3JY13 (fr. Zem13) abnormal		-	G
25D19B	Y	Y	-
48D19	Y	Y	-
48D19C	Y	Y	-

* all regenerants except Zem 13 and 3JY13 were derived from Domo protoplasts

** Chromosome number: 36 = normal

G = greater than 36

other values approximations

Y=yes, - = not determined

FIGURE 24 Pollen mother cell of B.juncea regenerate .
stained with acetocarmine, with a normal
number of chromosomes ($n=18$).

FIGURE. 25 Pollen mother cell of B.juncea regenerate
stained with acetocarmine, with an
abnormal number of chromosomes ($n=15$).

24

25

DISCUSSION

B.juncea var. Zem was originally used in this study because of its low erucic acid content. If a regenerate was obtained with a low glucosinolate content, then the recovered plant would likely be suitable material for the development of a capola quality B.juncea (an oilseed with low glucosinolates in the meal and low erucic acid in the oil). However, Zem performed poorly in culture, generally failing to survive and only producing one regenerate from many experiments. Fazekas et al. (1986) failed to achieve regeneration in Zem explant cultures, and found all Chinese origin B.juncea greatly inferior to Indian cultivars for regeneration from explants. Other researchers have noted genetic differences in tissue culture response; Spangenberg et al. (1986) observed a five fold reduction in microcallus formation in winter B.napus protoplasts, compared to that of spring B.napus protoplasts, and Hui et al. (1978) found only one of four cultivars of B.juncea to be very responsive in regeneration studies with hypocotyl and cotyledon explants. Dietert et al. (1982) observed the differences between B.oleracea cultivars in tissue culture to be as great as differences between species. In this study, B.juncea var. Domo proved superior to Zem for protoplast

culture, in terms of viability and plant regeneration, although both are of Chinese origin. However, large plant to plant differences were also seen, indicating the genetic variation within a cultivar may affect results.

The cortical layers of thin epidermal strips were chosen as a protoplast source after the method of Klimaszewska and Keller (1987) developed for B. napus. Since these layers were sterilized prior to stripping from the internodes, they suffered little damage. Also, a large number of protoplasts could be isolated from one plant. An alternative commonly used as a protoplast source is young leaves. Chatterjee et al. (1985) reported success with B. juncea mesophyll protoplasts, when the plants were grown under sterile conditions. However, several experiments in the present study showed leaves to be inferior to stem epidermal layers as a protoplast source, with mesophyll protoplasts being more fragile and impossible to isolate successfully from donor plants grown in growth chambers. Cotyledons (Wenbin et al., 1986), hypocotyls (Glimelius and Ottoson, 1983), and roots (Xu et al. 1982) have been used as sources for Brassica protoplasts. All three sources require large numbers of seeds, and therefore diverse genetic makeup, for each preparation. Also, the possibility of choosing an adult

plant with useful agronomical characteristics cannot be realized with these sources. The epidermal layers used in this study can be isolated from individual adult plants, and need not be grown under sterile conditions. Although the plants were normally grown under controlled conditions in a growth chamber, protoplasts have also been successfully isolated from greenhouse grown material, and some shoots were recovered from protoplasts derived from a greenhouse grown plant.

The tissue density, or weight of material, used when enzymatically isolating Brassica protoplasts is generally not considered closely. However, of the numerous pretreatments and enzyme solutions tested in this study, only the tissue density within the enzyme solution had a significant effect. Differences were found between cultivars and individual plants, but success was greatest when the density was approximately 0.25g f.wt./10ml of enzyme solution - slightly lower than most reported densities, e.g. Gatenby and Cocking (1977) : 0.5g f.wt./10ml, Bidney et al. (1983): 0.3g f.wt./10 ml. Zem was less susceptible than Domo to overloading, up to 1g f.wt./10ml enzyme solution could be successfully protoplasted when Zem was the source plant.

Pelleting the protoplasts proved to be superior to floatation for washing B.juncea protoplasts. When 0.5M sucrose was used to wash the B.juncea protoplasts, over 75% of the viable protoplasts continued to pellet. Shillito et al. (1983), and Bidney et al. (1983), have used the floatation technique to separate the intact protoplasts from cell debris. However, in this study the floated protoplasts did not show a higher survival rate than protoplasts washed by pelleting, and the practice of washing with sucrose was discontinued. Debris was kept to minimum by the low density of tissue in the enzyme solution; generally, there was almost complete digestion of the cell walls.

Agarose was shown to be beneficial to protoplast culture in this study. Following the method used by Keller (unpublished) for culturing microspores, the B.juncea protoplasts were floated in liquid media above an agarose solidified media underlayer. Others have reported the beneficial effects of agarose; Shillito et al. (1983) compared agarose with other gelling agents and found agarose to be superior for a number of species. They embedded the protoplasts, then floated them in liquid medium, which was renewed periodically. Adams and Townsend (1983) remelted the agarose to dilute the

embedded colonies. Shillito has hypothesized that embedding stabilizes fragile protoplasts. However, it is possible there is some factor other than stabilization for the B. juncea protoplasts were cultured in a liquid overlayer and yet seemed to benefit from the underlayer (Table 4). Eliminating direct contact with the plastic petri dish is one possibility; more probable is the ability to culture in very thin overlayers - 1.5 ml spreads easily over an underlayer. Maeda et al. (1983) suggests that an agar-liquid method supports protoplasts due to greater availability of oxygen. Embedded B. juncea protoplasts also survived, although it was necessary to leave them several days before adding liquid media, contrary to Shillito's findings for B. rapa which needed immediate transfer to liquid for survival.

Most of the changes in the media seemed to have little effect on the viability of the Brassica protoplasts. For example, the addition of Ficoll, the addition of folic acid, biotin, glycine, glutamine, serine, and glutathione as found in Lichter's medium, or using Lichter's growth regulator concentration in S₁, all had no noticeable effect on survival. However, when observations between different media led to manipulation of the calcium and magnesium levels, effects were obvious

and almost immediate. Within a few days of culturing, it was possible to distinguish between various salt concentrations by the appearance of the protoplasts.

Arnold and Eriksson (1977) reported a high requirement for calcium (1760mg/l) in Pisum sativum mesophyll protoplast cultures. Interestingly, Hoffman et al. (1979) noted that the addition of calcium to the medium was detrimental to Brassica protoplasts. However, both Lichter's and Pelletier's media have high levels of calcium as compared to S₁, and both support very high initial viabilities with B. juncea protoplasts. When the calcium levels in S₁ were increased, the viability also increased. The magnesium salt concentration affected the formation of tight, compact divisions; low levels resulted in loose, budding microcolonies. There are few reports of manipulation of specific inorganic elements in the literature, in fact, Schenck and Robbelen (1982) have specifically recommended against "detailed experiments on the improvement of nutrient media", believing time could be better spent on other factors. However, a variation of their medium, "X" medium with 0.12mM MgSO₄ 7H₂O and 6mM CaCl₂ 2H₂O, clearly increased viability and greatly improved consistency between preparations. Indeed, the only mesophyll protoplast isolation which survived to callus stage was cultured in the X medium.

The protoplast derived calli from B.juncea proved to be very recalcitrant to attempts at shoot initiation. The developing calli were first transferred to a broad range of auxin:cytokinin ratios. Although a noticeable increase in morphogenesis was noted at low levels of cytokinin, the morphogenic response invariably resulted in roots. Numerous variations on the media were tested, to no effect. Some changes, for example, the sucrose concentration, affected the survival of the calli, but increased calli survival did not improve the frequency of shoot regeneration which remained at the level of 1% of plated calli. This frequency is low compared to reports from other Brassicas: 20% for B.napus (Xu et al.1982, Klimaszewska and Keller, 1987), 11-20% for B.oleracea - cabbage (Xu et al.1982, Fu et al. 1985, respectively) and over 70% of surviving calli for B.oleracea - broccoli (Robertson and Earle, 1986). In later experiments with medium X, the regeneration frequency decreased relative to total number of calli plated (to 0.1%) but remained at approximately 1% on the control medium (3mg/l BA: 1mg/l NAA). The decrease might be related to the hormone regime, or the increased viability and plating efficiency of the X medium might allow many more colonies to survive which have no morphogenic capacity.

Several alternative cytokinins were tested in this study, but increased shoot regeneration frequencies were not observed. Kinetin has been reported as beneficial in stimulating shoots in B.juncea explants (Hui et al., 1978, Sayeedabanu et al., 1981, Singh et al., 1983, Pareek and Chandra, 1981), although George et al. (1980) and Mathews et al. (1985) report no success and no improvement with kinetin. In the present study, there was no improvement using kinetin, and kinetin riboside was inferior for survival when compared to growth of control calli. Glimelius and Ottosson (1983) and Klimaszewska and Keller (1987) report zeatin to be superior to BA for regeneration in Brassica, this finding was not reflected in the present study. Recently, Schering AG, Berlin, has been producing a new high potency cytokinin, thidiazuron, which has had reported success in promoting shoots in calli of Populus (Russell et al., 1986), apple (van Nieuwkerk et al. 1985), and Acer (Kerns and Meyer, 1985). A sample was obtained, but failed to promote regeneration in the B.juncea calli.

Addition of GA_3 to the regeneration medium did not stimulate any shoot formation in B.juncea. A review of Brassica literature shows several workers found the addition of GA_3 to be beneficial in stimulating shoot regeneration (Bidney et al., 1983, Pelletier et al., 1983,

Fu et al., 1985). Duplicating the above workers regeneration media also had no effect.

The present study indicated requirement of NAA survival of protoplast-derived calli. In contrast, Bidney et al. (1983) regenerated B.oleracea shoots using BA and GA₃ without NAA and Li et al. (1986) observed that even low levels of NAA were harmful while regenerating B.juncea. There was a requirement for NAA in this study whether the transferred calli were 'fresh' - just removed from the protoplast maintenance media - or 'established' - transferred from another regeneration media. Established calli were normally less sensitive to growth regulator treatments; although fresh calli had a reduced survival on media with 10 mg/l GA₃, established calli could withstand at least 40 mg/l GA₃ without any apparent difference from control.

Although Zem was consistently poorer in culture than Domo when grown from seed, the clones from the one Zem regenerate protoplasted well, and another shoot was obtained from culturing the protoplasts. Similarly, Robertson and Earle (1986) found B.oleracea plants from hypocotyl explant culture transferred to soil to be more amenable to tissue culture than seed grown plants grown under the same conditions. Regenerates from Domo also

protoplasted well, although no regenerate ever responded as well as the best seed grown plant which had a shoot regeneration frequency of 3%.

Various attempts to shock the calli, using high temperatures for several days, or no carbon source for several weeks, had no effect on survival or shoot regeneration. When half strength MS salts were employed, increased root production was seen, but no enhancement of shooting.

When modified S_1 (X') media was used as an induction media, the survival of the calli and the frequency of shoot regeneration was as high or higher than the MS control. It would appear that there is no disadvantage, and perhaps some advantage, in using modified S_1 for both protoplast and induction media. Klimaszewska (personal communication) noted an improvement in calli survival when protoplasts grown in B_5 were transferred to induction media composed of B_5 components.

All the regenerated plants which were transferred to soil flowered and produced pollen. However, these observations were not a good indication of cytogenetically normal plants, for of eight plants chromosomally examined only two had a normal somatic chromosome number of 36.

Altered chromosome numbers in B.napus plants regenerated from protoplasts have been reported (Newell et al. 1984), with only 40% of the recovered plants confirmed as diploid. Robertson and Earle (1986) noted genetic alterations in regenerated plants of B.oleracea - for example, inherited albinism - but provided no cytological data. The regenerated plants in this study provide material for additional cytological studies, for most of the regenerates set viable seed, and those which aborted their seed before maturity could be propagated by culturing the developing seed on sterile media.

The ability to regenerate plants from protoplasts, allows the protoplasts to be used in various genetic engineering schemes for plant improvement. The incorporation of foreign genetic material through transformation, fusion, electroporation, or microinjection have all been discussed previously with regard to other plants (see Literature Review, section A6 and B5). The basic protoplast technology for undertaking similar studies in B.juncea has been developed. However, more research is needed to improve plating efficiencies, and, more particularly, regeneration frequencies before this system can be used to full advantage.

CONCLUSION

This study has indicated that stem cortical tissue of Brassica juncea is a feasible source for protoplasts. Isolated protoplasts have regenerated cell walls, undergone sustained division, and achieved plant regeneration through organogenesis.

Successful protoplast isolation depended most heavily on the quantity of donor tissue placed in the enzyme solution; 0.25 g of tissue /10 ml enzyme solution consistently released viable protoplasts. Greater quantities often failed to release any viable protoplasts.

An agarose underlayer enhanced protoplast culture viabilities, when the protoplasts were cultured in a liquid medium, and embedding the protoplasts within an agarose-solidified medium was shown to have potential for increased plating efficiencies. For this potential to be realized, the optimum time for feeding, and possibly diluting, the dividing cultures must be ascertained.

Modifications to Schenck and Robbelen's S₁ medium proved to be highly advantageous for survival and colony formation (plating efficiency) in B. juncea

protoplast cultures. Increased calcium levels, combined with lowered magnesium, consistently supported high percentages of isolated protoplasts. Since increased magnesium levels were conducive to improved colony formation, it is possible that a two step culturing system, with increased magnesium levels after the initiation of cell division, could be an optimal procedure.

Efforts to increase regeneration frequencies demonstrated a requirement for NAA in the induction medium, but failed to increase the frequency beyond 1% of the calli plated. Induction medium with 4% sucrose was inferior to media with lower levels for callus survival. Experiments using a modified S_1 medium indicated that the medium was as good as, or superior to, the commonly used MS induction medium.

Regenerated plants were predominantly morphologically regular, all producing pollen, and most setting seed. However, cytological examination of the pollen mother cells revealed only 20% of the plants examined had a normal somatic chromosome number of 36.

REFERENCES

- Adams, T.L. and J.A.Townsend. 1983. A new procedure for increasing efficiency of protoplast plating and clone selection. Plant Cell Reports. 2: 165-168.
- Aggarwal, R.K., D.R.Sharma, H.O.Mehra, and R.K.Singh. 1983. Isolation and regeneration of the mesophyll protoplasts of Brassica juncea cultivar Prakash. Basic Life Sciences, 1983. 491-494.
- Alexander, R., and F.Dubert. 1983. The isolation and culture of Brassica napus cv. Lingot cotyledon protoplasts. In Protoplasts 1983, Poster Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 62-63.
- Arnold, S.V., and T.Eriksson. 1977. A revised medium for growth of pea mesophyll protoplasts. Physiol. Plant. 39: 257-260.
- Bates, G.W., and C.A.Hasenkampf. 1985. Culture of plant somatic hybrids following electrical fusion. Theor. Appl. Genet. 70: 227-233.
- Bhojwani, S.S., and E.C.Cocking. 1972. Isolation of protoplasts from pollen tetrads. Nature New Biology 239: 29-30.
- Bidney, D.L., J.F.Shepard, and E.Kaleikau. 1983. Regeneration of plants from mesophyll protoplasts of Brassica oleracea. Protoplasma 117: 89-92.
- Binding, H. 1982. protoplast regeneration in higher plants. In Plant Tissue Culture - Proc. 5th Intl.Cong. Edited by A.Fujiwana. Maruzen Co. Ltd. pp. 575-578.
- Bornman, C.H., and J.F.Bornman. 1985. Plant protoplast viability. In The physiological properties of plant protoplasts. Edited by P.E.Pilet. Springer-Verlag, Berlin. pp. 29-36.

- Bourgin, J.P.. 1983. Protoplasts and the isolation of plant mutants. In Protoplasts 1983, Lecture Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by J. Potrykus. Birkhauser, Basel. pp. 43-47.
- Burgess, J. 1983. Wall regeneration around isolated protoplasts. In Plant Protoplasts. Inter. Rev. Cytology. Suppl 16. Edited by K.L.Giles. Academic Press, New York. pp. 29-36.
- Butenko, R.G. 1979. Cultivation of isolated protoplasts and hybridization of somatic plant cells. International Review of Cytology. 59: 323-369.
- Chatterjee, G., S.R.Sikdar, S.Das, and S.K.Sen. 1985. Regeneration of plantlets from mesophyll protoplasts of Brassica juncea(L.) Czern. Plant Cell Reports. 4: 245-247.
- Chin, J.C., K.J.Scott. 1979. A large scale isolation procedure for cereal mesophyll protoplasts. Ann. Bot. 43: 23-32.
- Chuong, P.V., K.P.Pauls, and W.D.Beversdorf. 1987. Protoplast culture and plant regeneration from Brassica carinata cv Braun. Plant Cell Reports. In press.
- Chuong, .V., K.P.Pauls, and W.D.Beversdorf. 1985. A simple culture method for Brassica hypocotyl protoplasts. Plant Cell Reports. 4: 4-6.
- Chupeau, Y. C.Missonier, M.-C.Hommel, and J.Goujaud. 1978. Somatic hybrids of plants by fusion of protoplasts. Mol. gen. Genet. 165: 239-245.
- Cocking, E.C. 1960. A method for the isolation of plant protoplasts and vacuoles. Nature. 187: 962-963.
- Collins, W. 1986. Fire at the centre. Harrowsmith 67: 92-101.

- Davey, M.R. 1983. Recent developments in the culture and regeneration of plant protoplasts. In Protoplasts 1983, Lecture Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I. Potrykus. Birkhäuser, Basel. pp. 19-30.
- Dietert, M.F., S.A. Barron, and O.C. Yoder. 1982. Effects of Genotype on in vitro culture in the genus Brassica. Plant Sci. Lett. 26: 233-240.
- Dijak, M., D.L. Smith, T.J. Wilson, and D.C.W. Brown. 1986. Stimulation of direct embryogenesis from mesophyll protoplasts of Medicago sativa. Plant Cell Reports. 5: 468-470.
- Downey, R.K. 1985. Developing markets with biotechnology. Proceedings of 18th annual convention. Canola Council of Canada. 19-24.
- Engler, D.E., and R.G. Grogan. 1982/83. Isolation, culture and regeneration of lettuce leaf mesophyll protoplasts. Plant Science Letters. 28: 223-229.
- Evans, D.A., and J.E. Bravo. 1983. Plant protoplast isolation and culture. In Plant Protoplasts. Inter. Rev. Cytology. Suppl 16. Edited by K.L. Giles. Academic Press, New York. pp. 33-54.
- Fazekas, G.A., P.A. Sedmach, and M.V. Palmer. 1986. Genetic and environmental effects on in vitro shoot regeneration from cotyledon explants of Brassica juncea. Plant Cell, Tissue and Organ Culture. 6: 177-180.
- Flick, C.E., and D.A. Evans. 1983. Isolation, culture, and plant regeneration from protoplasts isolated from flower petals of ornamental Nicotiana species. Z. Pflanzenphysiol. 109: 379-383.
- Fluhr, R., D. Aviv, M. Edelman, and E. Galun. 1983. Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusions in Nicotiana. Theor. Appl. Genet. 65: 289-294.

- Fu, Y.-Y., S.-R.Jia, and Y.Lin. 1985. Plant regeneration from mesophyll protoplast culture of cabbage (Brassica oleracea var 'capitata'). Theor. Appl. Genet. 71: 495-499.
- Gatenby, A.A., E.C.Cocking. 1977. Callus formation from protoplasts of marrow stem kale. Plant Sci. Lett. 8: 275-280.
- Gamborg, O.L. 1984. Plant cell cultures: nutrition and media. Cell culture and somatic cell genetics of plants. 1: 19-26.
- George, L., and P.S. Rao. 1980. In vitro regeneration of mustard plants (Brassica juncea var RAI-5) on cotyledon explants from non-irradiated, irradiated and mutagen treated seed. Ann. Bot. 46: 107-112.
- Gleddie, S., W.A.Keller, and G.Setterfield. 1986. Somatic embryogenesis and plant regeneration from cell suspension derived protoplasts of Solanum melongena (eggplant). Can. J. Bot. 64: 355-361.
- Glimelius, K., M.Djupsjobaka, and H.Fellner-Feldegg. 1986. Selection and enrichment of plant protoplast heterokaryons of Brassicaceae by flow sorting. Plant Science. 45: 133-141.
- Glimelius, K. 1984. High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol. Plant. 61: 38-44.
- Glimelius, K., and A.Ottosson. 1983. Improved culture ability of the genus Brassica by using hypocotyls as the source for protoplasts. In Protoplasts 1983, Poster Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 64-65.
- Grun, P. and L.-J.Chu. 1978. Development of plants from protoplasts of Solanum (Solanaceae). Amer. J.Bot. 65(5): 538-543.

- Harms, C.T. 1983. Somatic hybridization by plant protoplast fusion. In Protoplasts 1983, Lecture Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 69-84.
- Hayashi, M., T.Nakajima. 1984. Genetic stability in regenerated plants derived from tobacco mesophyll protoplasts through three-step culture. Japan. J. Breed. 34: 409-415.
- Hoffman, F. and T.Adachi. 1981. "Arabidobrassica": Chromosomal recombination and morphogenesis in asymmetric intergeneric hybrid cells. Planta. 153: 586-593.
- Hoffman, F., H.Schenck, H.W.Kohlenbach, and Y.Y.Gleba. 1979. Regeneration and fusion of protoplasts from important crop plants of the Brassicaceae. In Advances in Protoplast Research. Proceedings of the 5th International Protoplast Symposium. Edited by L.Ferenczy and G.L.Farkas Pergamon Press, Oxford. pp. 287-292.
- Hui, L.H., and S.-Y.Zee. 1978. In vitro plant formation from hypocotyls and cotyledons of leaf mustard cabbage (Brassica juncea Coss). Z. Pflanzenphysiol. 89:77-80.
- Kao, K.N., and M.R.Michayluk. 1980. Plant regeneration from mesophyll protoplasts of alfalfa. Z.Pflanzenphysiol. 96:135-141.
- Kao, K.N., W.A.Keller, and R.A.Miller. 1970. Cell division in newly formed cells from protoplasts of soybean Exp. Cell.Res. 62: 338-340
- Kartha, K.K. 1975. Organogenesis and Embryogenesis. In Plant tissue culture methods. Edited by O.L.Gamberg and L.R. Wetter. National Research Council of Canada. Saskatoon. pp. 44-49.

- Kartha, K.K., O.L. Gamborg, F. Constabel, and N.K. Kao. 1974. Fusion of rapeseed and soybean protoplasts and subsequent division of heterokaryocytes. *Can. J. Bot.* 52: 2435-2437.
- Keller, W.A., G. Setterfield, G. Douglas, S. Gleddie, and C. Nakamura. 1982. Production, characterization, and utilization of somatic hybrids of higher plants. In *Application of Plant Cell and tissue culture*. Edited by D.T. Tomes, P.M. Harney, R.L. Peterson, B.E. Ellis, and K.J. Kasha. University of Guelph. pp. 81-114.
- Kerns, H.R., and M.M. Meyer. 1985. In vitro propagation of red-silver hybrid maples. *HortScience*. 20 (3): 115.
- Kirk, J.T.O., and R.N. Oram. 1981. Isolation of erucic acid-free lines of Brassica juncea: Indian mustard now a potential oilseed crop in Australia. *J. Austr. Instit. Agri. Sci.* 1981.
- Klimaszewska, K., and W.A. Keller. 1987. Plant regeneration from stem cortex protoplasts of Brassica napus. *Plant Cell Tissue Organ Culture*. In press.
- Klimaszewska, K., and W.A. Keller. 1986. Somatic embryogenesis in cell suspension and protoplast cultures of Brassica nigra (L.) Koch. *J. Plant Physiol.* 122: 251-260.
- Kohlenbach, H.W., G. Wenzel, and F. Hoffman. 1982. Regeneration of Brassica napus plantlets in cultures from isolated protoplasts of haploid stem embryos as compared with leaf protoplasts. *Z. Pflanzenphysiol.* 105: 131-142.
- Kondra, Z.P. 1985. Canola production and cultivar development in Canada. *Agr. & Forestry Bulletin*. University of Alberta. 8: 3-6.
- Kumar, P.M., P. Bhattacharya, N.M. Bhattacharya, and S.K. Sen. 1981. Induction of callus from the mesophyll protoplasts of Brassica campestris, an oilseed crop. *Indian J. of Exper. Bio.* 19: 656-657.

- Larkin, P.J. 1976. Purification and viability determinations of plant protoplasts. *Planta* 128: 213-216.
- Li, L.-C., and H.W.Kohlenbach. 1982. Somatic Embryogenesis in quite a direct way in cultures of mesophyll protoplasts of Brassica napus. *Plant Cell Reports* 1: 209-211.
- Linsefors, L., and P.Brodelius. 1985. Immobilization of plant protoplasts: viability studies. *Plant Cell Reports*. 4: 23-27.
- Maeda, Y., Y.Fujita, and Y.Yamada. 1983. Callus formation from protoplasts of cultured Lithospermum erythrorhizon cells. *Plant Cell Reports*. 2: 179-182.
- Manzara, T. and P.R.Lurquin. 1983. Systems for plant protoplast transformation. In *Protoplasts 1983, Lecture Proceedings*. 6th International Protoplast Symposium. *Experientia Supplementum* 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 57-60.
- Mathews, V.H., C.R.Bhatia, R.Mitra, T.G.Krishna, and P.S.Rao. 1985. Regeneration of shoots from Brassica juncea L.Czern and Coss cells transformed by Agrobacterium tumefaciens and expression of nopaline dehydrogenase genes. *Plant Science*:39:49-54.
- Medgyesy, P., R.Golling, and F.Nagy. 1985. A light sensitive recipient for the effective transfer of chloroplast and mitochondrial traits by protoplast fusion in Nicotiana. *Theor. Appl. Genet.* 70: 590-594.
- Miki, B.L.A., T.J.Reich, and V.N.Iyer. 1987. Microinjection: an experimental tool for studying and modifying plant cells. In *Plant Tissue and Cell Culture*. In press.
- Muhlbach, H.-P. 1980. Different regeneration potentials of mesophyll protoplasts from cultivated and a wild species of tomato. *Planta*. 148: 89-96.

- Nagamani, B., T.Kamala, T.Chandrasekhara Rao, and G.Lakshminarayana. 1981. Effect of irradiation and chemical treatment on yield and quality of Brassica juncea (L.) seed and oil. Proc. Indian Acad. Sci.(Plant Sci.) 90, No. 4: 281-284.
- Nagata, T., and I.Takebe. 1970. Cell wall regeneration and cell division in isolated tobacco protoplasts. Planta. 92: 301-308.
- Newell, C.A., M.L.Rhoads, and D.L.Bidney. 1984. Cytogenic analysis of plants regenerated from tissue explants and mesophyll protoplasts of winter rape, Brassica napus L. Can. J. Genet. Cytol. 26: 752-761.
- Oelck, M.M., V.A.Bapat, and O.Schieder. 1982. Protoplast culture of three legumes: Arachis hypogaea, Melilotus officinalis, Trifolium resupinatum. Z.Pflanzenphysiol. 106: 173-177.
- Orton, T.J. 1984. Somaclonal variation: theoretical and practical considerations. In Gene Manipulation in Plant Improvement. 16th Stadler genetics symposium. Edited by J.Gustafson. Plenum Press, New York. pp. 427-468.
- Pareek, L.K. and N.Chandra. 1981. Induced regeneration in vegetative and flowering internodal segments and ovary explants of Brassica juncea Coss. Indian Jour. of Exper. Bio. 19: 874-875.
- Paszkowski, J., B.Pisan, R.D.Shilito, B.Hohn, T.Hohn, and I.Potrykus. 1986. Genetic transformation of Brassica campestris var. rapa protoplasts with an engineered cauliflower mosaic virus genome. Plant Molec. Bio. 6: 303-312.

- Paszkowski, J., H.Shinshi, I.Koenig, G.B.Lazar, T.Hohn, V.Mandak, and I.Potrykus. 1983. Proliferation of cauliflower mosaic virus in protoplast-derived clones of turnip (Brassica rapa). In Protoplasts 1983, Poster Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 138-139.
- Pelletier, G., C.Primard, F.Vedel, P.Chérit, R.Remy, Rousselle, and M.Renard. 1983. Intergenic cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol. Gen. Genet. 191: 244-250.
- Pisan, B., I.Potrykus, and J.Paszkowski. 1983. Optimisation of turnip (Brassica rapa) protoplast culture for cauliflower mosaic virus transformation. In Protoplasts 1983, Poster Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46. Edited by I.Potrykus. Birkhauser, Basel. pp. 44-45.
- Robertson, D., and E.D.Earle. 1986. Plant regeneration from leaf protoplasts of Brassica oleracea var. italica CV Green Comet broccoli. Plant Cell Reports. 5 (1): 61-64.
- Rose, R.J. 1980. Factors that influence the yield, stability in culture and cell wall regeneration of spinach mesophyll protoplasts. Aust. J. Plant Physiol. 7: 713-725.
- Russell, J.A., and B.H.McCown. 1986. Culture and regeneration of Populus leaf protoplasts isolated from non-seedling tissue. Plant Science. 46: 133-142.
- Sayeedabanu, S., S.Singh, L.K.Pareek, and N.Chandra. 1981. Induced differentiation from cultured cotyledonary leaves of mustard (Brassica juncea var. Prakash). Current Science 50:412-414.
- Schenck, H.G., and G.Robbelen. 1982. Somatic hybrids by fusion of protoplasts from Brassica oleracea and B. campestris. Z.Pflanzenuchtg. 89: 278-288.

- Schubert, S., and H.Matzke. 1985. Influence of phytohormones and other effectors on proton extrusion by isolated protoplasts from rape leaves. *Physiol. Plant.* 64: 285-289.
- Shahin, E.A. 1985. Totipotency of tomato protoplasts. *Theor. Appl. Genet.* 69: 235-240.
- Shillito, R.D., J.Paszkowski, and I.Potrykus. 1983a. Culture in agarose improves protoplast plating and proliferation, and permits division in otherwise unresponsive systems. In *Protoplasts 1983, Poster Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46*. Edited by I.Potrykus. Birkhauser, Basel. pp. 266-267.
- Shillito, R.D., J.Paszkowski, and I.Potrykus. 1983b. Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. *Plant Cell Reports* 2:244-247.
- Shillito, R.D. and H.H.Steinbiss: 1983. Genetic transformation. In *Protoplasts 1983, Lecture Proceedings. 6th International Protoplast Symposium. Experientia Supplementum 46*. Edited by I.Potrykus. Birkhauser, Basel. pp. 131-132.
- Singh, R.J. 1984. The regeneration of milkweed (*Asclepias* spp.) plants from hypocotyl callus, cell suspension cultures and hypocotyl protoplasts. *Z. Pflanzenzuchtg.* 92: 95-101.
- Singh, S., S.Banu, L.K.Pareek, and N.Chandra. 1983. Morphogenesis in organ, tissue, and cell cultures of some species of *Brassica*. *Basic Life Sciences, 1983.* pp 441-444.
- Somers, D.A., K.R.Narayanan, A.Kleinhofs, S.Cooper-Bland, and E.C.Cocking. 1986. Immunological evidence for transfer of the barley nitrate reductase structural gene in *Nicotiana tabacum* by protoplast fusion. *Mol. Gen. Genet.* 204: 296-301.

- Spangenberg, G., H.U.Koop, R.Lichter, and H.G.Schweiger. 1986. Microculture of single protoplasts of Brassica napus. Physiol. Plant. 66: 1-8.
- Spangenberg, G., and H.G.Schweiger. 1986. Controlled electrofusion of different types of protoplasts and subprotoplasts including cell reconstitution in Brassica napus L. European Journal of Cell Biology. 41: 51-56.
- Spangenberg, G., H.U.Koop, and H.G.Schweiger. 1985. Different types of protoplasts from Brassica napus L.: analysis of conditioning effects at the single cell level. Eur. J. of Cell Bio. 39: 41-45.
- Sundberg, E., and K.Glimelius. 1986. A method for production of interspecific hybrids within Brassicaceae via somatic hybridization, using resynthesis of Brassica napus as a model. Plant Science. 43: 155-162.
- Takebe, I., G.Labib, and G.Melchers. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwiss 58: 318-320.
- Takeuchi, Y., and A.Komamine. 1982. Effects of culture conditions on cell divisions and composition of regenerated cell walls in Vinca rosea protoplasts. Plant & Cell Physiol. 23(2): 249-255.
- Tal, M., and J.W.Watts. 1979. Plant growth conditions and yield of viable protoplasts isolated from leaves of Lycopersicon esculentum and L. peruvianum. Z. Pflanzenphysiol. 92: 207-214.
- Tewari, J.P. 1985. Diseases of Canola caused by fungi in the Canadian prairies. Agr. & Forestry Bulletin. University of Alberta. 8: 13-20.

- Toriyama, K., T. Kameya, and K. Hinata. 1987a. Selection of a universal hybridizer in Sinapis turgida Del. and regeneration of plantlets from somatic hybrids with Brassica species. Planta. In press.
- Toriyama, K., K. Hinata, and T. Kameya. 1987b. Production of somatic hybrid plants, 'Brassicomoricaandia', through protoplast fusion between Moricaandia arvensis and Brassica oleracea. Plant Science Letters. In press.
- U, N. 1935. Genomic analysis in Brassica with special reference to the experimental formation of B.napus and peculiar mode of fertilization. Jpn J Bot. 7: 389-452.
- van Nieuwkerk, J.P., R.H. Zimmerman, and I. Fordham. 1985. Response of apple cultivars in vitro to thidiazuron. HortScience. 20 (3): 523.
- Van Slogteren, G.M.S., K. Planque, and J. Lekkerkerk. 1980. Evaluation of parameters affecting the initiation of division of protoplasts of haploid and diploid Nicotiana sylvestris and Nicotiana tabacum. Plant Sci. Lett. 20: 35-45.
- Vardi, A., P. Spiegel-Roy, and E. Galun. 1975. Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. Plant Sci. Lett. 4: 231-236.
- Von Arnold, S. and T. Eriksson. 1977. A revised medium for growth of pea mesophyll protoplasts. Physiol. Plant. 39: 257-260.
- Wenbin, L., C. Zhenghua, S. Yuhua, and Z. Dawei. 1986. Studies on plant regeneration from protoplasts of Brassica juncea. Acta Genetica Sinica. 13(3): 184-187.
- Xu, Z.-H., M.R. Davey, and E.C. Cocking. 1982. Plant regeneration from root protoplasts of Brassica. Plant Sci. Lett. 24: 117-121.

- Xu, Z.-H., M.R.Davey, and E.C.Cocking. 1981. Isolation and sustained division of Phaseolus aureus (Mung bean) root protoplasts. Z. Pflanzenphysiol. 104: 289-298.
- Yarrow, S.A., S.C.Wu, T.L.Barsby, R.J.Kemble, and J.F.Shepard. 1986. The introduction of CMS mitochondria to triazine tolerant Brassica napus L., var. "Regent", by micromanipulation of individual heterokaryons. Plant Cell Reports 5:415-418.

APPENDIX

TABLE 1

MS Medium	
components (mg/l)	
KNO ₃	1900
NH ₄ NO ₃	1650
CaCl ₂ 2H ₂ O	440
MgSO ₄ 7H ₂ O	370
KH ₂ PO ₄	170
FeIII EDTA Na salt	40
MnSO ₄ H ₂ O	22.3
ZnSO ₄ 7H ₂ O	8.6
H ₃ BO ₃	6.2
KI	0.83
NaMoO ₄ 2H ₂ O	0.25
CuSO ₄ 5H ₂ O	0.025
CoCl ₂ 6H ₂ O	0.025
nicotinic acid	1
pyridoxine HCl	1
thiamine HCl	10
myo-inositol	100
sucrose	20000

TABLE 2

Media components (mg/l)

	S ₁	Pell B	Lichter
NH ₄ NO ₃	800		
KNO ₃	2000	2500	125
(NH ₄) ₂ SO ₄		134	
NaH ₂ PO ₄		150	
CaNO ₃ 4H ₂ O			500
CaCl ₂ 2H ₂ O	200	750	
MgSO ₄ 7H ₂ O	1880	250	125
KH ₂ PO ₄	700		125
Fe SO ₄ 7H ₂ O		27.8	
FeIII EDTA Na salt	50		40
Na EDTA		37.3	
MnSO ₄ H ₂ O	10	10	22.3
ZnSO ₄ 7H ₂ O	10	2	8.6
H ₃ BO ₃	10	3	6.2
KI	1	0.75	
NaMoO ₄ 2H ₂ O		0.25	0.25
CuSO ₄ 5H ₂ O		0.025	0.025
CoCl ₂ 6H ₂ O		0.025	0.025
nicotinic acid		1	
pyridoxine HCl		10	0.5
sucrose	20000		65000
glucose	72000*	20000	
mannitol		70000	
2,4-D	0.2	0.25	0.5
NAA	1	1	0.5
BA	0.5	1	0.05

Lichter's also has:

folic acid	0.5
biotin	0.05
glycine	2
glutamine	800
serine	100
glutathione	30

* modified; original culture media 0.55M mannitol

APPENDIX

TABLE 3

Kao's Medium

mineral salt components (mg/l)

KNO ₃	1000
NH ₄ NO ₃	600
CaCl ₂ 2H ₂ O	600
MgSO ₄ 7H ₂ O	300
KH ₂ PO ₄	170
KCl	300
FeIII EDTA Na salt	28
MnSO ₄ H ₂ O	10
ZnSO ₄ 7H ₂ O	2
H ₃ BO ₃	3
KI	0.75
NaMoO ₄ 2H ₂ O	0.25
CuSO ₄ 5H ₂ O	0.025
CoCl ₂ 6H ₂ O	0.025

Sugars (mg/l)

glucose	68,400	mannose	125
sucrose	125	rhamnose	125
fructose	125	cellobiose	125
ribose	125	sorbitol	125
xylose	125	mannitol	125

Organic acids (mg/l) -adjusted to pH 5.5 with NH₄OH

sodium pyruvate	5	malic acid	10
citric acid	10	fumaric acid	10

.... continued on next page ..

APPENDIX

TABLE 3 -continued

Kao's Medium ...continued

Vitamins (mg/l)

inositol	100	biotin	0.005
nicotinamide	1	choline chloride	0.5
pyridoxine HCl	1	riboflavin	0.1
thiamine HCl	10	ascorbic acid	1
D-calcium pantothenate	0.5	vitamin A	0.005
folic acid	0.2	vitamin D	0.005
p-aminobenzoic acid	0.01	vitamin B ₁₂	0.01

Hormones (mg/l)

2,4-D	0.2
zeatin	0.5
NAA	1

Other components

vitamin-free casamino acid (mg/l) 125

coconut water (ml/l) 10

from mature fruits: heated to 60°C for 30 min and filtered

pH 5.7

APPENDIX

TABLE 4

Chatterjee's BG Culture Medium

Compounds	source or mg/l
Inorganics	B ₅
Fe EDTA	MS
inositol	100
glycine	2
thiamine	0.5
pyridoxine HCl	0.5
nicotinic acid	5
folic acid	0.5
biotin	0.05
2,4-D	0.3
NAA	0.3
BA	1
glucose	0.6M
coconut milk	5%

pH 5.8

APPENDIX

TABLE 5

Pelletier C media

Component (mg/l)

NH_4NO_3	200	H_3BO_3	3
KNO_3	1250	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	10
$(\text{NH}_4)_2\text{SO}_4$	67	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2
NaH_2PO_4	75	KI	0.75
KH_2PO_4	35	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	525	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
		$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
		Na_2EDTA	37.3
Inositol	100	Sucrose	20000
Nicotinic acid	1	D-mannitol	40000
Pyridoxine HCL	1	NAA	0.2
Thiamine	10	BA	1

pH 5.8

NAA - Naphthalene Acetic Acid

BA - 6 Benzyl aminopurine

APPENDIX

TABLE 6

B ₅ Medium	
components (mg/l)	
KNO ₃	2500
(NH ₄) ₂ SO ₄	134
NaH ₂ PO ₄	150
CaCl ₂ · 2H ₂ O	750
MgSO ₄ · 7H ₂ O	250
FeIII EDTA Na salt	40
MnSO ₄ · H ₂ O	10
ZnSO ₄ · 7H ₂ O	2
H ₃ BO ₃	3
KI	0.83
NaMoO ₄ · 2H ₂ O	0.25
CuSO ₄ · 5H ₂ O	0.025
CoCl ₂ · 6H ₂ O	0.025
nicotinic acid	1
pyridoxine HCl	1
thiamine HCl	10
myo-inositol	100
sucrose	20000

END

20/06/88

FIN