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**TISSUE CULTURE AND INTERGENERIC
SOMATIC
HYBRIDIZATION IN BRASSICACEAE**

by

ANNE-CHRISTINE DOMINIQUE BONFILS, B.Sc.

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

Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
July 1992
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submitted by

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in partial fulfilment of the requirements
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ABSTRACT

Plant regeneration from stem cortex explants of thirteen cultivars of Brassica juncea was assessed, and proved to be strongly affected by genotype, with up to 50% of explants producing shoots in the most responsive lines and absence of shoot organogenesis in the least responsive lines. Stem cortex protoplast culture success depended on genotype, donor plant age, plating density, dilution frequency, colony size upon transfer to regeneration media, and growth regulator content of the media. After one week of culture, up to 11-14% of the cells divided, and about 0.002% produced 1-2 mm colonies within 6 weeks. A maximum of 7% of these colonies gave rise to shoots upon transfer to shoot regeneration media.

The second objective of the study was to attempt somatic hybridizations between Brassica crop species, and the flea beetle resistant Thlaspi arvense (stinkweed), Lunaria annua (silver dollar) or Capsella bursa-pastoris (shepherd's purse). A preliminary assessment of explant and protoplast cultures of these wild species was undertaken. Shepherd's purse proved to be the most responsive, with rapidly growing embryogenic cell suspension cultures, established from leaf-derived calli. Viable protoplasts could readily be isolated from these cell suspensions. After one week of culture, protoplast viability was 62%, and 7% of the cells had divided. Embryos were regenerated from protoplast-derived microcolonies, plated on growth regulator-free medium. Although these embryos were difficult to root, some plants were recovered.

Shepherd's purse protoplasts were fused with B. napus protoplasts in the presence of PEG. Enrichment of hybrid cells consisted of treating B. napus protoplasts with iodoacetamide and X-irradiating shepherd's purse protoplasts. Hundreds of calli could be recovered from post-fusion protoplast cultures, however, numerous attempts to regenerate plants from these calli were unsuccessful. Therefore, cell lines were screened for hybridity, using colorimetric analyses of glutamate oxaloacetate transaminase (GOT) isozymes. Out of 1300 calli, 42 showed hybrid banding patterns. The DNA of some putative hybrid lines was analyzed by polymerase chain reaction (PCR) random amplification of DNA sequences (RAPDs) and by restriction fragment length polymorphism (RFLP) studies. The results have confirmed hybridity of all the analyzed cell lines, including some which had only exhibited B. napus GOT isozyme patterns.

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LIST OF ABBREVIATIONS

AS = adenine sulfate
ABA = abscisic acid
BA = benzyladenine
BARiboside = benzyladenine riboside
BSA = bovine serum albumin
cas. am. = acid hydrolysed casein
CMS = cytoplasmic male sterility
coxII = cytochrome oxidase, subunit II
CTAB = cetyltrimethylammonium bromide
2,4-D = 2,4-dichlorophenoxyacetic acid
DAPI = 4,6-diamidino-2-phenylindole
EDTA = ethylenediaminetetra-acetic acid
d.f. = degree of freedom
DTT = dithiothreitol
FACS = fluorescence activated cell sorting
FDA = fluorescein diacetate
FITC = fluorescein isothiocyanate
GA3 = gibberellic acid
GOT = glutamate oxaloacetate transaminase
G.R. = growth regulator
IAA = indole acetic acid
IBA = indole butyric acid
IOA = iodoacetamide
2ip = 2-isopentenyl-adenine
IPA = indole propionic acid
K = kinetin
NAA = 1-naphthalene acetic acid
p = significance level for statistical analyses
PCR = polymerase chain reaction
PEG = polyethylene glycol
I-PPT = phosphinotricin = ignite
PVP = polyvinylpyrrolidone
RAPDs = random amplification of polymorphic DNA
RFLP = restriction fragment length polymorphism
RITC = rhodamine isothiocyanate
s.e. = standard error
TZ = thidiazuron
X² = Chi-square value
Z = zeatin
Zrib = zeatin riboside

GENERAL
INTRODUCTION

The Brassicaceae contain a wide range of crop species which have been bred for centuries, mainly for human consumption. They include vegetable species of the genus Brassica, such as cabbage, cauliflower, broccoli, brussels sprouts, kale (B. oleracea L.), turnip (B. campestris L.) and leafy mustard (B. juncea (L.) Czern., B. carinata L.). Of greater economic importance are the oilseed species B. napus and B. campestris (rapeseed). Thanks to important cultivar improvements, B. napus, and to a lesser extent B. campestris, are now extensively grown for edible oil production, while the crushed seeds provide a protein-rich meal, which is competitive in quality with soybean seed cake, used to feed livestock. In "double-low" cultivars of rapeseed, known as canola, oil contains less than 2% erucic acid, a fatty acid (C22:1) suspected of causing heart lesions on mice, and one gram of seed meal contains less than 30 μ M glucosinolates, thioglucosides responsible for loss of palatability and digestibility of the meal (Kirk and Oram, 1978). The first "double-low" lines were obtained by crossing improved lines with B. napus cv. Bronowski, which contained low levels of glucosinolates. Improvements of traits such as earliness to flower, seed yield, the quality and quantity of seed oil and proteins, and male sterility, have also been important goals for breeders. Resistances of adult canola plants to major pathogens such as blackleg (Phoma lingam, also known as Leptosphaeria maculans), and clubroot (Plasmodiophora brassicae) have also been improved through breeding (Sippell *et al.*, 1991; Diederichsen and Sacristán, 1991). However, the B. napus gene pool available to breeders is limited, and some related species constitute desirable new gene sources.

Plant breeders are becoming increasingly interested in B. juncea (mustard), as a potential third canola species. Mustard is already an important crop plant, as Canada is a leading exporter of mustard seeds for condiment preparation (e.g. Dijon mustard), primarily to Europe and Japan. Some of the interesting traits of B. juncea are a superior level of drought tolerance relative to B. napus, lower susceptibility to silique shattering, and resistance to blackleg (Phoma lingam) (Kirk and Oram, 1981), while some lines are also resistant to white rust (Albugo candida) (Kolte et al., 1991). In addition, B. juncea is early maturing, higher seed yielding and oil comprises 38% of the seed dry weight (Love et al., 1990). Although mustard oil is widely used for human consumption on the Indian sub-continent (Pua, 1990), and in the ex-USSR (Kirk and Oram, 1978), it is not acceptable in Canada, due to the presence of high levels of erucic acid. The seeds also contain high levels of glucosinolates. Efforts are now underway to breed new glucosinolate and erucic acid-free B. juncea cultivars; "double-low" mustard varieties are available (Love et al., 1990), but not yet agronomically acceptable.

Breeders are also very interested in the "rustic" germplasm of wild Cruciferous species, which often contain genes for tolerance to adverse environmental conditions (drought, flooding, heat, cold, high salt concentrations), and disease or insect resistances. For example, wild B. oleracea varieties carry a resistance to blackleg (Mithen and Herron, 1991), yellow mustard (Sinapis alba) is not attacked by Heterodera schachtii, a white cyst nematode (Thierfelder et al., 1991), stinkweed (Thlaspi arvense), shepherd's purse (Capsella bursa-pastoris) and flaxweed (Descurainia sophia) are frost tolerant (J. Singh, personal communication).

Of particular interest is the resistance to flea beetles (Phyllotreta cruciferae or P. striolata) displayed by some Cruciferous weeds. Flea beetles are responsible for heavy losses in Cruciferous crops on the Canadian Prairies (Lamb, 1984). They are chemically controlled with a granular carbofuran (Furadan™, FMC), which is a systemic cholinesterase inhibitor with contact and digestive effects (Hartley and Kidd, 1987). Furadan is presently under a Special Review by the Pest Control Products Regulations of Agriculture Canada, due to high toxicity levels to bees, cattle, fish, to wildlife in general, and in particular to the burrowing owl (R.-M. Leblanc, personal communication). Its registration may not be continued, but no other efficient insecticide is available to farmers. The development of flea beetle resistant canola crops is therefore the ideal solution to this problem, and wild Cruciferae species are interesting gene sources. Stinkweed (Thlaspi arvense) is not attacked by flea beetles, is cold tolerant and has a short life cycle, while silver dollar (Lunaria annua) and white mustard (Sinapis alba) are also unattractive to flea beetles and may even repel these insects (Meisner and Mitchell, 1983; Lamb, personal communication). Shepherd's purse (Capsella bursa-pastoris) is not attacked by flea beetles either (Chew, 1988), is resistant to Alternaria brassicae (Tewari, 1991), and has a short life cycle. In these cases however, plant breeders are faced with a serious problem: except for S. alba, these Cruciferous species belong to different tribes than B. napus, and strong incompatibility barriers prevent sexual hybridization.

An alternative approach to the problem could be through protoplast fusion, i.e. somatic hybridization. Not only may protoplast fusion bypass sexual incompatibilities, it may also allow the introgression of only a part of the wild species' genome, through asymmetric hybridization. In asymmetric hybridizations, one of the fusion partners is treated with chromosome destabilizing procedures, such as X-ray irradiation, to induce unidirectional chromosome elimination from the hybrid cells (Dudits *et al.*, 1980). The resulting asymmetric hybrid plants are usually more fertile than symmetric hybrids (Hinnisdaels *et al.*, 1988), and since they only contain part of the donor's genome, the number of backcrosses to the parental crop species, necessary for restoring good agronomic quality, would be reduced. Somatic hybridizations have successfully been achieved in Brassica species, often used to resynthesize amphidiploid species such as B. napus (Schenck and Röbbelen, 1982; Glimelius *et al.*, 1986), but also to create new genomic combinations between sexually incompatible species, for example Arabidopsis thaliana + B. campestris (Hoffmann and Adachi, 1981), Eruc. sativa + B. napus (Fahleson *et al.*, 1988b), or Diplotaxis harra + B. napus (Klimaszewska and Keller, 1988).

These facts have motivated my study, which was conducted in pursuit of five major objectives. The first objective of this project was to study protoplast culture of B. juncea, as a potential approach to obtain mustard lines containing low levels of erucic acid and glucosinolates. Protoplast culture is a key technique for genetic engineering, as it provides for large numbers of isolated cells, that can be subjected to mutational treatments, serve as recipients to exogenous genes, or be fused to produce somatic

hybrids. For the last twenty years, numerous efforts have been dedicated to develop protoplast culture protocols in a variety of Brassica species, but B. juncea proved to be one of the least amenable to plant regeneration. I decided to concentrate on defining conditions which would allow good plant regeneration frequencies from protoplast cultures of B. juncea, since they did not exist at that time. Without plant recovery, the potential of protoplasts in genetic engineering could not be easily realized for breeding purposes.

To date, "in vitro" manipulation studies of Thlaspi arvense or Lunaria annua have not been reported, and only a few reports of in vitro manipulation of Capsella bursa-pastoris (Monnier and Lagriffol, 1986; Zilkah and Gressel, 1977) are available. The development of protoplast technology for these species was my second objective, since it is also a prerequisite to using them in somatic hybridization experiments.

The third objective of the project was to attempt somatic hybridization between one of the flea beetle-resistant wild species and either B. napus, B. campestris or B. juncea. For this purpose, it was necessary to develop suitable fusion and selection methods, and to establish appropriate conditions for plant regeneration.

Once calli or plants are regenerated from fusion experiments, it is imperative to determine their true hybrid nature. Hybrid identification was the fourth objective, using the molecular techniques of native isozyme polyacrylamide gel electrophoresis (PAGE), Southern blots and Polymerase Chain Reaction (PCR) amplification of polymorphic DNA sequences. The use of PCR for somatic hybrid characterization has not yet been reported, and I hoped to show that it is a simple and rapid method for analyzing the

DNA content of putative hybrids.

Finally, the ultimate objective of the study would be to test hybrid cell lines or plants for their resistance to flea beetle attacks, and eventually to include the plants as source material for a breeding programme involving backcrosses to the crop species.

CHAPTER I

PLANT REGENERATION FROM STEM CORTEX EXPLANT AND PROTOPLAST CULTURES OF BRASSICA JUNCEA.

LITERATURE REVIEW

A. BRASSICA JUNCEA: DESCRIPTION AND AGRONOMIC ASPECTS.

Brassica juncea is an amphidiploid or allotetraploid species of the A and B genomes ($n=18$, AB), resulting from the cross of B. nigra ($n=8$, B) and B. campestris ($n=10$, A), according to the taxonomic triangle of U (1935) reproduced on **Figure 1**. It includes varieties with swollen stems or roots, leafy vegetables, and oilseed types. In Canada, it is mainly known as belonging to the oilseed type mustard group, which also comprises B. carinata and Sinapis alba (Fazekas et al., 1986). For centuries, mustard has been used for its oil, as a vegetable, for seasoning food, for medicinal purposes and religious ceremonies. It became an important crop in Canada during World War II, resulting from its high demand in specialized lubricants for battle ships and other war equipment (Kirk and Oram, 1978). Canada is now growing mustard as a condiment crop, and provides most of the worldwide supply of mustard seeds.

a. The problem of glucosinolates and erucic acid.

The pungent taste and nose irritation, so typical of mustard, are mostly due to the presence of the glucosinolates sinigrin and gluconapin (Kirk and Oram, 1978). Glucosinolates are a class of thioglucosides, of general formula $R-C(=N-OSO_3)(S-C_6H_{11}O_5)$, where the side chain R can represent a variety of more or less complex alkyl, alkenyl or heterocyclic structures (Röbbelen and Thies, 1980).

Figure 1: Taxonomic triangle of U (1935) illustrating genetic relationships between *Brassica* species.

Glucosinolates can be found in ten Dicotyledonous families, namely the Limnanthaceae, Cariaceae, Tropaeolaceae, Salvadoraceae, Euphorbiaceae, and the five families of the Capparale order, the Capparaceae, Tovariaceae, Resedaceae, Moringaceae, and Brassicaceae, from which they are characteristically produced (Underhill, 1980). When the seeds are crushed, a thioglucosidase, myrosinase, which always appears to be in glucosinolate-producing species, is released. Myrosinase has been reported to be stored in idioblasts (Bones and Thangstad, 1991). Hydrolysis of glucosinolates by myrosinase results in the production of glucose and volatile compounds such as isothiocyanates (also called mustard oils), thiocyanates and nitriles (Nishie and Daxenbichler, 1980). Glucosinolates are not considered to be directly toxic, but some of their hydrolytic products are physiologically active. For example, thiocyanate ions compete with iodide for uptake by the thyroid, and are thus responsible for the formation of goitres (Röbbelen

and Thies, 1980; Nishie and Daxenbichler, 1980). A protein rich mustard meal consists of crushed seeds from which the oil was extracted. The growth of chickens, fed on a diet containing as low as 3% of mustard meal, was significantly decreased (Kirk and Oram, 1978). When mice were fed on a diet containing 20% of mustard meal, they gained one-third less weight after two weeks, than mice fed on a diet lacking thioglucosides (Bell *et al.*, 1971).

Biologically, glucosinolates probably play a role in repelling insects. Many of their hydrolysis products (isothiocyanates, nitriles, thiocyanates) are highly toxic to generalist insects (Erickson and Feeny, 1974), although they act as potent feeding stimulants for specialized insect pests of the Brassicaceae, such as flea beetles or *Pieris brassicae*, the cabbage butterfly (Feeny *et al.*, 1970). Isothiocyanates are also known to be antibacterial and antifungal agents, and seem to repel wild vertebrates such as deer or rabbit (Lerin, 1980), while nitriles cause renal and hepatic pathologies to vertebrates (Harborne, 1982).

There is no direct evidence for the toxicity of erucic acid to humans, but it has been shown to cause severe heart necrotic lesions on mice (Kirk and Oram, 1978), and is thus considered dangerous.

b. Alternatives to breeding for glucosinolate elimination.

Various methods have been assayed in order to reduce levels of glucosinolates in *B. juncea*. For example, an extremely low sulphur supply to the plants reportedly reduced glucosinolates by 35% (Röbbelen and Thies, 1980). Other methods include cooking crushed seeds prior to oil extraction in order to inactivate myrosinase, or on the

contrary, promoting hydrolysis of glucosinolates into volatile compounds, that would be removed in strong stream flows (Bell *et al.*, 1971). All these methods are either uneconomical, or markedly reduce protein quality or quantity (Röbbelen and Thies, 1980). Glucosinolates have been eliminated from canola meal, by grinding in methanol containing dissolved ammonia, and counter-current extraction of the slurry with hexane, but these are still pilot studies (Diosady and Rubin, 1991).

c. Conventional breeding for glucosinolate and erucic acid elimination.

Since the levels of glucosinolates and erucic acid are genetically determined, conventional breeding can result in the production of low glucosinolate and erucic acid varieties, through single plant selection. Indeed, erucic acid-free lines of *B. juncea* were isolated in Australia in 1981 (Kirk and Oram, 1981), whereas the selection of a Chinese zero-glucosinolate variety was reported once (Cohen *et al.*, 1983). Double-low lines are currently undergoing field trials in Canada (Love *et al.*, 1990). In theory, mutations could also be induced to increase genetic variation, by irradiating the plants or treating them chemically, but this approach is difficult due to the amphidiploid nature of *B. juncea*, where mutations in one genome would likely be hidden by the other genome. As a result, no double-low lines have thus far been produced via mutagenesis and selection.

B. CULTURE OF BRASSICA JUNCEA TISSUE EXPLANTS.

Published reports on B. juncea explant cultures are listed on Table 1. The importance of genotype for plant regeneration from in vitro cultures is well recognized, but seems particularly critical in the genus Brassica. Dietert et al. (1982) for example, compared the organogenic response of 6 Brassica species, and of 17 genotypes of B. oleracea: the variation between genotypes of the same species was as great as the variation between species! Brassica juncea genotypes are mainly classified into two geographical groups: the India/Pakistan group and the China/Eastern Europe group. The Indian genotypes have brown seeds, which contain high levels of allyl- and butenyl-isothiocyanates, 42 to 58% erucic acid in the oil, and have high fibre contents (Fazekas et al., 1986; Kirk and Oram, 1981). The seeds of Chinese genotypes are usually yellow, sometimes brown, contain allylisothiocyanates only, and the oil comprises 18 to 37% erucic acid (Kirk and Oram, 1981). Fazekas et al. (1986) have indicated that Indian genotypes are generally more responsive for plant regeneration from hypocotyl explant cultures, than are Chinese genotypes. However, Hui and Zee (1978), working with four Chinese cultivars, also reported tremendous differences between genotypes, South Wind being much superior to the other three. Similarly, Kirti and Chopra (1989a) observed major genotypic variations between three Indian cultivars, the least responding of which never gave rise to shoot organogenesis.

Table 1: Overview of plant regeneration from *Brassica juncea* explant culture.

explant source [variety]	growth regulators [mg/l]	regeneration response [frequency of responding calli]	reference
hypocotyls [South Wind]	a- 0.5 NAA + 2 BA b- 0.5 NAA + 4 K c- 0.5 NAA + 2 Z d- 0.5 NAA + 2 2ip	shoots [a- 70%]; [b- 40%] [c- 50%]; [d- 30%]	Hui and Zee, 1978
cotyledons [NM*]	a- 0.5 NAA + 2 BA b- 0.5 NAA + 4 K c- 0.5 NAA + 0.5 Z d- 0.5 NAA + 0.5 2ip	shoots [a- 70%]; [b- 70%] [c- 60%]; [d- 50%]	
cotyledons [RAI-5]	a- 1 NAA + 0.2 BA b- 0.2 BA c- 1 NAA + 1 BA	shoots [a- 66%]; [b- 17%]; [c- 96%]	George and Rao, 1980
internodal segments, ovary explants [NM]	a- 0.5 IAA b- 0.5 K c- 1 K	flowering shoots or single flowers [NM]	Pareek and Chandra, 1981
cotyledons [Prakash]	1 IAA + 2 K	shoots [NM]	Sayeedabanu et al., 1981
hypocotyls [NM]	2 NAA + 4 K + 0.5 2,4-D	shoots [2%]	Dietert et al., 1982

*NM = Not Mentioned.

Table 1 (cont.): Overview of plant regeneration from *B. juncea* explant culture.

source [variety]	growth regulators [mg/l]	regeneration [frequency of regenerating calli]	reference
microspores in anthers [RIK81-1][RIK80-1] late uninucleate	0.1 NAA + 0.1 2,4-D	embryos [1% of microspores]	Sharma and Bhojwani, 1985
cotyledons [Zem 1] [Zem 2] [Vniimk 351]	1 NAA + 1 BA	shoots from Vniimk 351 only [4%]	Fazekas <i>et al.</i> , 1986
cotyledons [RH36]	a- 2.3 IAA + 1.8 BA b- 2.3 IAA + 3.5 BA c- 0.5 NAA + 1 BA d- 2 NAA + 1.8 BA	shoots [a- 66%]; [b- 6.4%] [c- 59%]; [d- 18%]	Narasimhulu and Chopra, 1988
hypocotyls [RLM 198] [RLM 514] [RLM 619]	0.25 2,4-D + 0.5 NAA + 0.5 BA riboside	embryos [RLM198= 32.2%] [RLM514= 0.7%] [RLM 619= 0]	Kirti and Chopra, 1989a
cotyledons with apical bud [NM*]	a- 1 BA b- 1 BA + 0.1 NAA	a- shoots [50%] b- no shoots	Sharma <i>et al.</i> , 1990

*NM = Not Mentioned.

Seedling explants have been preferred by most investigators, perhaps because they are younger, more metabolically active, and less differentiated. Hui and Zee (1978) obtained high percentages of plant regeneration, ranging from 30 to 70%, both for cotyledon and hypocotyl explants. However, other results were not always as encouraging. For instance, the success of George and Rao (1980) with plant regeneration from cotyledon explants (up to 96%) was accompanied with a complete lack of regeneration from hypocotyl explants. Dietert *et al.* (1982) obtained only 2% plant regeneration from hypocotyls, and Fazekas *et al.* (1986) observed a 4% regeneration frequency from cotyledons, indicating that other factors need consideration.

Cytokinin/auxin ratios are known to affect *in vitro* organogenesis markedly, with the general rule that high ratios favour shoot development, whereas low ratios tend to promote root development (Margara, 1984). This is not so simple however, and although comparisons of growth regulators were performed by several research teams, the best combinations to be used for *B. juncea* plant regeneration, vary between laboratories. Hui and Zee (1978) obtained best results with a medium containing 0.5 mg/l NAA and 2 mg/l BA (Table 1). The growth regulators NAA and BA were also found superior for plant regeneration from cotyledons of *B. juncea* cv. RA15 by George and Rao (1980), but at 1 mg/l each; in their hands, kinetin and zeatin inhibited shoot initiation, contrasting with Hui and Zee (1978), whose kinetin-containing medium supported good shoot regeneration. Shoots were obtained, also from cotyledons, by Sayeedabanu *et al.* (1981), using IAA and kinetin, but the frequency was not mentioned. Narasimhulu and Chopra (1988) also assessed NAA, IAA and BA, and found IAA to be

superior. They observed a 10 fold decrease in regeneration frequency (66% to 6.4%, with 2.3 mg/l IAA mg/l + 1.8 BA mg/l and 2.3 mg/l IAA + 3.5 mg/l BA, respectively), just by doubling the amount of BA used. An increase in BA concentration was also found to inhibit shoot regeneration by George and Rao (1980).

C. PROTOPLAST CULTURE OF BRASSICA SPECIES, WITH EMPHASIS ON B. JUNCEA.

The regeneration of plants from isolated protoplasts of B. napus was first reported by Kartha *et al.* (1974), and the regeneration of plants from B. juncea protoplasts was first reported by Chatterjee *et al.* (1985). Since then, plant regeneration has been commonly reported for a number of Brassica species, with varying degrees of success. Advances in protoplast culture of Brassica species have shown that several factors influence cell division and plant regeneration. As with explants, genotype determines the success of protoplast culture. Glimelius (1984) has subjected hypocotyl-derived protoplasts of several Brassica species to the same culture conditions. While 70% of the protoplast-derived calli from B. napus gave rise to numerous shoots, less than 1% of those from B. campestris (var. Tyko) produced shoots. Jourdan and Earle, in 1989, using mesophyll protoplasts, also found B. napus superior to other Brassica species. Kao and Séguin-Swartz (1987) reported that about 1% of protoplast-derived calli of B. juncea cv. Varuna regenerated shoots, whereas no shoots were obtained from B. juncea cv. Cutlass. Important variations between B. juncea cultivars were also reported by Eapen

et al. (1989), where 2-3% of the protoplast-derived embryos from cv. RAI-5 matured into plantlets, whereas no shoots were obtained from cv. TM4. Finally, 15-20% of protoplast-derived calli of *B. juncea* cv. Indian Mustard produced embryos, compared to only 3-6% in *B. juncea* cv. Leaf Heading, under the same conditions (Pua, 1990).

Mesophyll tissue, often collected from young aseptic plantlets, has often been used as a *B. juncea* protoplast source (Eapen *et al.*, 1989; Kao and Séguin-Swartz, 1987; Chatterjee *et al.*, 1985). High numbers of protoplasts could be isolated from a single plant, but they were fragile, released large quantities of brown substances that seemed to be correlated with high polyphenol oxidase activity, and that were toxic to the protoplasts and to small colonies (Carlberg *et al.*, 1983). As a result, investigators have turned towards other donor material. For example, with *B. napus*, Klimaszewska and Keller (1987) successfully used cortical cells from actively growing stems, Xu *et al.* (1982) isolated protoplasts from *in vitro*-grown roots, and Kohlenbach *et al.* (1982) obtained regenerable protoplasts from stem embryogenic cultures. Somatic embryos developed from suspension-derived protoplasts of *B. nigra* (Klimaszewska and Keller, 1986). For *B. juncea*, best results were obtained with seedling material, cotyledons and hypocotyls (Table 2). Kirti and Chopra (1989b) obtained 43% shoot regeneration from hypocotyl-derived protoplasts.

Most commonly used media for initial protoplast plating are a medium described by Glimelius (1984), derived from 8p medium of Kao and Michayluk (1975), and a medium based on B5 (Gamborg *et al.*, 1968), described by Pelletier *et al.* (1983).

Table 2: Overview of plant regeneration from Brassica juncea protoplast culture.

source [cultivar]	G.R.[mg/l] for plant regeneration	regeneration [frequency]	reference
hypocotyls 1.[Leaf Heading] 2.[Indian Mustard]	0.2 BA, 0.2 2,4-D	1. proembryos [3-6%] 2. proembryos [15-20%]	Pua, 1990
hypocotyls [RLM 198]	a. 0.1 IAA, 2 BA, 2 Zrib. b. 0.5 2,4-D, 0.5 NAA, 0.5 BA riboside c. 0.5 NAA, 2 BA	a. shoots [35%] b. embryos [6-14%] c. embryos [22%]	Kirti and Chopra, 1989b
mesophyll 1.[RAI-5] 2.[TM-4]	0.05 ABA, 1.0-3.0 GA3	1. embryos [2-3% regenerated plants] 2. no organogenesis	Eapen et al., 1989
seedling mesophyll [Southern Curled]	1.0 NAA, 1.0 IPA, 0.02 GA3	no callus no organogenesis	Jourdan and Earle, 1989
mesophyll 1.[Cutlass] 2.[Varuna]	2 Zrib., 0.1 NAA	1. no organogenesis 2. shoots [<1%]	Kao and Séguin- Swartz, 1987
stem cortex [Domo]	1 NAA, 3 BA	shoots [2%]	Sproule, 1987
mesophyll [RLM 514]	a. 0.5 2,4-D, 0.5 NAA, 3 BA, 0.1 GA3 b. 1 NAA, 2 BA	a. embryos [20-25%] b. shoots [5-10%]	Chatterjee et al., 1985

G.R. = growth regulator

Commonly used growth regulators are 2,4-D at 0.2-1.0 mg/l, NAA at 0.5-1.0 mg/l, and BA at 0.1-0.5 mg/l, in the initial culture medium (Schenck and Hoffmann, 1984). Subsequent introduction of fresh medium with reduced levels of osmotica is necessary for further development; the appropriate feeding and dilution frequencies must be assessed, in order to avoid medium exhaustion, over-population, and accumulation of inhibitory products (Chuong *et al.*, 1985). If dilution is too high, on the other hand, cells might lose necessary metabolites, or certain compounds in the medium might be toxic, because their relative concentration would be too high compared to the number of cells.

D. OBJECTIVES.

The objectives of this project were to assess the major experimental factors for B. juncea protoplast culture, with the view of improving cell division, callus proliferation and plant regeneration. Induced or spontaneous variants could then be selected from such a system, based on their phenotype (e.g. low glucosinolate production). The genetic base of B. juncea could consequently be broadened, and some lines might be included in canola breeding programmes. From previously published reports of plant regeneration from Brassica spp. protoplasts, it is clear that induction of organogenic responses depends mainly on the genotype used, the protoplast source, media compositions and the overall culture method.

The regeneration potential of various genotypes of B. juncea needed to be

investigated. Stem cortex explants were chosen as the source material for this investigation. Callus formation and root or shoot organogenesis from explant cultures are much more rapid than from protoplast cultures, which are also more labour intensive. Stem cortex tissue is young, metabolically active, and large quantities can be retrieved from single plants. Although explants might differ from protoplasts with regard to their morphogenic response, it was reasonable to expect that if a genotype could not be induced to regenerate plants from explants, it would probably be a poor source of protoplasts capable of division and regeneration. On the other hand, a genotype responsive to explant culture, might not provide regenerable protoplasts, due to higher levels of stress and complete loss of organization associated with protoplast culture. Still, the response of explants would give a good indication on the regeneration potential of given genotypes.

The culture of mesophyll-derived protoplasts of B. juncea has generally not resulted in high plant regeneration frequencies (Kao and Séguin-Swartz, 1987; Sproule, 1987). Seedling-derived protoplasts have later proved to be much more responsive (Pua, 1990), but their disadvantage is that many seeds are required for each preparation. Stem cortex cells were thus chosen as donor material for protoplast culture, for the reasons just mentioned, but also because Klimaszewska and Keller (1987) have shown that B. napus stem cortex-derived protoplasts were less fragile than mesophyll-derived ones, divided with high frequencies, and had a good regeneration potential. The protoplast culture was then to be optimized for the chosen genotype and source material, by comparing various treatments and culture media, for each critical step of the general protocol.

MATERIALS AND METHODS

A. EXPLANT CULTURES.

a. Donor material. Seeds were obtained from E. Bevis (Plant Research Centre, Agriculture Canada, Ottawa). The cultivars Forge, UC Davis #11 and Zem were yellow-seeded and of Chinese origin. The cultivars Cutlass, Domo, Donskaja, Jubilejnaja, Vniimk-351, and Zaria were yellow-seeded and of Eastern Europe origin, whereas the cultivars Blaze, RLM 198, RLM 514 and RLM 619 were brown-seeded and of Indian origin. Seeds were sown in 12 cm pots containing a mixture of soil:peat:sand (1:2:1), and maintained in a growth chamber under a 16 h photoperiod, with $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ light intensity derived from cool white fluorescent tubes and incandescent bulbs (400-700nm), 20:15°C day:night temperature, and 70% relative humidity. Plants were watered twice a day and fertilized once a week with a 20:20:20 (nitrogen : phosphorus : potassium) liquid solution, and with Hoagland's solution (Appendix 1). The main stem was pinched before elongation, to eliminate apical bud dominance, and stimulate bolting and flowering of axillary buds. Stems were collected after approximately one month, when the inflorescences had one to three open flowers. Some cultivars bolted and flowered later than others. The time of stem harvest was arbitrarily based on the physiological stage of the plants, rather than on their actual age. The three terminal internodes were referred as "terminal internodes", while the next three (fourth, fifth, and sixth internodes) were referred as "basal internodes".

b. Sterilization. When necessary, internodes were cut to a convenient size, usually

around 4 cm long, and placed into autoclaved MagentaTM containers. They were surface sterilized in 70% ethanol for one minute, and in a super-saturated solution of calcium hypochlorite for 20 minutes. They were then rinsed in sterile distilled water, three times for five minutes each.

c. Explant isolation and plating. Longitudinal strips, comprising the epidermis, a few cortex cell layers, and occasionally some phloem and xylem vessels, were cut with BeaverTM 75 micro-sharp blades (Waltham, MA), and pulled away from the stems with fine forceps. These 2 to 5 mm wide strips were cut into segments of 5 mm length, and rapidly plated in 60x20 mm plastic Petri dishes, with cortex cells touching the culture medium. Culture media consisted of autoclaved MS (Murashige and Skoog, 1962) as modified by Douglas *et al.*, 1981 (Appendix 3), pH 5.8, containing 3% w/v sucrose, and solidified with 0.8% w/v Difco-Bacto agar. Growth regulators (prepared as described in Appendix 2) were either 1 mg/l NAA and 3 mg/l BA in N1 medium, 0.5 mg/l NAA and 5 mg/l BA in N2 medium, 0.1 mg/l IBA and 1.5 mg/l BA in I1 medium, or 0.2 mg/l IBA and 3.0 mg/l BA in I2 medium. After plating, dishes were wrapped with Parafilm (Am. Nat. Can, Greenwich, N.Y.), and maintained under continuous fluorescent light ($70 \mu\text{Em}^{-2}\text{sec}^{-1}$) at 25°C.

d. Rooting of plantlets and transfer to greenhouses. Regenerated shoots were transferred to 'baby food' jars containing growth regulator-free B5 medium (Gamborg *et al.*, 1968, Appendix 4) with 2% sucrose, and solidified with 0.8% w/v Difco-Bacto agar. These shoots were kept at 20°C with a photoperiod of 8 h and a light intensity of $70 \mu\text{Em}^{-2}\text{sec}^{-1}$. Regenerated material was maintained in conditions described, with

monthly transfers to fresh medium. Well rooted plantlets were acclimated to greenhouse conditions, by transferring them to peat pellets (Jiffy 7, Shippegan, N.B.) which were placed in a mist chamber for two weeks, before potting them in soil and placing them in the greenhouse.

e. Data collection, analysis and presentation. Results were taken from a minimum of 360 explants per treatment. Callus proliferation and organogenesis were recorded after one month, and expressed as percentages of responding explants over the total number of explants plated. Since these data were all binomial (presence or absence of calli, roots or shoots on known numbers of explants), a logistic regression analysis with a logit link function, with associated Chi-square values (X^2), were used to predict the occurrence of events, and calculate standard errors. These two values are presented in the results. The significance level for all analyses was set at $p < 0.01$.

B. PROTOPLAST CULTURE.

a. Protoplast isolation and washing. Donor material, sterilization and explant isolation were as described above (paragraph A), but the stem cortex strips were not cut into 5 mm segments. Approximately 0.5 g of the strips was plated per 100x15 mm plastic Petri dish, and pretreated in 20 ml of autoclaved growth regulator-free liquid MS medium, supplemented with 2% sucrose, pH 5.8, at 4°C in the dark, for 24 h. The pretreatment medium was replaced with 20 ml of enzyme solution, consisting of 0.5% cellulase "Onozuka" RS (Yakult Honsha, Tokyo), 1% macerozyme R-10 (Yakult Honsha, Tokyo),

and 0.4 M mannitol, pH 5.8, filter-sterilized through a 0.2 μm Nalgene™ filter. Strips were digested at 25°C, in the dark, for 18 h. Protoplasts were filtered through 88 μm and 44 μm nylon mesh to eliminate large debris and undigested material. They were collected into 15 ml glass tubes, and centrifuged for 6 min at 400 RPM (100g). The enzyme solution was discarded, and the pelleted protoplasts were resuspended in culture medium, and washed twice by centrifugation.

b. Protoplast plating and initial culture conditions. Protoplasts were counted with a haemocytometer, and density was adjusted to 10^5 protoplasts per ml, unless mentioned otherwise. Basic culture medium was filter-sterilized X (Sproule, 1987, Appendix 5), or B (Pelletier *et al.*, 1983, Appendix 6). Three ml of the protoplast suspension were plated into 15x60 mm plastic Petri dishes. When dishes were coated with a 2 ml underlayer consisting of culture medium solidified with 0.4% agarose SeaPlaque (FMC, Rockland), only 1.5 ml of the protoplast suspension were plated per dish. The dishes were wrapped with Parafilm and incubated at 25°C in darkness for one week.

c. Medium changes for protoplast cultures. After one week, the volume of protoplast suspensions was halved, and supplemented with 0.75 ml of fresh culture medium. Protoplasts plated on B received C medium (Pelletier *et al.*, 1983), those plated on X received X0.2 medium (X with 0.2 M glucose), and the cultures were transferred to a light intensity of $24 \mu\text{Em}^{-2}\text{sec}^{-1}$. After two more weeks, protoplast-derived microcolonies were supplemented with 1 ml of D medium (Pelletier *et al.*, 1983), and transferred to a light intensity of $70 \mu\text{Em}^{-2}\text{sec}^{-1}$. Colonies were left in those conditions until they reached 0.5 to 1.0 mm diameter, and were considered transferrable to regeneration medium.

d. Regeneration media. Colonies were large enough for transfer after approximately five weeks of culture. Regeneration media were based on MS or B5 media, supplemented with various compositions of gelling agents, carbohydrates and growth regulators. These regeneration media will be listed in the results section.

e. Rooting of plantlets and transfer to greenhouses. Regenerated plantlets were treated as described above (paragraph A.d.) for rooting and transfer to greenhouse conditions.

f. Data collection and analysis. Protoplast viability and plating efficiency were recorded after one week. Plating efficiency was defined as the percentage of dividing protoplasts divided by the total number of protoplasts plated, after one week. Viability and division frequencies were estimated by observing fluorescein diacetate (FDA) stained cells (as described in Widholm, 1972), with a Carl Zeiss inverted photomicroscope equipped with the Fluorescein filter combination (BP546/12 (green excitation)/LP590). For each treatment, the cells were counted in three microscopic fields (x100) per dish, of 3 random dishes, and the experiment repeated at least twice. These data were also binomial (alive or dead protoplasts, dividing or not). A logistic regression analysis with a logit link function was therefore undertaken, to predict the occurrence of given events, and calculate standard errors. These two values were presented in result tables. Cultures were monitored after two weeks, but at that stage, plates were overcrowded, and precise counts were impossible to obtain. The number of transferrable colonies was recorded after five weeks. Callus growth and organogenesis frequency were recorded after one month on regeneration media.

RESULTS

A. BRASSICA JUNCEA EXPLANT CULTURES.

A total of 8502 stem cortex explants, from 13 mustard genotypes, were plated on media N1, N2, I1 and I2, from either the three terminal internodes (labelled terminal internodes) or the fourth, fifth, and sixth internodes (labelled basal internodes). Data collected from all explants were combined for the comparison of medium capacity to sustain callus proliferation, root and shoot development. This was possible because the logistic regression on the percentages of explants producing callus, roots and shoots showed non-significant triple interactions between genotype, internode and medium ($X^2 = 45.88$, $X^2 = 10.75$, $X^2 = 16.09$, respectively, d.f. = 36, $p < 0.01$). Similarly, data collected from all explants were combined for the estimation of the source material effect on callus and shoot development.

a. **Effect of medium on callus, root and shoot induction.** N1 and N2 were the most favourable to callus growth, with 90% and 85% of explants producing callus, respectively. Although the assessed media, N1, N2, I1 and I2, had a highly significant effect on callus proliferation from explants ($X^2 = 14.35$, d.f. = 3, $p < 0.01$), they were all more or less suitable to callus initiation (Table 3). Growth regulator composition had more striking effects on callus quality. Explants cultured on N1 and N2 media, both containing NAA and BA, rapidly developed wet, loose, yellowish calli, which commonly reached a diameter of 3 cm within one month, and from which the original explant's

shape and organization had totally disappeared (**Fig. 2c**). In contrast, explants cultured on I1 and I2, both containing IBA and BA, developed very compact, small, dark green calli, corresponding to swelling and division of cells inside the explant, while the epidermis and general shape of explants were maintained (**Fig. 2b**). Compact callus would eventually develop after 4 to 5 weeks, from the explant's cut edges only. These calli also had a marked tendency to oxidize, become black at ends, and release oxidation products into culture media, which became brownish.

Growth regulators had drastic effects on root development ($X^2=661.87$, d.f.=3, $p<0.01$), as shown on **Figs. 2a-3d**. Large numbers of roots and root hairs (**Fig. 2a**) rapidly developed from 65.7% and 55.5% of the explants cultured on NAA-containing media (N1 and N2), covered callus surface after one month (**Fig. 2c**), and eventually covered the whole dish surface after two months (**Fig. 2e**). Not a single root was produced in IBA-containing media, I1 and I2 (**Figs. 2b, 3b, 3c, 3d**).

Table 3: Effect of culture medium and source tissue on percentage of *B. juncea* stem cortex explants giving rise to callus, root and shoot development.

medium	tissue	percentages of explants giving rise to		
		callus [s.e.]	roots [s.e.]	shoots [s.e.]
N1	T	93 [2]	70 [3]	6 [1]
	B	82 [4]	57 [4]	4 [1]
N2	T	86 [2]	57 [3]	4 [1]
	B	81 [4]	53 [4]	3 [1]
I1	T	87 [2]	0 [0]	5 [1]
	B	67 [4]	0 [0]	1 [1]
I2	T	85 [2]	0 [0]	4 [1]
	B	79 [3]	0 [0]	3 [1]

Media were modified MS (Appendix 3) with 3% sucrose and solidified with 0.8% Difco-Bacto agar. Medium N1 contained 1 mg/l NAA and 3 mg/l BA, medium N2 0.5 mg/l NAA and 5 mg/l BA, medium I1 0.1 mg/l IBA and 1.5 mg/l BA, and medium I2 contained 0.2 mg/l IBA and 3 mg/l BA. T = terminal explants. B = basal explants. Data collected from 8502 explants were combined. Data are presented as predictions (followed by standard errors), from a logistic regression model using the logit link function. The effect of medium on callus and root production was highly significant; its effect on shoot regeneration was not significant ($p < 0.01$).

Figure 2a: Callus proliferation and root development from stem cortex explants of B. juncea cv. Cutlass, after three weeks of culture on medium N1.

Figure 2b: Compact callus formation typical of stem cortex explants of B. juncea, after one month of culture on medium I2. Note the complete lack of root development.

Figure 2c: Shoot and root development, on large fluffy calli, proliferated from stem cortex explants of B. juncea cv. Blaze, after one month of culture on medium N1.

Figure 2d: Massive root production covering most callus surfaces, after five weeks of culture of B. juncea (cv. RLM 198) stem cortex explants on medium N2.

Figure 2e: Stem cortex explants after five weeks of culture on medium N1. Roots covered most of the Petri dish surface, and some abnormal leafy structures had formed. Most of these structures did not have an apex, and therefore died upon transfer to rooting medium.

Figure 2f: Regenerated explant-derived plantlet of B. juncea cv. Zaria, with elongated vitrified blades, after five weeks of culture on medium N2.

Figure 3a: Highly vitrified shoot of B. juncea cv. Blaze, regenerated from stem cortex explant culture, after five weeks on medium N2.

Figure 3b: Normal looking shoot of B. juncea cv. Blaze, regenerated from stem cortex explant culture, after five weeks on medium I2.

Figure 3c: Multiple shoot production from stem cortex explants of B. juncea cv. Zaria, after five weeks of culture on medium N2.

Figure 3d: Premature flowering of a B. juncea cv. Domo plantlet after five weeks of culture on medium I2.

The percentages of explants producing shoots depending on the culture medium, are shown on **Table 3**. These percentages are low, as they include data from all genotypes, some of which responding poorly or not at all. Shoot induction was not affected by modifications in the nature and concentration of growth regulators ($X^2=5.88$, d.f. = 3, $p < 0.01$), in contrast with their tremendous effect on root induction. However, the quality of shoots produced was clearly dependent upon the growth regulators used. Media N1 and N2, containing NAA, induced the regeneration of highly turgescient, translucent, vitrified shoots (**Fig. 2c, 3a**), often thin and elongated (**Fig. 2f**), with leaves of abnormal shape and consistency. An additional problem of N1 and N2 media was that, for certain genotypes (particularly RLM198 and RLM619, **Fig. 2e**), roots were over-produced and covered the whole callus surface, before the formation of dark small leafy structures, which often lacked a shoot meristem and consequently necrosed upon transfer to rooting medium. Media I1 and I2, containing IBA, sustained development of normal looking shoots, with no sign of vitrification, nor morphological abnormalities (**Fig. 3b, 3c, 3d**).

b. Effect of source material on callus, root and shoot induction. Callus proliferated from 88% of the 3 terminal internode explants, and from 77% of the basal internode explants, which was a highly significant difference ($X^2=24.60$, d.f. = 1, $p < 0.01$). The size, shape, consistency and colour of responding calli were not affected by the source material.

Although 33% of the terminal internode-derived explants, and 28% of the basal internode-derived explants developed roots, this difference was not significant ($X^2=4.31$,

d.f. = 1, $p < 0.01$).

Shoots developed from 48% of the terminal internode explants, and from 26% of the basal internode explants (highly significant difference: $X^2 = 16.41$, d.f. = 1, $p < 0.01$). Shoot quality was not affected by the source material.

c. Effect of genotype on callus, root and shoot proliferation. For the same reasons as above, all explants were considered to analyze the role of genotype in callus, root and shoot development. The effect of genotype on callus formation was highly significant ($X^2 = 79.35$, d.f. = 12, $p < 0.01$). The percentages of terminal explants producing callus, per genotype, indicate that the three best responding genotypes were Vniimk351 (97%), Colman's (96%), and Cutlass (94%). Callus was still induced from 69% of explants from the least responding genotype, Zem. Genotype also had a marked effect on root development ($X^2 = 113.85$, d.f. = 12, $p < 0.01$). The percentages of terminal explants producing roots on media N1 and N2, per genotype, indicate that the three best responding genotypes were Colman's (96%), Cutlass (90%) and RLM198 (84%). UC-Davis11 was the least responding genotype, with only 14% of the terminal explants producing roots.

Interestingly, genotype also had a marked effect on shoot regeneration ($X^2 = 172.41$, d.f. = 12, $p < 0.01$). It contributed most to the total X^2 value for shoot production, followed by internodes, then by media. The percentages of terminal explants producing shoots per genotype (**Figure 4**), indicate that the three best responding genotypes were Blaze (14%), Forge (11%) and Zaria (11%). No explant from the genotypes Zem and Vniimk351 produced shoots.

The percentages of organogenic calli, classified with regards to the number of shoots regenerated per callus, are shown on Table 4. As would be expected, genotypes from which less explants produced shoots, also sustained less shoots per explant; UC-Davis 11, Jubilejnaja and Donskaja never developed more than 4 shoots per explant. However, there was no statistical difference between genotypes from which explants yielded 5 to 10, and 10 or more shoots (Fig. 3c), with regards to numerous shoot production ($X^2=12.07$ and $X^2=13.34$, d.f.=10, $p<0.01$). The production of 5 to 10, and 10 or more shoots per explant was not influenced either by media ($X^2=2.81$ and $X^2=5.14$, d.f.=3, $p<0.01$), nor by internodes ($X^2=0.045$ and $X^2=5.16$, d.f.=1, $p<0.01$).

The estimated percentages of Fig. 4 were based on the number of responding explants, over the total number of plated explants. When results were expressed as percentages of the total number of shoots regenerated, over the number of explants proliferating callus, it was evident that Blaze was quite superior to other cultivars, with a shoot regeneration frequency of 51%, but the overall genotype classification for shoot initiation remained unchanged.

Plantlets were transferred to short day conditions (8 h photoperiod) as soon as the two first leaves were formed, to avoid premature flowering, which was otherwise commonly observed (Fig. 3d).

Figure 4: Effect of genotype on shoot production from *B. juncea* terminal stem cortex explants.

Table 4: Percentages of organogenic calli from *B. juncea* stem cortex explants, classified with regards to the number of shoots regenerated per callus.

genotype	shoot producing calli (%)							
	number of shoots per callus							
	1	2	3	4	5	5+	10	10+
Blaze	34	19	13	2	2	10	1	19
Zaria	33	17	11	4	0	15	0	19
Forge	32	14	14	4	7	23	2	4
RLM 619	42	19	10	10	0	10	0	10
Cutlass	50	23	12	6	6	3	0	0
RLM 198	42	17	0	8	0	17	0	17
RLM 514	18	27	27	9	0	9	0	9
Donskaja	55	27	18	0	0	0	0	0
Domo	0	60	20	0	0	20	0	0
Jubilejnaja	50	0	0	50	0	0	0	0
UC Davis 11	100	0	0	0	0	0	0	0

Data from 8502 explants were combined.

Table 5: *Brassica juncea* stem cortex explant-derived calli, regenerated shoots, and percentage of shoot regeneration per genotype as expressed by the number of shoots regenerated from one hundred calli.

genotype	total number of calli	total number of shoots	% shoot regeneration
Blaze	987	499	51
Zaria	670	304	45
Forge	396	159	40
RLM 619	418	97	23
RLM 198	318	47	15
Cutlass	558	69	12
RLM 514	435	38	9
Domo	442	21	5
Donskaja	492	18	4
Jubilejnaja	430	5	1
UC Davis 11	621	2	0
Vniimk 351	498	0	0
Zem	892	0	0

d. Rooting of regenerated shoots and acclimation to growth chamber conditions.

Except for stunted vitrified leaf structures such as those shown on Fig. 2e, regenerated shoots were easily rooted in growth regulator-free medium, even though two to three transfers were required for rooting heavily vitrified material. They all survived transfer to Jiffy 7 peat pellets, and were successfully acclimated to growth chamber conditions.

B. BRASSICA JUNCEA PROTOPLAST CULTURES.

Stem cortex explant culture allowed the selection of genotypes with good shoot regeneration potential, namely Blaze, Zaria and Colman's, according to Tables 4-5 and Fig. 4. Blaze was the most commonly used genotype for protoplast culture, since it was the best responding.

a. Wall-digesting enzymes. Overnight incubation in wall-digesting enzyme mixtures of 1% macerozyme and 0.5% cellulase resulted in high protoplast yields, as shown on Table 6. Protoplast populations were heterogeneous in size, ranging from 15 to 40 μm , and in appearance, from highly cytoplasmic (usually the small ones) to highly vacuolated. Protoplast yield was highly affected by the addition of salts, but not by halving enzyme concentrations. Cell viability and divisions after one week were not different ($X^2=3.39$, d.f.=4, $p<0.01$), however, the colony development after 45 days was much superior in cultures digested with 1% macerozyme and 0.5% cellulase, rather than with 0.5% macerozyme and 0.25% cellulase (Table 6).

Table 6: Effect of enzyme solution on *B. juncea* (cv. Blaze) protoplast yield, cell viability after one week, and colony formation after 45 days of culture.

enzyme solution	protoplasts/g (f.w.)	cell viability (%)	standard error (%)	colony formation after 45 days (per dish)
N	$4 \times 10^6 - 8 \times 10^6$	80	3	4000 yellow-green col. (100-200 cells)
N + salts	$3 \times 10^6 - 6 \times 10^6$	73	3	3200 yellow-green col. (100-200 cells)
N + 0.05 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0	-	-	-
1/2 N	$4 \times 10^6 - 8 \times 10^6$	68	4	isolated cells preponderant. 2000 translucent col. (20-40 cells)
1/2 N salts	$5 \times 10^5 - 1 \times 10^6$	80	3	isolated cells preponderant. small translucent col. (up to 20 cells)

N = 0.5% cellulase RS, 1% macerozyme R10, 0.4 M mannitol. 1/2 N = 0.25% cellulase RS, 0.5% macerozyme R10, 0.4 M mannitol. salts = 3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 mM KH_2PO_4 . Yield was calculated from haemocytometer counts, with each Petri dish containing 0.3 to 0.6 g (f.w.) of material in 15 ml of enzymes.

Numbers of colonies formed after 45 days were estimated by counting colonies visible to the bare eye, on 1/4 of the dish surface. 1.5 ml of protoplast suspension were plated per dish. There was no significant effect of enzyme solution on cell viability after one week ($p < 0.01$).

Interestingly, a calcium chloride concentration of 0.05 M totally inhibited cell wall digestion (Table 6).

b. Protoplast washing procedure. The suspensions were routinely filtered through nylon mesh and centrifuged, resulting in the separation of a protoplast pellet, and the supernatant, in which most dead cells would remain. Still, a fair amount of debris and some xylem vessels also pelleted, which were suspected to impair further cell viability and division. Flotation protocols were therefore assayed, over a 0.4 M or a 0.6 M sucrose solution, or between Percoll discontinuous gradients, with the aim of obtaining purified protoplast preparations. The use of a 0.4 M solution resulted in all the protoplast pelleting. Viability of protoplasts floated over 0.6 M sucrose was 92%, and debris were virtually eliminated. Figure 5a shows viable protoplasts, which fluoresce after FDA staining; when protoplasts were faintly illuminated with visible light, dead protoplasts could also be counted (Fig. 5b). On the other hand, yield was 40 times lower than for pelleted protoplasts, as three quarters of the protoplasts did not float and were lost (Table 7). Protoplasts floated over sucrose had a cell viability after one week of 87%, which was significantly higher than the 75% viability of pelleted protoplasts ($X^2=19.76$, d.f=3, $p<0.01$), but cell division after one week was not significantly different (Table 7). In addition, the appearance of brown exudate (Fig. 5c), the swelling and budding of some giant cells which would never divide (Fig. 5d), were not affected by the initial purity of the protoplast suspension, and were still problematic. Floating over Percoll was not advantageous either, since all cell material collected at one interface, between 20% and 30% Percoll, debris were not separated from viable

protoplasts, while viability and division after one week were lower (Table 7).

c. Protoplast density. Densities of 2×10^4 protoplasts/ml or less, were too low to sustain viability and high division frequencies (Table 8). Cell viability after one week was highest for a 5×10^5 protoplasts/ml plating density, but so were problems of swelling protoplasts and brown exudate production, and after two weeks, the plates became overcrowded with small colonies that eventually turned brown and died. A protoplast density of 1×10^5 was thus routinely plated.

d. The use of an agarose-solidified underlayer. The presence of an agarose-solidified underlayer was significantly beneficial to cell viability after one week ($X^2=20.44$, d.f.=1, $p<0.01$), and doubled division rates (Table 9; $X^2=26.08$, d.f.=1, $p<0.01$). Underlayers composed of X or B media, with either 0.2 M or 0.4 M glucose, were compared. The molarity of glucose had a significant effect on cell viability after one week ($X^2=6.725$, d.f.=1, $p<0.01$), however, the logistic regression also showed a significant double interaction between molarity and medium ($X^2=11.95$, d.f.=1, $p<0.01$), therefore, it could not be decided which of the two molarities was most beneficial. There was no significant difference between media X and B, however, medium X with 0.4 M glucose sustained slightly higher division rates (Table 9), and was therefore chosen as a routine medium for underlayers.

Figure 5a: Protoplasts stained with FDA after plating into culture medium, seen under a Zeiss fluorescent microscope equipped with the Hoechst filter combination (x100).

Figure 5b: Protoplasts stained with FDA after plating into culture medium, seen under a Zeiss fluorescent microscope equipped with the Hoechst filter combination, slightly illuminated with visible light in order to visualize dead protoplasts and cell debris (x100).

Figure 5c: Budding giant cell, which floated to the medium surface and never divided (x400).

Figure 5d: Brown precipitate, suspected to consist of polyphenol oxidation products (x400).

Table 7: Effect of washing procedure at isolation on *B. juncea* (cv. Blaze) protoplast yields, cell viability and division rates.

cleaning	pp yield/g (fw)	protoplast viability at isolation (%)	cell viability 1 week [s.e.(%)]	cell division 1 week (%) [s.e.(%)]
percoll ⁽¹⁾	4-8 x 10 ⁶	74	69 [6]	12 [2]
sucrose ⁽²⁾	1-2 x 10 ⁵	92	87 [2]	16 [1]
pellet ⁽³⁾	4-8 x 10 ⁶	86	75 [1]	18 [3]
sucrose pellet ⁽⁴⁾	3-6 x 10 ⁶	64	76 [4]	14 [2]

Culture medium was B, described on Appendix 6.

⁽¹⁾ interface between 20% and 30% Percoll.

⁽²⁾ interface between 0.6 M sucrose and CPW salt solution (Appendix 7).

⁽³⁾ pellet after centrifugation in B medium.

⁽⁴⁾ pellet after centrifugation in a 0.6 M sucrose solution.

Washing procedure had a significant effect on protoplast viability but not on division rates ($p < 0.01$).

Table 8: Effect of plating densities (protoplasts/ml) on viability and division of *B. juncea* (cv. Zaria) protoplasts.

density (protoplasts/ml)	cell viability (%)	cell division (%)
5×10^3	0	0
1×10^4	19	5
2×10^4	27	15
5×10^4	59	25
7×10^4	54	27
1×10^5	57	24
5×10^5	75	17

Protoplasts were plated over an agarose underlayer. Results were taken after two weeks, from two replicate experiments.

Table 9: Effect of agarose underlayer on viability and division of *B. juncea* (cv. Blaze) protoplasts, after one week of culture.

underlayer	cell viability (%) [s.e.]	cell division (%) [s.e.]
no underlayer	53 [2]	8 [1]
X 0.4 M glucose	62 [2]	15 [1]
X 0.2 M glucose	60 [2]	12 [1]
B 0.4 M glucose	55 [2]	11 [1]
B 0.2 M glucose	65 [2]	14 [1]

Data were recorded from two independent experiments. 1.5 ml of protoplast suspensions were plated over 2 ml of agarose-solidified (0.4%) underlayer. 3 ml were plated for the control. The presence of an agarose-solidified underlayer was significantly beneficial to viability and division ($p < 0.01$). The nature of medium had no influence.

Table 10: Effect of initial volume of protoplast suspension plated per dish, on viability and cell division of *B. juncea* protoplasts (cv. Blaze, Zaria, Colman's and Domo).

variety	volume (ml)	cell viability (%) [s.e.]	cell division (%) [s.e.]	visible colonies after 60 days (per dish)	transferable colonies after 60 days (per dish)
Blaze	1.5	61 [2]	17 [2]	3200	450
	3.0	52 [4]	16 [3]	8000	25
Domo	1.5	48 [6]	14 [4]	1800	83
	3.0	36 [4]	11 [2]	3500	78
Forge	1.5	67 [2]	9 [1]	2500	500
	3.0	64 [3]	12 [2]	7000	52
Zaria	1.5	64 [2]	22 [2]	2600	240
	3.0	68 [2]	18 [2]	8500	31

Viability and divisions were recorded after one week in medium B. Protoplasts were plated over an agarose-solidified underlayer. Initial volume had a significant effect on protoplast viability and division rates ($p < 0.01$).

e. Effect of initial volume of protoplast suspension plated per dish. Highly significant differences were recorded between viabilities and division rates after one week of cultures plated at 1.5 ml or 3.0 ml per dish ($X^2=64.88$ and $X^2=35.39$, d.f. = 3, $p < 0.01$; Table 10). Moreover, dishes in which 3 ml of protoplast suspension had been plated, rapidly became overcrowded with small colonies, most of which would never exceed a 0.5 mm size, and would necrose upon transfer to regeneration medium.

Consequently, although the number of visible colonies per dish after two months was proportional to the plated volume, transferrable colonies were up to eighteen fold higher in 1.5 ml containing dishes. Colonies frequently grew faster and greener at the edge of petri dishes, where they just skimmed the culture medium without being covered.

f. Comparison of two culture media for initial plating. Medium X was developed by Sproule (1987) for B. juncea (cv. Domo) stem cortex-derived protoplasts, and is described in Appendix 5. Medium B was developed by Pelletier *et al.* (1983) for B. napus mesophyll protoplasts, and is described in Appendix 6. No significant difference was detected between these two media for sustaining protoplast viability and division after one week ($X^2=0.655$ and $X^2=1.909$, d.f. = 1, $p < 0.01$). Giant distorted cells that do not divide were more often observed in medium X, and colonies tended to agglutinate, so that accurate cell counts were difficult. In medium B, cells were often elongated, developing into loose colonies. Medium B was routinely used thereafter.

Table 11: Viability and division of B. juncea (cv. Blaze) protoplasts after one week of culture, depending on medium.

medium	cell viability (%) [s.e]	cell division (%) [s.e]
X	63 [1]	18 [1]
B	65 [1]	20 [1]

Data from 7 independent experiments were combined. Media X and B are described on Appendices 5 and 6. 1.5 ml of protoplast suspension were plated over agarose-solidified underlayers. Medium had no effect on cell viability nor division rates ($p < 0.01$).

g. Variations of calcium and magnesium concentrations in initial culture

medium. Media supplemented with various concentrations of calcium chloride, and either 0.2 mM or 2.0 mM magnesium sulphate were assessed (Table 12). Medium had highly significant effects on cell viability and divisions after one week ($X^2=26.93$, d.f.=9, $p<0.01$), the calcium and magnesium-free medium being non-supportive. However, the logistic regression model also showed a highly significant interaction between media and the replicate dishes for viability ($X^2=75.54$, d.f. = 10, $p<0.01$) and division ($X^2= 38.04$, d.f. = 10, $p<0.01$), meaning that internal variations amongst dishes for the same medium, were at least as high as variations observed between media. The number of visible colonies after 45 days was highly variable (Table 12), however, without a clear correlation with divalent ion concentrations.

h. Transfer to plant regeneration media. Only 6% of 0.5 mm transferred colonies survived and proliferated into callus, whereas 80% of the older, larger transferred colonies would develop callus (Table 13 and Fig. 6c). Thereafter, colonies reached at least 1.0 mm diameter, after 45 days of culture, before being transferred onto regeneration medium. When solidifying agents were tested for their capacity to sustain callus growth and plant regeneration, 1% of agarose SeaPlaque proved to be most beneficial to the development of large green calli ($X^2=29.29$, d.f. = 1, $p<0.01$), as shown on Table 14 and Fig. 6d, although the percentage of calli produced was unchanged ($X^2=0.005$, d.f. = 1, $p<0.01$). The media tested on Table 14 had no effect on the frequency of callus production ($X^2=7.215$, d.f.=3, $p<0.01$),

Table 12: Effect of various concentrations of calcium and magnesium ions in initial culture medium on viability and division of *B. juncea* (cv. Blaze) protoplasts.

MgSO ₄ ·7H ₂ O (mM)	CaCl ₂ ·2H ₂ O (mM)	cell viability(%) [s.e.]	cell division(%) [s.e.]	colonies/dish after 45 days [s.e.]
0.2	0.5	60 [3]	2 [0]	500 green [15.8] 32 transferable
0.2	1.0	59 [3]	4 [0]	250 brown [11.2]
0.2	3.0	51 [2]	1 [0]	175 small brown [9.4]
0.2	6.0	57 [2]	1 [0]	450 green [15.0] 56 transferable
2.0	0.5	50 [4]	0 [0]	96 small yellow [6.9]
2.0	1.0	57 [3]	2 [0]	150 small yellow [8.7] 8 transferable
2.0	3.0	55 [2]	2 [0]	135 small yellow [8.2] 40 transferable
2.0	6.0	64 [2]	1 [0]	30 small green [3.9]
0.0	0.0	19 [2]	0 [0]	1 large green [0.7] 1 transferable
0.25*	8.0*	61 [2]	4 [0]	600 green [17.3] 42 transferable

Transferable colonies were green, and 1.5 mm of diameter or more. Basal medium was S1 (Schenck and Röbbelen, 1982). *Control: medium X. Protoplasts were then fed with C and D, described on Appendix 6. The effect of calcium and magnesium was not significant after one week ($p < 0.01$).

but larger calli were produced in the 2,4-D containing medium solidified with agarose. No significant interaction between medium and solidifying agent was detected. **Figure 6e** shows that microcolonies developed into actively growing calli upon transfer to medium E (Pelletier, 1983), but organogenesis was not stimulated. Media containing B5 salts and vitamins, with 3% sucrose, 1 mg/l NAA and 3 mg/l BA, did not stimulate more than 1.7% shoot production.

Tables 15-18 show various regeneration media, all based on MS, where the effects of gelling agents, carbohydrates, and growth regulators, were assessed. Callus development was best achieved with 3% sucrose, or 1% sucrose with 2% glucose or mannitol. Typical calli were produced depending on the medium (**Fig. 7a-7g**). Shoot development was promoted by NAA and BA-containing media, or by IBA and either BA, zeatin or TZ-containing media. The maximum shoot regeneration frequency was 7.5% (**Table 15**). The addition of 0.02 mg/l GA3 to N1 (agar-solidified) did not affect organogenesis. All shoots were highly vitrified, but were easily rooted on growth-regulator-free B5 medium, then acclimated to growth chamber conditions.

Table 13: The effect of age and size of *B. juncea* protoplast-derived colonies, when transferred to regeneration medium, on callus proliferation.

age (days)	colony diameter (mm)	colonies developing callus (%)	standard error (%)
30	0.5 - 1.0	6	3
45	0.8 - 1.2	81	5
60	1.0 - 3.0	79	6

Data were collected three weeks following transfer of microcolonies to regeneration medium. Medium was N1 (described in Table 3), solidified with 1% agarose SeaPlaque. A minimum of 100 colonies was plated per treatment. The effect of diameter on callus development was highly significant ($p < 0.01$).

Table 14: The effect of medium and solidifying agent on callus development from *B. juncea* protoplast-derived colonies.

medium	colonies developing callus (%)		callus diameter (mm)*	
	agar [s.e.]	agarose [s.e.]	agar [s.e.]	agarose [s.e.]
1	71 [6]	67 [7]	8 [0]	13 [0]
2	70 [6]	72 [6]	7 [0]	14 [0]
3	67 [7]	66 [7]	5 [0]	14 [0]
4	80 [6]	84 [5]	3 [0]	24 [0]

50 colonies were plated per medium. Media were MS with 1% sucrose and 2% mannitol, solidified with 1% agarose SeaPlaque or 0.8% Difco-Bacto agar. Growth regulators were, in mg/l: medium 1 = 1 NAA + 3 BA; medium 2 = 1 NAA + 1 BA; medium 3 = 0.2 IBA + 1 BA; medium 4 = 0.05 2,4-D + 0.5 NAA + 3 BA. Data were collected 3 weeks after transfer to regeneration media. The solidifying agent and culture medium had no significant effect on the percentage of callus production, but had a highly significant effect on callus diameter ($p < 0.01$).

* Regression analysis: Gamma distribution, reciprocal link function.

Figures 6a to 6e: Brassica juncea cv. Blaze protoplast-derived colonies.

Figure 6a: After 45 days of culture. Plating efficiency was such, that the development of overcrowded colonies was reduced (x1.6).

Figure 6b: After 45 days of culture. Colony size was inversely proportional to their number (x1.6).

Figure 6c: Top dish: After 60 days of culture. Bottom dish: After 60 days of culture, and two weeks after transfer to regeneration medium E (x1).

Figure 6d: Left: After 3 weeks on regeneration medium (N1, solidified with 1% SeaPlaque agarose). They had been transferred as soon as they had reached a diameter of 0.5 mm, after 30 days of culture. Right: After 3 weeks on regeneration medium (N1, solidified with 1% SeaPlaque agarose). They had been transferred as soon as they had reached a diameter of 1.0 mm, after 30 days of culture (x1).

Figure 6e: Left: After 3 weeks on regeneration medium N1, solidified with 0.8% Difco-Bacto agar. Right: After 3 weeks on regeneration medium N1, solidified with 1.0% SeaPlaque agarose (x1).

Figure 7a to 7g: Brassica juncea cv. Blaze protoplast-derived calli, depending on the regeneration medium.

Figure 7a: Callus typically produced after one month of culture on media containing low levels of carbohydrates. Medium was N1, solidified with 0.8% Difco-Bacto agar, with 0.5% glucose (x3.5).

Figure 7b: Callus typically produced after one month of culture on media containing IBA as an auxin source (x3.5).

Figure 7c: Root-producing callus typically produced after one month of culture on media containing NAA as an auxin source (x3).

Figure 7d: Light green compact dry callus, typically produced after one month of culture on media containing 2,4-D as an auxin source (x4).

Figure 7e: Humid shiny compact globular callus, typically produced after one month of culture on media containing 2ip (x4).

Figure 7f: Light green fluffy humid callus, typically produced after one month of culture on media containing NAA and BAP as growth regulators (x3).

Figure 7g: Early shoot development after one month of culture on N1, solidified with 1% SeaPlaque agarose (x7).

Table 15: The effect of carbohydrate composition of regeneration medium on callus proliferation and differentiation from *B. juncea* (cv. Blaze) protoplast-derived colonies.

#	% sucrose	% glucose	% mannitol	% callus	% roots	% shoots
1	1	-	-	67	43	0
2	3	-	-	80	53	7
3	-	1	-	43	13	0
4	-	3	-	63	3	0
5	-	-	1	23	*	0
6	-	-	3	13	*	0
7	1	2	-	93	30	0
8	3	1	-	97	63	3
9	1	3	-	87	33	0
10	3	3	-	53	20	0
11	1	-	1	*	*	0
12	1	-	2	85	45	8
13	1	-	3	63	*	0
14	3	-	1	63	*	0
15	3	-	3	*	*	0
16	-	1	2	57	0	0
17	-	2	2	73	33	0

Medium was modified MS, 3 mg/l BA, 1 mg/l NAA, 0.8% Difco-Bacto agar. At least 30 colonies were plated per medium.

Table 16: The effect of solidifying agent on callus proliferation and differentiation from *B. juncea* (cv. Blaze) protoplast colonies.

#	% agar	% agarose	% gelrite	% callus	% roots	% shoots
18	0.4	-	-	62	*	0
19	0.8	-	-	76	*	6
20	1.0	-	-	68	*	2
21	-	0.4	-	72	*	0
22	-	0.8	-	60	*	0
23	-	1.0	-	78	*	4
24	-	-	0.2	60	*	0
25	-	-	0.4	18	*	0
26	-	-	0.8	56	*	0

Medium was modified MS, 3% sucrose, 3 mg/l BA, 1 mg/l NAA. 50 colonies were plated per medium.

Tables 15 and 16: - represents absence of growth regulator. * represents missing data.

Table 17: The effect of auxin and cytokinin combinations on callus proliferation and differentiation from *B. juncea* protoplast-derived colonies.

#	auxins (mg/l)				cytokinins (mg/l)			% callus	% roots	% shoots
	N	Ib	Ia	D	B	Z	TZ			
solidified with 0.8% (w/v) Difco-Bacto agar; 3% (w/v) sucrose										
N1	1	-	-	-	3	-	-	67	27	4
N2	0.5	-	-	-	5	-	-	74	34	4
I1	-	0.1	-	-	1.5	-	-	89	0	6
I2	-	0.2	-	-	3	-	-	77	0	0
27	1	-	-	-	1	-	-	63	13	3
28	0.02	-	-	1	1	-	-	83	0	3
solidified with 1% (w/v) SeaPlaque agarose; 3% (w/v) sucrose										
29	0.5	-	-	-	3	-	-	72	10	0
30	0.5	-	-	0.05	3	-	-	75	2	0
31	0.5	-	-	-	5	-	-	60	18	5
32	1	-	-	-	1	-	-	65	35	0
33	1	-	-	-	3	-	-	57	10	0
34	2	-	-	-	1	-	-	72	45	0
35	2	-	-	-	2	-	-	38	10	0
36	2	-	-	-	3	-	-	65	18	0
37	3	-	-	-	1	-	-	62	38	0
38	3	-	-	-	3	-	-	67	23	0
39	-	0.1	-	-	1.5	-	-	87	*	0
40	-	1	-	-	-	1	-	77	*	3
41	-	2	-	-	-	1	-	53	*	0
42	-	1	-	-	-	3	-	70	*	0
43	-	2	-	-	-	3	-	77	*	0
44	1	-	-	-	-	3	-	93	17	3
45	2	-	-	-	-	1	-	73	10	0
46	2	-	-	-	-	3	-	83	*	0
47	1	-	-	-	-	-	5	83	*	0
48	2	-	-	-	-	-	5	87	*	0
49	-	1	-	-	-	-	5	97	*	0
50	-	2	-	-	-	-	5	73	*	0
51	-	-	1	-	1	-	-	80	0	0
52	-	-	2	-	3	-	-	67	*	7
53	1	-	-	1	1	-	-	87	*	0
54	-	-	1	1	1	-	-	80	*	0
55	-	-	1	1	-	1	-	77	*	0

Table 17 (cont.): The effect of auxin and cytokinin combinations on callus proliferation and differentiation from *B. juncea* protoplast-derived colonies.

#	auxins (mg/l)				cytokinins (mg/l)			% callus	% roots	% shoots
	N	Ib	Ia	D	B	Z	TZ			
solidified with 0.8% Difco-Bacto agar; 1% sucrose, 2% mannitol										
56	0.5	-	-	-	3	-	-	77	17	3
57	0.5	-	-	0.05	3	-	-	77	0	0
58	1	-	-	-	3	-	-	67	13	0
59	-	0.1	-	-	1.5	-	-	77	0	3
60	-	0.5	-	-	1	-	-	60	0	0
61	-	1	-	-	1	-	-	53	0	0
52	-	2	-	-	1	-	-	*	*	0
63	-	0.5	-	-	3	-	-	63	0	0
64	-	2	-	-	3	-	-	60	*	0
solidified with 1% SeaPlaque agarose; 1% sucrose, 2% mannitol										
65	0.5	-	-	-	3	-	-	68	25	0
66	0.5	-	-	0.05	3	-	-	78	12	2
67	-	0.1	-	-	1.5	-	-	63	0	7
68	-	1	-	-	-	3	-	87	0	0
69	1	-	-	-	-	3	-	90	13	3
70	-	1	-	-	-	-	5	67	3	3
71	1	-	-	-	-	-	5	93	0	0
72	0.2	-	-	-	0.2	-	-	53	7	3
73	0.4	-	-	-	0.2	-	-	67	27	0
74	0.6	-	-	-	0.2	-	-	73	17	0
75	0.2	-	-	-	0.6	-	-	67	13	0
76	0.4	-	-	-	0.6	-	-	67	13	0
77	0.6	-	-	-	0.6	-	-	73	33	3

Medium was modified MS. N=NAA; Ib=IBA; Ia=IAA; D=2,4-D; B=BA; Z=zeatin; TZ=thidiazuron. 30 colonies were plated per medium.

- represents absence of growth regulator. * represents missing data.

Table 18: The effect of cytokinins on *B. juncea* (cv. Blaze) protoplast-derived callus production and shoot regeneration.

#	Z	2ip (mg/l)	B	K	TZ	% callus	% shoots
78	-	1	-	-	-	*	0
79	-	2	-	-	-	*	0
80	1	1	-	-	-	53	7
81	1	2	-	-	-	63	3
82	3	1	-	-	-	*	0
83	3	2	-	-	-	*	0
84	-	-	1	-	-	*	0
85	-	-	3	-	-	*	0
86	1	-	-	-	-	*	0
87	3	-	-	-	-	*	0
88	1	-	3	-	-	*	0
89	1	-	0.5	-	-	63	7
90	-	-	-	-	1	*	0
91	-	-	-	-	5	77	3
92	-	-	-	-	10	*	0
93	-	-	1	-	5	*	0
94	-	-	3	-	5	*	0
95	-	-	-	1	-	*	0
96	-	-	-	3	-	*	0
97	-	-	0.5	3	-	*	0

Medium was modified MS. Z=zeatin; B=BA; K=kinetin; TZ=thidiazuron.

A minimum of 30 colonies was plated per medium.

- represents absence of growth regulator. * represents missing data.

DISCUSSION

Brassica juncea stem cortex explant culture. The first step of the project consisted in assessing the effect of genotype on the plant regeneration potential of B. juncea. Dale and Jones (1982) have used explant cultures to assess the potential response of protoplasts from various tissues of Lolium multiflorum, with the assumption that non-responsive tissues in explant cultures were unlikely to produce responsive protoplasts. Thirteen genotypes of B. juncea were surveyed under the same conditions, for their regeneration potential from stem cortex explants, with the assumption that it would provide a good indication of stem cortex protoplast behaviour, for a given genotype. Explants from all genotypes were simultaneously cultured on four media, differing in their growth regulator composition, in order to examine potential interactions between culture condition and genotype.

Growth regulators had a marked effect on the quality of calli and quantity of roots produced: NAA-containing media sustained the development of larger, more prolific calli, than those sustained by IBA-containing media. Spectacular root formation, stimulated by NAA-containing media, has been reported before. Dietert *et al.* (1982), working with hypocotyl explants of a number of Brassica species, noticed that NAA would promote root over-production, unless 0.5 μ M 2,4-D was also included in the regeneration medium. The auxin NAA also induced rooting of B. juncea (var. RAI-5) cotyledonary explants (George and Rao, 1980), but has nevertheless been commonly considered essential to shoot regeneration from explant cultures of Brassica species

(Klimaszewska and Keller, 1985; Kirti and Chopra, 1989a) usually in combination with BA (Hachey *et al.*, 1991; Yang *et al.*, 1991; Narasimhulu and Chopra, 1988). On the other hand, Sharma *et al.* (1990) reported that concentrations as low as 0.5 μ M NAA favoured the formation of friable callus that would not regenerate, from *B. juncea* cotyledonary explants. The auxin IBA has not been commonly used for *Brassica* explant cultures. However, in this report, IBA-containing media also sustained good shoot regeneration, without the inconveniences of root over-production and of shoot vitrification. Vitrification is a common anomaly of *in vitro*-grown shoots, which are swollen, water logged, deficient in chlorophyll and lignification. It seems to be correlated with a decreased activity of the enzyme p-coumarate CoA-ligase, a key enzyme for lignification (Phan, 1991). Phan (1991) reported a strong impact of BA on the occurrence of vitreous plants from *Malus sylvestris* explant cultures. In this study, media N1 and I2 both contained 3 mg/l BA, and only differed in the nature and concentration of auxins. Still, the shoots produced on N1 were highly vitrified, those produced on I2 were normal, which suggests that NAA might favour vitrification.

All genotypes reacted similarly to modifications in growth regulator composition, and no interaction between medium and genotype was detected.

The superiority of the three terminal stem internode explants over basal internodes for rhizogenesis and organogenesis was not surprising, and in accordance with Klimaszewska and Keller (1985), for *B. napus* stem cortex explants. Less differentiated and more metabolically active tissues are recognized to respond better to *in vitro* culture. Indeed, successful explant cultures of *B. juncea* have always involved seedling material,

either cotyledons or hypocotyls (Table 1). This is the first time successful stem cortex explant culture of B. juncea has been reported.

Callus proliferation, and mostly root and shoot production, were all influenced by genotype. Interestingly, the behaviour of genotypes concerning callus proliferation or root initiation did not reflect shoot production. Although genetic variations have frequently been reported for the plant regeneration potential of B. juncea cultures, the only thorough comparison has been reported by Fazekas *et al.* (1986), who compared 21 lines (8 brown-seeded and 13 yellow-seeded) for regeneration from cotyledonary explants. They showed that there is a clear correlation between taxonomic group and shoot regeneration from explants plated *in vitro*: 20 to 50% of cotyledon explants from brown-seeded Indian lines gave rise to shoots (except for one, which was of Chinese accession), as compared to only 0 to 12% from yellow-seeded Chinese lines. In the present study, the brown-seeded Blaze produced 47 shoots from 100 explants (50.6 shoots from 100 calli, Table 5). However, the second and third best responding genotypes, Zaria (45.4 shoots from 100 calli) and Forge (40.2 shoots from 100 calli), were yellow-seeded, and of Chinese/Eastern Europe origin. The other Chinese/Russian lines tested behaved poorly. This is in accordance with Hui and Zee (1978), who assessed the response of hypocotyl explants from four B. juncea cultivars, namely South Wind, Haka, Swatow, and Mooi Ts'ol, all Chinese and closely related, and noticed a much superior regeneration potential of South Wind, while the three others performed poorly. Kirti and Chopra (1989a) also noticed major differences amongst closely related Indian cultivars, with 32% regeneration for cv. RLM198, whereas only 1% of the

explants gave rise to plants for cv. RLM514, and none for cv. RLM619, respectively. In this study, however, 100 calli of RLM619, RLM198 and RLM514 produced 23, 15 and 9 shoots, respectively. The discrepancies between these results and those obtained by Kirti and Chopra (1989a) stress the importance of comparing genotypes simultaneously under strictly identical conditions, in order to eliminate environmental factors such as medium composition and the physiological status of explants. A generalized statement such as expressed by Fazekas *et al.* (1986), directly linking taxonomic origin and shoot differentiation, could not be drawn from the results presented here.

Brassica juncea stem cortex protoplast culture. The stem cortex protoplast culture system was developed for B. napus by Klimaszewska and Keller (1987), and first assessed for B. juncea by Sproule (1987). The factors studied by Sproule have included pretreatment before digestion; tissue density during enzymatic digestion; physical culture conditions; organic supplements such as folic acid, biotin, glycine, glutamine, serine and glutathione in the initial culture medium; various media for callus survival and plant regeneration. However, most of the results have been difficult to interpret due to the use of a non-supportive basal medium in which few protoplasts would survive. Good protoplast survival has been obtained after the basal medium was improved by modifying concentrations of calcium and magnesium ions, and microcalli could be transferred to plant regeneration medium. These microcalli were very recalcitrant to shoot regeneration, despite numerous varying compositions of growth regulators, and with shock treatments such as high temperature or absence of a carbon source for several

weeks (Sproule, 1987).

The limiting factors for plant regeneration from B. juncea stem cortex protoplasts were unexplained, and might have resulted from the choice of genotype, the source material, or the experimental procedure. The genotype used in this system was chosen from the stem cortex explant survey described earlier. Stem cortex proved to be an excellent protoplast source for B. napus (Klimaszewska and Keller, 1987). I therefore concentrated on assessing critical factors of the experimental protocol.

Protoplast viability was first monitored with FDA, which is a non-polar molecule and readily enters the cells. In live cells, esterases cleave off the acetate groups, and the polar fluorescein molecules accumulate in the cell (Widholm, 1972). Although esterases can still be active in dead cells, this technique was considered accurate enough for the purposes of this study. With practice, it was possible to recognize most dead cells without FDA staining, due to their shrunken, dense appearance, and irregular shapes, so that counts were carried out under visible light. Viable protoplasts were readily isolated from stem cortex tissue of B. juncea, cv. Blaze, Zaria and Colman's. They always consisted of heterogeneous populations of varying sizes and cytoplasmic density, as is commonly observed in Brassica (Pua, 1990; Jaiswal et al., 1990; Kirti and Chopra, 1989b; Glimelius, 1984). A few giant distorted cells, which never divided, always appeared after a few days, but did not hinder division of the other cells. Glimelius (1984) and Pua (1990) observed that large, vacuolated protoplasts would divide slowly, if at all. The enzymatic digestion was routinely carried out overnight. For such long periods, it is commonly advised to add salts, or culture medium components to the

enzymatic mixture in order to stabilize the plasma membrane, and to stimulate cell division (Schenck and Hoffman, 1984; Pelletier *et al.*, 1983; Cassells and Cocker, 1982). In the present system, however, the addition of calcium and magnesium salts led to a decrease in protoplast yield (Table 6), and colony formation after 45 days was not improved. This contrasts with Rose (1980), who observed that 7 mM Ca^{++} , or 7 mM Mg^{++} in the enzyme solution increased yield and viability of mesophyll protoplasts of spinach. Since magnesium could substitute for calcium, he concluded that protoplast stability was due to a general divalent cation effect, for example through their binding to negatively charged membrane groups such as phosphates. Enzyme concentrations should be as low as possible, to minimize cellular damage due to impurities found in commercial enzymes. Mesophyll protoplasts of *B. juncea* have been isolated with as little as 0.25% R10 cellulase and 0.05% macerozyme (Eapen *et al.*, 1989), and Kirti and Chopra (1989b) have isolated hypocotyl protoplasts with 1% R10 cellulase and 0.1% pectolyase Y-23. In my system, the negative impact of salts on protoplast yield was more obvious when the enzyme concentration was halved, suggesting that salts had an inhibitory effect on enzymatic activity. Indeed, no digestion occurred when enzymes were supplemented with 50 mM calcium chloride, which might block enzyme access to active sites on cell walls. Reduced concentrations of enzymes led to good protoplast yields, however, further development into microcolonies was not achieved.

Protoplasts recovered after flotation over 0.6 M sucrose were free of cellular debris and showed an increased viability (92%) at isolation, relative to pelleted protoplasts (86%). However, the buoyant density of 0.6 M sucrose was not sufficient

to separate all the viable protoplasts from the debris. As a result, 75% of the viable protoplasts were found in the pelleted debris (Table 7). Brassica protoplasts have been successfully purified by floatation over 0.6 M sucrose by Eapen *et al.* (1989) and Klimaszewska and Keller (1987).

Percoll is composed of colloidal silica particles which have been coated with PVP, is non-toxic to cells, and has a low osmolarity, so that iso-osmotic density gradients can be formed (Somerville *et al.*, 1981). All protoplasts collected at one interface after centrifugation in a discontinuous Percoll gradient, which defeated cleaning purposes (Table 7). Pelleting remained the routine washing procedure in the present system.

Protoplast density has been recognized as being critical for their survival in culture. A minimum plating density of 5×10^4 was required for good viability of stem cortex B. juncea protoplasts (Table 8), which agrees with most protoplast culture reports, where densities vary between 2×10^5 and 5×10^5 pp/ml. This high density requirement might be related to the diffusion of essential metabolites from the protoplasts into the culture medium, or to excessive amounts of some medium nutrients which become toxic to the cells (Chuong *et al.*, 1985). This latter suggestion seems less likely though, since low density protoplast cultures are possible, provided that the medium is enriched, for example with vitamins (Koop *et al.*, 1983). On the other hand, protoplasts compete for available nutrients when plating densities are too high, and colony development is restrained.

SeaPlaque agarose-solidified underlayers were highly beneficial to B. juncea stem cortex protoplast viability and division rates (Table 9). It was possible to culture 1.5 ml

of protoplast suspension over these underlayers, but 3 ml were required to completely cover a petri dish lacking an underlayer. An obvious observation is that underlayers reduce superficial tensions of the petri dishes, thus allowing small amounts of medium to spread into thin layers, resulting in better oxygen availability to a majority of cells. Indeed, greener and larger colonies of *B. juncea* were observed on the edges of petri dishes. Oxygen availability has been important for *B. napus* (Chuong *et al.*, 1985) and *B. juncea* hypocotyl protoplasts (Kao and Séguin-Swartz, 1987). However, no differences in viability and division after one week were detected, when plating 1.5 ml or 3 ml of protoplast suspensions over agarose-solidified underlayers were compared (Table 10), and the number of colonies obtained was proportional to the volume plated, suggesting that there is more to the underlayer effect, than just oxygen availability. Agarose underlayers have been recommended for the culture of *B. napus* and *B. juncea* stem cortex protoplasts (Klimaszewska and Keller, 1987; Sproule, 1987). Shillito *et al.* (1983) reported that plating and bead cultures in SeaPlaque agarose drastically improved plating efficiencies in all species tested, and that agarose was superior to other solidifying agents tested. Jaiswal *et al.* (1990) also preferred SeaPlaque agarose over other types of agarose. They actually noticed that Sigma Type I, Seaprep and Sigma Type IX agarose inhibited division of *B. carinata* cotyledon protoplasts. Kao *et al.* (1990) reported that agarose underlayers resulted in lower divisions in *B. oleracea*, but without mentioning the type of agarose used. Takeuchi and Komamine (1982) studied cell wall regeneration after enzymatic digestion, and noticed that the use of an agar-solidified underlayer led to a 4-fold increase in cell division after 35 days, compared to liquid

cultures. When they studied wall components, they found that extracellular polysaccharides such as arabans or galactans accumulated in the protoplast liquid culture medium. However, when the protoplasts were sustained by an agar underlayer, these polysaccharides deposited into the regenerated cell walls. They concluded that the physical state of agar prevented the diffusion of cell wall components, which could thus deposit into the cell wall.

Medium X has been developed by Sproule (1987), by varying calcium and magnesium concentrations from the Schenck and Röbbelen (1982) medium. It possesses lower concentrations of vitamins and micronutrients than B (Pelletier *et al.*, 1983), which is based on B5 and successfully used for a number of Brassica species (Robertson *et al.*, 1983; Jourdan and Earle, 1989). Table 11 shows no difference between media X and B for their ability to sustain protoplast viability and division. Protoplasts cultured in medium B were easier to monitor, since they did not agglutinate as in medium X. Regardless of the medium used, brown exudates, suspected to consist of oxidized polyphenols (Glimelius, 1984), were often observed (Fig. 5d). High levels of brown particles were always correlated with low protoplast viability and division rates. High polyphenol oxidation was also observed by Schenck and Hoffmann (1984) with B. napus, Robertson *et al.* (1988) with B. oleracea mesophyll protoplasts, Kirti and Chopra (1989b) with B. juncea hypocotyl protoplast-derived colonies, and Jaiswal *et al.* (1990) with B. carinata cotyledon protoplasts.

Modifications of calcium and magnesium ion ratios had no effect on protoplast viability and division rates after one week (Table 12), except for poor responses when

both ions were omitted. Tomato protoplasts also died when not supplied with calcium ions (Bellini *et al.*, 1990). Calcium has been related to cell wall synthesis, since it accumulates in the cytoplasm near the growing regions of the cell (Reiss and Herth, 1979). Rose (1980) has suggested that calcium was important for cell wall regeneration of spinach mesophyll protoplasts, and 12 mM calcium chloride in the culture medium of pea mesophyll protoplasts has stimulated their survival and division rates (Von Arnold and Ericksson, 1977). An intracellular increase of calcium has been shown to mediate cytokinin action, and therefore cell division (Tanimoto and Harada, 1986).

Plant regeneration has been a major problem in *B. juncea* protoplast cultures (Jourdan and Earle, 1989; Eapen *et al.*, 1989; Kao and Séguin-Swartz, 1987; Sproule, 1987). Chatterjee *et al.* (1985) were the only ones to report good shoot regeneration from mesophyll-derived protoplasts. Later studies with seedling-derived protoplasts, not available at the time this study was carried out, led to much higher regeneration frequencies (Pua, 1990; Kirti and Chopra, 1989b). Glimelius (1984), and Klimaszewska and Keller (1987), recommended to transfer *Brassica* protoplast-derived colonies as soon as they reach a diameter of 0.5 mm, in order to maintain good regeneration potential. In the culture system described here, however, callus would not develop unless the transferred colonies had reached a diameter of 1.0 mm (Table 13).

Agarose is a purified form of agar, consisting of repeating agarobiose units with hydroxyethyl groups, from which most of the sulphate ester side groups have been removed (Shillito *et al.*, 1983). Regeneration media solidified with SeaPlaque agarose promoted better callus proliferation than did agar-solidified media (Table 14). Agar

might thus contain impurities that could hinder cell divisions. The use of gelrite also resulted in large green calli, however, neither agarose nor gelrite improved shoot regeneration frequencies (Table 16).

The media B5 and MS were compared for their ability to sustain callus proliferation and stimulate shoot regeneration. No more than 2% of the calli regenerated shoots on B5, and MS was chosen as a base medium for further investigations. Medium B5 has a higher $\text{NO}_3^-/\text{NH}_4^+$ ratio than MS medium, and MS ammonium level has been reported to inhibit shoot regeneration in *B. napus* stem cortex explants (Klimaszewska and Keller, 1985). However, shoot regeneration has frequently been observed on MS-based media with *B. napus* (Barsby et al., 1986; Chuong et al., 1985) and with *B. juncea* (Sproule, 1987). Most efforts were dedicated to determine the appropriate growth regulator combinations for shoot regeneration (Tables 17-18). Auxins were first assessed in conjunction with cytokinins, since both are usually described as necessary to shoot production in *B. juncea* (see Tables 1 and 2 for references). Callus development was usually good, ranging from 38 to 97% of the transferred colonies, with an average of 71%. The morphological characteristics were very typical of the growth regulator used. Although many NAA-containing media did not promote shoot development, most shoots did arise when NAA constituted the main auxin source, in combination with BA (Table 17). The auxin NAA was specifically required for plant regeneration from *B. napus* stem cortex explants (Klimaszewska and Keller, 1985), and from *B. juncea* stem cortex protoplasts (Sproule, 1987). On the other hand, NAA was highly detrimental to *B. napus* stem cortex protoplast-derived colonies (Klimaszewska and Keller, 1987). Table 17 also

shows that IBA, with either BA, zeatin, or TZ, could stimulate shoot production from up to 3% of the calli. The auxin IBA is not commonly used for plant regeneration from Brassica protoplast cultures. In this system, GA3 did not influence organogenesis, although it had been considered essential for shoot regeneration from B. napus protoplasts (Pelletier et al., 1983; Kartha et al., 1974). My results are in accordance with Klimaszewska and Keller (1985) and Sproule (1987), who did not observe any GA3-related improvements of plant regeneration. Cytokinins as sole growth regulator sources also stimulated shoot regeneration, zeatin and 2ip being the most effective, with a maximum of 7% regenerating calli. Zeatin was also beneficial to stem cortex protoplasts of B. napus (Klimaszewska and Keller, 1987).

Concluding remarks. Stem cortex explant culture of B. juncea was extremely genotype dependent, and allowed the selection of good responding genotypes, namely Blaze, Zaria and Colman's, which produced respectively 51, 45, and 40 shoots per one hundred calli. Blaze was therefore chosen for stem cortex protoplast cultures. Modifications from Pelletier's (1983) culture media, did not lead to drastic improvements in protoplast viability and plating efficiencies, except for the use of agarose underlayers. I was hoping to correlate explant behaviour with protoplast response, but all genotypes tested (Blaze, Zaria, Colman's, RLM514, and Zem) had excellent plating efficiencies, and plant regeneration frequencies were too low to draw conclusions (data not shown).

CHAPTER II

TISSUE AND PROTOPLAST

CULTURE OF THE WILD

CRUCIFERS

Lunaria annua,

Thlaspi arvense,

and Capsella bursa-pastoris.

LITERATURE REVIEW

A. THE PROBLEM OF FLEA BEETLES.

The most serious insect pests of Cruciferous crops on the Canadian Prairies are flea beetles (Phyllotreta cruciferae and P. striolata). The insects overwinter, attack seedling crops during May and June, feed on the cotyledons and first true leaves (Burgess *et al.*, 1977), and continue feeding on mature plants (Lamb, 1984). Olfactory stimuli in the form of mustard oils are believed to trigger the feeding behaviour of adult P. cruciferae; once the biting response is elicited, glucosinolates, and possibly nutrients, may induce continued feeding (Hicks, 1974). The only efficient control of flea beetles on the Canadian Prairies today is with FuradanTM (FMC), a granular carbofuran whose use might be discontinued due to high toxicity levels to wildlife. The development of flea beetle resistant canola crops would be the ideal solution to flea beetle attacks

B. SOURCES OF FLEA BEETLE RESISTANCE.

Flea beetles attack most wild and cultivated species of the Brassicaceae, but Lunaria annua (silver dollar), Thlaspi arvense (stinkweed), and Capsella bursa-pastoris (shepherd's purse), are not attacked (Feeny *et al.*, 1970), even though they also produce substantial amounts of glucosinolates. Since a correlation is not always found between the level of flea beetle attacks and the type and quantity of glucosinolates produced,

feeding deterrents are believed to play a major role in resistance mechanisms (Meisner and Mitchell, 1983). Leaves of Lunaria annua (silver dollar, $x=7$; $2n=28$) contain saponins (Meisner and Mitchell, 1983), which might inhibit flea beetle feeding. Thlaspi arvense (stinkweed, $x=7$; $2n=14$), a common weed of unpleasant odour, which has a short life cycle but can also overwinter as vegetative compact rosettes (Best and McIntyre, 1975), is found in cultivated fields throughout the world, and is adapted to a wide range of environments. Methanol extracts of stinkweed leaves suppressed feeding of flea beetles when added to the surface of susceptible radish leaves. Meisner and Mitchell (1983) attributed this feeding inhibition to saponins. Capsella bursa-pastoris (shepherd's purse, $x=8$; $2n=32$) is a wild Cruciferous species originating from the Mediterranean lands, and is found on wastelands and on cultivated fields throughout the world, except in Polynesia (British herbal pharmacopoeia, 1983). In addition to being unattractive to flea beetles, it is resistant to the fungus Alternaria brassicae (Tewari, 1991), and relatively tolerant to frost, both of which are desirable traits for Brassica crop improvement.

Lunaria annua belongs to the tribe Lunarieae, T. arvense and C. bursa-pastoris to the tribe Lepidieae, and crop species such as B. napus, B. campestris, or B. juncea belong to the tribe Brassiceae (Darlington and Wylie, 1955). The flea beetle resistant species are therefore sexually incompatible with the Brassica crop species, though interesting traits might be transferred through asymmetric hybridization, or by protoplast fusion, provided that good protoplast systems are available for both fusion partners.

C. TISSUE AND PROTOPLAST CULTURE OF WILD SPECIES.

Table 19 lists the wild Cruciferous species for which protoplast isolation protocols have been established as a prerequisite for somatic hybridization with Brassica crop species. Although plants have been regenerated from mesophyll-derived protoplasts of Diplotaxis muralis and Eruca sativa, mesophyll tissue frequently yields protoplasts which do not divide, nor can be regenerated into plants, thus requiring the initiation of cell suspensions as alternative protoplast sources. Indeed, embryogenic cell suspensions have been the source of regenerable protoplasts for recalcitrant species such as Arabidopsis thaliana (Ford, 1990).

The development in vitro of shepherd's purse zygotic embryos has been well characterized (Monnier and Lagriffol, 1986; Monnier, 1984), and C. bursa-pastoris is now considered a model species for studies of zygotic embryogenesis. The proliferation of Capsella callus, followed by regeneration of shoots from seed, cotyledonary and hypocotyl explants, has been previously reported (Zilkah and Gressel, 1977). However, protoplast isolations from L. annua, T. arvense or C. bursa-pastoris have not been described previously.

2

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Table 19: Protoplast culture studies in wild Cruciferous species.

species	traits of interest	regeneration	reference
<u>Arabidopsis thaliana</u>	fundamental studies	40% shoots	Karesch <i>et al.</i> , 1991
<u>Arabidopsis thaliana</u>	not mentioned	yes	Ford, 1990
<u>Brassica spinescens</u>	white rust resistance; tolerance to salt	no	Kirti <i>et al.</i> , 1991
<u>Diplotaxis harra</u>	possible cytoplasmic male sterility; possible insect tolerance	no	Klimaszewska and Keller, 1988
<u>Diplotaxis muralis</u>	drought tolerance cytoplasmic male sterility	6 % shoots 8 % embryos	Sikdar <i>et al.</i> , 1990b
<u>Eruca sativa</u>	stress tolerance	16 % shoots 11 % embryos	Sikdar <i>et al.</i> , 1987
<u>Moricandia arvensis</u>	C3-C4 intermediate	no	Toriyama <i>et al.</i> , 1987a
<u>Raphanus sativus</u>	cytoplasmic male sterility	no	Sakai and Imamura, 1990
<u>Sinapis turgida</u> (mutant)	increase of genetic variability	no	Toriyama <i>et al.</i> , 1987b

D. OBJECTIVES.

The objective of this study was to develop a protoplast culture system from at least one of the wild species mentioned above, which would allow protoplast fusion with Brassica crop species. As with B. juncea (Chapter I), tissue culture was considered a convenient approach to detect the species potential to produce dividing protoplasts, and to establish optimal medium compositions for plant regeneration. Finally, cell suspension cultures were initiated as alternative protoplast sources, in the event that mesophyll tissue would not yield viable protoplasts.

MATERIALS AND METHODS

A. EXPLANT CULTURES

a. Plant material. Seeds of Lunaria annua, Thlaspi arvense and Capsella bursa-pastoris were obtained from Dr. R.K. Downey (Agriculture Canada, Saskatoon, Sask.). Plants were obtained and maintained as described in Chapter I. When planted in vitro, the seeds were surface-sterilized for 1 min. in 70% ethanol, 30 min. in a super-saturated solution of calcium hypochlorite containing a few drops of Tween 80, and rinsed three times in sterile distilled water. Sterile seeds were then germinated on growth regulator-free B5 medium (Appendix 4) solidified with 0.8% agar, in sterile plastic Magenta boxes (100 mm high), at 20°C, 16 h photoperiod, with a light intensity of $100 \mu\text{Em}^{-2}\text{sec}^{-1}$.

b. Culture of seedling and leaf explants. Leaves from 3 to 5 week-old in vitro plantlets, cotyledons and hypocotyls from one to two week-old in vitro-grown seedlings, were cut into 5 mm squares and segments, plated in 20x60 mm plastic Petri dishes onto various MS-based (Appendix 3) solidified media as discussed in the results section, and maintained at 20°C, 16 h photoperiod, with a light intensity of $100 \mu\text{Em}^{-2}\text{sec}^{-1}$. The growth regulators tested were adenine sulphate, BA, kinetin, TZ (NorAm, Wilmington, DE), zeatin, 2,4-D, NAA, IAA, and GA3, alone or in combination. Media were solidified with 0.8% Difco-Bacto agar, 0.8% SeaPlaque or 0.7% SeaKem agarose (FMC, Rockland).

B. CELL SUSPENSION INITIATION AND MAINTENANCE.

Cotyledons of L. annua and leaf pieces of three week old in vitro-grown plantlets of T. arvense and C. bursa-pastoris, were cultured on agar-solidified MS medium containing 0.5 to 2 mg/l 2,4-D, in order to obtain friable callus. Cell suspensions were initiated and maintained as described by Gleddie (1989).

For T. arvense cell suspensions, attempts to reduce phenolic oxidation, or to precipitate out polyphenols, included adding 0.1 g PVP 40, 0.1 g Polyclar AT, or 1.0 g activated charcoal in small nylon mesh bags, per 33 ml flask; dissolving 0.7% PVP 40 or 0.3 mM ascorbic acid in the culture medium; growing the cell suspensions in darkness.

One shepherd's purse cell suspension had been initiated by S. Gleddie, three years before protoplast isolation and plant regeneration experiments were initiated. This line was designated as SS cell suspensions.

Fresh cell suspensions were initiated from leaves of in vitro grown shepherd's purse plantlets, without a callus propagation phase. To produce these suspensions, the leaves were finely lacerated and cultured directly in liquid MS medium, 2% sucrose, 2 mg/l 2,4-D, and maintained as described in Gleddie (1989). Five week old cultures were blended (Wareing blender, high setting) for 5 to 10 seconds, and twice more, at monthly intervals. These shepherd's purse suspension cultures were four to six months old when used, and were designated as SPP cell suspensions.

C. PLANT REGENERATION FROM CELL SUSPENSIONS.

a. Embryogenesis in liquid media. Both SS and SPP shepherd's purse cell suspensions were used for regeneration experiments in liquid cultures, where 2,4-D was omitted or replaced with various growth regulators. The effects of the growth regulators NAA, BA, and TZ, were evaluated, alone or in combination, in modified MS media containing 2% sucrose.

b. Embryogenesis on solidified media. The shepherd's purse cell suspension culture medium was filtered through a 44 μm nylon mesh. Cell aggregates of 1.0 to 2.0 mm diameter collected on filters, were either rinsed with 100 ml of growth regulator-free MS medium, or transferred directly onto 15 ml of solidified regeneration MS medium in 20x60 mm plastic Petri dishes. Various growth regulators and solidifying agents were compared for their effect on embryogenesis and organogenesis. The colonies were maintained at 20°C, 16 h photoperiod, with a light intensity of 100 $\mu\text{Em}^{-2}\text{sec}^{-1}$ composed of incandescent and fluorescent light. Calli were transferred onto fresh medium after 4 weeks.

D. PROTOPLAST ISOLATION AND CULTURE.

a. From mesophyll tissue. Leaves were cut into 1-2 mm strips, and when necessary, brushed with a sterile toothbrush or peeled from the lower epidermis. Approximately 0.25 g of leaf tissue was digested as described in Chapter I.

b. From shepherd's purse SS cell suspensions. Approximately 0.5 g (fresh weight) of cells, collected on 44 μ m nylon mesh three to four days after transfer, were digested in 15 ml of enzyme solution. Although several enzymatic solutions were assessed, the routine mixture contained 2% macerozyme R10, 1% cellulase "Onozuka" RS (Yakult Honsha, Tokyo), 0.05% pectolyase Y-23 (Seishin Pharmaceuticals, Tokyo), 0.4 M mannitol, at pH 5.8. The Petri dishes (100x15 mm) were incubated at 28°C on a reciprocating shaker (40 rpm) for 3 to 6 h in the dark. Protoplasts were filtered through 44 and 22 μ m nylon mesh, and washed two times in salt solution (0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5% KCl, pH 6.9). Protoplast density was adjusted to 10^5 protoplasts/ml of culture medium. Protoplast suspensions were plated and maintained as described in Chapter I.

Culture media were B (Pelletier *et al.*, 1983, Appendix 6), L (Lichter, 1981, Appendix 8), 8p (Kao and Michayluk, 1975, Appendix 9), X (Sproule, 1987, Appendix 5) or S1 (Schenck and Röbbelen, 1982, Appendix 5). Additives such as 1% PEG M.W. 4000 (Merck, Darmstadt), 0.25 g/l acid hydrolysed casein (Sigma, St. Louis, MO), 2 g/l bovine serum albumin (Fraction V, Sigma, St. Louis, MO), and 20 ml/l coconut milk (heated at 60°C for one hour and filter sterilized) were assessed. Once they reached 0.5-2.0 mm diameter, colonies were transferred onto agarose-solidified callus propagation MS medium, supplemented with 0.1 mg/l 2,4-D and 3 mg/l BA. After 3 weeks, they were transferred onto regeneration growth regulator-free medium.

E. EMBRYO MATURATION AND ROOTING

Well developed embryos with clear bipolar axes were transferred onto agar-solidified B5, MS, or Monnier's (Monnier, 1978, Appendix 10) media, full or half strength. These media were either growth regulator-free, or supplemented with various combinations of 0.2 mg/l NAA, 0.05 mg/l GA3, 0.2 mg/l IBA, 2.0 mg/l kinetin or BA, and 0.5 to 2.0 mg/l ABA. The addition of 5% (w/v) activated charcoal was assessed, and the sucrose concentration varied from 2 to 12% (w/v). Embryos were plated into Petri dishes or baby food jars, with or without paper filters, and sealed either with Parafilm or Saranwrap. Regenerated shoots and embryos were transferred onto growth regulator-free B5 medium for root initiation.

Plantlets were planted in Jiffy 7 peat pellets, left in a mist chamber for 2 weeks, then transferred to soil in a growth cabinet, 20/15°C (day/night) temperature, 16 h photoperiod ($100 \mu\text{Em}^{-2}\text{sec}^{-1}$).

F. DATA COLLECTION AND ANALYSIS.

Unless explained otherwise, organogenesis frequencies are expressed as the number of explants or cell clusters exhibiting root, shoot or embryo development, over the total number plated. The growth curve of the SS cell suspension was established by measuring the packed cell volume of the suspension at daily intervals.

In order to monitor cell wall digestion and regeneration, cells and protoplasts

were stained with 0.001% calcofluor white for ten minutes, centrifuged at 100xg, and rinsed with culture medium. Nuclei were counted after protoplasts were fixed with 1% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 6.0, for 15 min., and stained with 5 μ g/ml DAPI, as described in Coleman and Goff (1985). Observations were made with a Carl Zeiss inverted photomicroscope equipped with the H $\ddot{o}$ echst filter combination (G365 (UV excitation)/LP420).

For electron microscopic observations, leaves of regenerated and seed-derived plants were frozen in liquid N₂ on the cryostage, critical point dried, and coated with gold. Scanning electron micrographs were obtained with an AMRay 1000A scanning electron microscope.

Viability and division frequencies of protoplasts were estimated after 7 days, by observing FDA stained cells, as described in Chapter I. Protoplast data were collected and analyzed as in Chapter I.

RESULTS

A. LUNARIA ANNUA (SILVER DOLLAR).

a. **Organogenesis from leaf explants.** Leaf segments of 5 week-old *in vitro* grown plantlets, were plated on agar-solidified MS medium supplemented with various growth regulators. Callus proliferated throughout the explant surface in presence of the auxins NAA or 2,4-D, while it hardly developed, and only around cut veins, in presence of 1 mg/l zeatin, 1 mg/l IBA, 0.5, 1, 2, 5 mg/l TZ, or 5 mg/l BA. Shoots were produced by 8% of the explants cultured on 5 mg/l BA.

b. **Organogenesis from cotyledonary explants.** Two week-old cotyledon pieces were plated onto various MS-based solidified media, as shown on Table 20. After two months, calli were light green, very compact and dry (Fig. 8a); regenerated shoots were morphologically normal and extremely hairy (Fig. 8b). The auxin IBA clearly reduced frequencies of shoot induction ($X^2=44.48$, d.f. = 1, $p<0.01$). Explant orientation did not affect organogenic responses ($X^2=5.02$, d.f. = 1, $p<0.01$). On the other hand, shoot production was remarkably more active when culture media were solidified with agarose than with agar (Table 20; $X^2=17.15$, d.f. = 1, $p<0.01$). The many shoots obtained could not be rooted, even after addition of 1 mg/l NAA to the rooting medium.

c. **Cell suspension initiation.** Calli obtained from cultures of mesophyll tissue and of cotyledonary explants never became sufficiently friable to successfully initiate cell suspensions. They rapidly oxidized upon transfer to 2,4-D containing liquid medium,

Table 20: Effect of growth regulator, solidifying agent, and explant orientation on plant regeneration from *Lunaria annua* cotyledons.

orientation	growth regulator (mg/l)	solid. agent (0.8 % w/v)	number of explants	number of calli	number of shoots
U	0.2 IBA + 3 BA	agar	12	4	4
U	0.2 IBA + 3 BA	agarose	12	5	6
L	0.2 IBA + 3 BA	agar	12	3	0
L	0.2 IBA + 3 BA	agarose	12	3	1
U	3 BA	agar	12	11	14
U	3 BA	agarose	12	11	51
L	3 BA	agar	12	12	23
L	3 BA	agarose	12	12	33

U = upper epidermis in contact with culture medium.

L = lower epidermis in contact with culture medium.

Medium had a highly significant effect on callus proliferation and shoot regeneration. Agarose was superior to agar for shoot production. Orientation had no effect ($p < 0.01$).

and necrosed after 2 months, without further development.

d. Protoplast isolation from leaves of 5 week-old plantlets grown *in vitro*. Enzyme solutions of macerozyme and cellulase concentrations varying from 0.5% to 2.0%, and mannitol concentration varying from 0.3 to 0.6 M, were assessed for protoplast isolation. None of these combinations could lead to cell wall digestion. Leaves were thereafter brushed with a sterile toothbrush, peeled (with great difficulty), or cut into 1-2 mm strips, only to result in the release of hundreds of trichomes, and of a few dead protoplasts. Pretreatment of the leaf tissue such as described in Chapter I for *B. juncea*, did not improve isolation. Further experimentation was not undertaken.

B. THLASPI ARVENSE (STINKWEED).

a. Organogenesis from leaf explant cultures. A list of culture media on which leaf segments from 3 week old *in vitro*-grown plantlets of stinkweed have been plated in order to stimulate plant regeneration is given (Tables 21, 22). In general, NAA as the sole growth regulator gave rise to excellent rhizogenesis (Fig. 8c), and 2,4-D to friable callus proliferation. Shoots did not develop in presence of auxins only, except when 0.5 mg/l NAA was added to the culture medium, on which 3.6% of explants produced shoots (Table 21). The best auxin/cytokinin combinations (up to 12.5% shoot regeneration), involved 0.1-0.2 mg/l 2,4-D with 2 mg/l kinetin or 2-5 mg/l TZ, and 0.1 mg/l NAA with 3 mg/l TZ and 0.1 mg/l GA3 (Fig. 8d; Table 21). However, the media which stimulated highest frequencies of shoot regeneration were supplemented with the cytokinins adenine sulphate or TZ, alone or in combination with kinetin or zeatin (Table 22). Neither kinetin, zeatin, nor BA alone were able to initiate shoot regeneration. Unless highly vitrified, the produced shoots were easily rooted on growth regulator-free medium (Fig. 8e).

Table 21: Effect of auxins and cytokinins on organogenesis from leaf explants of stinkweed and shepherd's purse.

	auxins			cytokinins					% shoot regeneration	
	2,4-D	IAA	NAA	AS	BA	K	TZ	Z	GA3	stinkweed shepherd's p.
0.1	-	-	-	-	-	-	-	-	-	0 0
1.0	-	-	-	-	-	-	-	-	-	0 0
-	0.5	-	-	-	-	-	-	-	-	0 0
-	2.0	-	-	-	-	-	-	-	-	0 0
-	-	0.5	-	-	-	-	-	-	-	4 0
-	-	2.0	-	-	-	-	-	-	-	0 0
0.2	-	-	-	2.0	-	-	-	-	-	0 0
0.1	-	-	-	-	1.0	-	-	-	-	0 0
0.1	-	-	-	-	3.0	-	-	-	-	0 4
0.2	-	-	-	-	3.0	-	-	-	-	0 0
0.2	-	-	-	-	3.0	-	-	-	0.1	0 0
0.2	-	-	-	-	3.0	-	-	-	1.0	0 0
0.2	-	-	-	-	-	2.0	-	-	-	12 0
0.1	-	-	-	-	-	-	5.0	-	-	6 15
0.2	-	-	-	-	-	-	2.0	-	-	12 0
0.2	-	-	-	-	-	-	2.0	-	0.1	0 0
0.2	-	-	-	-	-	-	2.0	-	1.0	0 0
0.2	-	-	-	-	-	-	-	2.0	-	0 0
-	1.0	-	-	-	2.0	-	-	-	-	0 0
-	3.0	-	-	-	2.0	-	-	-	-	0 0
-	-	1.0	-	2.0	-	-	-	-	-	0 0
-	-	0.1	-	-	0.5	-	-	-	-	0 0
-	-	0.1	-	-	1.0	-	-	-	-	0 8
-	-	0.1	-	-	3.0	-	-	-	-	0 14
-	-	0.1	-	-	3.0	-	-	-	0.1	0 0
-	-	0.1	-	-	3.0	-	-	-	1.0	0 0
-	-	1.0	-	-	3.0	-	-	-	-	0 0
-	-	1.0	-	-	5.0	-	-	-	-	0 0
-	-	1.0	-	-	-	2.0	-	-	-	0 12
-	-	1.0	-	-	-	-	3.0	-	-	0 0
-	-	-	-	-	-	-	3.0	-	0.1	12 0
-	-	0.1	-	-	-	-	10.0	-	-	0 0
-	-	1.0	-	-	-	-	-	2.0	-	0 0

Media were MS, with 2% sucrose and solidified with 0.7% SeaKem agarose. Data were collected after 2 months. - represents absence of corresponding growth regulator.

Table 22: Effect of cytokinins as sole growth regulator sources, on organogenesis from explants of stinkweed and shepherd's purse.

AS	BA	K	TZ	Z	% shoot regeneration stinkweed	regeneration shepherd's purse
0.5	-	-	-	-	0	0
1.0	-	-	-	-	0	0
2.0	-	-	-	-	25	0
5.0	-	-	-	-	0	0
10.0	-	-	-	-	20	0
-	0.5	-	-	-	0	0
-	1.0	-	-	-	0	0
-	2.0	-	-	-	0	0
-	5.0	-	-	-	0	0
-	-	0.5	-	-	0	0
-	-	1.0	-	-	0	6
-	-	3.0	-	-	0	0
-	-	-	0.5	-	25	0
-	-	-	1.0	-	25	12
-	-	-	2.0	-	20	15
-	-	-	5.0	-	12	0
-	-	-	10.0	-	0	0
-	-	-	-	0.5	0	0
-	-	-	-	1.0	0	0
-	-	-	-	2.0	0	0
-	-	-	-	5.0	0	0
-	1.0	-	1.0	-	0	0
-	3.0	-	1.0	-	0	0
-	1.0	-	2.0	-	0	0
-	3.0	-	2.0	-	0	0
-	2.0	-	-	1.0	0	0
-	2.0	-	-	3.0	0	0
-	-	0.1	1.0	-	0	0
-	-	3.0	1.0	-	0	6
-	-	0.1	2.0	-	38	38
-	-	3.0	2.0	-	0	15
-	-	-	2.0	0.1	38	25
-	-	-	2.0	1.0	40	0
-	-	-	2.0	3.0	25	0

Media were MS, with 2% sucrose and solidified with 0.7% SeaKem agarose. Data were collected after 2 months. - represents absence of corresponding growth regulator.

Figure 8a: Callus of Lunaria annua with shoot meristems, developed from a cotyledonary explant after one month of culture onto MS regeneration medium supplemented with 3 mg/l BA. x8.

Figure 8b: Lunaria annua shoot developed from a cotyledonary explant after two months of culture onto MS-based agarose solidified regeneration medium supplemented with 3 mg/l BA. x4.

Figure 8c: Rhizogenesis from leaf explants of Thlaspi arvense after two months of culture on MS regeneration medium supplemented with 0.5 mg/l NAA. x2.

Figure 8d: Shoots obtained from leaf explants of T. arvense after two months on MS regeneration medium supplemented with 3 mg/l TZ and 0.1 mg/l GA3. x2.

Figure 8e: Thlaspi arvense regenerant from leaf explant culture, after three weeks on growth regulator-free rooting medium. x2.

Figure 8f: Green compact calli of Capsella bursa-pastoris after two months on MS medium supplemented with 0.2 mg/l 2,4-D. x2.

b. Cell suspension cultures. Once transferred to liquid cultures, friable calli rapidly released single cells of variable sizes and shapes, which were often thin, elongated, curly, and did not divide (**Fig. 9b**). When cell suspensions were initiated directly from lacerated leaves, stinkweed cells did divide, and formed small colonies. However, heavy polyphenolic oxidation occurred, was rapidly correlated with growth inhibition, and could not be reduced by supplementing the culture medium with PVP40, Polyclar AT, ascorbic acid, nor activated charcoal. When grown in darkness, the cell clusters became yellow, although cells did not divide. Cultures were disposed of, after 7 months of efforts to induce cell division.

c. Protoplast isolation. Most of the experiments involving growth chamber stinkweed plants were ruined by acute bacterial contamination problems. Severe sterilization with 7% calcium hypochlorite and a few drops of Tween 80 for 30 min, resulted in damage to leaves, without solving the problem. Three week-old in vitro-grown plantlets were therefore used as tissue source, and viable protoplasts were readily isolated without pretreatment, after overnight digestion in 0.5% cellulase, 1% macerozyme, and 0.4 M mannitol (**Fig. 9a**). Approximately $3 \cdot 10^6$ viable protoplasts, representing an initial viability of about 70%, could be generated from one gram of mesophyll tissue, with very little debris except for the floating cuticle.

d. Protoplast culture. Various initial culture media were assessed: S1 (Scnenck and Röbbelen, 1982), X (Sproule, 1987), B (Pelletier, 1983), and L (Lichter, 1981), with or without 2% Ficoll, but were not successful in stimulating protoplast division.

Protoplasts were cultured at 28, 25, 20, 10 and 2°C in medium X.

A viability of 37% was recorded after one week at 2°C, 17% at 10°C, while protoplasts died within 2 days at 28°C. Protoplasts did not divide, regardless of the temperature.

Nurse culture systems were examined, where cell suspension cultures of Nicotiana debneyii and Brassica juncea were embedded in 0.8% agar or 0.6% SeaPlaque agarose, covered with sterile filter paper (Whatman #2, Balston Ltd.), over which L. arvense mesophyll protoplasts were plated. Cell division of stinkweed mesophyll protoplasts could not be induced.

C. CAPSELLA BURSA-PASTORIS (SHEPHERD'S PURSE).

a. Organogenesis from leaf explant cultures. Leaf segments from 3 week old in vitro-grown plantlets of shepherd's purse were plated on several media, in order to stimulate plant regeneration (Tables 21, 22). As with stinkweed, NAA as sole growth regulator gave rise to excellent rhizogenesis, while 2,4-D led to green friable callus proliferation (Fig. 8f). Shoots did not develop in presence of auxins alone (Table 21), but cytokinins stimulated shoot organogenesis. The best auxin/cytokinin combinations for up to 15% shoot regeneration involved 0.1 mg/l 2,4-D, with 3 mg/l BA or 5 mg/l TZ, and 0.1-1 mg/l NAA, with 1-3 mg/l BA or 2 mg/l kinetin. As with stinkweed, the media which stimulated highest frequencies of shoot regeneration were supplemented with the cytokinin TZ, in combination with 0.1-3 mg/l kinetin or 0.1 mg/l zeatin (Table 22). Neither kinetin, zeatin, TZ, nor BA alone, were able to initiate shoot regeneration. The shoots appeared normal, and easily rooted on growth regulator-free medium.

b. Protoplast culture from mesophyll tissue. Viable protoplasts were readily isolated from 3 week-old *in vitro*-grown plantlets, however, protoplast division could not be stimulated in B nor in 8p media.

c. Cell suspension initiation and maintenance. Most experiments were carried out with the 3 year old SS cell suspensions, which remained clumpy and slow growing for about one year after initiation. It was necessary to homogenize the cell aggregates several times in a blender in order to reduce their size. **Figure 9c** shows flasks of clumpy SPP suspensions, then 4 months old, before and after blending, compared with a flask of SS cell suspensions, then 3 years old, which was fine, with most aggregates containing approximately 20 to 100 cells. In one week, the packed volume of the SS suspension increased from 1.75 ml (1 g fresh weight inoculum) to 8.85 ml, following a most typical sigmoidal growth curve (**Fig. 10**). Most cells were isodiametric, had a dense cytoplasm, and small vacuoles. Xylem differentiation occurred frequently (**Fig. 12a**), but organogenesis was not observed in the presence of 2,4-D (**Fig. 12b**).

d. Embryogenesis in liquid media. Upon transfer to growth regulator-free liquid MS medium, SS cells elongated typically into finger-like shapes around compact colonies (**Fig. 9e**). Then, 1 to 14 root apices developed rapidly from these cell aggregates, and grew for one month, to a maximum length of 12 mm, after which they browned and necrosed despite weekly feedings. **Figure 9f** shows the clear differentiation of a vascular system within such a root. Attempts were made to induce direct embryogenesis in liquid cultures by varying the growth regulator composition, as shown in **Table 23**. Root differentiation was induced at a similar rate when the culture medium was supplemented

with either 1.0 or 2.0 mg/l NAA, as when growth regulators were lacking. The cytokinins BA and TZ induced the formation of many green cell clusters or primordia, with well-defined outlines and compact, organized cores (**Fig. 9g**). Occasional bipolar structures were encountered, but somatic embryos never developed. Instead, these cultures turned brown after 4 transfers (5 weeks), except in the presence of 2,4-D. On the other hand, after 2,4-D was omitted from SPP cell suspensions for 5 weeks, 16% of the cell colonies had formed dark green compact structures, while 86% had formed roots (**Fig. 9d**).

Figure 9a: Mesophyll protoplasts from 3 week old stinkweed in vitro-grown plantlets, in enzymes. x400.

Figure 9b: Elongated, curly cells, released from stinkweed friable calli cultured in 2,4-D containing liquid medium, in unsuccessful attempts to initiate cell suspensions. x 200.

Figure 9c: Top flask: four month old clumpy SPP suspension, before blending. Middle flask: SSP suspension after blending. Bottom flask: three year old fine SS cell suspension, with most aggregates containing approximately 20 to 100 cells. x0.5

Figure 9d: Dark green compact structure and several roots produced from SPP cell clusters in liquid, after 2,4-D was omitted for 5 weeks. x5.

Figure 9e: Typically elongated cells, around a compact colony, observed three weeks after 2,4-D was removed from SS cell suspension cultures. x 100.

Figure 9f: Root with a clearly defined vascular system, five weeks after 2,4-D was removed from SS cell suspension cultures. x 100.

Figure 9g: Green cell cluster induced by BA and TZ, after one month in liquid culture of SS suspensions. x100.

f

g

Figure 10: Growth curve of shepherd's purse SS cell suspension over 10 days.

Table 23: Effect of various growth regulators on cell viability and organogenesis of C. bursa-pastoris cell suspensions, in liquid cultures.

growth regulator	conc. mg/l	packed cell vol (mg/l)	viability	organogenesis
-	-	7.5	++	100 % roots
2-4,D	2.0	7.0	+++	none
NAA	1.0	7.5	+	100 % roots
NAA	0.2	6.0	+	100 % roots
BA	3.0	6.0	++	green cell clusters with dense cores. Bipolar structures
TZ	3.0	6.0	+	green cell clusters with dense cores
NAA + BA	0.2 3.0	5.5	++	root apices. Green cell clusters with dense cores
NAA + TZ	0.2 3.0	5.5	++	a few green cell clusters

Basal medium was MS salts with 2 % sucrose. Observations were taken after 3 weeks of treatment. +++, ++, +: good (>70%), average (50-70%), low (<50%).

e. **Shoot production and embryogenesis on solid media.** A different approach was to plate cells from the suspension cultures onto solidified media. The 6 month old SPP suspensions regenerated shoots within one month, moderately in presence of 5 mg/l BA and 0.5 mg/l NAA, but mainly on growth regulator-free media with frequencies up to 50% (Table 24). Growth regulator-free medium was significantly superior for shoot regeneration ($X^2=60.75$, d.f. = 3, $p<0.01$), while the type of carbohydrate had no effect ($X^2=3.92$, d.f. = 1, $p<0.01$). Although highly significant differences were detected between media solidified with agar or agarose ($X^2=34.58$, d.f. = 1, $p<0.01$), the interaction between solidifying agent and medium was also significant ($X^2=84.32$, d.f. = 3, $p<0.01$) so that the best solidifying agent could not be identified. The shoots were normal or slightly vitrified, but easily rooted.

Table 24: Effects of carbohydrates, growth regulators and solidifying agents on plant regeneration from SPP shepherd's purse cell suspension.

G.R. (mg/l)	carbohydrate (% w/v)	gelling agent (% w/v)	callus (%)	shoots [s.e.] (%)
-	2 sucrose	0.6 agarose	100	50 [8]
-	2 sucrose	0.8 agar	100	15 [1]
-	1 suc. + 1 mann.	0.6 agarose	100	13 [11]
-	1 suc. + 1 mann.	0.8 agar	98	7 [5]
5B .5N	2 sucrose	0.6 agarose	100	3 [2]
5B .5N	2 sucrose	0.8 agar	100	10 [4]
5T 1N	1 suc. + 1 mann.	0.6 agarose	100	0 [0]
5T 1N	1 suc. + 1 mann.	0.8 agar	100	3 [1]
5T 1N 0.05G	1 suc. + 1 mann.	0.6 agarose	100	0 [0]
5T 1N 0.05G	1 suc. + 1 mann.	0.8 agar	100	0 [0]

Growth regulators had a highly significant effect on shoot regeneration ($p<0.01$).

Excellent embryo formation was triggered from SS cell clusters plated onto growth regulator-free medium, or with a combination of 1.0 mg/l NAA and 0.5 mg/l BA (Table 25). Rinsing with growth regulator-free medium before plating did not affect embryogenesis. Although numerous embryos developed (Fig. 11a,c), only a few were morphologically normal. Multiple cotyledons (Fig. 11b), fused in a funnel (Fig. 11d), premature elongation of the hypocotyl region, necrosis of the suspensor, or swelling and vitrification occurred often, and complete embryo maturation was usually not achieved. Secondary embryogenesis, or the development of embryos on the primary embryos, was a major problem (Fig. 11e). As a result, roots did not develop on most of the embryos upon transfer to rooting medium, despite their clear bipolar nature and the presence of well defined root apices. None of the media, additives, and physical conditions assessed were successful in inducing maturation of aberrant embryos, and the most drastic effect was high levels of anthocyanin production among these embryos. However, non-vitrified and leafy material (Fig. 11f) was readily rooted, and plants were regenerated, transferred to soil, and acclimated to growth chamber conditions. Plants regenerated from the 6 month old suspension all had morphologically normal flowers (Fig. 11g), however, shapes and sizes of vegetative parts varied tremendously from plant to plant. Most surprising were the trichome variations of SPP regenerants, with only one quarter of the plants having 5 branched trichomes on leaves and stems, and straight hairs on leaf edges only, which is the normal trichome constitution of shepherd's purse; other regenerants had 3-6 branched trichomes, or straight ones over the entire leaf surface, sometimes absent from stems and present on sepals. Triangular seed-pods were formed from

Table 25: Effect of growth regulators on rhizogenesis and embryogenesis from suspension cultures of *C. bursa-pastoris*, plated on various solidified media.

SOLIDIFIED MEDIUM ^a	not rinsed		rinsed ^b	
	R(%) ^c	E(%) ^d	R(%)	E(%)
G.R. ^e -free	0	73	0	74
1.0 mg/l NAA 0.5 mg/l BA	0	50	18	52
1.0 mg/l NAA 0.5 mg/l BA 0.05 mg/l GA3	20	10	24	7
1.0 mg/l NAA 5.0 mg/l TZ 0.05 mg/l GA3	11	11	17	9

a. Medium was modified MS solidified with 1% agarose. Percentages represent the mean of at least 60 colonies per treatment, in two separate experiments. Data were recorded after two months. b. Cells were rinsed with 100 ml of growth regulator-free MS medium prior to plating. c. R: calli exhibiting rhizogenesis. d. E: calli exhibiting embryo development.

e. G.R.: growth regulator.

Figure 11a: Embryo developed from a shepherd's purse SS cell cluster cultured onto growth regulator-free solidified medium. x10.

Figure 11b: Abnormal embryo with multiple fused cotyledons. x10.

Figure 11c: Cell cluster from SS suspension, with clearly bipolar embryo x6.

Figure 11d: Scanning electron micrograph of an embryo with funnel shaped cotyledons. x25.

Figure 11e: Secondary embryogenesis on a vitrified embryo. Arrow shows secondary embryo. x20.

Figure 11f: Leafy non-vitrified shoot regenerated from embryogenic SS clusters, 2 months after transfer to growth regulator-free medium. x1.

Figure 11g and 11h: Normal fruits and flowers of SPP regenerant.

11g: x10. 11h: x1.

flowers of all regenerants, but viable seeds were produced only from SPP regenerants (Fig. 11g-h). Abnormal flower development such as aberrant numbers of petals or sepals, poor pistil and anther development was common among regenerants from the 3 year old SS suspension.

f. Protoplast isolation from SS cell suspensions. At the time protoplast isolations were first attempted, SS cell suspensions were two years old and still contained large cell aggregates, requiring complex enzymatic solutions. Acceptable yields of viable protoplasts were obtained only after digestion with 2% macerozyme and 2% cellulase at 28°C on a shaker, and yields from 3×10^6 to 3×10^7 pp/g fresh weight required the use of pectolyase Y-23, or potent enzymes such as driselase and rhozyme. However, after being blended a few times, and after six additional months of weekly transfers, the suspensions became fine, and were digested within 3-6 h with an enzymatic solution of 2% macerozyme, 1% cellulase and 0.05% pectolyase Y-23 (Fig. 12d). Some aggregates of 10 to 100 cells still remained intact, but these were eliminated by filtration through the nylon filters. Many spontaneous fusions occurred during enzymatic digestion. Polynucleated protoplasts were easily recognized by their large size, irregular humped shape, and the presence of several small vacuoles (Fig. 12c). When stained with DAPI, these protoplasts appeared to contain up to eight nuclei. A few cells escaped digestion and filtration, as seen with calcofluor white staining of the walls, but FDA staining indicated that most of the undigested cells were dead.

g. Culture of cell suspension-derived protoplasts. Both 8p and B media sustained cell wall regeneration within 3 days, with 62% viability and 7% divisions after one week of

culture (**Fig. 12e and f**), while microcolonies developed within three weeks (**Fig. 12g**). The effects of 1 % PEG MW 4000, 0.25 g/l acid hydrolysed casein, 2 g/l BSA, and 20 ml/l coconut milk are shown in **Table 26**. A highly significant difference between media was shown for protoplast viability ($X^2=531.09$, d.f. = 9, $p<0.01$), with the media 8p, 8p with coconut milk and 8p with casamino acids and BSA contributing 84 % to the total X^2 value. Media were also significantly different for division frequencies ($X^2= 367.64$, d.f. =9, $p<0.01$), with 8p alone contributing more than 52% to the total X^2 value. However, after one month, thousands of microcolonies developed, regardless of the additives. Some preparations were unsuccessful, and the cultured protoplasts became swollen and eventually died without dividing. However, once first divisions were initiated, plating efficiencies were high and consistent between experiments. Embryos regenerated on a growth regulator-free solidified medium, from which plantlets developed. These plantlets were rooted, transferred to peat pellets (**Fig. 13a**), and 4 plants were recovered (**Fig. 13b**).

Regenerants had typical lanceolate leaves with basal acute auricles, but also displayed a variety of shapes from entire to deeply lobed (**Fig. 13c**). **Figure 13d** shows the candelabrum-shaped trichomes of a seed-grown *C. bursa-pastoris* plant; trichomes of one regenerant (**Fig. 13e**) showed this typical shape but with variable numbers of branches. All regenerants had male sterile flowers with odd petal and sepal numbers; silicles developed but never reached maturity and did not produce seeds (**Fig. 13f**).

Table 26: Viability and division rates of suspension-derived *C. bursa-pastoris* protoplasts.

MEDIUM	VIABILITY (%) [s.e]	DIVISION (%) [s.e.]	COLONIES AFTER 5 WEEKS
L ficoll sucrose	56 [5]	2 [0]	TN ^a
L ficoll sucrose PEG	56 [5]	4 [9]	TN
8p	62 [7]	7 [1]	TN
8p PEG	58 [6]	3 [0]	TN
8p casamino ac.	60 [11]	3 [1]	TN
8p BSA	56 [12]	4 [0]	TN
8p coco. milk	69 [8]	6 [2]	TN
8p casamino ac. + BSA	51 [9]	3 [1]	TN
8p casamino ac. + coc. milk	54 [6]	4 [0]	TN
8p BSA + coco. milk	60 [5]	2 [1]	TN

a. TN: Too Numerous to count. Viability and division rates were recorded after one week in culture. Additives were: 2% (w/v) ficoll, 13% (w/v) sucrose, 1% (w/v) PEG M.W. 4000, 0.25 g/l acid hydrolysed casein, 2 g/l BSA, fraction V, 20 ml/l coconut milk. 8p medium from Kao and Michayluk, 1975, L medium from Lichter, 1981. Medium had a highly significant effect on protoplast viability and division ($p < 0.01$).

Figure 12a: Xylem differentiation in SS cell suspensions cultured in liquid medium supplemented with 2 mg/l 2,4-D. x200.

Figure 12b: Undifferentiated small cell clusters in SS cell suspensions cultured in liquid medium supplemented with 2 mg/l 2,4-D. x400.

Figure 12c: Arrow shows an SS suspension-derived polynucleated protoplast arising from multiple spontaneous protoplast fusions. x200.

Figure 12d: Suspension-derived shepherd's purse protoplasts, after 6 h of enzymatic digestion. x400.

Figure 12e: First protoplast division after 5 days of culture in 8p medium. x400.

Figure 12f: Second protoplast division after one week of culture in 8p medium. x400.

Figure 12g: Protoplast-derived colony after 21 days of culture. x400.

Figure 13a: Protoplast-derived shepherd's purse plantlet acclimated to growth chamber conditions in a peat pellet. x0.5.

Figure 13b: Protoplast-derived shepherd's purse plant, potted in soil and transferred to a growth chamber. x0.25.

Figure 13c: Variety of leaf shapes, from entire to deeply lobed, produced on a shepherd's purse protoplast-derived regenerant. x1.

Figure 13d: Scanning electron micrograph of the upper surface of a leaf of a shepherd's purse seed-derived plant. Bar represents 100 μm .

Figure 13e: Scanning electron micrograph of the upper surface of a leaf of a regenerant from suspension-derived protoplasts. Bar represents 100 μm .

Figure 13f: Lanceolate leaf, abnormal male sterile flower and immature fruit of a shepherd's purse protoplast-derived regenerant. x3.

DISCUSSION

Response similarities were detected between stinkweed and shepherd's purse leaf explant cultures. In both species, media containing NAA induced prolific root development, while 2,4-D stimulated callus proliferation, as was observed for *B. juncea* (Chapter I), and has been often reported (e.g. Gleddie, 1989; Kao and Séguin-Swartz, 1987). Cytokinins were necessary to shoot regeneration, with highest frequencies obtained with TZ, adenine sulfate, kinetin or zeatin (Table 22), although no shoots were obtained in media containing kinetin, zeatin, or BA alone. This is in agreement with Pelletier *et al.* (1983), who selected adenine sulphate as the best cytokinin for plant regeneration from protoplasts of *B. napus*, and with Gleddie (1989), who selected TZ as the best cytokinin for plant regeneration from cell suspensions of *A. thaliana*. Gleddie (1989) observed that TZ induced the production of morphologically abnormal shoots and embryo-like structures; in the present study, the shoots produced in presence of TZ were normal to slightly vitrified, and easily rooted. Combinations of auxins and cytokinins are generally recommended for plant regeneration from explant cultures (Margara, 1984), however, the addition of auxins to silver dollar, shepherd's purse and stinkweed regeneration media largely decreased shoot production, in accordance with previous reports for Cruciferous species (Klimaszewska and Keller, 1987; Gleddie, 1989).

Most strikingly, the parallel manipulations of these three wild Cruciferous species provided further evidence for the importance of both the source of plant material and the genotype, for plant regeneration from tissue and protoplast cultures. Under strictly

identical culture conditions, responses have ranged from absence of callus production to shoot regeneration from explants, and from dead or non-dividing protoplasts, to embryogenic cultures and protoplast-derived plants. Sustained divisions could not be obtained from mesophyll protoplasts of silver dollar, stinkweed, nor shepherd's purse, although protoplasts derived from mesophyll tissue of axenic plants have proved to be readily amenable to culture for a number of wild Crucifers, including Arabidopsis thaliana (Damm and Willmitzer, 1988), Eruca sativa (Fahleson et al., 1988b), Sinapis alba (Primard et al., 1988), and Diplotaxis muralis (Sikdar et al., 1990b). For shepherd's purse, protoplast culture was dramatically improved by using cell suspensions rather than mesophyll protoplasts, resulting in protoplast division, callus proliferation, embryogenesis, and plant regeneration. Ford (1990) also reported that protoplasts of A. thaliana undergo sustained cell divisions when isolated from cell suspension cultures, whereas leaf or root-derived protoplasts from axenic plants failed to divide. Cells in suspension are in a low state of differentiation, have rapid division cycles, are adapted to in vitro conditions, and should therefore be quite amenable to protoplast culture. On the other hand, stinkweed cells in suspensions, under the same conditions as shepherd's purse cells, did not divide and underwent polyphenolic oxidation that could not be reduced with the antioxidant ascorbic acid, nor by polyphenol chelators such as Polyclar AT, PVP 40, or activated charcoal.

Capsella bursa-pastoris SS suspension cultures, once stabilized, divided quickly and remained fine and unorganized in the presence of 2,4-D. In an attempt to induce embryogenesis in liquid cultures, 2,4-D was removed from the medium, which stimulated

root development. When NAA was added to the liquid medium, root organogenesis was also promoted, while the cytokinins BA and TZ only stimulated the early stages of cell organization into clusters or primordia (Table 23). These cell clusters and primordia which formed in response to cytokinins, browned after five weeks with no further development.

On the other hand, these suspension cultures were very embryogenic once they were plated onto solidified growth regulator-free medium. Rinsing the cells before plating, in order to remove as much 2,4-D as possible did not affect the frequency of embryogenesis, which suggests that a supply of exogenous 2,4-D was not required for embryo development, and that low levels of 2,4-D did not inhibit embryo development. Both 1 mg/l NAA or 0.5 mg/l BA in the solid medium, reduced the frequency of embryogenesis, and were therefore considered slightly inhibitory (Table 25). In contrast with the findings of Sikdar *et al.* (1990b) with *D. muralis* protoplasts, GA3 was found to be inhibitory to embryogenesis. Cell clusters and root meristems previously cultured in growth regulator-free liquid medium for two to three weeks did not respond in terms of embryo production when plated on solid medium.

The embryogenic response of SS shepherd's purse cell suspensions was, thus, extremely dependent on physical culture conditions. In liquid cultures, 2,4-D containing medium was only gradually diluted out at each weekly transfer, since fresh medium was added to one third of the old culture medium. This might partly explain the lack of embryogenesis observed in liquid cultures. The initial presence of 2,4-D in culture media is often found to be essential to stimulate embryogenesis in Crucifers (Sikdar *et*

al., 1990b), but it can become strongly inhibitory at later stages (Klimaszewska and Keller, 1986). Also, gas exchange conditions between cells and their environment are more favourable when cells are plated onto solidified medium. These shepherd's purse suspensions differ dramatically from the *B. nigra* cell suspension described by Klimaszewska and Keller (1986). *Brassica nigra* cell suspensions, when plated onto solidified media, did not produce any somatic embryos, whether they were rinsed with growth regulator-free medium or not; however, somatic embryos were produced in all liquid media tested.

The six month old *C. bursa-pastoris* SPP cell suspensions, on the other hand, produced embryos in liquid culture, shoots when plated onto solidified media (Table 24), and normal plants were easily and rapidly recovered. However, somaclonal variations were detected by trichome abnormalities, mainly the presence of straight trichomes throughout the leaf surface and on sepals, which suggest that one should be cautious when utilizing trichome type as a characterization marker for somatic hybrids. Genetic abnormalities are a well recognized problem associated with cell suspension or callus cultures. The older suspension cultures might have contained numerous abnormal aneuploid or polyploid cells, as is often described (Karp, 1989), reducing their plant regeneration potential.

Three to six hours of digestion were sufficient to obtain up to 3×10^7 protoplasts/g of cells from SS cell suspensions, from which 2% to 7% had divided after one week (Table 26). Organic additives such as BSA, coconut milk and casamino acids, supplemented to 8p-based media, had no positive effect on the number of transferable

colonies obtained after five weeks, in contradiction with previous reports. Casamino acids contain metabolic intermediates for growth, and are thought to possess detoxifying properties. They are beneficial to *B. nigra* cell suspension, and to *B. juncea* mesophyll protoplasts (Kao and Séguin-Swartz, 1987). On the other hand, Davies *et al.* (1989) detected inhibitory compounds in casein hydrolysate and coconut water, which had detrimental effects to leaf protoplasts of alfalfa.

Excellent embryogenic potential was retained in shepherd's purse cell suspension cultures for more than 3 years, as was reported for *A. thaliana* cell suspensions (Ford, 1990; Gleddie, 1989). This is in contrast with *B. nigra* cell suspensions, that displayed a marked reduction in embryogenic potential after 50 weeks (Klimaszewska and Keller, 1986). However, many of the *Capsella* embryos derived from the 3 year old suspensions were abnormal and did not mature, despite the many culture conditions assessed. Large numbers were multicotyledonary, or developed into the shape of a funnel, which was also observed by Monnier (1978) from zygotic embryos cultured *in vitro*. Monnier (1978) recommended to culture embryos with high sucrose concentrations (up to 12%) to simulate the seed coat and prevent precocious embryo germination. Loh *et al.* (1983) observed that secondary embryos grew into plants when cultured in presence of 10^{-4} M kinetin, or of 10^{-7} to 10^{-4} M BA. The *C. bursa-pastoris* embryogenic calli had been transferred to growth regulator-free medium for 4 months before embryo maturation was attempted. Loh *et al.* (1983) believed that the capacity for further embryogenesis, rather than for shoot regeneration, could be selected for by repeatedly subculturing secondary embryogenic calli. This might explain that all attempts to stimulate embryo maturation

from shepherd's purse calli were unsuccessful, and that plant regeneration seemed to occur spontaneously with low frequencies.

Concluding remarks. This study describes, for the first time, the initiation and maintenance of embryogenic cell suspension cultures of C. bursa-pastoris, which were still embryogenic after 3 years, provided large populations of regenerable protoplasts, and could thus be considered as an acceptable protoplast source. Another unique feature of these suspensions was the high levels of root organogenesis obtained upon removal of 2,4-D from liquid cultures. This could provide an interesting system to be used in root development studies. Wild relatives have been considered useful in providing genetic sources for improving Brassica crops (Chatterjee *et al.*, 1988; Primard *et al.*, 1988; Toriyama *et al.*, 1987b). The ability to isolate protoplasts and regenerate plants from C. bursa-pastoris may allow the transfer of genetic traits from this species to agronomically important Brassica species, through protoplast manipulation and somatic hybridization.

CHAPTER III

SOMATIC HYBRIDIZATION

BETWEEN BRASSICA

CROP SPECIES AND THE WILD

CRUCIFERS

THLASPI ARVENSE

AND CAPSELLA BURSA-PASTORIS.

LITERATURE REVIEW

A. INTRODUCTION.

Crosses between crop species and wild relatives, or unrelated species, constitute a desirable approach for increasing genetic diversity from which breeders can select, but they are often limited, due to strong incompatibility barriers such as a blockage of pollen tube development, or lack of endosperm development. Brassica crop species cannot be sexually crossed to many species outside the phylogenetic triangle of U (Glimelius, 1988). Somatic hybridization, or the fusion of protoplasts isolated from somatic cells, may overcome sexual barriers between species. Novel genetic combinations can also be created due to the possibility of transferring only part of one parent's nuclear genome (asymmetric hybrids), of transferring organelles without the nucleus (cybrids), and of organellar genomic recombination (Keller *et al.* 1982). Protoplast fusion is therefore the method of choice for the transfer of uncharacterized complex polygenic traits, such as yield or pest resistance, and somatic hybrids can serve as genetic bridges for the transfer of variable amounts of genetic information from one species to another.

B. SOMATIC HYBRIDIZATION IN BRASSICA.

a. Intraspecific fusions and cytoplasmic hybridizations.

Intraspecific fusions have been used to create alloplasmic lines (i.e. same nucleus substituted in different cytoplasms), mostly for B. napus. Cytoplasmic traits of interest include atrazine-resistance encoded in chloroplast DNA of some B. campestris lines (Pelletier *et al.*, 1983), and above all, cytoplasmic male sterility (CMS). Cytoplasmic male sterility systems are of major practical interest for the production of F1 hybrid populations, and have been the main issue for most intraspecific somatic hybridization studies in Brassica napus, as listed in Table 27.

b. Interspecific fusions.

The resynthesis of B. napus, by fusion of the parental diploid ancestors B. oleracea and B. campestris, has been achieved many times (Table 28), with the main objective of increasing genetic variability of B. napus. Somatic hybridizations between many Brassica species have also been undertaken, in order to combine all three Brassica genomes A, B and C, from the triangle of U (Glimelius, 1988). Most of these studies have resulted in successful regeneration of somatic hybrids, although aneuploidy and sterility were frequent (Terada *et al.*, 1987; Sundberg *et al.*, 1987). Other interspecific hybrids, however, such as B. juncea + B. spinescens (Kirti *et al.*, 1991), or B. oleracea + B. napus (Kao *et al.*, 1992), have retained full chromosome complements of both parental species, and were at least female fertile, thus allowing backcrosses to the crop species. Traits of agronomic interest to Brassica crop species include: resistance to white

Table 27: Intraspecific somatic hybridizations of Brassica napus for transfer of cytoplasmic male sterility or restoration of normal chloroplasts in CMS lines.

parent 1 (P1)	parent 2 (P2)	selection	reference
chloroplasts from <u>Raphanus sativus</u>	normal <u>B. napus</u> chloroplasts	none	Pelletier <i>et al.</i> , 1983
chloroplasts from <u>Raphanus sativus</u> . CMS.	normal <u>B. napus</u> chloroplasts	X-rays. density gradient fractionation to form P1 cytoplasts	Bornman <i>et al.</i> , 1986
CMS mitochondria	triazine resistant chloroplasts	micromanipulation	Yarrow <i>et al.</i> , 1986
CMS Polima spring type	fertile winter type <u>B. napus</u> chloropl.	P1 = X-rays P2 = IOA	Barsby <i>et al.</i> , 1987
CMS Ogura	fertile <u>B. napus</u> chloroplasts	P1 = X-rays	Menczel <i>et al.</i> , 1987
CMS Ogura	normal <u>B. napus</u> chloroplasts	P1 = X-rays P2 = IOA	Morgan and Maliga, 1987
CMS Ogura	winter type <u>B. napus</u> chloroplasts	plant morphology	Jarl and Bornman, 1988
haploid CMS	haploid atrazine resistant	none	Chuong <i>et al.</i> , 1988
various winter cultivars	triazine resistant cv. OAC Triton	P1 = triazine P2 = X-rays	Tomzik and Hain, 1988

CMS = cytoplasmic male sterility.

rust (Albugo candida), tolerance to salt, and high photosynthetic efficiency, displayed by B. spinescens (Kirti et al., 1991); cytoplasmic male sterility of B. napus (Kao et al., 1992); drought tolerance and resistance to the fungus Alternaria brassicae, displayed by Sinapis alba (Primard et al., 1988); atrazine resistance, encoded in the chloroplasts of B. campestris (Jourdan et al., 1989).

c. Intergeneric fusions.

Somatic hybridizations have been attempted to create genetic bridges for the transfer of agronomically interesting traits from sexually incompatible wild species to the Brassica crop species (Table 29). Traits of agronomic interest to Brassica crop species include: aphid and drought tolerance displayed by Eruca sativa (Fahleson et al., 1988b; Sikdar et al., 1990a); cold tolerance in Barbarea vulgaris (Glimelius et al., 1991); high concentration of nervonic acid, used as lubricant, produced by Thlaspi perfoliatum (Glimelius et al., 1991); cytoplasmic male sterility that could arise in hybrids with Diplotaxis harra (Klimaszewska and Keller, 1988); intermediate C3-C4 photosynthetic properties, exhibited by Moricandia arvensis (Toriyama et al., 1987a).

Many stable lines of intergeneric and intertribal hybrid plants have been produced, such as B. juncea + D. muralis (Chatterjee et al., 1988), B. juncea + Eruca sativa (Sikdar et al., 1990a) and B. napus + Eruca sativa (Fahleson et al., 1988b), although some were highly abnormal and sterile, and therefore could not be used in breeding programmes. Arabidobrassica produced through fusion of A. thaliana and B. campestris protoplasts, displayed normal vegetative morphology, but developed abnormal flowers and were therefore sterile (Hoffmann and Adachi, 1981);

Table 28: Interspecific somatic hybridizations in *Brassica* sp.

parent 1 (P1)	parent 2 (P2)	selection	regeneration of hybrids	fertile plants*	reference
<i>B. oleracea</i>	<i>B. campestris</i>	hybrid vigour	yes	yes	Schenck and Röbbelen, 1982
<i>B. oleracea</i>	<i>B. campestris</i>	none	no	no	Menczel and Wolfe, 1984
<i>B. oleracea</i>	<i>B. campestris</i>	flow sorting	no	no	Glimelius et al., 1986
<i>B. oleracea</i>	<i>B. campestris</i>	inability to regenerate of P1 and P2	yes	NM*	Taguchi and Kameya, 1986
<i>B. oleracea</i> CMS Ogura	<i>B. campestris</i> atrazine ^R	P1 = atrazine P2 = inability to regenerate	yes	no	Robertson et al., 1987
<i>B. oleracea</i>	<i>B. campestris</i>	micromanipul. flow cytometry	yes	yes	Sundberg et al., 1987
<i>B. oleracea</i>	<i>B. campestris</i>	P1 = IOA P2 = inability to regenerate	yes	yes	Terada et al., 1987
<i>B. nigra</i> hygromycin ^R	<i>B. napus</i>	P1 = X-rays P2 = hygrom.	yes	no	Sacristán et al., 1989
<i>B. nigra</i>	<i>B. napus</i>	flow cytometry	yes	yes	Sjödin and Glimelius, 1989b
<i>B. oleracea</i>	<i>B. napus</i> atrazine ^R	P1 = atrazine	yes	yes	Jourdan et al., 1989
<i>B. oleracea</i>	<i>B. nigra</i>	P1 = IOA	yes	yes	Jourdan and Salazar, 1991
<i>B. juncea</i>	<i>B. spinescens</i>	none	yes	yes	Kirti et al., 1991
<i>B. oleracea</i>	<i>B. napus</i> CMS Ogura	P1 = IOA	yes	yes	Kao et al., 1992

a: Fertile plants = relate to female fertility; some "fertile" hybrids were male sterile.

*NM = not mentioned.

Table 29: Intergeneric hybridizations in *Brassica*.

parent 1 (P1)	parent 2 (P2)	selection	regeneration of hybrids	fertile plants ^a	reference
<u>B. campestris</u>	<u>Arabidopsis thaliana</u>	hybrid vigour of colonies	yes	no	Gleba and Hoffmann, 1980
<u>B. oleracea</u>	<u>Moricandia arvensis</u>	inability of P1 and P2 to regenerate	yes	no	Toriyama et al., 1987a
<u>B. oleracea</u>	<u>Sinapis turgida</u>	P1 = inability to regenerate P2 = 5MT ^R and nitrate-reductase deficient	no	no	Toriyama et al., 1987b
<u>B. napus</u>	<u>S. alba</u>	none	yes	yes	Primard et al., 1988
<u>B. napus</u>	<u>Eruca sativa</u>	micromanipulation flow cytometry	yes	yes	Fahleson et al., 1988b
<u>B. juncea</u>	<u>Diplotaxis muralis</u>	P2 = X-rays plant morphology	yes	NM*	Chatterjee et al., 1988
<u>B. napus</u>	<u>D. harra</u>	P1 = IOA P2 = inability to regenerate	yes	no	Klimaszewska and Keller, 1988
<u>B. oleracea</u>	<u>Raphanus sativus</u>	P1 = IOA P2 = inability to regenerate	yes	yes	Kameya et al., 1989
<u>B. juncea</u>	<u>E. sativa</u>	P2 = X-rays plant morphology	yes	yes	Sikdar et al., 1990a
<u>B. napus</u>	<u>Barbarea vulgaris</u>	flow sorting	yes	no	Glimelius et al., 1991
<u>B. napus</u>	<u>Thlaspi perfoliatum</u>	flow sorting	yes	yes	Glimelius et al., 1991

a: Fertile plants relate to female fertility, even partial; some of the hybrids might have been male sterile. *NM = not mentioned. B. napus + Diplotaxis harra hybrids

(Klimaszewska and Keller, 1988), and *B. napus* + *A. thaliana* hybrids (Bauer-Weston, 1990) were also reported to be sterile.

C. SELECTION OF HETEROKARYONS.

A combination of two or more of the following selection methods is often used, to restrict growth from both fusion partners and to increase the chances of recovering somatic hybrids.

a. Use of selectable markers.

Hybrid selection schemes have been based on the inability of one (or both) parental protoplasts to undergo division or plant regeneration, using hybrid vigour, or morphological differences between parents. Carlson *et al.* (1972) used the expression of auxin autotrophy following hybridization of two *Nicotiana* species, while both parents required auxin in the culture medium. Non-regenerable protoplasts were fused to a regenerable species in some studies (Chatterjee *et al.*, 1988; Klimaszewska and Keller, 1988), while neither parental population could develop colonies in fusions between *B. oleracea* and *Moricandia arvensis* (Toriyama *et al.*, 1987a). Kamata and Nagata (1987) have retrieved 70%-80% of heterokaryons after centrifugation in a density gradient of Percoll, between mesophyll *Nicotiana glauca* protoplasts and epidermal *N. langsdorfii* protoplasts, which had different specific gravities; although this strategy seemed to be a widely applicable method for protoplasts which do not possess any selectable marker, it has not been exploited by other authors. *Brassica oleracea* and *B. campestris*

protoplasts released toxic substances, presumably polyphenolics, that allowed selective growth of only hybrid cells (Schenck and Röbbelen, 1982). Ideal selection strategies utilize the traits to be transferred, through fusion, as selection pressures. The transfer of resistance to the blackleg fungal pathogen *Phoma lingam* from *B. juncea* or *B. nigra* to *B. napus*, is a good example: irradiated mesophyll protoplasts of *B. juncea* and *B. nigra*, resistant to *P. lingam*, were fused to hypocotyl protoplasts of *B. napus*, a susceptible species (Sjödin and Glimelius, 1989a). They were selected in presence of 3 μ M sirodesmin PL, a phytotoxin specifically produced by *P. lingam*. Resistance to blackleg was obtained in most of the toxin-selected plants, whereas unselected somatic hybrid plants were sensitive to the fungus.

Double mutants of *Sinapis turgida*, containing both dominant and counter-selectable markers, have been isolated and characterized by Toriyama *et al.* (1987b). These mutants failed to grow on media containing nitrate as sole nitrogen source (selected by a resistance to chlorate), and were resistant to 5-methyltryptophan (5MT). An *Arabidopsis thaliana* mutant cell line, non regenerable and resistant to the sulfonylurea herbicide chlorsulfuron (DuPont), was used in somatic hybridizations with *B. napus* stem cortex protoplasts (Bauer-Weston, 1990). An other example would be the incorporation of hygromycin resistance in *B. nigra*, by transformation with a disarmed *Agrobacterium tumefaciens* strain, and its use as a selectable marker in protoplast fusions with *B. napus* (Sacristán *et al.*, 1989).

A very valuable strategy is the combination of two antibiotic or herbicide resistant transgenics, so that only hybrid cells can grow on selective media supplemented with the

appropriate antibiotics or herbicides. Such a selection system was utilized for the recovery of somatic hybrids between diploid clones of potato, using a kanamycin resistant line and a hygromycin resistant line (Masson *et al.*, 1989). Dual-antibiotic resistance was also exploited in somatic hybridizations by Sproule *et al.* (1991), using methotrexate resistant *N. tabacum* and kanamycin resistant *N. debneyi* transgenics, both obtained by co-cultivation with a disarmed *A. tumefaciens* strain.

b. Metabolic inactivation.

Protoplast inactivation is a widely applicable selection procedure, and is frequently employed, since the development of mutants or transformants is time consuming. Iodoacetamide (IOA), a commonly used metabolic inhibitor reacting with sulfhydryl groups, has successfully inhibited protoplasts of *Nicotiana* sp. (Sirodov *et al.*, 1981), of *B. oleracea* in fusions with *B. campestris* (Terada *et al.*, 1987), of *B. napus* in interspecific fusions (Barsby *et al.*, 1987; Morgan and Maliga, 1987) and in fusions with *Diplotaxis harra* (Klimaszewska and Keller, 1988).

c. Nuclear inactivation.

Gamma or X-irradiation at sub-lethal doses provide good selection means against one of the parental species by preventing protoplast division, but also induces the formation of asymmetric hybrids by causing directional chromosome elimination after fusion from the irradiated partner. Dudits *et al.* (1987) transferred methotrexate and 5-methyltryptophane (5-MT) resistance from *Daucus carota* to *Nicotiana tabacum* by irradiating the carrot cell suspension-derived protoplasts. In *Brassica*, irradiation has successfully prevented protoplast division of *B. napus* in intraspecific fusions (Menczel

et al., 1987), and of *B. nigra* prior to fusion with *B. napus* (Sjödin and Glimelius, 1989b). Little is known on the precise effect of irradiation; it is believed to result in breaks of DNA sugar-phosphate bonds (Veleminsky and Gichner, 1978), and in reactions between hydrogen ions and DNA purine and pyrimidine bases (Holroyd and Glass, 1969).

Irradiation and IOA treatment have been used in conjunction, for example by Barsby et al. (1987) to obtain cybrids of *B. napus*, by Dudits et al. (1987) to transfer methotrexate and 5-MT resistance from *Daucus carota* to *Nicotiana tabacum*, and by Bates et al. (1987) to transfer kanamycin resistance and genes for nopaline synthesis from *N. plumbaginifolia* to *N. tabacum*.

d. Mechanical isolation.

Fluorescence activated flow cytometry has been adapted to sort large numbers of heterokaryons. Fusion partners are stained with fluorescent dyes emitting at distinct wavelengths, such as carboxyfluorescein (green fluorescence) or RITC (red fluorescence). Alternatively, one of the parents is stained with a dye emitting in the green spectrum, while red epifluorescence emitted by chloroplasts of the other partner is exploited; heterokaryons are then distinguished from parental cells by their dual fluorescence, and selectively collected by the cell sorter (Glimelius, 1988). This technique requires expensive equipment and considerable technical expertise, however, once established, it has been widely applicable (Glimelius et al., 1986). Micromanipulation is probably the most precise selection system, but also the most tedious. Once identified, hybrid cells are individually isolated with micropipettes. One

difficulty with this approach is the necessity to culture protoplasts at low densities, however, it has been achieved with the use of nurse cultures. Nurse culture with a nitrate reductase deficient mutant of Nicotiana tabacum was used by Bauer (1990), to grow B. oleracea micromanipulator-isolated heterokaryotes. Although much work has been dedicated to the optimization of micromanipulation, it does not seem to be a method of choice.

D. CHARACTERIZATION OF PUTATIVE HYBRIDS.

The selection of hybrid products usually leads to an enrichment of hybrid cells, but does not constitute proof of hybridity. Putative hybrids must therefore be characterized, in order to detect eventual parental escapes.

Floral and leaf characteristics, usually intermediate to those of the parents, have often been analyzed (e.g. Toriyama *et al.*, 1987a; Kirti *et al.*, 1991; Sproule *et al.*, 1991; Kao *et al.*, 1992). An other marker is the nature and shape of trichomes (Gleddie, 1984). Chromosome counts in root tip cell mitoses have been commonly used, however, the chromosome origin can accurately be determined only when parental chromosomes are of different sizes, as was the case for A. thaliana and B. oleracea fusion products (Nagl and Hoffmann, 1980). Chromosome numbers could also be estimated by flow cytometry, since they have been correlated with measurements of nuclear DNA content in somatic hybrids of some Brassicaceae (Fahleson *et al.*, 1988a).

Electrophoretic isozyme studies, using starch or polyacrylamide gels, have been

used extensively with regenerated plant material (Wetter and Dyck, 1983). Examples of the most common isozymes employed to assess somatic hybrid material are: aspartate aminotransferase, esterases, peroxidases, phosphodiesterase, leucine aminopeptidase and glucose-6-phosphate dehydrogenase (Wetter and Dyck, 1983). Ribulose bisphosphate carboxylase (RBPCase; fraction-I protein; Rubisco), the most abundant protein of green chloroplasts, has also been frequently analyzed, by isoelectric focusing (Gleddie, 1984). Criticisms of these methods are that they only represent gene expression rather than the actual genetic content (G. Bates, personal comm.), and that only a few genes can be identified with zymograms (Glimelius, 1988).

Recombinant DNA techniques have provided powerful tools for somatic hybrid analysis, mainly with restriction fragment length polymorphism (RFLP) studies. Probes can code for species-specific repetitive DNA, facilitating screening for the presence of genetic material from a given species. For example, cloned Eruca sativa-specific DNA sequences were used as probes to determine the extent of E. sativa genome transfer in E. sativa + B. napus somatic hybrids (Fahleson *et al.*, 1991). Probes can code for genetic markers, or unique genes to be introgressed; for example, the cloned A. thaliana-specific acetolactate synthase gene to be transferred to B. napus, was used to characterize B. napus + A. thaliana asymmetric hybrids (Bauer-Weston, 1990). Finally, probes can code for chloroplast- or mitochondrial-specific genes, thus allowing the analysis of cytoplasmic genotype (e.g. Jourdan *et al.*, 1989).

E. FATE OF PARENTAL NUCLEAR GENOMES: SPONTANEOUS AND INDUCED ASYMMETRIC HYBRIDIZATIONS.

Chromosome elimination is very common among fusion products between phylogenetically remote species, such as soybean + tobacco (Kao, 1977), although the extent and direction of the loss has been considered unpredictable (Hinnisdaels *et al.*, 1988). Even interspecific somatic hybrids, which could be obtained sexually, have frequently shown aneuploidy, with chromosome numbers that differ widely from the sum of both parental genomes (Glimelius, 1988). These variations may depend upon the length of culture necessary to recover plants, or incompatibilities between nuclear and cytoplasmic genes (Evans *et al.*, 1983). Chromosome elimination from one species rather than the other has been attributed to different culture condition requirements, the source of protoplasts (e.g. hypocotyl vs cell suspension), or their stage in the mitotic cycle. Szabados and Dudits *et al.* (1980) observed that fusion of interphase and mitotic protoplasts resulted in premature chromosome condensation in the interphase nuclei, resulting in their pulverization and elimination. Asymmetric hybridization is, therefore, often spontaneous. The only thorough comparison of nuclear DNA content in several hybrid combinations, comprising interspecific, intertribal crosses, and crosses with species of various ploidy levels, was performed in Brassicaceae by Sundberg and Glimelius (1991). They concluded that both the genetic distance and ploidy level differences between the parental species had an effect on chromosome elimination; more DNA was eliminated in intertribal than in interspecific crosses, and chromosomes were

lost from the diploid parental species in diploid/tetraploid fusions (Sundberg and Glimelius, 1991).

Asymmetric hybridizations can also be performed, with the aim of creating hybrids which carry the whole genome of the recipient species (the crop species) and only a few chromosomes of the donor (wild) species, thus reducing the linkage drag to be eliminated by repeated backcrosses to the crop species (recurrent parent). Several methods can be used to develop asymmetric somatic hybrids. A common technique is to irradiate one of the parents before fusion. Bates *et al.* (1987) have efficiently eliminated most Nicotiana plumbaginifolia chromosomes in 40% of regenerants derived from fusions between gamma-irradiated kanamycin-resistant N. plumbaginifolia with N. tabacum. Although a debated observation, chromosome elimination has been correlated with irradiation doses. For example, analyses of asymmetric somatic hybrids between leaf protoplasts from L. esculentum and gamma-irradiated leaf protoplasts from L. peruvianum, has been performed by Wijbrandi *et al.* (1990), using 29 RFLP markers. Eight "low dose" hybrids were irradiated with 50 gray, and seven "high dose" hybrids were irradiated with 300 gray, resulting in a 14-69% elimination of alleles from the irradiated species, and dose-dependent loss of donor DNA, since low dose hybrids contained more alleles and complete chromosomes than high dose ones. Chatterjee *et al.* (1988) have gamma-irradiated D. muralis protoplasts before fusion with B. juncea. This was not used as a selection method, since D. muralis protoplasts had a very low plating efficiency, but indeed to favour asymmetric fusions. Sikdar *et al.* (1990a) have used a similar strategy by irradiating Eruca sativa, in fusions with B. juncea.

F. FATE OF PARENTAL CYTOPLASMIC GENOMES.

a. Chloroplasts.

Most studies report complete segregation of parental chloroplasts, sometimes random, but very often strongly biased towards one of the parents. For example, non-random segregation occurred in somatic hybrids between B. napus and E. sativa, in favour of B. napus chloroplasts (Fahleson *et al.*, 1988a). In B. napus + S. alba somatic hybrids, 12 had B. napus chloroplasts, while only 2 had S. alba plastids (Primard *et al.*, 1988). The other parental plastid population might still be present, but at undetectable levels (Moll *et al.*, 1990). Analyses of chloroplast DNA complements of various Brassica hybrid combinations, led Sundberg and Glimelius (1991) to conclude that, in case of distantly related species, the chloroplasts of the species whose nucleus predominated, would be favoured. On the other hand, segregation was more random in cases of amphidiploid hybrids between closely related species; the strong bias towards B. napus chloroplasts in hybrids with B. juncea remained unexplained (Sundberg and Glimelius, 1991). A single case of chloroplast recombination has been reported to date in Nicotiana (Medgyesy *et al.*, 1985).

b. Mitochondria.

Mitochondrial DNA readily undergoes intragenomic recombination, but in somatic hybrids, they can recombine intergenomically, and characteristically contain fragments from both parents as well as new fragments (Pelletier *et al.*, 1988). Random or biased

inheritance of mitochondria have also been observed, independent of chloroplasts (Primard *et al.*, 1988). For example, mitochondrial recombinations were not detected in hybrids between *B. napus* and *B. oleracea* (Jourdan *et al.*, 1989), which all contained only *B. napus* mitochondria. The non-random segregation of mitochondria could be due to different ploidy levels of parents, different organelle replication rates, protoplast source and unequal input of organelles, or parental phylogenetic distance (Landgren and Glimelius, 1990).

G. INTROGRESSION OF LIMITED AMOUNTS OF DONOR DNA INTO RECIPIENT CELLS.

A desirable objective of somatic hybridization would be the introduction of specific genes rather than the production of addition lines, is desirable for reducing linkage drag (hybrid drag), increasing genetic stability, and the transfer of useful genes to future generations. For this to occur, homology must exist between chromosomal regions from both species (Hinnisdaels *et al.*, 1988). An anticipated problem would be that chromosome complements of each species remain physically separated within the hybrid nucleus. Gleba *et al.* (1987) have studied metaphase plates of somatic hybrids, and observed that chromosomes of each species remained as distinct groups within the nucleus, even after long periods of culture. Unless the two genomes are intermixed, for example by colchicine treatments which disorganize the spindle microtubules, chromosomal recombination between the two species would be impossible. In contrast, Chatterjee *et al.* (1988) observed that true somatic hybrids between *B. juncea* and *D.*

muralis formed three quadrivalents and one hexavalent at meiosis, suggesting homology between at least three linkage groups, and between one chromosome of one species and two chromosomes of the other species. Precise selection pressures have allowed transfer of specific genes; the transfer of resistance against Phoma lingam from B. juncea or B. nigra to B. napus, was indeed achieved by selecting fusion products with sirodesmin PL, a phytotoxin specific of Phoma lingam (Sjödín and Glimelius, 1989b).

H. OBJECTIVES.

The isolation of viable protoplasts from Thlaspi arvense (stinkweed) leaves, and Capsella bursa-pastoris (shepherd's purse) cell suspensions was described in Chapter II. The objective of this study was to achieve PEG-mediated fusion between these species and the crop species B. napus, B. juncea, and B. campestris, and to regenerate and characterize viable somatic hybrids.

Several approaches were taken to select and regenerate somatic hybrids between these wild crucifers and the crop species. A very powerful selection system is the use of fluorescence activated flow cytometry, which was attempted. It was shown that the protoplast culture medium used for B. napus could be selective against stinkweed mesophyll protoplasts. Two mutant B. napus cell suspensions, resistant to the Hoechst herbicide phosphinotricin (l-PPT) or to chlorsulfuron (DuPont) were available for selection of heterokaryons. Different levels of natural resistance to the American Cyanamid imidazolinone herbicides AC263.499 and AC252.214, by wild and cultivated

Cruciferous species (Miki *et al.*, 1990), was another potential selection scheme for the selection of somatic hybrids. A more general selection strategy was to treat the Brassica species with iodoacetamide, while stinkweed and shepherd's purse were X-irradiated prior to fusion for selection purposes, but also to encourage asymmetric hybridization.

The regeneration and characterization of putative somatic hybrid cell lines were also important objectives. Various regeneration media were assessed, and hybridity was verified by morphological, biochemical and molecular means.

MATERIALS AND METHODS

A. SELECTION SYSTEMS FOR ENRICHMENT IN HYBRID CELL LINES.

a. Flow sorting. Concentrated solutions of 5 mg/ml RITC and FITC, pH=7.5, were prepared in acetone or 95% ethanol, as described in Redenbaugh *et al.* (1982). Stem cortex protoplasts of *B. napus* cv. Westar were isolated as described in Chapter I, except that they were recovered by flotation over a 0.6 M sucrose solution, and *T. arvense* mesophyll protoplasts were isolated from 3 week old axenic plantlets as described in Chapter II. The enzymatic solution for stinkweed protoplasts was supplemented with 36 $\mu\text{g/ml}$ RITC, and the enzymatic solution for *B. napus* protoplasts was supplemented with 18 $\mu\text{g/ml}$ FITC. For detection purposes, fusion products were mimicked by double-staining a protoplast population of *B. napus* with RITC and FITC. Protoplast density was adjusted to at least 10^6 per ml. Flow sorting was performed by M. Bergeron (Health and Welfare, Ottawa) using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) with a Coherent (Palo Alto, CA) Innova 90 argon-ion laser, adjusted to a 488 nm excitation wavelength and 500 mW power output. Prior to sorting, the collecting chamber and the system were surface sterilized with 70% ethanol. The sheath fluid consisted of a sterile solution of 0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.5% KCl, pH=6.9, and sheath pressure was 13-15 psi. The nozzle tip had a diameter of 76 μm , but was replaced after a few experiments with a 110 μm tip. A piezoelectric quartz crystal, vibrating at 32 kHz, divided the flow stream into droplets, which were deflected by charged plates:

protoplasts displaying both RITC and FITC fluorescences were retrieved into a glass tube, while all other cells were sent to a waste container. Data were analyzed with a Coulter Easy 88 Data Management System (Coulter Electronics, Hialeah, FL), and plotted onto two dimensional graphs of red vs green fluorescence. Sorted protoplasts were then centrifuged, concentrated to one thousand protoplasts per ml of medium B (Pelletier *et al.*, 1983), and plated over a 0.4% SeaPlaque™ agarose (FMC, Rockland) underlayer, or on filter paper over *B. napus* cell suspensions, embedded in 0.4% SeaPlaque agarose.

b. Specific herbicide resistance. Autoclaved B5 medium containing 2% sucrose, 0.8% Difco-Bacto agar, was cooled, supplemented with 0 to 250 $\mu\text{g/l}$ of the imidazolinone herbicides AC252.14 or AC263.499, and poured into Magenta™ boxes. Seeds of *B. napus* spp. *oleifera* cv Westar, *B. juncea* cv. Blaze, and *T. arvense* were sterilized as described in Chapter I, plated onto these solidified media, and maintained at 20°C, with a 16 h photoperiod under fluorescent and incandescent lights. Seedling growth was recorded after one week of culture. Cell suspensions of *B. juncea* cv. Domo (provided by A. Sproule, Agriculture Canada, Ottawa), *B. napus* cv. Tower (provided by M. Oelk, Hoechst Canada, Saskatoon) and *T. arvense* (see Chapter II) were also plated in Petri dishes, onto solidified MS media containing 2% sucrose, 2 mg/l 2,4-D, and supplemented with 0 to 250 $\mu\text{g/l}$ of AC252.14 or AC263.499. Cell divisions were recorded after one month.

A *B. napus* (cv. Topas) mutant cell suspension, labelled C3, and displaying a resistance to the herbicide chlorsulfuron (DuPont), was provided by J. Grainger,

Agriculture Canada, Ottawa. Chlorsulfuron was added to the B. napus C3 and the C. bursa-pastoris SS suspension-derived plated protoplasts, for final concentrations of 0.1, 0.25, 0.5, 1.0 and 2.0 $\mu\text{g/ml}$. Viability and division frequencies were recorded after one week, in view of determining the optimal chlorsulfuron concentration that would selectively inhibit division of shepherd's purse protoplasts.

Another B. napus (cv. Tower) cell suspension, labelled PPT334, and displaying a resistance to the herbicide phosphinotricin (l-PPT, Amer. Cyanamid), was provided by M. Oelck, Hoechst Canada, Saskatoon. A filter-sterilized 1 mM l-PPT stock solution was prepared in CPW salts (Appendix 7), and added to the B. napus C3 and the C. bursa-pastoris SS suspension-derived plated protoplasts, for final concentrations of 10, 20, 50 and 100 μM . Viability and division frequencies were recorded after one week, in view of determining the optimal l-PPT concentration that would selectively inhibit division of shepherd's purse protoplasts.

c. Iodoacetamide treatment of Brassica protoplasts. A 30 mM IOA stock solution was prepared in salt wash solution (0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.5% KCl, $\text{pH}=6.9$), and stored at -20°C . Stem cortex and cell suspension protoplasts were isolated as described in Chapters I and II, respectively, except that 50 ml glass tubes (instead of 15 ml) were used. Wall-digestive enzymes were discarded, and replaced with B medium containing from 0.25 to 6.0 mM IOA. Protoplasts were treated for 20 min. with regular swirling of the tubes, then centrifuged at 100xg and rinsed with 0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.5% KCl, $\text{pH}=6.9$, twice. Protoplasts were counted with a haemocytometer, centrifuged, and their density adjusted to 2.10^5 protoplasts per ml of B medium. They were plated and

maintained as described in Chapter I. Cell viability and division rates were determined as described in Chapter I.

d. Irradiation of C. bursa-pastoris SS cell suspension-derived protoplasts.

Shepherd's purse protoplasts were isolated as described in Chapter II, except that they were X-irradiated in the enzyme solution, after digestion, but before the first centrifugation. A Picker Scanray X-Ray tube (AC 128 Automatic, Scanray, Copenhagen) was set at 240 kV and 5 mA, and run for varying times to obtain the desired dosages, as indicated by the manufacturer.

e. Morphology. Regardless of the selection used, and when no selection pressure was applied, the greenest and largest colonies were preferentially transferred to the regeneration medium.

B. FUSION PROTOCOL.

The plant material consisted of: B. juncea (cv. Blaze) stem cortex; B. napus (cv. Westar) stem cortex; the C3 B. napus (cv. Tower) mutant cell suspension; the PPT334 B. napus (cv. Tower) cell suspension; B. campestris (canola cv. Tobin) stem cortex; B. campestris hybrid chinese cabbage mesophyll, from one month old greenhouse plants; T. arvense mesophyll from 3 week old *in vitro*-grown plantlets, and C. bursa-pastoris SS cell suspensions. Stem cortex-derived parental protoplast populations were isolated as described in Chapter I, except that B. napus protoplasts were recovered by flotation over 0.6 M sucrose; mesophyll and cell suspension-derived protoplasts were isolated as

described in Chapter II. With greenhouse material, contamination was controlled with 100 mg/l cefotaxime and 100 mg/l carbenicillin, added to the enzymes during protoplast isolation. In all cases, counted protoplasts were resuspended in salt wash solution (0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 2.5% KCl, pH=6.9). One million protoplasts of each parental species were distributed per 50 ml glass tube, gently mixed, and centrifuged at 100xg for 5 minutes. Supernatant was discarded, leaving a volume of 0.5 ml per tube, including the pellet, which was carefully resuspended. An equal volume of 25% PEG MW' 4000 (Merk, Darmstadt) or 8000 (EM Science, Cherry Hill, NJ), dissolved in 3% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH=6.9, filter-sterilized and stored at 4°C, was slowly added, drop by drop, surrounding the resuspended pellet. The tubes were angled for a greater contact surface between PEG and the protoplasts, and left for 15 min. with occasional gentle agitation. Salt wash solution was added, starting with a few drops and progressively increasing the volume, every two minutes, for a total of 30 minutes. The fused protoplasts were allowed to stand for another 10 min., centrifuged at 100xg for 5 min., and resuspended in culture medium B (Pelletier *et al.*, 1983) at a density of 2×10^5 protoplasts per ml. They were cultured as described in Chapter I. Four sets of controls were included per experiment, consisting of: the non-fused parents (irradiated or treated with IOA, if applicable); a mixture of the unfused parents; homofusions of treated parental protoplasts, that is fusion of each population to itself; a mixture of both homofused treated parental protoplast populations. Table 35 lists the fusion experiments undertaken. Attempts to estimate fusion frequencies included observing a fusion aliquot under the microscope, during the whole fusion and elution processes; staining one fusion

partner with FITC, and the other one with RITC, before the fusogen treatment; counting colonies from heterofusion dishes, and comparing their numbers and morphology to those of control dishes.

C. CALLUS PROLIFERATION AND PLANT REGENERATION.

As soon as colonies had reached one millimetre diameter on medium D, they were transferred onto solidified medium for plant regeneration. The initial media assessed were selected from published papers reporting good plant regeneration from B. napus or B. juncea protoplasts, and from my own observations with B. juncea, L. annua, T. arvense and C. bursa-pastoris (Chapters I and II). Other attempts included: growth regulator-free medium; media containing up to 10 mg/l BA, for a 2 week high cytokinin shock, followed by transfer to 0.1 NAA and 0.5 BA, 0.5 BA, or growth regulator-free media; addition of 3 % (w/v) activated charcoal; addition of metabolic inhibitors such as 1 to 10 $\mu\text{g/l}$ actinomycin-D and 10^{-5} to 10^{-4} M ABA; solidifying media with 0.8% SeaPlaque agarose, 0.8% agar or 0.2% gelrite; temperature shocks at 4°C, or 32.5°C for 3 days; replacing parafilm with Saranwrap or millipore membranes to seal dishes; transferring calli to Magenta™ boxes.

Table 30: Fusion experiments between Brassica crop species and T. arvense or C. bursa-pastoris, undertaken in the present study.

parent 1 (P1)	treatment of P1	parent 2 (P2)	treatment of P2	number of fusions
<u>B. napus</u> cortex	none	<u>T. arvense</u> mesophyll	none	4
<u>B. juncea</u> cortex	none	<u>T. arvense</u> mesophyll	none	2
<u>B. napus</u> cortex	none	<u>C. bursa-pastoris</u> SS cell suspension	none	3
<u>B. campestris</u> canola, cortex	none	<u>C. bursa-pastoris</u> SS cell suspension	none	2
<u>B. campestris</u> cabbage, mesophyll	none	<u>C. bursa-pastoris</u> SS cell suspension	none	2
<u>B. napus</u> C3 cell suspension	none	<u>C. bursa-pastoris</u> SS cell suspension	none	1
<u>B. napus</u> PPT334 cell suspension	none	<u>C. bursa-pastoris</u> SS cell suspension	none	1
<u>B. napus</u> cortex	1.5 mM IOA	<u>T. arvense</u> mesophyll	none	4
<u>B. napus</u> C3 cell suspension	1.5 mM IOA	<u>C. bursa-pastoris</u> SS cell suspension	10 kRad X-rays	3
<u>B. napus</u> C3 cell suspension	3.0 mM IOA	<u>C. bursa-pastoris</u> SS cell suspension	10 kRad X-rays	2
<u>B. napus</u> PPT334 cell suspension	1.5 mM IOA	<u>C. bursa-pastoris</u> SS cell suspension	10 kRad X-rays	5
<u>B. napus</u> PPT334 cell suspension	3.0 mM IOA	<u>C. bursa-pastoris</u> SS cell suspension	10 kRad X-rays	1
<u>B. napus</u> PPT334 cell suspension	6.0 mM IOA	<u>C. bursa-pastoris</u> SS cell suspension	10 kRad X-rays	1

D. BACKFUSIONS.

Cell suspensions were initiated from a callus line, obtained from a herterofusion between the B. napus l-PPT resistant cell line and SS shepherd's purse cell line, that strongly displayed shepherd's purse and B. napus isozyme and RFLP bands. Two fusion experiments were carried out, where suspension-derived hybrid protoplasts were fused with B. napus stem cortex protoplasts. Hybrid cells were irradiated with 10 kRad, and B. napus cortex cells were treated with 1.5 mM IOA. They were fused and cultured as described above.

E. CHARACTERIZATION OF PUTATIVE HYBRIDS.

a. Isozyme analyses. Isozyme analyses were performed on putative hybrid calli of B. napus cv. Westar, chlorsulfuron or l-PPT resistant cv. Tower, and C. bursa-pastoris, and from the respective parental tissue. Plant tissue (0.75 g) was placed in a 1.5 ml microfuge tube containing sea sand and 0.5 ml of protein extraction buffer (30 mM DTT, 0.2 M Tris.HCl, pH=8.5), ground with a plastic homogenizer, and centrifuged at 12 000xg for 10 min. at 4°C. Polyacrylamide mini-gels and running buffers were prepared as described in the Hoefer (San Francisco, CA) electrophoresis catalog (1990-91), using a vertical BioRad (Richmond, CA) Protean II mini-gel apparatus. The separating gel contained 6.25% polyacrylamide. The apparatus was placed on ice, and gels run for 45 min. at 10 watts (constant power, yielding approximately 200 volts). Glutamate

oxaloacetate transaminase (GOT) isozymes were visualized by a colorimetric reaction as described in Thorpe *et al.* (1987), using α -ketoglutaric acid and L-aspartic acid as the substrates and Fast Blue BB as the stain. Patterns became visible after 10 to 20 min. at 32.5°C. When desired, gels were fixed in 5% acetic acid, or dried with a Bio-Rad gel drier, according to the manufacturer's instructions.

As 1300 calli were examined, a first approach was to pool 5 calli per sample, and 260 such samples were analyzed for their GOT pattern. When a sample displayed characteristic banding patterns from both parents, the five callus lines were run individually, and results recorded in sketch form.

b. Random amplification of DNA fragments (RAPDs). Genomic DNA was extracted from 1 to 2 g of potential hybrid calli and of parental cell suspensions. Plant material was frozen in liquid nitrogen and extracted in phenol:chloroform as described in Maniatis *et al.* (1982). Alternatively, DNA was extracted using CTAB as described in Doyle and Doyle (1990). Nucleic acids were treated with 100 μ g/ml RNase for 1 h at 37°C, resuspended in low TE buffer (10 mM Tris, 0.1 mM EDTA, pH=8.0), then diluted in water for the polymerase chain reaction (PCR). Estimations of DNA concentrations were obtained with an UV spectrophotometer (Hitachi, Tokyo). The PCR reaction mix and Taq polymerase were from Promega and as specified in Promega's technical bulletin number 49 (1989). Random 10mer primers were obtained from J. Carlson, U. of British Columbia, Vancouver, Canada. Denaturation was at 95°C for 1 min., annealing at 32.5°C for 1 min., and chain extension at 72°C for 2.5 min., and 40 such cycles were run on a Coy Tempcycler (Ann Arbor, MI), overnight. Post-amplification samples were

run at 50 volts, for 4 h, in 0.8% SeaKem agarose (FMC, Rockland)-solidified gels composed of TBE buffer (Maniatis *et al.*, 1982) and 25 $\mu\text{g/l}$ ethidium bromide (EtBr).

c. Restriction Fragment Length Polymorphism (RFLP) analyses. Five to ten μg of DNA from putative hybrids, *B. napus* and *C. bursa-pastoris*, were digested overnight, at 37°C, with 50 units of the restriction enzymes BamHI, EcoRI, HaeIII, HindIII, PstI, PvuII, SacI and XhoI (Promega, Madison, WI), in presence of Promega buffer, 25 mM spermidine, 20 $\mu\text{g/ml}$ BSA and 200 $\mu\text{g/ml}$ boiled RNase. Digested DNA samples were run overnight, at 300 volts, in 0.8% SeaKem agarose gels, which were then photographed, trimmed, and blotted against Nytran membranes (Schleider and Schuell, Keene, NH), according to Southern's protocol (Southern, 1975, described in Maniatis *et al.*, 1982). The DNA was fixed to Nytran by a two minute exposure to long wavelength UV light. Filters were prehybridized, hybridized to ^{32}P -CTP radiolabelled probes, and washed, as described by the manufacturer of Nytran filters (Schleider and Schuell). Probes were random-primed with the "Prime-a-gene" kit of Promega, according to the provided instructions. Nuclear probes were pTA71, constructed from ribosomal RNA gene repeating units (coding for structural genes for 26S, 18S and 5.8S ribosomal RNA) cloned in the 4 kb plasmid pAC184 (Gerlach and Bedbrook, 1979), and pTA655, a wheat 5S ribosomal DNA gene, cloned into pBR322 (Gerlach and Dyer, 1980). The probe used to analyze chloroplast DNA was pBA1-9, constructed from *N. tabacum* chloroplast PvuII fragments P2 (3.4 kb), P7 (13.5 kb) and P10 (22 kb), inserted into *E. coli* plasmid pBR322 (Aviv *et al.*, 1984). Mitochondrial probes were: E33, a 2.4 kb fragment of the

5'end of the *coxII* gene, coding for cytochrome oxidase subunit II (*coxII*) in wheat mitochondria, and E369, a 4 kb fragment of the 3'end of the *coxII* gene, both inserted in a pUC9 (2.565 kb) vector (Bonen et al., 1984). Radioactivity of the filters was measured, and the filters were exposed to X-Omat AR X-ray film (Kodak, Rochester, NY) in Kodak cassettes equipped with two intensifying screens, at -70°C, for one hour to two weeks, depending on the intensity of radioactivity.

RESULTS

A. SELECTION SYSTEMS FOR ENRICHMENT IN HYBRID CELL LINES.

a. **Flow cytometry.** Protoplasts stained with FITC or RITC were able to divide and numerous colonies were obtained. The flow cytometer of Health and Welfare, Ottawa, is usually run with a blood diluent which contains EDTA and has a total molarity of 0.21 M. All protoplasts were killed with such a sheath fluid. It was therefore replaced with a 0.4 M calcium and potassium wash solution, which allowed protoplast survival. The nozzle tip replacement, from 76 to 110 μm of diameter, also increased the viability of sorted protoplasts. **Fig. 14a** shows a graph of red (y axis) vs green (x axis) fluorescence, emitted by RITC-stained stinkweed protoplasts and FITC-stained B. napus protoplasts, respectively. Both emission spectra were clearly distinct at the same excitation wavelength of 488 nm. Double staining (**Fig. 14b**) was plotted on a diagonal corresponding to cells fluorescing on both vertical and horizontal axes. When all protoplast populations were mixed, the double-stained population was easily distinguishable from parental cells (**Fig. 14c**) and could be sorted. However, the sheath fluid pressure was such that only a few droplets contained cells, even from a concentrated protoplast suspension of a million per ml. As a result, only a few hundreds viable protoplasts were retrieved in 6 ml of sheath fluid and diluted culture medium. Culture conditions for these protoplasts could not be established, as all sorted material was contaminated with bacteria.

Figure 14: Two dimensional graphs of red versus green fluorescence emitted by protoplasts stained with fluorescent dyes (RITC and FITC), while run through a flow cytometer. **a:** The x axis represents the RITC-stained protoplast population (red fluorescence), and the y axis represents the FITC stained protoplasts (green fluorescence). **b:** Protoplasts were double-stained with FITC and RITC, and are plotted on a diagonal. **c:** This graph represents a 2:1 mixture of the protoplast populations from graphs a and b, to mimic a heterofusion experiment. Only protoplasts on the diagonal would be sorted as putative hybrids.

b. Specific herbicide resistance. No specific resistance of stinkweed seedlings to the imidazolinone herbicides AC252.14 and AC263.499 could be demonstrated, compared to B. napus and B. juncea seedlings in similar conditions. In presence of 50 $\mu\text{g/l}$ of AC252.14, stinkweed seedling growth was reduced to 22% of the control, while B. napus and B. juncea seedlings grew to 35% and 25% of the controls (Fig. 15). Figure 16 shows a similar trend for AC263.499. At this concentration, actually, the crop species remained greener than stinkweed seedlings, which had completely bleached.

Cells from B. napus and B. juncea suspensions were still capable of dividing, one month after plating on solidified media supplemented with 100 $\mu\text{g/l}$ AC252.14 or AC 263.499. Plated stinkweed cells, on the other hand, did not divide at any of the tested herbicide concentrations, including the controls. Therefore, specific T. arvense resistance to these herbicides could not be demonstrated.

Cell suspension-derived protoplasts of B. napus cv. Tower (line PPT334) and C. bursa-pastoris were compared for their resistance to I-PPT. The B. napus cell suspension could tolerate up to 100 μM I-PPT, however, 20 μM were sufficient to decrease protoplast division more than two fold (from 6% to 2% after one week), while 50 μM I-PPT reduced cell viability from 89% to 62%, and cell division to less than 1% after one week. Rare colonies were obtained after five weeks, when B. napus protoplasts had been plated for one week in presence of 50 or 100 μM I-PPT. Considering that protoplasts isolated from the SS shepherd's purse cell suspension could still divide in presence of 50 μM I-PPT, resistance to I-PPT could not be a suitable selection marker.

Figure 15: Effect of the herbicide AC252.14 on seedling growth of *B. juncea*, *T. arvense* and *B. napus*, after 3 weeks of culture.

Figure 16: Effect of the herbicide AC263.499 on seedling growth of *B. juncea*, *T. arvense* and *B. napus*, after 3 weeks of culture.

One week of culture in a medium containing up to 0.25 mg/l chlorsulfuron did not affect viability of protoplasts isolated from the chlorsulfuron resistant B. napus cv. Topas C3 cell suspension, nor did it affect viability of shepherd's purse protoplasts. On the other hand, shepherd's purse cell division was completely inhibited after a week of culture in 0.1 mg/l chlorsulfuron, as compared to 2% dividing B. napus protoplasts. If required, chlorsulfuron could, therefore, be supplemented to the culture medium of heterofusion products, to select against unfused shepherd's purse protoplasts.

c. Iodoacetamide (IOA) treatment of Brassica protoplasts. Figure 17 shows that protoplast viability and division from B. napus cv. Westar stem cortex were effectively controlled by a treatment of 1.5 mM IOA. Solution stocks of 30 mM IOA were first conserved in the fridge, which proved to be inadequate as IOA activity was reduced within two weeks, resulting in high survival and division rates of protoplasts. Very few protoplasts escaped from the IOA treatment, after solution stocks were stored at -20°C.

d. Irradiation of shepherd's purse SS suspension-derived protoplasts. Increasing irradiation doses significantly decreased cell division after one week, although cell viability remained high (Fig. 18). A 10 kRad dose, although allowing cell division after one week, was sufficient to prevent colony formation and was therefore routinely administered to shepherd's purse protoplasts.

Therefore, a combination of IOA treatment and X-ray irradiation was chosen as a general selection strategy, which might also encourage asymmetric hybridization.

Figure 17: Effect of IOA on *B. napus* cv. Tower protoplast viability and division, after one week in culture.

Figure 18: Effect of X-irradiation on viability and division of shepherd's purse cell suspension-derived protoplasts.

B. PROTOPLAST FUSION.

Polyethylene glycols of molecular weight 4000 (Merk) and 8000 (EM Science) were compared for their capacity to fuse stem cortex B. napus protoplasts to suspension-derived C. bursa-pastoris protoplasts, with minimal damage. The 8000 MW PEG proved to be superior to the 4000 MW PEG. It sustained a viability of 71% and 11% divisions after one week, compared to 52% and 6% after PEG 4000 fusions. Drastic protoplast agglutination (**Fig. 19a**) and isolated fusion events (**Fig. 19b and c**) were observed during PEG treatment. These cell clumps redissociated slowly during elution; obvious fusion products were rarely seen after PEG treatments had ended, so that fusion rates could not be directly assessed. Brassica napus protoplasts were stained with FITC, in order to visualize fusion products, however, the emitted fluorescence was too diffuse to allow accurate identification. In one experiment, twelve shoots were obtained out of 220 PEG 8000 fusion-derived colonies, while no shoots developed from the 40 colonies plated after the PEG 4000 fusions. No colony developed from any of the other experiments involving stinkweed mesophyll protoplasts.

When no selection was applied (see **Table 30**), colonies would develop both from controls and heterofusions; the Brassica species developed large green colonies, shepherd's purse developed yellow colonies with occasional green ones, while hundreds of Brassica and shepherd's purse-like colonies were observed in heterofusion plates. Therefore, hybrid cell lines could not be morphologically distinguished from parental escapes.

Figure 19a: Protoplast agglutination during PEG treatment of a 1:1 mixture of B. napus stem cortex protoplasts and T. arvense mesophyll protoplasts (x100).

Figure 19b: Homofusion and heterofusion events during PEG treatment of a 1:1 mixture of B. napus stem cortex protoplasts and T. arvense mesophyll protoplasts (x100).

Figure 19c: Heterofusion events during PEG treatment of a 1:1 mixture of B. campestris cv. Tobin stem cortex protoplasts and shepherd's purse SS cell suspension protoplasts (x200).

Figure 19d: Fusion experiment between IOA-treated B. napus cv. Tower cell suspension protoplasts, and X-irradiated C. bursa-pastoris cell suspension protoplasts.

Top dishes: right = irradiated shepherd's purse protoplasts (control)
left = homofusion of irradiated shepherd's purse protoplasts (control)

Middle dishes: two heterofusion dishes.

Bottom dishes: right = IOA-treated B. napus protoplasts (control)
left = homofusion of IOA-treated B. napus protoplasts (control)

Figure 19e: Fusion experiment between B. campestris cv. Tobin stem cortex protoplasts, and X-irradiated C. bursa-pastoris cell suspension protoplasts.

Top dishes: right = irradiated shepherd's purse protoplasts (control)
left = homofusion of irradiated shepherd's purse protoplasts (control)

Middle dish: a heterofusion dish.

Bottom dishes: right = B. campestris protoplasts (control)
left = homofusion of B. campestris protoplasts (control)

The combination of 1.5 mM IOA treatment with 10 kRad X-irradiation was found to be extremely effective, since parental protoplasts, or their homofusions, generally did not divide. Only once did a homofusion of shepherd's purse irradiated protoplasts give rise to colonies. Brassica protoplasts and homofusions also escaped occasionally, in which case the whole experiment was discarded. From 12 to about 700 colonies developed per heterofusion plate, representing plating efficiencies from 0.004% to 0.23%. Similar colony outcomes were obtained from heterofusions involving Brassica protoplasts treated with 3.0 and 6.0 mM IOA.

C. PLANT REGENERATION FROM PEG-TREATED PROTOPLASTS.

Fusion-derived colonies were plated onto MS regeneration media containing 3% sucrose, supplemented with 3 mg/l zeatin, with 1 mg/l NAA and 3 mg/l BA, or with 0.1 mg/l kinetin and 2 mg/l TZ, and solidified with 0.8% agar or SeaPlaque agarose. One shoot was obtained from fusions between B. juncea stem cortex and shepherd's purse SS suspension protoplasts, seventeen shoots were obtained from fusions between B. napus stem cortex and shepherd's purse SS protoplasts (Fig. 20d), and many embryos developed from fusions between B. campestris cv. Tobin stem cortex and shepherd's purse SS protoplasts (Fig. 20a-c). The shoots were readily rooted, however, the embryos were often vitrified, their maturation could not be stimulated, and they were eventually lost after five months of unsuccessful attempts. A few hundred calli were obtained from heterofusions involving shepherd's purse and C3 B. napus suspension-

Figure 20a-c: Somatic embryos developed from heterofusions between B. campestris cv. Tobin stem cortex protoplasts and C. bursa-pastoris SS cell suspension protoplasts (x8).

Figure 20d: Calli and shoots produced from heterofusions between IOA-treated B. napus stem cortex protoplasts and C. bursa-pastoris SS cell suspension protoplasts (x1).

Figure 20e: Small calli developed from heterofusions between IOA-treated B. napus cv. Topas cell suspension protoplasts and irradiated C. bursa-pastoris SS cell suspension protoplasts (x1).

Figure 20f and g: Large green calli developed from heterofusions between IOA-treated B. napus cv. Tower cell suspension protoplasts and irradiated C. bursa-pastoris SS cell suspension protoplasts (x1). Figure 22f = hybrid line 1A from Table 33

Figure 22g = hybrid line 10A from Table 33.

derived protoplasts, which bleached rapidly and died. On the other hand, large, loose, green, and rapidly growing calli developed from heterofusions between B. napus cv. Tower PPT334 and SS shepherd's purse suspension-derived protoplasts, upon transfer to plant regeneration medium (Fig. 20 f-g). These calli were transferred monthly to a wide variety of media and conditions, as described in Materials and Methods, but organogenesis was not stimulated.

D. HYBRID CHARACTERIZATION.

a. Morphological analysis. Twelve plants from heterofusions between B. napus and C. bursa-pastoris, and one from B. juncea and C. bursa-pastoris fusions, were acclimated to growth chamber conditions. They all displayed morphological characters from their Brassica parent only, flowered normally, and set seeds.

b. Isozyme : lysis. The plants described above were tested for their GOT isozyme patterns; they all exclusively displayed the Brassica specific banding patterns.

As no plants were regenerated from fusion experiments performed between X-irradiated SS shepherd's purse cell suspension derived protoplasts, and IOA-treated I-PPT resistant B. napus cv. Tower cell suspension derived protoplasts, and no morphological trait allowed the identification of hybrid cell lines, isozyme analysis was used to rapidly screen for heterokaryotic cell lines among 1300 heterofusion calli. Following separation by electrophoresis on polyacrylamide gels, parental glutamate oxaloacetate transaminase (GOT) isozymes displayed distinct species-specific migration patterns, which were

consistent within each species, whether the analyzed cells were from cell suspensions, stems, roots, shoots, petioles, or leaves. In particular, C. bursa-pastoris produced two lower molecular weight proteins, which migrated faster than B. napus GOT isozymes, and were quite diagnostic. Examples of isozyme patterns from parental cell lines and putative hybrid calli are shown in Fig. 21; the calli which displayed bands from both parents were retained for further analysis, along with a few "escapes", for comparison purposes. Out of 1300 calli, 42 displayed GOT isozyme bands from both parents, 1258 displayed patterns similar to those of B. napus, although usually sharper and less smeary, while none showed shepherd's purse pattern. According to GOT patterns, 30%, 53.3% and 24% fusion colonies were B. napus escapes, after protoplasts had been inactivated with 1.5 mM, 3 mM or 6 mM IOA.

Among the 42 hybrid cell lines, all had the complete isozyme set of B. napus, although with variable banding intensities, while only 19 showed both complete isozyme parental sets. Hybrid cell lines were classified according to the relative intensity of bands for the two fastest migrating shepherd's purse specific isozymes. Cell lines A showed an intense band, lines B showed a clearly visible band, yet relatively less intense than for lines A, and lines C showed a visible but faint band, for shepherd's purse lowest molecular weight isozyme. Lines D, E, and F showed an intense, a band of medium intensity, and a faint band, respectively, for shepherd's purse second lowest molecular weight isozyme, while the fastest migrating band was absent. Cell lines which only displayed B. napus pattern were not assigned a letter.

Figure 21: A few examples of glutamate oxaloacetate transaminase (GOT) patterns after electrophoretic separation.

Black squares indicate B. napus specific bands.

Black arrows indicate C. bursa-pastoris specific bands.

B.n = B. napus

C.b = C. bursa-pastoris

Cell lines which were further analyzed are:

69.1 = 16B from Table 33

69.2 = 27

80.2 = 30

80.3 = 25F

89.1 = 1A

105.1 = 9A

110.5 = 17B

154.2 = 18B

B n 685 691 692 693 694 695 C b

B n 1057 1058 1059 1064 1065 1081 C b

B n 1103 1104 1105 1541 1542 1543 C b

c. Random amplification of polymorphic DNA fragments (RAPDs). Callus DNA was extracted with phenol and chloroform, however. DNA precipitates were very gelatinous, difficult to dissolve, and average yield was 5.2 μg DNA per gram of callus. The extraction method involving CTAB (Doyle and Doyle, 1990), on the other hand, resulted in white fibrous DNA precipitates which would readily dissolve in TE buffer, with average yields of 15.9 μg DNA per gram of callus. No problems of DNA digestion with restriction enzymes were detected, so that the CTAB extraction method was preferred.

Polymerase chain reactions were carried out, using random 10mer primers, to amplify random DNA sequences of the hybrid cell lines selected after isozyme analysis. **Table 31** shows the search for an appropriate primer, which would create diagnostic and consistently repeatable parental patterns. The primer of sequence CCTGGGTTCC was chosen as the most reliable and diagnostic; it amplified a few species-specific bands, and in particular a 0.6 kb band specific to B. napus, faint but consistent, and a strong 2.8 kb band specific to shepherd's purse (**Fig. 22**). Estimated 5 ng and 50 ng of template parental DNA were amplified, without modifying the resulting patterns (**Fig 22**). Parental DNA was also mixed at various ratios before amplification (1:1, 1:4, 1:8, 1:10, 10:1, 8:1 and 4:1), which verified that the simultaneous presence of both types of DNA did not result in changes of species-specific bands, despite the appearance of some minor novel bands.

Table 31: Number of bands and diagnostic patterns obtained after amplification of B. napus and C. bursa-pastoris genomic DNA, with random sequence primers.

primer sequence	number of major (minor) bands		diagnostic patterns
	<u>C. bursa-pastoris</u>	<u>B. napus</u>	
CCTGGGCTGG	5 (many)	3 (5 to many)	-
CCTGGGTTCC	4 or 5 (3)	3 (5 to many)	+ + +
TTTGGGGGGA	3 (many)	8 (many)	-
TTAACCCGGC	2 (3)	2 (5)	+ + +
TTACCCCGGC	3	1 (6)	+
CTCGGGTGGG	3 (2)	5 (4)	+
CTGTCCCTTT	0 (1)	*	*
TGTACGTGAC	2 (6)	*	*
TGTCGGTTGC	7 (many)	5 (5)	+
ATGTGTTGCG	3 (many)	3 (many)	-
AGCAGCGTGG	many	many	-
CGCACCGCAC	4 (many)	3 (many)	+
CTGGCGGCTG	many	7 (many)	-
GCCTGGTTGC	3 (9)	5 (6)	+

* = missing data

- = non diagnostic patterns

+ = diagnostic patterns

+ + + = highly diagnostic patterns

Figure 22: Random Amplified Polymorphic DNA (RAPDs) of B. napus and C. bursa-pastoris and putative hybrids, using the 10mer primer of sequence CCTGGGTTCC (Table 33).

The top lane of both gels was loaded with the λ -HindIII marker.

Top gel:

1 Bn = amplification of 5 ng B. napus DNA

1 Cp = amplification of 5 ng C. bursa-pastoris DNA

2 Bn = amplification of 50 ng B. napus DNA

2 Cp = amplification of 50 ng C. bursa-pastoris DNA

Bottom gel: amplification of 50 ng of all DNA samples.

Bn = B. napus

Cb = C. bursa-pastoris

110.5-B = 17B of Table 33

59.5-C = 19C

blank = reaction mix without DNA

d. Southern hybridization of nuclear rDNA. Table 32 and Fig. 23 show the search for appropriate restriction enzymes with the probes pTA71 and pTA655 (=5S), which would create diagnostic parental patterns. For pTA71, the *Sac*I patterns clearly differentiated parental ribosomal DNAs, with one *B. napus* specific band (about 1.7 kb) and 2 *C. bursa-pastoris*-specific bands (about 3.1 and 1.9 kb). Fifteen cell lines were analyzed with pTA71, among which 12 had been classified from A to E according to the presence and intensity of shepherd's purse specific GOT isozyme bands, and 3 had displayed *B. napus* banding patterns only. Each analyzed line contained bands specific to both parents (Table 33), with similar relative banding intensities (Fig. 24). Except for some lines missing the 2.3 kb fragment (common to both parents), hybrids had the sum of both parental sets of bands. For pTA655, the *Bam*HI patterns clearly differentiated parental ribosomal DNAs, with 3 major *B. napus* specific bands (about 4.2, 3.0 and 1.9 kb) and 3 *C. bursa-pastoris* specific bands (about 2.4, 1.6 and 1.4 kb). Eighteen cell lines were analyzed with pTA655, among which 11 had been classified from A to F according to the presence and intensity of shepherd's purse specific GOT isozyme bands, and 7 had displayed *B. napus* banding patterns only. Each analyzed line contained bands specific to both parents (Fig. 25 and Table 33). The relative intensity of bands from both parents was comparable, except for cell lines 1A and 29, where the *B. napus* genome seemed to predominate. Hybrids had the sum of both parental sets of bands, and 10 out of 18 exhibited a new band of about 1.5 kb (Fig.25).

e. Southern hybridization of cytoplasmic DNA.

1. Chloroplast DNA. The restriction enzyme EcoRI allowed to differentiate both parental cpDNAs, as shown on **Fig. 26**, with 4 B. napus specific and 3 shepherd's purse specific bands. **Table 33** shows that all cell lines exhibited the B. napus pattern, except for line 26, which displayed shepherd's purse pattern. The novel pattern displayed by line 7A on **Fig. 27** was due to partial digestion of the DNA.

2. Mitochondrial DNA. The parental mitochondrial DNA could be differentiated with all the restriction enzymes assayed, when hybridized with the E33 probe (**Fig. 26**).

As summarized on **Table 33**, BamHI showed 5 hybrids having the sum of parental bands, and 19 hybrids exhibiting novel bands (**Fig. 28**). When hybridized to the probe E369, however, 2 lines displayed B. napus patterns, 4 had shepherd's purse bands, 4 had a sum of both, and 9 exhibited novel bands. Only line 23D displayed the sum of parental bands with both E33 and E369 probes.

E. PROTOPLAST BACKFUSIONS.

Fusions between B. napus stem cortex protoplasts treated with 1.5 mM IOA, and X-irradiated protoplasts (10kRad) derived from the 5A cell line, in suspension, were carried out. Several hundred putative hybrid calli were retrieved from two experiments, but could not be regenerated, and were not further analyzed.

Table 32: Number of bands and diagnostic patterns of *B. napus* and *C. bursa-pastoris* genomic DNA, depending on the probe and restriction enzyme used.

probe	enzyme	number of bands		diagnostic patterns
		<i>B. napus</i>	<i>C. bursa-pastoris</i>	
pTA71	BamH1	8	4	-
	EcoR1	8	5	-
	HaeIII	6	6	-
	HindIII	2	2	-
	PstI	2	2	+
	PvuII	3	2	+
	SacI	6	5	+++
	TaqI	many	many	-
	XhoI	4	4	-
pTA655 (= 5S)	BamH1	6	5	+++
	EcoR1	2	2	-
	HindIII	1	1	-
	XhoI	1	1	-
pBA1-9	BamH1	1	2	+
	EcoR1	6	8	+++
	HaeIII	5	5	+++
	PstI	*	2	*
	PvuII	4	2	-
	SacI	2	1	-
	TaqI	many	many	-
E33	BamH1	2	2	+++
	EcoR1	4	4	+++
	HaeIII	4	3	+++
	PstI	4	2	+
	PvuII	3	3	+++
	SacI	2	3	+++

* = missing data

- = non diagnostic patterns

+ = diagnostic patterns

+++ = highly diagnostic patterns

Table 33: Random amplification of DNA sequences (RAPDs) and Restriction Fragment Length Polymorphism (RFLP) analyses of putative hybrid cell lines between B. napus and C. bursa-pastoris.

CLONE	RAPDs	pTA71	pBA1-9	5S	E33	E369
1A	*	H	N	H	H	N
2A	*	H	N	H	R	H
3A	H	*	*	*	*	*
4A	*	H	N	H	H	N
5A	*	H	N	H	R	*
6A	*	H	N	H	R	*
7A	*	*	N	*	*	*
8A	*	*	N	*	*	*
9A	*	H	N	H	R	*
10A	H	H	N	H	R	R
11B	*	H	N	H	H	*
12B	H	*	N	*	*	*
13B	H	H	N	H	R	R
14B	*	H	N	H	R	R
15B	H	H	N	H	*	R
16B	*	H	N	H	R	S
17B	H	H	N	H	R	R
18B	*	H	N	H	R	*
19C	H	*	*	*	*	*
20D	H	H	N	H	R	R
21D	*	*	N	*	*	*
22D	*	H	N	H	R	R
23D	*	H	N	H	H	H
24E	*	H	N	H	R	S
25F	H	H	*	H	*	*
26	*	H	S	H	R	S
27	*	H	N	H	R	S
28	*	H	N	H	R	H
29	*	H	N	H	R	R
30	H	H	N	H	R	*
31	*	H	N	H	R	R
32	H	H	N	H	H	H

* = missing data.

Figure 23: Restriction Fragment Length Polymorphisms of *B. napus* and *C. bursa-pastoris* total genomic DNA, digested with various restriction enzymes, and hybridized with the wheat rDNA probe pTA71, or the pTA655 (=5S) rDNA probe, on Southern blots.

B = *B. napus*

C = *C. bursa-pastoris*

Top blot:

H = HaeIII

P_s = PstI

P_v = PvuII

S = SacI

T = TaqI

Second blot:

B = BamHI

E = EcoRI

H = HindIII

X = XaeIII

Bottom blot:

B = BamHI

Probe: pTA 71

Probe: 5S

3 of/de 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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Figure 24: Restriction Fragment Length Polymorphisms of parental and putative hybrid total genomic DNA, digested with *Sac*I, and hybridized with the wheat rDNA probe pTA71 on Southern blots.

Cb = C. bursa-pastoris

lane 1 = 29 of Table 33

lane 2 = 23D

lane 3 = 20D

lane 4 = 18B

lane 5 = 10A

lane 6 = 9A

Bn = B. napus

lane 7 = 30

lane 8 = 28

lane 9 = 24E

lane 10 = 21D

lane 11 = 14B

lane 12 = 13B

lane 13 = 10A

lane 14 = 5A

lane 15 = 2A

Probe: pTA71
Enzyme: SacI

Figure 25: Restriction Fragment Length Polymorphisms of parental and putative hybrid total genomic DNA, digested with BamH1, and hybridized with the rDNA probe pTA655 (=5S) on Southern blots.

Bn = B. napus

Cb = C. bursa-pastoris

lane 1 = 1A of Table 33

lane 2 = 2A

lane 3 = 4A

lane 4 = 5A

lane 5 = 6A

lane 6 = 9A

lane 7 = 24E

lane 8 = 25F

lane 9 = 20D

lane 10 = 22D

lane 11 = 23D

lane 12 = 28

lane 13 = 26

lane 14 = 27

lane 15 = 29

lane 16 = 30

lane 17 = 31

lane 18 = 32

Probe: 5S

Enzyme: BamH1

Figure 26: Restriction Fragment Length Polymorphisms of B. napus and C. bursa-pastoris total genomic DNA, digested with various restriction enzymes, and hybridized with the chloroplast DNA probe pBA1-9 (top), or the mitochondrial DNA probe E33 (bottom), on Southern blots.

B = B. napus

C = C. bursa-pastoris

B = BamH1

E = EcoR1

H = HaeIII

P_s = Pst1

P_v = PvuII

S = Sac1

T = Taq1

Probe: pBA1-9

Probe: E33

Figure 27: Restriction Fragment Length Polymorphisms of parental and putative hybrid total genomic DNA, digested with EcoR1, and hybridized with the chloroplast DNA probe pBA1-9 on Southern blots.

lane 1 = 8A of Table 33

lane 2 = 6A

lane 3 = 2A

lane 4 = 5A

lane 5 = 1A

lane 6 = 7A

lane 7 = 4A

lane 8 = 3A

Cb = C. bursa-pastoris

Bn = B. napus

lane 9 = 31

lane 10 = 30

lane 11 = 27

lane 12 = 26

lane 13 = 28

lane 14 = 25F

lane 15 = 24E

lane 16 = 22D

lane 17 = 21D

Cb = C. bursa-pastoris

Bn = B. napus

Probe: pBA1-9

Enzyme: EcoR1

Figure 28: Restriction Fragment Length Polymorphisms of parental and putative hybrid total genomic DNA, digested with BamHI, and hybridized with the mitochondrial DNA probe E33 on Southern blots.

lane 1 = 1A of Table 33

lane 2 = 2A

lane 3 = 4A

lane 4 = 5A

lane 5 = 10A

lane 6 = 16B

lane 7 = 13B

lane 8 = 31

Cb = C. bursa-pastoris

Bn = B. napus

lane 9 = 15B

lane 10 = 17B

lane 11 = 24E

lane 12 = 20D

lane 13 = 22D

lane 14 = 23D

lane 15 = 28

lane 16 = 26

lane 17 = 27

lane 18 = 30

lane 19 = 32

lane 20 = 33

Probe : E33
Enzyme : Bam H1

DISCUSSION

Selection of heterokaryons.

Fusions were attempted without a selection strategy, however, plating efficiencies of parental protoplasts were such that thousands of colonies developed per dish, in both control and heterofusion plates. Such large numbers of calli were difficult to handle, and small percentages of heterofusion products could not be visually identified. Numerous embryos developed from "unselected" fusions, between B. campestris cv. Tobin stem cortex and shepherd's purse protoplasts, but they did not mature and were eventually lost without being analyzed. Eighteen shoots were also obtained from fusions between B. juncea or B. napus stem cortex and shepherd's purse suspension protoplasts, which were all morphologically identical to the Brassica crop parent, and which proved not to be hybrids according to GOT isozyme patterns. Selection systems against both fusion partners, and in particular against the Brassica partner, were thus considered essential.

Fluorescence activated cell sorting (FACS) was assessed, as a general system to select for heterokaryons. The fluorescent stains RITC and FITC proved to be viable, and readily distinguishable at the same excitation wavelength (488 nm), while double-stained populations could be separated from parental cells when run through a fluorescence activated cell sorter (Fig. 14). The use of a 110 μm nozzle tip increased viability of the sorted protoplasts, however, the sheath fluid pressure, of 13-15 psi, and the droplet rate frequency, were much higher than those described by Glimelius *et al.* (1986), and too strong to permit retrieval of acceptable protoplast densities. In addition, all sorted

material became contaminated with bacteria during sorting manipulations. Under these conditions, which could not be modified, FACS did not constitute an appropriate selection strategy. Once established, however, this technique has been generally applicable, and has allowed a Swedish group to recover many Brassica somatic hybrids (Glimelius *et al.*, 1991; Sjödin and Glimelius, 1989b; Fahleson *et al.*, 1988b; Sundberg *et al.*, 1987).

Potential selection systems against stinkweed or shepherd's purse protoplasts included the lack of stinkweed protoplast division in culture medium adapted to the Brassica species, specific resistance of some B. napus cell lines to the herbicide chlorsulfuron, and X-irradiation. Protoplasts isolated from the shepherd's purse cell suspensions were able to divide in the presence of 50 μ M 1-PPT, compared to less than 1% division of protoplasts isolated from the 1-PPT resistant B. napus cv. Tower, therefore, resistance to 1-PPT was not a suitable selection marker. On the other hand, shepherd's purse cell division was completely inhibited after a week of culture in 0.1 mg/l chlorsulfuron, while B. napus cv. Topas protoplasts were still dividing, so that chlorsulfuron might have been efficient in selecting against unfused shepherd's purse protoplasts. Chlorsulfuron-based selection was not exploited, since a 10 kRad X-irradiation, mostly used to encourage asymmetric hybridization, was also sufficient to prevent colony formation.

Differential levels of resistance to the imidazolinone herbicides AC252.14 or AC263.499, as described by Miki *et al.* (1990), could not be demonstrated, and this approach could therefore not be used in hybrid selection. Division of protoplasts from

B. napus cv Westar and B. juncea cv. Blaze stem cortex, or from B. napus cell suspensions, was effectively controlled by a treatment of 1.5 mM IOA. A double inactivation protocol was therefore routinely used, involving IOA-treatment of protoplasts from the Brassica species, and X-irradiation of shepherd's purse protoplasts. This combination has originally been described by Sidorov *et al.* (1981) for the selection of Nicotiana heterokaryons, and has successfully been used in Brassica by Morgan and Maliga (1987), and Barsby *et al.* (1987).

Characterization of putative B. napus + C. bursa-pastoris hybrid cell lines.

Isozyme analyses. In fusion experiments between IOA-treated B. napus protoplasts and X-irradiated shepherd's purse protoplasts, the parental protoplast populations did not survive, while hundreds to thousands of colonies were recovered following fusions. Cell division and proliferation of hybrid calli were thus possible in spite of the genetic distance between fusion partners. Another possibility was that the shepherd's purse protoplasts acted as a nurse culture to the IOA-treated B. napus protoplasts, as was described by Yarrow *et al.* (1986). No plant could be regenerated from these calli, so that the analysis of fusion products were performed at the callus level. Isozyme analyses are simple, rapid, reproducible, sensitive, and were thus considered adequate for screening hybrid calli from parental escapes. Glutamate oxaloacetate transaminase (GOT), an important enzyme involved in the Krebs cycle and gluconeogenesis, has four major alleles in B. napus, and five in C. bursa-pastoris, yielding very diagnostic patterns after electrophoretic separation (**Fig. 21**). Still, it is important to stress that a single marker was used, and therefore asymmetric hybrids that lost all coding regions for GOT

isozymes of one of the parental species were not selected. Such was the case with Eruca sativa + B. napus hybrids, which showed hybrid isozyme patterns for only one or two, out of three investigated enzymes (Fahleson *et al.*, 1988), even though there was no irradiation nor other chromosome destabilizing treatment. Out of 1300 calli analyzed in the present study, 42 exhibited hybrid GOT banding patterns, but only 19 showed complete isozyme sets of B. napus and C. bursa-pastoris, with bands of various relative intensities, suggesting that there was, indeed, asymmetric hybridization. These results could also suggest the existence of a nurse culture effect of X-irradiated cells, allowing division of IOA-treated B. napus protoplasts. According to GOT isozyme patterns, 30%, 53.3% and 24% of fusion colonies were B. napus escapes, after protoplasts had been inactivated with 1.5 mM, 3 mM or 6 mM IOA, respectively. This contrasts with observations by Terada *et al.* (1987), who showed that 94 % of fusion-derived regenerants were B. oleracea parental escapes after protoplasts had been treated with 2.5 mM IOA, 57% were escapes after a 7.5 mM IOA treatment, while only 10% escaped after a 15 mM IOA treatment.

Random amplification of polymorphic DNA fragments (RAPDs). The polymerase chain reaction (PCR) allows amplification of specific DNA fragments flanked by oligonucleotide primers, from a DNA template (White *et al.*, 1989). Several random primers were tested to search for diagnostic amplified patterns for both fusion partners. The primer of sequence CCCTGGGTTCC consistently presented diagnostic parental patterns, even after drastic modifications of template DNA concentration (Fig. 22), or when both parental DNA samples were mixed at various ratios, and was therefore used

for analysis. All analyzed fusion-derived callus lines displayed both diagnostic parental bands (**Fig. 22** and **Table 33**), including lines which had only shown B. napus GOT isozyme patterns. These results were interpreted as an indication for the inadequacy of GOT isozyme analyses to detect all hybrid lines.

Southern hybridization of nuclear rDNA and cytoplasmic DNA. Fifteen cell lines, including some which had only shown B. napus GOT patterns, were analyzed with the ribosomal DNA probe pTA71. They all contained bands specific to both parents (**Table 33**), with similar relative banding intensities (**Fig. 24**). Except for some lines missing the 2.3 kb fragment (common to both parents), hybrids had the sum of both parental sets of bands. All eighteen cell lines analyzed with the ribosomal DNA probe pTA655 also contained bands specific to both parents (**Fig. 25** and **Table 33**), and relative banding intensities from both parents were comparable; hybrids exhibited the sum of both parental sets of bands, and 10 out of 18 showed a new band of about 1.5 kb (**Fig.25**), indicating that DNA rearrangements might have occurred. The complete hybridity of all analyzed cell lines for these two nucleolar markers probably result from the presence of large copy numbers of nuclear-encoded ribosomal genes, per genome. Therefore, although ribosomal probes are valuable for somatic hybrid characterization, they did not allow the detection of chromosome elimination, correlated with the lack of gene expression for shepherd's purse GOT isozymes. It seemed nevertheless reasonable to expect chromosome elimination, since shepherd's purse protoplasts were X-irradiated to favour asymmetric fusions, since B. napus and C. bursa-pastoris are not closely related, and since GOT isozyme patterns showed incomplete hybridization patterns. Irradiation

is largely considered as a valuable tool for eliminating chromosomes (Hinnisdaels *et al.*, 1988; Sikdar *et al.*, 1990a). For example, Chatterjee *et al.* (1988) have gamma-irradiated *D. muralis* protoplasts before fusion with *B. juncea*, resulting in elimination of up to 10 of the 42 *D. muralis* chromosomes, as opposed to no chromosome elimination from fusions that did not involve irradiation. According to a comparative study between various *Brassica* somatic hybrids by Sundberg and Glimelius (1991), more DNA was eliminated in intertribal than in interspecific crosses.

Table 33 shows that only the chloroplasts of *B. napus* were maintained in all cell lines, except for line 26, which displayed shepherd's purse chloroplasts (**Fig. 27**). Most investigations report rapid and complete segregation of parental chloroplasts, sometimes random, but very often strongly biased towards one parent (Fahleson *et al.*, 1988b; Primard *et al.*, 1988; Sundberg *et al.*, 1987; Morgan and Maliga, 1987), as was observed in the present study. Random segregation of plastids has been observed mostly among somatic hybrids between sexually compatible species, while hybrids between incompatible species often show biased chloroplast segregation (Landgren and Glimelius, 1990). Analyses of chloroplast DNA complements of various *Brassica* hybrid combinations, led Sundberg and Glimelius (1991) to conclude that, in case of distantly related species, the chloroplasts of the species with the largest nucleus would be favoured. Shepherd's purse and *B. napus* are both tetraploids ($2n=38$ and $2n=32$), while their actual genome size cannot be compared due to lack of information for shepherd's purse. On the other hand, if some shepherd's purse chromosomes were eliminated, as was suggested by GOT isozyme patterns, then the *B. napus* nucleus would

predominate. Biased chloroplast segregation has been attributed to unequal input of organelles from the fusion partners, due to different genome sizes or parental cell type used (Landgren and Glimelius, 1990). In this study, however, both protoplast partners were derived from cell suspensions. Differences in plastid reproduction rates have been reported to influence the average plastid ratios (Moll *et al.*, 1990). The bias towards *B. napus* chloroplasts might also be due to irradiation of shepherd's purse protoplasts, however, Sidorov *et al.* (1981) observed preferential maintenance of the *Nicotiana* irradiated chloroplasts.

Antibiotic resistances have been used as chloroplast screening markers in heterokaryons of a *N. tabacum* line resistant to streptomycin and a *N. plumbaginifolia* resistant to lincomycin, by Moll *et al.* (1990). By growing those *Nicotiana* heterokaryons on streptomycin or lincomycin, they have demonstrated that, in contact with a given antibiotic, the chloroplasts which carried the corresponding marker were efficiently selected for, while the other chloroplast population was undetectable. Interestingly, the effect was reversible upon selection with the other antibiotic, so that the previously undetectable chloroplast population became dominant (Moll *et al.*, 1990). Therefore, when only one population can be detected, as was the case in this study, low numbers of the other chloroplast type might actually be present.

As summarized on Table 33, BamH1 restriction digests hybridized with the mitochondrial probe E33 showed the sum of parental bands for 5 hybrids, and novel bands for 19 hybrids (Fig. 28). Upon hybridization to the probe E369, however, 2 cell lines displayed *B. napus* patterns, 4 had shepherd's purse bands, 4 had the sum of both,

and 9 exhibited novel bands. Only line 23D displayed the sum of parental bands with both E33 and E369 probes. Various rearrangements were displayed with no obvious preferential interactions at specific sites (Fig. 28), unlike previous reports of repeated recombination patterns of somatic hybrid mitochondrial DNA (Landgren and Glimelius, 1990; Primard *et al.*, 1988). Mitochondrial DNA has been reported to undergo modifications following tissue culture (Morgan and Maliga, 1987), but Landgren and Glimelius (1990) did not find any mtDNA rearrangements in the *B. napus* plants regenerated from protoplast culture. Mitochondrial DNA of *Brassica* somatic hybrids has commonly undergone intergenomic recombination (Primard *et al.*, 1988; Morgan and Maliga, 1987; Robertson *et al.*, 1987; Chetrit *et al.*, 1985). Landgren and Glimelius (1990) demonstrated an inverse correlation between the taxonomic distance between fusion partners and mitochondrial DNA recombination rates, since significantly fewer rearrangements occurred in hybrids between *B. napus* and *E. sativa* than in hybrids between *B. napus* and *B. nigra* and between *B. campestris* and *B. oleracea*. In the present study, however, the majority of cell lines analyzed displayed intergeneric mtDNA recombinations, and the discrepancies between patterns obtained with the mitochondrial probes E33 and E369 once again emphasized the importance of assessing several regions of the mitochondrial genome, before drawing general conclusions.

Lack of plant regeneration of *B. napus* + *C. bursa-pastoris* hybrid cell lines.

Plant regeneration attempts included modifications of growth regulators, cold treatments, as successfully employed to regenerate plants from *Arabidopsis thaliana* cell suspensions (Gleddie, 1989), and use of metabolic inhibitors. Sethi *et al.* (1990) showed

radical improvements in organogenesis of Brassica, Nicotiana and Datura explants, correlated with inhibition of unorganized cell division, by supplementing culture media with metabolic inhibitors such as ABA and actinomycin-D. No plants were obtained from hybrid calli between B. napus and C. bursa-pastoris. Schieder *et al.* (1985) obtained non-regenerable calli from fusion products of Datura innoxia and Atropa belladonna that retained all parental chromosomes, however, hybrid plants could be regenerated from plants that had lost most Atropa chromosomes, implying that asymmetry could have a major impact on plant regeneration potential. On the other hand, functional true somatic hybrid plants have been recovered between distantly related plants with no or little chromosome loss (Glimelius *et al.*, 1991). Somatic incompatibility, although not sufficient to prevent cell division of B. napus + C. bursa-pastoris heterokaryons, might have inhibited organized development, which require "more balanced gene activities" (Hinnisdaels *et al.*, 1988). However, it was not possible to conclude whether lack of plant regeneration was due to genetic incompatibilities, or to culture conditions. Both parental protoplasts were isolated from cell suspensions, which probably displayed unstable chromosome compositions, and from which plants were regenerated with difficulty.

Concluding remarks.

Double inactivation combining IOA treatments and X-irradiation, proved to be the most efficient selection system for heterokaryons. Hundreds of calli were recovered from fusions between B. napus and C. bursa-pastoris cell suspension-derived protoplasts, which were screened for hybridity by isozyme analyses. The results of RAPD and RFLP

DNA analyses have confirmed hybridity of all the examined cell lines, including some which had only exhibited B. napus GOT isozyme patterns. Therefore, although isozyme analyses allowed a rapid screening for hybridity, they obviously did not permit detection of large numbers of asymmetric hybrids.

***SUMMARY AND
GENERAL CONCLUSIONS***

Plant regeneration from Brassica juncea stem cortex explant culture was strongly affected by genotype, and ranged from 50% for the most responsive lines (Blaze, Zaria, Colman's), to no shoot production for the least responsive lines (Zem, Vniimk351). Blaze was chosen for stem cortex protoplast isolation. After one week of culture, a maximum of 11-14% of the cells had divided, and about 0.002% produced 1-2 mm colonies within 6 weeks. Numerous modifications to the regeneration medium could not increase the frequency of plant regeneration above 7% of the colonies plated. Plant regeneration from protoplasts has been a major problem in B. juncea (Jourdan and Earle, 1989; Eapen et al., 1989; Kao and Séguin-Swartz, 1987; Sproule, 1987), as compared with regeneration from explant cultures, suggesting that the actual protoplast isolation and culture protocols are disrupting the cells, to the extent that their potential for organized development is largely reduced.

The undeniable practical potential of somatic hybridization first relies on the ability to isolate and culture protoplasts from both parental species. Stem cortex protoplast cultures of B. juncea (Chapter I) and B. napus (Klimaszewska and Keller, 1987) have been developed. Culture of Thlaspi arvense and Lunaria annua protoplasts was not achieved, however, high yields of viable Capsella bursa-pastoris protoplasts were isolated from rapidly growing embryogenic cell suspension cultures. These protoplasts divided, formed colonies and calli, from which a few plants could be regenerated. Capsella protoplasts could thus be used in fusion experiments with B. napus, B. juncea and B. campestris protoplasts.

Efficient selection strategies for heterokaryon cell lines are also required.

Iodoacetamide treatment of *B. napus* protoplasts and X-irradiation of shepherd's purse protoplasts were successfully combined to select for heterokaryons. When screened for their GOT isozyme patterns, only one out of thirty fusion-derived calli exhibited bands from both parents, however, total genomic DNA analyses through RAPDs and Southern hybridizations showed hybridity of all cell lines. Therefore, the selection strategy was more efficient than was first estimated, and nurse culture effects from the irradiated protoplast population, rescuing IOA-treated *B. napus* protoplasts, might have been negligible.

Finally, certainly the most important factor for the practical application of protoplast fusion, is the capacity to regenerate hybrid plants, that are at least partially fertile. This represented the major bottleneck of the present study, since no plant was ever regenerated from selected heterokaryotic cell lines of *B. napus* + *C. bursa-pastoris*, despite numerous attempts. Plant regeneration from fused protoplasts has been reportedly problematic, or regenerants could be highly abnormal and sterile (Hinnisdaels *et al.*, 1988). On the other hand, large numbers of true and partial hybrid plants, with unique cytoplasmic combinations, have been obtained from interspecific and intergeneric combinations in the *Brassicaceae* (Glimelius *et al.*, 1991; Pelletier *et al.*, 1988). Most of these hybrids were at least partially fertile, and could therefore be backcrossed to the parental crop species, and integrated into breeding programmes (Glimelius *et al.*, 1991). Other examples of fertile somatic hybrids successfully used in a breeding programme include *N. rustica* + *N. tabacum* (Douglas *et al.*, 1981), from which new varieties, resistant to blue mold (*Peronospora tabacina*), were created after backcrosses to tobacco

(Pandeya *et al.*, 1991); Solanum tuberosum + S. circaefolium, exhibiting resistance to the pathogens Phytophthora infestans and Globodera pallida (Mattheij *et al.*, 1992).

In the present study, the ideal situation would have been to preserve the entire genome of B. napus, with introgression of gene clusters involved with flea beetle resistance, from shepherd's purse. However, since no specific selection was applied, and since the characterization markers chosen were few and random, these highly asymmetric hybrids would have probably been overlooked and rejected as B. napus parental escapes. It would be an interesting curiosity to submit hybrid cell lines to the attack of flea beetles, provided they are attracted to B. napus callus, with the view of screening resistant clones. Although these clones could then be backfused with napus protoplasts, the lack of a specific selection strategy would again preclude the screening of those resistant cell lines or regenerants. Data for genetic markers linked to flea beetle resistance, obtained through RFLP, would provide a rapid and precise selection system, but are not available, as no morphological or biochemical relationships have yet been established for flea beetle resistance in shepherd's purse, and resistance under insect pressure has not been screened.

APPENDICES

APPENDIX 1

Composition of Hoagland's solution:

493 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
136 mg/l KH_2PO_4
506 mg/l KNO_3
1180 mg/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
Iron Chelate
2 mg/l $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
3 mg/l H_3BO_3
0.1 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
0.2 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
0.02 mg/l $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$
6 mg/l KCl

APPENDIX 2

Preparation of growth regulator stocks

All cytokinins were dissolved in a few drops of 6 N HCl.
All auxins were dissolved in a few drops of absolute ethanol.
Gibberellins were dissolved in absolute ethanol.
Absciscic acid was dissolved in water.

Stocks were 1 mg growth regulator in 10 ml H_2O , and were kept at 4°C in darkness.

APPENDIX 3

Composition of MEDIUM MS, from Murashige and Skoog (1962), as modified by Douglas *et al.*, (1981).

Stock solutions: **Final concentration (g/l)**
For a final volume of 2 l of MS salt
and B5 vitamin stock (x5):

macronutrients

KNO ₃	19.0 g	1.90
NH ₄ NO ₃	16.5 g	1.65
MgSO ₄ .7H ₂ O	3.7 g	0.37
CaCl ₂ .2H ₂ O	4.4 g	0.44
KH ₂ PO ₄	1.7 g	0.17
Fe-330	0.4 g	0.04

vitamins

B5 vit. stock (x100)	100.0 ml	
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micronutrients

MS μ nutrient stock (x100)	100.0 ml	
KI stock (x1000)	10.0 ml	0.00083

For a final volume of 1 l of B5 (Gamborg *et al.*)
vitamin stock (x100):

myo-inositol	10.0 g	0.100
nicotinic acid	0.1 g	0.001
pyridoxine HCl	0.1 g	0.001
thiamine HCl	1.0 g	0.010

For a final volume of 1 l of MS
micronutrient stock (x100):

MnSO ₄ .4H ₂ O	2.2300 g	0.0223
H ₃ BO ₃	0.6200 g	0.0062
ZnSO ₄ .7H ₂ O	0.8600 g	0.0086
Na ₂ MoO ₄ .2H ₂ O	0.0250 g	0.00025
CuSO ₄ .5H ₂ O	0.0025 g	0.000025
CoCl ₂ .6H ₂ O	0.0025 g	0.000025

APPENDIX 4

Composition of MEDIUM B5, from Gamborg et al., 1962.

Stock solutions: **Final concentration (g/l)**
For a final volume of 2 l of
B5 salt and vitamin stock (x5):

macronutrients

KNO ₃	25.0 g	2.500
MgSO ₄ .7H ₂ O	2.5 g	0.250
CaCl ₂ .2H ₂ O*	7.5 g*	0.750*
NaH ₂ PO ₄	1.5 g	0.150
Fe-330	0.4 g	0.040

vitamins

B5 vit. stock (x100) (appendix 3)	100.0 ml	
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micronutrients

B5 μ nutrient stock (x100)	100.0 ml	
KI stock (x1000)	10.0 ml	0.00083

For a final volume of 1 l of
B5 micronutrient stock (x100):

MnSO ₄ .H ₂ O	10.000 g	0.010000
H ₃ BO ₃	3.000 g	0.003000
ZnSO ₄ .7H ₂ O	2.000 g	0.002000
Na ₂ MoO ₄ .2H ₂ O	0.250 g	0.000250
CuSO ₄ .5H ₂ O	0.025 g	0.000025
CoCl ₂ .6H ₂ O	0.025 g	0.000025

*: Modified. Original concentration was 0.150 mg/l (Gamborg et al., 1962).

APPENDIX 5

Composition of MEDIUM X, from Spoule (1987), derived from Schenck and Röbbelen (1982).

For a final volume of 2 l of X stock (x5):		Final concentration (g/l)
	(g)	
NH ₄ NO ₃	8.00	0.800
CaCl ₂ .2H ₂ O	8.80	0.880
MgSO ₄ .7H ₂ O	2.95	0.295
KH ₂ PO ₄	7.00	0.700
Fe(III)EDTA	0.50	0.500
diNa salt		
myso-inositol	1.00	0.100
MnSO ₄ .H ₂ O	0.10	0.010
ZnSO ₄ .7H ₂ O	0.10	0.010
H ₃ BO ₃	0.10	0.010
KI	0.01	0.001
sucrose		26.00
glucose		39.64
2,4-D		0.0002
NAA		0.001
BA		0.0005

Filter sterilize.

Composition of medium S1, from Schenck and Röbbelen (1982). Same as X, but with 0.2 g/l CaCl₂.2H₂O and 1.88 g/l MgSO₄.7H₂O.

APPENDIX 6

Composition of MEDIA B,C and D, from Pelletier *et al.*, 1983.

(g/l)	B	C	D
KNO ₃	2.500	1.250	1.250
(NH ₄) ₂ SO ₄	0.134	0.067	0.067
NH ₄ NO ₃	-	0.200	-
NaH ₂ PO ₄ ·H ₂ O	0.150	0.075	0.075
KH ₂ PO ₄	-	0.035	0.035
MgSO ₄ ·7H ₂ O	0.250	0.250	0.250
CaCl ₂ ·2H ₂ O	0.750	0.525	0.525
Fe-330	0.040	-	-
FeSO ₄ ·7H ₂ O.	-	0.0373	0.0373
Na ₂ EDTA			
myo-inositol	0.100	0.100	0.100
nicotinic acid	0.001	0.001	0.001
pyridoxine.HCl	0.001	0.001	0.001
thiamine.HCl	0.010	0.010	0.010
MnSO ₄ ·H ₂ O	0.010000	0.010000	0.003360
H ₃ BO ₃	0.003000	0.003000	0.001240
ZnSO ₄ ·7H ₂ O	0.002000	0.002000	0.002100
Na ₂ MoO ₄ ·2H ₂ O	0.000250	0.000250	0.000050
CuSO ₄ ·5H ₂ O	0.000025	0.000025	0.000005
CoCl ₂ ·6H ₂ O	0.000025	0.000025	0.000005
KI	0.00083	0.00083	0.00166
glucose	20.0	-	-
sucrose	-	20.0	20.0
mannitol	70.0	40.0	40.0
2,4-D	0.25 mg	-	1.0 mg
NAA	1.00 mg	0.2 mg	-
BA	1.00 mg	1.0 mg	-
adenine sulfate	-	-	30.0 mg

APPENDIX 7

Composition of CPW salts, from Frearson *et al.*, 1973.

	(mg/l)
KH ₂ PO ₄	27.20
KNO ₃	101.00
CaCl ₂ .2H ₂ O	1480.00
KI	0.16
CuSO ₄ .5H ₂ O	0.25
pH=5.8, filter-sterilized.	

APPENDIX 8

Composition of MEDIUM L, from Lichter, 1981.

	(mg/l)		
KNO ₃	125	glycine	2.00
MgSO ₄ .7H ₂ O	125	myo-inositol	100.00
CaNO ₃ .4H ₂ O	500	nicotinic acid	5.00
KH ₂ PO ₄	125	pyridoxine.HCl	0.50
FeEDTA Na salts	40	thiamine.HCl	0.50
MnSO ₄ .4H ₂ O	22.300	folic acid	0.50
H ₃ BO ₃	6.200	biotin	0.05
ZnSO ₄ .7H ₂ O	8.600	glutamine	800.00
Na ₂ MoO ₄ .2H ₂ O	0.250	glutathione	30.00
CuSO ₄ .5H ₂ O	0.025	serine	100.00
CoCl ₂ .6H ₂ O	0.025		
2,4-D	0.250	mannitol (0.4M)	36.4 g
NAA	0.250		
BA	0.025		

APPENDIX 9

Composition of MEDIUM 8p, from Kao and Michayluk, 1975, as modified by Glimelius, 1984.

salts (mg/l)

NH ₄ NO ₃	600
KNO ₃	1900
CaCl ₂ .2H ₂ O	600
MgSO ₄ .7H ₂ O	300
KH ₂ PO ₄	170
KCl	300
Fe-330	28

sugars (mg/l)

fructose	250
ribose	250
xylose	250
mannose	250
rhamnose	250
cellobiose	250
sorbitol	250
mannitol	250
glucose	79270

micronutrients (mg/l)

H ₃ BO ₃	3.000
MnSO ₄ .7H ₂ O	10.000
ZnSO ₄ .7H ₂ O	2.000
Na ₂ MoO ₄ .2H ₂ O	0.250
CuSO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
KI	0.750

casamino acid (mg/l) 250

coconut milk 20 ml
from mature fruits, heated to 60°C
for 30 min., and filter-sterilized.

organic acids (mg/l)

Na pyruvate	5.0
citric acid	10.0
malic acid	10.0
fumaric acid	10.0

growth regulators

2,4-D	1.0
NAA	0.1
BA	0.5

This medium is filter-sterilized.

vitamins (mg/l)

myo-inositol	0.100
nicotinic acid	0.001
pyridoxine HCl	0.001
thiamine HCl	0.010

APPENDIX 10

Composition of MEDIUM Mo, from Monnier, 1978.

	(mg/l)		
KNO ₃	1900.0	H ₃ BO ₃	12.40
CaCl ₂ ·2H ₂ O	880.0	MnSO ₄ ·H ₂ O	33.60
NH ₄ NO ₃	825.0	ZnSO ₄ ·7H ₂ O	21.00
MgSO ₄ ·7H ₂ O	370.0	KI	1.66
KCl	350.0	Na ₂ MoO ₄ ·2H ₂ O	0.55
KH ₂ PO ₄	170.0	CuSO ₄ ·5H ₂ O	0.05
Na ₂ EDTA	14.9		
FeSO ₄ ·7H ₂ O	11.1	glutamine	400.00
glucose	120 g		

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