THE UNIVERSITY OF CALGARY

Regeneration and Transformation of *Brassica rapa* (Canola)

by

John Hachey

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I believe in aristocracy...Not an aristocracy of power, based upon rank and influence, but an aristocracy of the sensitive, the considerate and the plucky. Its members are to be found in all nations and classes, and all through the ages, and there is a secret understanding between them when they meet. They represent the true human condition, the one permanent victory of our queer race over cruelty and chaos. Thousands of them perish in obscurity, a few are great names. They are sensitive for others as well as for themselves, they are considerate without being fussy, their pluck is not swankiness but the power to endure, and they can take a joke.

E.M. Forster [1939]

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Regeneration and Transformation of *Brassica rapa* (Canola)" submitted by John E. Hachey in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

A system has been developed for efficient regeneration of shoots from *Brassica rapa* in vitro that is also susceptible to *Agrobacterium* - mediated transformation. Using 4-day old cotyledons with petioles as explants and a combination of BA ($2mgL^{-1}$)and NAA ($1mgL^{-1}$) in the regeneration media, up to 70% of explants produced shoots after 2 weeks in culture. This method was found to be widely applicable to *B. rapa* cultivars. Histological studies indicated the development of multiple shoot primordia from cells about 100 µm from the cut site within 2 days of culture initiation. The wounded surface of these explants was found to be susceptible to *Agrobacterium* - mediated transformation and shoots resistant to kanamycin emerged from the transformed explant. However, these shoots were not transformed and survived selection through cross-protection from the selective agent by surrounding transformed cells. Possible factors preventing the recovery of transformed shoots from this system are discussed.

ACKNOWLEDGEMENTS

Thanks are due to my parents and family for their support through the years, without which this thesis would not have been completed.

I would also like to thank Dr. M.M. Moloney for his expert advice and guidance and stalwart patience.

I am also indebted to Dr. T.A. Thorpe who gave me the confidence and incentive to pursue graduate studies

Finally, I would like to thank all the other aristocrats in and around the lab for their help, encouragement and humor.

THIS BUD'S FOR ME

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LIST OF ABBREVIATIONS

20X SSC	3 molar sodium chloride, 0.3 molar sodium citrate (pH 7.0)
$AgNO_3$	silver nitrate
AMV	alfalfa mosaic virus
AS	acetosyringone
ATP	adenosine triphosphate
BA	N^6-benzyladenine
C	centigrade
CaMV 35S	35 Svedberg transcript of the cauliflower mosaic virus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpm	disintegrations per minute
dTTP	deoxythymidine triphosphate
g	1 gravity unit
$gent^r$	gentamycin resistant
h	hour
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
kan^r	kanamycin resistant
KCl	potassium chloride
Kinetin	6-Furfurylaminopurine
$MgCl_2$	magnesium chloride
mgL^{-1}	milligram per litre
MG/L	medium consisting of 50% of a mannitol-glutamate-salts medium (Garfinkel and Nestor, 1980) and 50% Luria broth (Maniatis *et al.* , 1982)

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List of Abbreviations
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ml	millilitre
mM	millimolar
mm	millimeter
MS	a plant tissue culture medium (Murashige and Skoog, 1962)
Na_2EDTA	disodium ethylenediaminetetraacetic acid
NAA	α-naphthaleneacetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NH_4Cl	ammonium chloride
nos	nopaline synthase
NPT II	neomycin phosphotransferase II
PCR	polymerase chain reaction
RNA	ribonucleic acid
Sarkosyl	N-dodecanoyl-N-methylglycine
SDS	sodium dodecyl sulphate
T-DNA	transfer-DNA
T_L-DNA	left transfer-DNA
Tris HCl	2-amino-2- (hydroxymethyl)-1,3-propandiol-hydrochloride
TWEEN-20	polyoxyethylenesorbitan monolaurate
w/v	weight -to-volume
μCi	microcurie
$\mu Em^{-2}s^{-1}$	microEinstein per square meter per second
μg	microgram
μl	microlitre
μM	micromolar
μm	micrometer

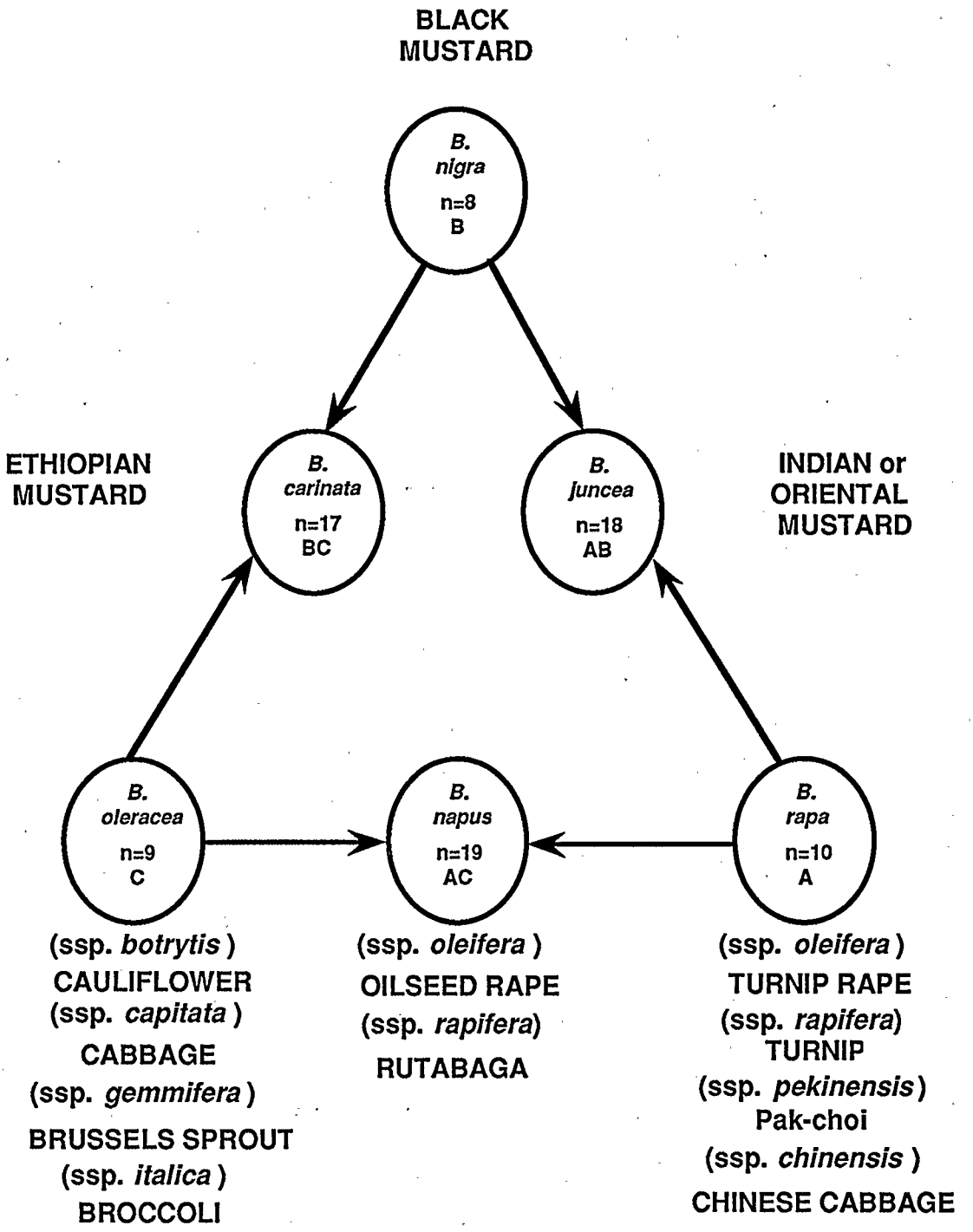
CHAPTER 1 :
INTRODUCTION

1.1 THE GENUS *BRASSICA*

The genus *Brassica* contains many economically important crop plants cultivated for edible seed oil, vegetables and fodder. The relationship of these plants to each other is most easily summarized by U's triangle (Fig. 1), which he developed based on interspecific hybridization and cytogenetic evidence (U, 1935). U's findings have since been corroborated and extended through the use of many techniques, the most recent being RFLP (Restriction Fragment Length Polymorphism) analysis of the six species (Song *et al.*, 1988; Song *et al.*, 1990). Further, the analysis of the restriction pattern of chloroplastic DNA has allowed identification of the maternal parent for most of the amphidiploids of this genus (Palmer *et al.*,1983).

Many species of the *Cruciferae* (including the genus *Brassica*) contain a sporophytic self-incompatibility (SI) system which forces natural outcrossing (Nasrallah and Nasrallah, 1989). In sporophytic systems, the SI phenotype of the pollen is determined by the genotype of the sporophyte (*i.e.* the plant producing the pollen). This is thought to occur as a result of the relevant genes being expressed pre-meiotically by the pollen mother cells or in the cells of sporophytic tissues surrounding the developing pollen (Nasrallah and Nasrallah, 1989). In its most simplified form, contact between pollen and stigma of the same genotype will result in inhibition of pollen grain germination, thereby preventing the pollen tubes of such grains from penetrating the stigmatic surface and effecting fertilization (Downey *et al.*, 1980). In the *Brassicas* , self pollination occurs in the amphidiploids, whereas the diploid species are generally self-incompatible. The self-compatible phenotype of the

Figure 1. Phylogenetic relationships of *Brassica* species. Letters indicate the genomes of the three primary species. Arrows indicate the origin of the amphidiploid genomes. n= the haploid number of chromosomes in a genome. Included are some common names of important crop subspecies (ssp.). (Adapted from Erickson *et al.* , 1983 and U, 1935).


BLACK MUSTARD
B. nigra n=8 B
ETHIOPIAN MUSTARD
B. carinata n=17 BC
B. juncea n=18 AB
INDIAN or ORIENTAL MUSTARD
B. oleracea n=9 C
B. napus n=19 AC
B. rapa n=10 A
(ssp. botrytis) CAULIFLOWER
(ssp. capitata) CABBAGE
(ssp. gemmifera) BRUSSELS SPROUT
(ssp. italica) BROCCOLI
(ssp. oleifera) OILSEED RAPE
(ssp. rapifera) RUTABAGA
(ssp. oleifera) TURNIP RAPE
(ssp. rapifera) TURNIP
(ssp. pekinensis) Pak-choi
(ssp. chinensis) CHINESE CABBAGE

amphidiploids would have been essential in order that the product of the rare event of a fertile interspecific cross could survive past the first generation. While the basis of self-compatibility in the amphidiploids is not understood, it has been hypothesized that it may be the result of the co-supression of the S-locus genes from each genome (Nasrallah *et al.*, 1991).

In the diploid *Brassicas* significant loss of vigor and fertility usually occurs when they are self-pollinated (inbreeding depression). However, self-compatible strains do occur naturally. Self-compatibility in these strains is a result of the action of supressor genes that are unlinked to the S-locus (Nasrallah and Nasrallah, 1989).

Of the six *Brassica* species described in U's triangle, three are diploid species (*Brassica oleracea, Brassica nigra,* and *Brassica rapa*) and three are amphidiploid species (*Brassica carinata, Brassica juncea,* and *Brassica napus*). The latter three species were formed by interspecific hybridization between the diploid species (Downey and Röbbelin, 1989; Song *et al.*, 1988).

1.2 ECONOMIC IMPORTANCE

1.2.1 *Brassica oleracea*

Brassica oleracea contains the cole crops cabbage, cauliflower, brussels sprouts, broccoli, kohlrabi and kale. Cole crops are among the most widely grown vegetables in the temperate zones. In Europe, over 30% of the land growing vegetables is occupied by cole crops. They are also among the leading vegetables grown in North America (Nieuwhof, 1969).

1.2.2 *Brassica carinata*

Brassica carinata (Ethiopian mustard) is an interspecific hybrid between *Brassica oleracea* and *Brassica nigra* . It is exclusively cultivated in Ethiopia and neighboring countries and therefore has had little exposure to modern plant improvement. However, it has recently gained recognition, because it possesses several agronomically important traits (*eg.* drought, insect and disease resistance; Anand *et al.*, 1985, c.f. Yang *et al.*, 1991) and has the potential to fit rain-fed agriculture as evidenced by its ability to outyield the traditional Indian oilseed crops *Brassica juncea* and *Brassica rapa* in both seed and oil yield (Anand *et al.*, 1985).

1.2.3 *Brassica nigra*

Brassica nigra (black mustard) was an important spice in early times but, because of many characters considered unsuitable for a crop plant (including pre-harvest seed-shedding and the propensity of the seed to remain dormant in the soil and so become a weed in succeeding crops), it has been largely replaced as a crop by high yielding domestic forms of *Brassica juncea* in Britain as the main source of hot mustard flour or paste (Gill and Vear, 1980).

1.2.4 *Brassica juncea*

Brassica juncea (Indian mustard) is an amphidiploid between *Brassica nigra* and *Brassica rapa* . It is cultivated widely in India (along with *Brassica rapa*) for its seed oil. In Canada, it is grown for the condiment mustard market but it is also being bred so that its oil will be suitable for edible oil processing in Western countries (*i.e.* that it is Canola quality, see below). Since *Brassica*

juncea is more heat and drought tolerant than the other species of Canola (*i.e. Brassica napus* and *Brassica rapa*) it could extend the growing range of *Brassica* oilseed production into areas of Western Canada currently unable to cultivate Canola (Woods *et al.*, 1991).

1.2.5 *Brassica rapa* (syn. *campestris*)

The major cultivated forms of *Brassica rapa* can be classified either morphologically (as turnip, oilseed or leafy vegetable) or geographically with respect to their area of origin (Europe, India or Asia). Turnips (ssp. *rapifera*) are found in all three regions, but historically have been more important to European and Asian economies. Oilseeds are mainly of European and Indian origin, with a distinct subspecies being associated with each region. European oilseed forms (ssp. *oleifera*), commonly known as turnip rape or colza, are the primary *Brassica rapa* oilseed types grown in temperate regions. The Indian oilseeds are known as toria (ssp. *dichotoma*) and sarson (ssp. *trilocularis*). The leaf vegetable types of *Brassica rapa* are mainly of Asian origin. These include Pak-choi (ssp. *chinensis*) and Chinese cabbage (ssp. *pekinensis*) (McGrath and Quiros,1991).

1.2.6 *Brassica napus*

Brassica napus is the result of a crossing event between *Brassica rapa* and *Brassica oleracea* . It exists in two commercially important forms. Rutabaga (ssp. *rapifera*) is an important vegetable crop and oilseed rape (ssp. *oleifera*) is the primary oilseed crop in China, Northern Europe and Canada (Downey and Röbbelin, 1989).

1.3 The Oilseed Brassicas

Over 13% of the world's edible oil supply comes from the oilseed Brassicas (rapeseed and mustard), making them the third most important edible oil source after soybean and palm (Downey and Röbbelen, 1989). Due to their ability to germinate and grow at low temperatures, the oilseed Brassicas are one of the few edible oil crops that can be cultivated in the cooler agricultural regions and at higher elevations. Within the rapeseed species of *Brassica napus* and *Brassica rapa*, both spring and winter varieties occur. The winter forms are more productive than the spring forms, but are less winter hardy than the winter cereals. The winter form of *Brassica napus* predominates in Northern Europe and Central and Southern China. Under the severe winter conditions encountered in Western Canada and Western China, as well as in Northern Sweden, the spring forms of *Brassica napus* and *Brassica rapa* must be grown.

The harvested part of the plant, the seed, normally yields on extraction over 40% oil on a dry weight basis and a meal containing 38%-44% of high quality protein. The meal is used as an organic fertilizer in Asian countries or, in the Western World, as a high protein feed for livestock and poultry. About 80% of the monetary value of the seed is derived from the extracted oil and the rest is from the meal (Downey and Röbbelin, 1989).

In Canada, oilseed varieties of *Brassica napus* and *Brassica rapa* are grown extensively. The use of *Brassica rapa* extends the growing region for this oilseed crop because it takes less time to mature than does *Brassica napus*. Many years of plant breeding have transformed the chemistry of the seed so

that the oil is considered suitable for human consumption in Western countries and the meal can be used for animal feed. The oil was improved significantly for human consumption by modifying its fatty acid composition to reduce the levels of erucic acid (Downey, 1964), a compound that was a potential health hazard for humans consuming the oil (Daun, 1984; cf Lamb, 1989). Glucosinolates were reduced in the meal because this class of compounds was found to be repellant or toxic to some farm animals (Röbbelen and Thies, 1980; cf Lamb, 1989). The resultant 'double low' varieties of rapeseed are known as Canola, which are this nation's most important oilseed crop (Lennox,1984).

1.4 TISSUE CULTURE OF THE GENUS *BRASSICA*

Tissue culture techniques for crop species have become a valuable addition to the plant breeder's arsenal of tools for the improvement of a crop's performance. Not only are these techniques valuable in themselves but are also a prerequisite for the application of genetic engineering to that crop. The applications of plant cell and tissue culture are numerous. For instance, *in vitro* meristem culture has been used for the elimination of viruses from plants in order to improve yield (Hu and Wang, 1983) and shoot tip culture is used extensively for the micropropagation of floricultural crops (Sagawa and Kunisaki, 1990). Cultured plant cells can be used for secondary product synthesis (Whitaker and Hashimoto, 1986), to study plant physiology at the cell level (Moloney *et al.* , 1983; Seresinhe and Oertl, 1991; Tan and Liang, 1991; Messner *et al.*, 1991) or for the selection of mutants *in vitro* (*eg.* herbicide resistance; Chaleff and Ray, 1984). Haploid plants recovered from cultured anthers or microspore derived haploid cells are important tools for breeders

following diploidization. When these plants are integrated into a conventional breeding program they have resulted in the rapid development of new varieties (Morrison and Evans, 1988; Morrison *et al.*, 1991). Cultured microspores themselves can be used for a variety of purposes. They can be used to select for novel mutants (*eg.* herbicide resistance; Swanson *et al.*, 1988; Swanson *et al.*, 1989) or to examine basic developmental phenomenon in plants (*eg.* embryogenesis; Taylor *et al.* , 1990). Microspore derived embryos are also being utilized in the development of synthetic seed technology (Datla and Potrykus, 1989).

Embryo rescue is another important tool for the plant breeder. Interspecific and intergeneric crosses are frequently carried out in plant breeding (most commonly in cereal crop breeding, but also in *Brassica* - *eg.* Kumar *et al.*, 1988) in order to transfer valuable genes from wild species to cultivated species. Incompatibility is often encountered in such crosses and results in shriveled seeds containing aborted embryos. By excising embryos from such seeds and growing them on a simple inorganic medium with an energy source, the progeny of these wide incompatible crosses can be rescued (Hu and Wang, 1986).

Protoplast fusion (somatic hybridization) has been suggested as an alternate means to overcome species barriers to sexual hybridization so that useful traits can be introduced into established crops. This technique can extend the range of possibilities for gene transfer among or between species that can be achieved via embryo rescue. For instance, it allows the organellar genomes of both progenitors to be represented among the hybrids, whereas in

sexual hybridization the male parent's organelles are usually excluded (Glimelius *et al.*, 1991).

Most proposed benefits of plant tissue culture require regeneration of a genetically modified or cloned plant. Without consistent regeneration of important crop species, the agricultural application of plant tissue culture will be limited (Flick *et al.* , 1983). Regeneration from plant cells or tissues *in vitro* can occur through two routes; organogenesis or embryogenesis. Embryogenesis results in the production of bipolar structures bearing both root and shoot apices in one step while organogenesis usually requires mutually exclusive shoot and root development regimes. While somatic embryos are produced in greater number per culture vessel than are adventitious shoots, the list of species that have been shown to undergo somatic embryogenesis is short when compared to those that will regenerate via organogenesis (Ammirato, 1983).

Once effective regeneration protocols are achieved, tissue culture can then be used by the plant breeder. Regeneration *in vitro* is used for the maintenance of self-incompatible parent lines where the planted crop is a hybrid variety (F_1), as is the case for many of the *Brassica* cole crops (Zee and Johnson, 1983). Another application is to increase the variability within a crop species through somaclonal variation (Larkin and Scowcroft, 1981). This variation can be exploited by screening for useful traits such as disease-, stress-, and herbicide-resistance (Knowles,1989). Reliable resistance to a pathogen, for example, can increase yields by up to 60% (Ratafia and Purinton, 1988).

Regeneration protocols have been developed for all the species within the *Brassica* genus using a variety of explants. These include microspores (from which haploid somatic embryos are regenerated), mature plant parts (*eg.* leaf, root, stem), juvenile plant parts (*eg.* hypocotyl and cotyledon), and protoplasts derived from these plant parts (*eg.* hypocotyl and leaf). Comparative studies of regeneration ability among the different species of the *Brassica* genus have consistently shown that *Brassica rapa* is the least amenable to regeneration *in vitro* . These consistent differences in organogenic response *in vitro* have been ascribed to genetic determinants (Murata and Orton, 1987) or environmental influences (represented by growth media and hormonal interactions, Narasimhulu and Chopra, 1988). The recently reported high level regeneration response obtained in certain subspecies of *Brassica rapa* (Chi and Pua,1989) argues against certain genotypes being inherently of low morphogenic potential. Rather, efficient regeneration from recalcitrant species may require the proper combination of explant, hormones and culture conditions. The oleiferous subspecies of *Brassica rapa* still remain the most consistently recalcitrant of the various *Brassica* species to date.

Most studies have involved optimizing a regeneration protocol for a single genotype. As a result, they may be of limited value upon extrapolation to other genotypes (*i.e.* varieties or subspecies) of the same species or to other species within the *Brassica* genus. Also, many of the regeneration protocols were developed for tissue culture applications, such as the exploitation of somaclonal variation (*eg.* callus culture), or for plant breeding purposes (*eg.* microspore-

derived embryogenesis). These protocols may not be suitable for use in a genetic transformation system because of the special needs required for this procedure. It has been shown (Moloney *et al.*, 1989; Colby *et al.*, 1991) that often the cells competent for transformation via *Agrobacterium* are not necessarily competent for regeneration and vice versa.

1.4.1 Tissue Culture of *B. rapa*

There have been many reports of regeneration from various tissues of *B. rapa* . Dunwell (1981) achieved 2%-5% regeneration from 2 turnip varieties of *B. rapa* (ssp. rapifera) using a combination of BA (1-10 mg/L) and NAA (10 mg/L). Singh and Chandra (1984) obtained 100% shoot regeneration from hypocotyl derived callus cultures of *B. rapa* cv. Yellow Sarson (an Indian oilseed) using only cytokinins (22.1 μM BA or 23.2 μM Kinetin). Although regeneration was fairly rapid from the callus cultures (30 days), callus culture initiation required 6 months.

Murata and Orton (1987) were able to regenerate shoots from cotyledon or hypocotyl derived callus of two varieties of *B. rapa* (Canola). Regeneration frequencies were 7.6% for cotyledons and 2.3% for hypocotyls. No specific information was presented regarding the hormone requirements for *B. rapa* regeneration but the authors found that medium containing BA alone was the most efficient and that auxin supplementation generally inhibited shoot formation.

Most other published protocols for the regeneration of shoots from explants of *B. rapa* involve the use of cotyledons. Jain *et al.* (1988), using 6 day old

cotyledons from Indian cultivars of *B. rapa* , (including a Yellow Sarson and a Brown Sarson oilseed variety), were able to regenerate shoots at a frequency of between 8% and 24%. They found that BA was ineffective as a cytokinin and that an auxin (IAA) was essential for regeneration to occur. Regarding the use of cotyledons as an explant source, the authors noted that a large number of sterile explants could be obtained in a short time, any time throughout the year.

Narasimhulu and Chopra (1988) and Narasimhulu *et al.* (1988), using six day old cotyledons also, were able to regenerate shoots from 12.6% of explants from a Brown Sarson variety of *B. rapa* and from 32% of explants from a Yellow Sarson variety of *B. rapa* using a combination of BA (4.4 µM) and NAA (5.3 µM). These authors noted that regeneration consisted of two phases : (1) callus formation and (2) shoot bud regeneration from the petiolar end of the explant.

Chi and Pua (1989) obtained regeneration from 2 varieties of chinese cabbage (*B. rapa* ssp. chinensis cv. Speedy and cv. 2B-21-64) at an efficiency of 13 % and 30 % respectively using cotyledon explants (with petiole intact) and a combination of BA (8.8 µM) and NAA (5.4 µM). Again, regeneration occured at the cut end of the explant. In this case regeneration was improved markedly (to >80%) by the addition of silver compounds.

As can be gleaned from the above experiments, intact cotyledons are a good explant source for shoot regeneration from various *B. rapa* subspecies. These explants have also been shown to provide for efficient shoot regeneration in other species of *Brassica* as well, including *B. carinata* (Jaiswal *et al.*, 1987), *B. juncea* (Sharma *et al.*, 1990) and *B. napus* (Moloney *et al.*, 1989). In the latter case regeneration from this tissue was shown to be

compatible with *Agrobacterium* mediated transformation, resulting in the efficient production of transformed shoots. As a result cotyledonary petiole explants were deemed the most likely to yield efficient shoot regeneration from Canola varieties of *B. rapa* in a way that was also compatible with *Agrobacterium* mediated transformation.

1.5 GENETIC ENGINEERING OF THE GENUS *BRASSICA*

There are now a wide variety of approaches to introducing exogenous DNA into plant cells (Potrykus, 1991), though *Agrobacterium tumefaciens* remains the transformation method of choice for most plant species (Weising *et al.*, 1988). Genetic engineering techniques offer great potential for adding specific characteristics to existing varieties of crops, thereby hastening the introduction of improved versions of established varieties into the field. Application of these techniques to Canola varieties could include the introduction of herbicide (Oxotoby and Hughes, 1990), virus (Nelson *et al.*, 1988; Harrison *et al.*, 1987) or insect (Meeusen and Warren, 1989) resistance. Yield losses due to weeds in Canola fields for example, especially to the broad leaf cruciferous species, can amount to 12%-14% even under optimum cultural conditions (Miki *et al.*, 1990). Other improvements that could be obtained through genetic engineering include improvement of the quality of the seed proteins (Kriz and Larkins, 1991), alteration of the content and composition of the seed oil and the production of valuable or therapeutic proteins in large amounts (Knauf, 1987; Knowles,1989).

By far the most intensely investigated *Brassica* species with respect to genetic engineering is *Brassica napus* , owing to its dominant position economically among *Brassica* crop species. Fertile transgenic plants have

been obtained through transformation with *Agrobacterium tumefaciens* of transverse stem segments (Fry *et al.*, 1987), longitudinal stem segments (Pua *et al.*, 1987), thin cell layers (Charest *et al.*, 1988), hypocotyls (Radke *et al.*, 1988),microspore embryos (Swanson and Erickson, 1989), cotyledonary petioles (Moloney *et al.*, 1989), and inflorescence stalks (Boulter *et al.*, 1990). Fertile transformed plants have also been obtained through transformation of stem segments with *Agrobacterium rhizogenes* (Guerche, 1987) and microinjection of microspore embryos (Neuhaus, 1987).

Two other species, *Brassica juncea* and *Brassica oleracea*, have also been transformed successfully with *Agrobacterium tumefaciens* resulting in the production of transgenic plants (Barfield and Pua, 1991; Mathews *et al.*, 1990; Srivastova *et al.*, 1988). However, although *Brassica rapa* tissues have been shown to be susceptible to *Agrobacterium tumefaciens* transformation (ssp. oleifera : Ohlsson and Eriksson, 1988; Grimsley *et al.*, 1989; ssp. rapifera : Hille *et al.*, 1986; Godwin *et al.*, 1991), no transformed plants have ever been regenerated from this species. As *Brassica rapa* comprises approximately 1/2 of the Canola crop in Canada (by acreage), a reliable transformation system for this species is desirable so that any agronomic improvement to varieties of the other Canola crop, *Brassica napus*, obtained through genetic engineering could also be available to *Brassica rapa*.

1.6 OBJECTIVES

The objective of the present study was to develop a reliable regeneration system for turnip rape (*Brassica rapa* ssp. oleifera) that also has the potential to be used in a system to genetically engineer this species through *Agrobacterium tumefaciens* mediated transformation.

Specific goals were therefore :

1) Evaluate the potential of the cotyledonary petiole method (Moloney *et al.*, 1989) to the transformation of *B. rapa* .

2) Optimize conditions for high level regeneration.

3) Determine whether there is a significant genotype specificity to the regeneration protocol.

4) Estimate the efficiency of *A. tumefaciens* -based transformation protocols for the transformation of cotyledonary petioles in *B. rapa* .

CHAPTER 2 :
MATERIALS AND METHODS

2.1 PLANT MATERIALS

Brassica rapa (Canola) varieties used in this study were Tobin, Colt, Parkland, Horizon, and Echo. A high glucosinolate variety (R-500; an Indian Sarson, ssp. trilocularis) was also used. All seeds were supplied by Dr. Keith Downey, Agriculture Canada, Saskatoon, Saskatchewan. All seeds were maintained at 4° C until used for experimental purposes.

Seeds were sterilized in 50 ml of a 10% solution of commercial bleach (Javex™) with 1-2 drops of TWEEN-20® for 10 minutes, followed by 3 rinses in 25-30 ml of sterile distilled water. The seeds were placed on germination medium (Murashige and Skoog, 1962) (MS) salts and vitamins, 3% sucrose, 0.7% phytagar (Gibco)) at a density of 20 seeds per plate and maintained at 24° C in a 16h light/8h dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$.

2.2 SHOOT REGENERATION

Cotyledons were excised from 4 day old seedlings (or from 4, 5, and 6 day old seedlings in one experiment) so that they included ≈ 2mm of petiole at the base. The petioles were embedded into the regeneration media tested here to a depth of ≈ 2mm. The culture vessels used were 100 x 15 mm sterile disposable petri dishes for germination and regeneration, and Magenta® jars (Sigma) for elongation and rooting. Petri dishes were sealed with 2 layers of Parafilm®.

Regeneration media comprised MS salts and vitamins, 1%, 2% or 3% sucrose, and various concentrations of α-naphthaleneacetic acid (NAA, Sigma) and 6-benzylaminopurine (BA, Sigma). BA at 2 mgL^{-1} (8.8 μM) was used in

conjunction with NAA at 0, 0.5, 1 and 2 mgL^{-1} (0, 2.7, 5.4, and 10.8 μM respectively). Also, NAA at 1 mgL^{-1} was used with 0, 1, 2, and 4 mgL^{-1} BA. Explants (10 per plate) were maintained at 24° C in a 16h light/8h dark photoperiod at a 'low' light intensity (30-40 $\mu Em^{-2}s^{-1}$). After 3 weeks, shoots and roots arising from the explants were counted. Percentage regeneration per plate (number of explants regenerating/total number of explants) was averaged for 6-7 plates for each treatment. A score of 'one' was given to an explant even when multiple regenerants were produced.

Rooting and acclimatization were performed on 2 week old explants containing regenerating shoots by placing them on germination media (see above) under low light intensity conditions. Two weeks later the regenerated shoots with accompanying root mass were transferred to potting mix (Peat Moss:Vermiculite(Terra-Lite® [W.R. Grace]:Terra-Green®[Oil-Dri], 2:1:2) supplemented with Osmocote®[14-14-14, Grace-Sierra] granular fertilizer and placed in a misting chamber (average relative humidity of ~40%). After another two weeks, plants were transferred to a greenhouse for further growth and flowering.

Since all data presented here are in the form of percentages, which have error variances that are a function of the mean and are not normally distributed (Ahrens *et al.*, 1990), an arcsine transformation of the data was performed (Zar, 1984) before they were subjected to statistical analysis. Significant treatment differences were selected by Tukeys Multiple Comparison Test (Zar, 1984).

2.3 HISTOLOGICAL STUDIES

To study the ontogeny of adventitious shoot bud differentiation in culture, petioles from cotyledons at day 0 and those cultured on shoot regeneration medium (MS + 2% sucrose, 2 mgL^{-1} BA, 1 mgL^{-1} NAA and 0.7% phytagar) were fixed after 2, 5, 7 and 11 days and processed for glycol methylacrylate (GMA, Aldrich) sectioning. After fixing the material in a freshly prepared, chilled solution of 2% glutaraldehyde and 2% formaldehyde in 50 mM sodium phosphate buffer (pH 6.8), the material was dehydrated in methyl cellosolve followed by two changes of absolute alcohol (O'Brien and McCully, 1981). The specimens were then infiltrated with the LKB infiltration solution containing GMA. After the tissue had been infiltrated it was embedded and polymerized in LKB embedding solution. Sections (3 µm thick) were obtained on a Reichert-Jung rotary microtome using dry glass knives. All sections were stained with periodic acid Schiff's reagent (PAS) for total insoluble polysaccharides and counterstained with amido black 10B for proteins (Yeung, 1984).

2.4 PLANT TRANSFORMATION

2.4.1 *Agrobacterium* Strains

Transformations of *Brassica rapa* were attempted according to the protocol of Moloney *et al.* (1989) with one of the following *Agrobacterium* strains :

1) Strain EHA101 (kan^r ; Hood *et al.* , 1986) containing the binary plasmid pCGN-783 ($gent^r$; Houck *et al.* , 1988).

2) Strain EHA101 containing one of a series of binary plasmids (pCGN-1547,-1548,-1549,-1557,-1558,-1559,-1578 ; gent^r; McBride and Summerfelt, 1990).

3) Strain EHA101 containing the plasmid pCa2AMVGN-1548 (gent^r, Oancia and Roeckel, unpublished).

4) Strain MP90 (gent^r; Koncz and Schell, 1986) containing the binary plasmid pITG-15 (kan^r; Babic, 1991).

5) Strain A281 (which contains the oncogenic Ti plasmid pTiBo542 ; Sciaky *et al.* , 1978) containing the binary plasmid pCGN-763 (Houck *et al.* , 1988).

The plasmid pCGN-783 contains between its T-DNA borders a gene construct comprised of a CaMV 35S promoter, a neomycin phosphotransferase II (NPT II) coding region and a transcription terminator obtained from the gene coding for transcript 7 of the T_L- DNA of the *A. tumefaciens* octopine plasmid pTiAch5. The 1500 series of pCGN binary vectors contain an origin of replication that is highly stable in *A. tumefaciens* and contain between their T-DNA borders one of two plant selectable markers. The first is made up of a CaMV 35S promoter, NPT II gene and *tml* transcription terminator (from *gene6b* of the tumor morphology large locus of the octopine plasmid pTiA6; Ream *et al.*, 1983). This selectable marker is orientated so that its direction of transcription is from the right T-DNA border inwards (in pCGN-1558 and -1559) or outwards toward the right T-DNA border (pCGN-1557 and -1578). The second selectable marker comprises a mannopine synthase (mas) promoter, NPT II gene and mas

transcription terminator (the mas gene fragments are also from the octopine plasmid pTiA6, Ellis *et al.*, 1984). Again, transcription of the marker gene is either inwards from the right border (pCGN-1549) or outwards towards the right border (pCGN-1547 and-1548). The binary plasmid pCa2AMVGN-1548 is a derivative of pCGN-1548. It has an insert that contains a plant selectable marker comprised of a fusion gene that encodes a protein having both NPT II and ß-glucuronidase activity (Datla *et al.* , 1991). Upstream from this gene is a tandem CaMV-35S promoter (Kay *et al.* , 1987) fused to 35 bp of the 5' untranslated leader region from RNA 4 of the alfalfa mosaic virus (AMV), which acts as a translational enhancer (Jobling and Gehrke, 1987). Downstream from this gene is the the nopaline synthase (nos) translation termination and polyadenylation sequence (Herrera-Estrella *et al.*, 1983A). pITG-15 is a derivative of pBIN-19 (Bevan, 1984) that contains as a plant selectable marker the same promoter-fusion gene combination that was inserted into pCa2AMVGN-1548. It also contains as a plant selectable marker a mutated form of the gene coding for acetolactate synthase (ALS) from *Brassica napus* that is resistant to sulfonylurea herbicides (Babic, 1991). Upstream from this gene is the tandem CaMV-35S : AMV leader promoter and downstream is the nos transcription terminator. These *Agrobacterium* strains were maintained on AB minimal media (Chilton *et al.*, 1974) plates solidified with 1.5% Bacto-Agar (Difco) and containing 100 μgml^{-1} gentamycin sulfate (Sigma) and 100 μgml^{-1} kanamycin sulfate (Boehringer Mannheim).

2.4.2. Transformation

Single colonies of *Agrobacterium tumefaciens* were grown for 48 hours at 28°C in 5 mls of AB liquid media supplemented with the same antibiotics as the plates but at concentrations of 50 $\mu g ml^{-1}$. This bacterial suspension was centrifuged at 15,000 x g for 1 minute, resuspended in 1 ml of AB media, and 100 µl of this concentrate was diluted into 10 mls of AB media (final dilution = 1/20) in a 60mm x 15mm disposable petri dish. The cut end of freshly excised cotyledonary petioles (with cotyledons intact) were dipped in this suspension for a few seconds, then placed into regeneration media as described above. After 72 hours of cocultivation with *Agrobacterium* , explants were transferred to selection media (= regeneration media supplemented with 20 $\mu g ml^{-1}$ kanamycin sulfate and 500 $\mu g ml^{-1}$ carbenicillin (Pyopen®, Ayerst, filter sterilized and added after media was autoclaved and cooled to approximately 50°C). In experiments testing the effect of acetosyringone (AS; 3', 5'-dimethoxy 4'-hydroxyacetophenone, Aldrich) treatment of *Agrobacterium* on transformation efficiency, bacterial growth conditions were altered. From an overnight culture of the *Agrobacterium* strain grown in AB media with appropriate antibiotics, 100 µL was used to inoculate 5 ml of MG/L medium (consisting of 50% of a mannitol-glutamate-salts medium (Garfinkel and Nestor, 1980) and 50% Luria broth (Maniatis *et al.* , 1982)) that had been filter sterilized after the addition of AS and adjustment of the pH to 5.6. An overnight growth in this medium was used for transformations.

In some experiments, initial exposure conditions of the explant to *Agrobacterium* were modified. Explants (≈20 at a time) were floated on a bacterial suspension and placed within a sterile 250 ml Nalgene® disposable filterware container. The container was then attached to a vacuum line for 5 minutes. Explants were then blotted dry on sterile filter paper and placed on regeneration medium. This treatment was tried in order to increase infiltration of the *Agrobacterium* into the explant. If access to regenerable cells within the explant is a limiting factor then this treatment could enhance access.

After 2-3 weeks in culture, any regenerated shoots that remained green and healthy were transferred to elongation medium with kanamycin and carbenicillin (= 1X MS salts and vitamins, 1% sucrose, 0.7% phytagar, pH 5.8, supplemented with antibiotics as in selection media) to allow apical dominance to develop. Shoots that exhibited normal apical dominance and remained unbleached after a further 1-2 weeks were transferred to elongation media (as above but unsupplemented with antibiotics) for rooting. Rooted plants were cultivated to flowering as described above (Section 2.2).

2.5 IDENTIFICATION OF TRANSFORMANTS

2.5.1 NPT II Assays

Putative transgenic plants or calli were assayed for neomycin phosphotransferase (NPT-II) activity essentially as described by Radke *et al.* (1988), but modified according to Staebell *et al.* (1990). Approximately 150 mg of callus tissue or leaf tissue taken from regenerated shoots was homogenized (on ice) in an equal volume (w/v) of extraction buffer (50 mM sodium phosphate

buffer (pH 7.0), 1 mM Na_2EDTA, (pH 8.0), 0.1% Triton® X-100, 10 mM ß-mercaptoethanol, 0.1% Sarkosyl). Homogenization was performed in 1.5 ml disposable plastic tubes (Bioplastics) using disposable pestles (KONTES) and an electric drill. The homogenate was centrifuged at 15,000 x g at 4°C for 15 min and the supernatant assayed for NPT-II activity. For every sample, two reactions were performed : one which contained kanamycin (at 0.4 $mgml^{-1}$) and the second, a negative control, which lacked this substrate. To a 20 µl aliquot of tissue extract was added 10 µl of reaction buffer (67 mM Tris-Maleate (pH 7.1), 42 mM $MgCl_2$, 400 mM NH_4Cl, 1.7 mM dithiothreitol). Reactions were initiated by adding 10 µl of a radiolabeled ATP mix (1.5 mM unlabeled ATP and 40 µCi of HPLC purified [γ-^{32}P] ATP (4500 $CimM^{-1}$; ICN) and were incubated for 45 min at 37°C. The reaction catalysed by NPT II is as follows :

Kanamycin + ATP $\Rightarrow$ Kanamycin-P + ADP (Haas and Dowding, 1975)

Entire reaction volumes were pipetted onto cellulose phosphate paper (P81, Whatman) and the P81 paper was subjected to six washing steps. The paper was washed in 300 ml of 10 mM sodium phosphate (adjusted to pH 7.5) for 5 min at room temperature, then for 1 hr at 65°C in 10 ml of 1% SDS. After this, the filter was washed with 300 ml of 10 mM sodium phosphate (pH 7.5) at 65°C for 5 min and then with another 300 mls of the same buffer at 65° for 1 hour. The final two washes were with 300 ml of 10 mM sodium phosphate (pH 7.5) at room temperature for 5 min. The paper was then air dried and subjected to autoradiography overnight at -80°C using Kodak XAR-5 film and Dupont Cronex® Lightening-Plus intensifying screens.

2.5.2 PCR Assays

Some putatively transformed tissue was also subjected to PCR (polymerase chain reaction) analysis in order to ascertain whether or not DNA had been incorporated into the plant genome through *Agrobacterium* mediated transformation. This was done according to the protocol of Edwards *et al.* (1991). A leaf disc from the plant to be tested was pinched into an eppendorf tube using the lid of the tube. The tissue was macerated using a disposable teflon pestle for 15 seconds. Then 400 µl of extraction buffer (200 mM Tris HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the sample was vortexed for 5 seconds. The sample was left at room temperature for 1 hour and then centrifuged at 15,000 x g for 1 minute. 300 µl of the resultant supernatant was transferred to a new tube, mixed with an equal volume of isopropanol and left at room temperature for 2 minutes. This solution was centrifuged at 15,000 x g for 5 minutes, the supernatant discarded and the pellet vacuum dried. The pellet, containing the plant DNA, was then resuspended in 100 µl of TE (10 mM Tris·HCl (pH 8.0), 1 mM Na_2EDTA (pH 8.0)). 2.5 µl of this sample was used for a standard 100 µl PCR containing 10 µl 10x PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin), 16 µl dNTP mix (10 mM each of dATP, dGTP, dTTP and dCTP; final concentration of 200 µM), 7.5 µl of each primer (specific for the 5' and 3' ends of the NPT II gene-20 µM stock; final concentration of 1.5 µM) and 2.5 units Taq polymerase (BRL). The reactions were covered with 100 µl of mineral oil to prevent evaporation and were incubated in a programmable thermocycler (Techne) using the following conditions: an initial incubation at 94° for four minutes, 30 cycles of 92° for 1 minute, 48° for 1 minute and 72° for 3 minutes and a final

incubation at 72° for 10 minutes. Samples of the reactions (20 µl) were electrophoresed on a 0.7% agarose gel containing 1X TAE buffer (40 mM Tris-acetate, 1 mM Na_2EDTA (pH 8.0)). After electrophoresis, the gels were stained in 1X TAE buffer containing 1 $\mu g ml^{-1}$ ethidium bromide for approximately 15 minutes.

2.5.3 Southern Blots

In order to check the identity of the amplified bands, gels containing samples of PCR amplifications were subjected to Southern analysis. Gels were placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes, and then in neutralizing solution (1.5 M NaCl, 0.5 M Tris·HCl (pH 7.2), 1 mM Na_2EDTA (pH8.0)) for another 30 minutes The DNA was then transferred to a nylon membrane (Hybond N, Amersham) by capillary blotting as described in Maniatis *et al.* (1982). The filter was prehybridized for about 16 hours at 65°C in prehybridization solution (6X SSC, 5X Denhardt's solution and 0.5% SDS), then hybridized for about 16 hours at 65°C in the same solution containing ^{32}P-labelled probe. The probe was a 1 kb Pst I fragment of the NPT II gene (obtained by PCR) that had been labelled by the random-primer synthesis method (Feinberg and Vogelstein, 1984) and had a specific activity of about 10^8 dpm μg^{-1} . The filter was washed twice for 15 min in 2X SSC at 65°C, and once for 15 min in 2X SSC, 0.1% SDS at 65°C. The filter was autoradiographed for about 16 hours at -80°C using Kodak XAR-5 film and Dupont Cronex® Lightening-Plus intensifying screens.

CHAPTER 3 :
RESULTS

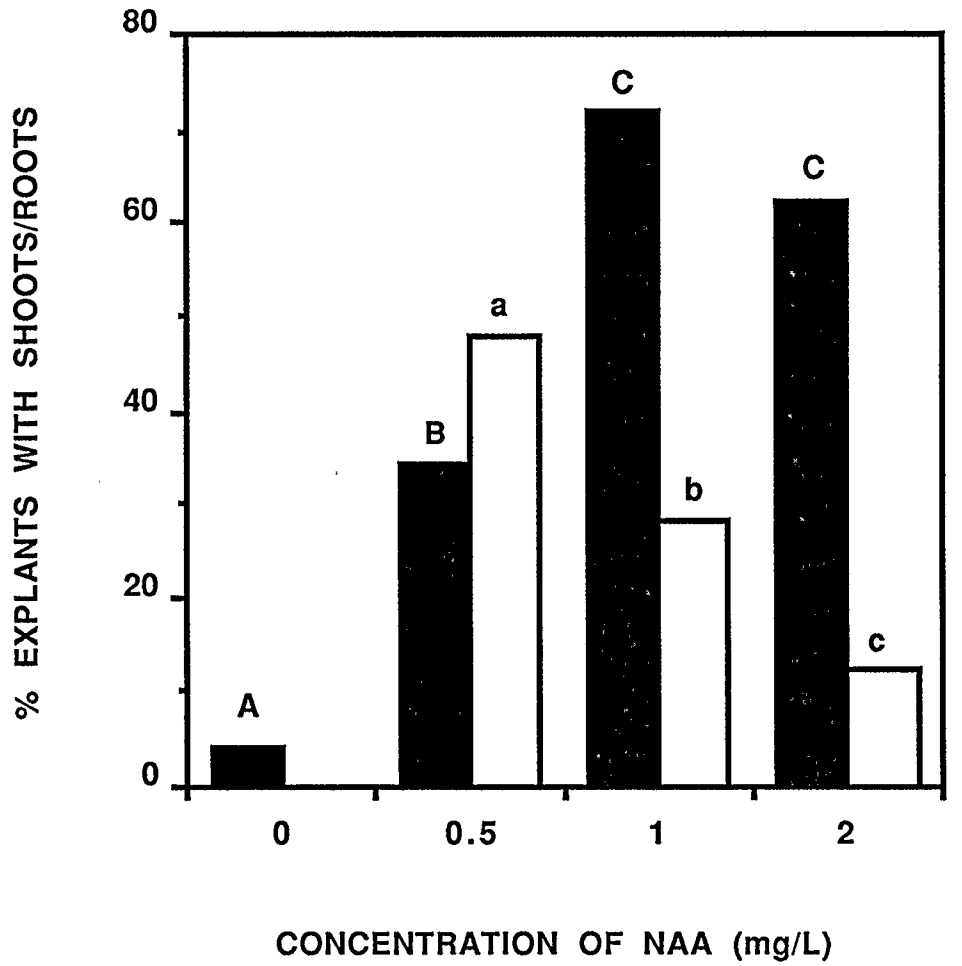
3.1 Regeneration of *B. rapa* Cotyledonary Explants

3.1.1 Effect of Media Composition

Initial experiments on regeneration from *B. rapa* cotyledon explants were aimed at reducing the necrosis of explants. This was approximately 90% after 3 weeks using the unmodified protocol previously developed for *B. napus* regeneration (Moloney *et al.*,1989). In an attempt to decrease explant necrosis, the light intensity was reduced from 60-80 $\mu Em^{-2}s^{-1}$ to 30-40 $\mu Em^{-2}s^{-1}$. Supraoptimal light intensities have been shown to be detrimental to both callus growth and shoot initiation (Seibert *et al.*, 1975). This resulted in improved survival of explants but had no effect on regeneration. Lowering the sucrose concentration from 3% to 2% increased explant survival considerably and callus formation was favoured, but regeneration was still a rare event. Sucrose at 2% was used in this case because of the findings of Murata and Orton (1987), using cotyledon explants of *Brassica* species, that almost no shoot formation occured on their MS media with 3% sucrose present.

At this point it was reasoned that hormonal factors might be the the most critical factor limiting regeneration from this system. As a result, the manipulation of both cytokinin and auxin concentrations in the medium was evaluated for its effect on regeneration. Lowering the BA concentration from 4 mgL^{-1} to 2 mgL^{-1} increased regeneration to 5%. The most critical factor for enhancement of shoot regeneration was the inclusion of NAA in the medium. With NAA, regeneration frequency increased to an optimum of ≈70% at 1 mgL^{-1} NAA and 2 mgL^{-1} BA. Figure 2 shows the dose-response relationship of these explants to increasing NAA concentrations. In the presence of NAA some root

Figure 2. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on 2 mg/L BA and various concentrations of NAA. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


80
60
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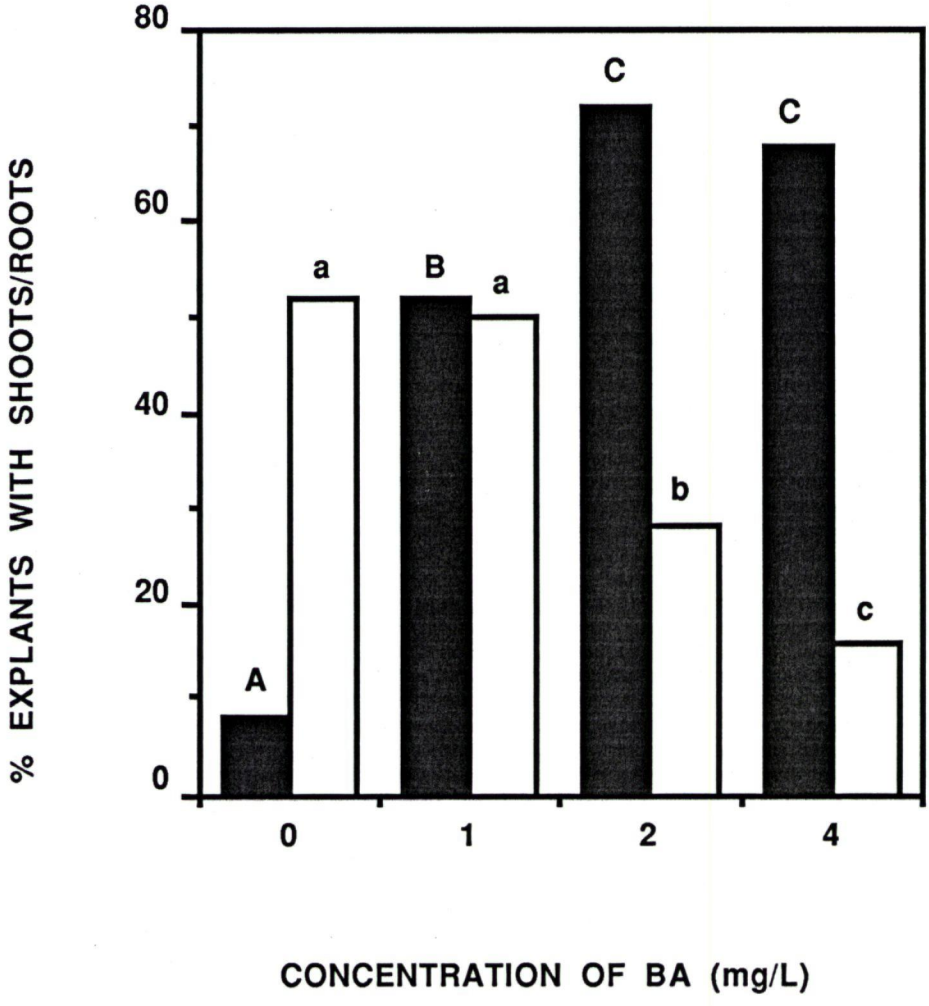
20
0
% EXPLANTS WITH SHOOTS/ROOTS
A
B
a
C
b
C
c
0
0.5
1
2
CONCENTRATION OF NAA (mg/L)

Figure 3. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on 1 mg/L NAA and various concentrations of BA. Data for 1 mg/L NAA and 2 mg/L is from the same experiment as in Figure 2. BA Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.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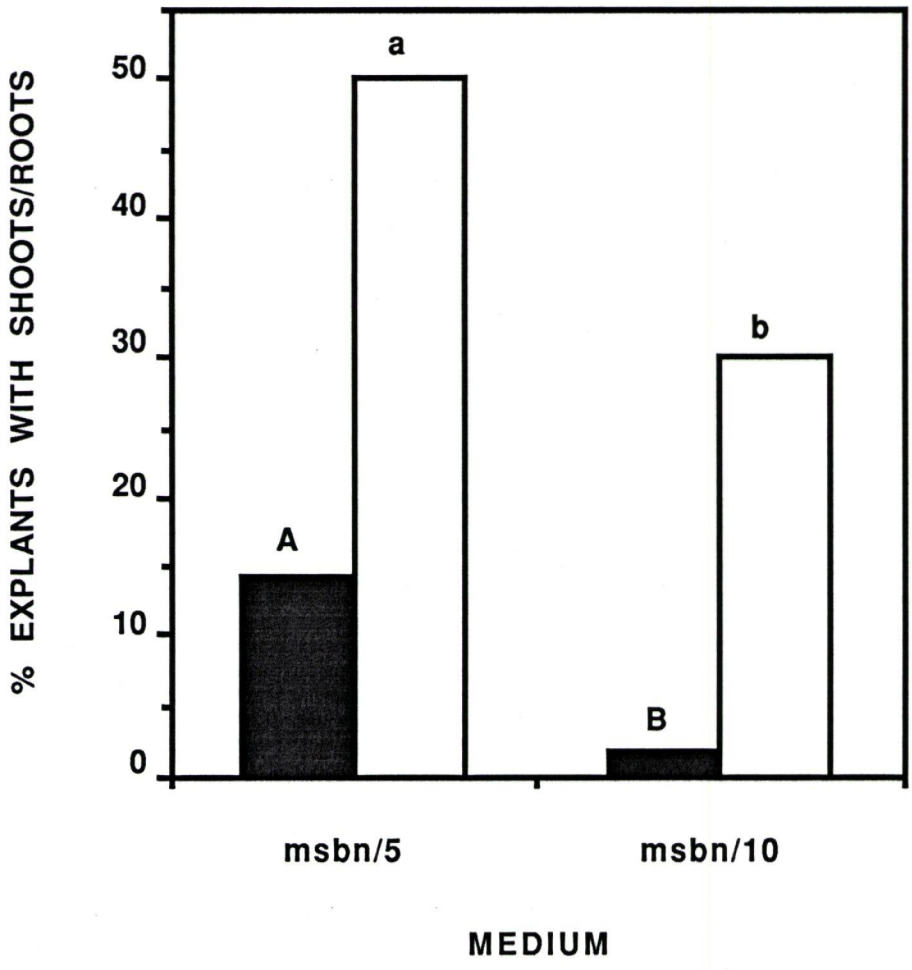
% EXPLANTS WITH SHOOTS/ROOTS
A
a
B
a
C
b
C
c
0
1
2
4
CONCENTRATION OF BA (mg/L)

formation was also promoted, but this did not adversely affect the appearance of shoot primordia.

The amount of BA in the medium was also varied to establish the concentration of this growth regulator required for maximum shoot regeneration (Figure 3). It was found that BA at a concentration of 2 mgL^{-1} or 4 mgL^{-1} was optimal for shoot regeneration among the concentrations tested. While 4 mgL^{-1} BA was also effective for shoot regeneration, the shoots arising on this medium were of a lesser quality often being more vitreous than those arising from explants on 2 mgL^{-1} BA. Cytokinins have been implicated as a causal factor in the occurrence of the vitreous state among *in vitro* cultured plants (Phan, 1991). It was also difficult to obtain shoots with well defined apical dominance at the higher BA concentration. It was unclear from this whether the auxin - cytokinin ratio was the major determinant for shoot regeneration or whether the absolute concentrations of BA and NAA were most important. Therefore, the concentrations of NAA and BA were reduced while maintaining the same molar ratio (1 : 1.63 NAA : BA). This resulted in a reduction in regeneration frequency to 14% after a five-fold reduction in absolute concentrations of these growth substances. A ten-fold reduction in absolute concentration practically abolished shoot regeneration (down to 1.6%, Figure 4).

The presence of NAA in the medium provoked the concurrent formation of roots and shoots from the explant. Increasing the concentration of NAA from 0.5 to 2 mgL^{-1} caused an increase in the production of shoots relative to the production of roots (Figure 2). Similarly, an increase in the concentration of BA from 0 to 4 mgL^{-1} caused an increase in shoot production relative to root

Figure 4. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on MS medium with 2% sucrose and either 1/5 of the optimum NAA and BA concentrations (MSBN/5; 0.4 mg/L BA and 0.2 mg/L NAA) or 1/10 the optimum concentration of these hormones (MSBN/10; 0.2 mg/L BA and 0.1 mg/L NAA). Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


% EXPLANTS WITH SHOOTS/ROOTS
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10
0
a
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A
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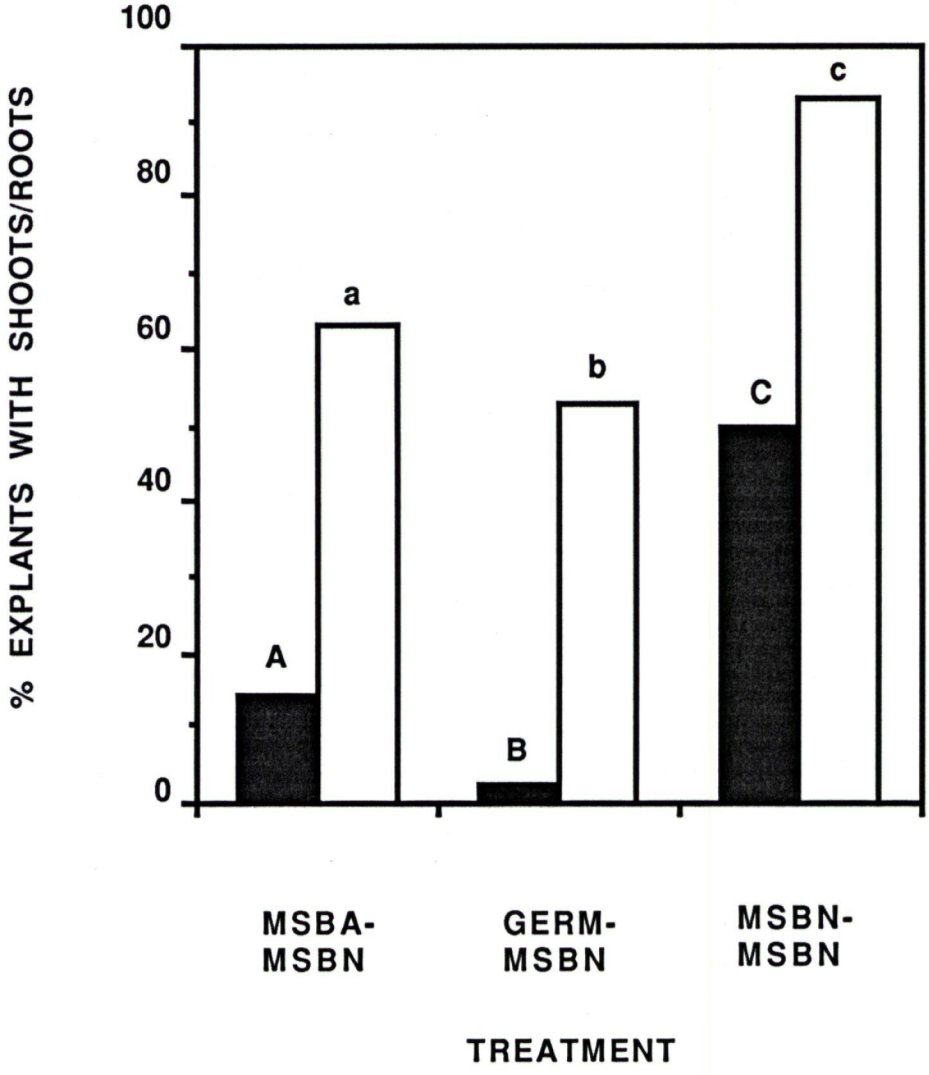
msbn/5
msbn/10
MEDIUM

production (Figure 3). These roots appeared at the base of the regenerating shoots and frequently transfer of regenerated shoots to a rooting medium was not required. Instead, transfer to a medium devoid of growth regulators allowed elongation of both roots and shoots to occur. In fact, maintenance of the explants on regeneration medium for longer than two weeks was detrimental and resulted in the vitrification of the newly formed shoots. The presence of both BA and NAA was essential within the first few days after explant excision for efficient regeneration to occur. If NAA was absent from the regeneration media during the first 4 days of culture, regeneration dropped by 36%. Also, if both BA and NAA were missing from the regeneration media during the first four days of culture, regeneration dropped by 48% (Figure 5).

Experiments were performed to establish whether there was a significant effect of silver ions on regeneration. Such an effect has been previously reported for members of the *Brassicaceae* (Chi and Pua,1989; De Block *et al.*, 1989; Chi *et al.*, 1991) and is presumed to be due to this ion's property as an antagonist of ethylene action (Beyer, 1976). In this system, using optimal NAA : BA ratios, treatment with 25 mgL^{-1} $AgNO_3$ (147 μM) and 500 mgL^{-1} carbenicillin, as suggested by De Block *et al.* (1989), was actually detrimental reducing regeneration frequencies to a value 10% below that obtained without these supplements (Table 1). This treatment also resulted in an increase in callus formation at the cut end of the explant and the production of vitreous shoots with an aberrant morphology.

Figure 5. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin from MSBN media after 4 days preculture on medium lacking NAA (MSBA) or on medium lacking hormones (GERM). Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots


100
80
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40
20
0
% EXPLANTS WITH SHOOTS/ROOTS
a
b
c
A
B
C
MSBA-
MSBN
GERM-
MSBN
MSBN-
MSBN
TREATMENT

TABLE 1 : REGENERATION OF SHOOTS FROM COTYLEDONARY PETIOLES OF *Brassica rapa* ON MSBN MEDIA IN THE PRESENCE (+) OR ABSENCE (-) OF 25 mgL^{-1} $AgNO_3$ and 500 mgL^{-1} CARBENICILLIN

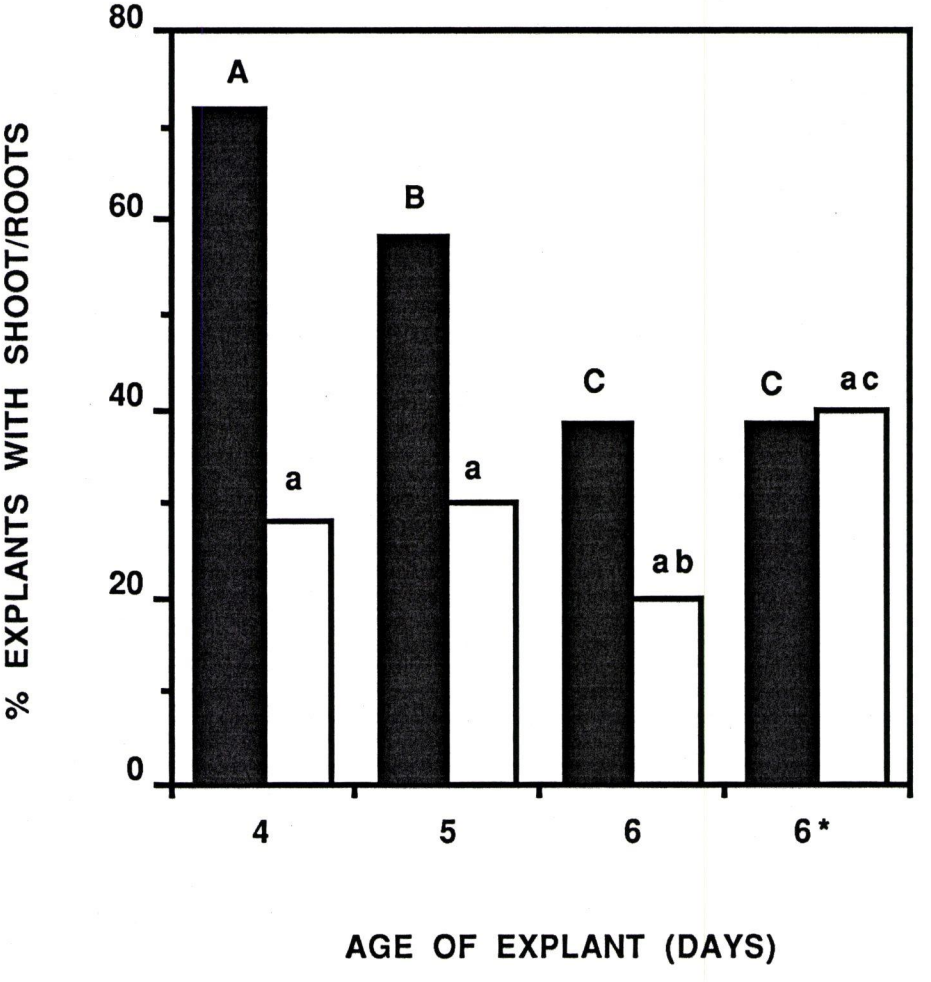
MEDIUM	% REGENERATION
MSBN (-)	51 A*
MSBN (+)	41 B

*Values with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test).

3.1.2 Explant Age

The age of the explant is another important factor that can affect shoot regeneration (Sharma *et al.* ,1990; Yang *et al.* , 1991; Sharma and Thorpe, 1989). A steady decrease in shoot regeneration was apparent from explants derived from seedlings older than 4 days (Figure 6). While explants younger than 4 days old were not tested for their regeneration response, it has been shown for *B. juncea* that a steady decline in regeneration efficiency occurs from explants younger than 4 days old (Sharma *et al.*, 1990).

Figure 6. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin obtained from donor seedlings of different ages. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots. * = high light (*i.e.* explants were incubated with light at an intensity of 60-80 $\mu Em^{-2}s^{-1}$).


80
60
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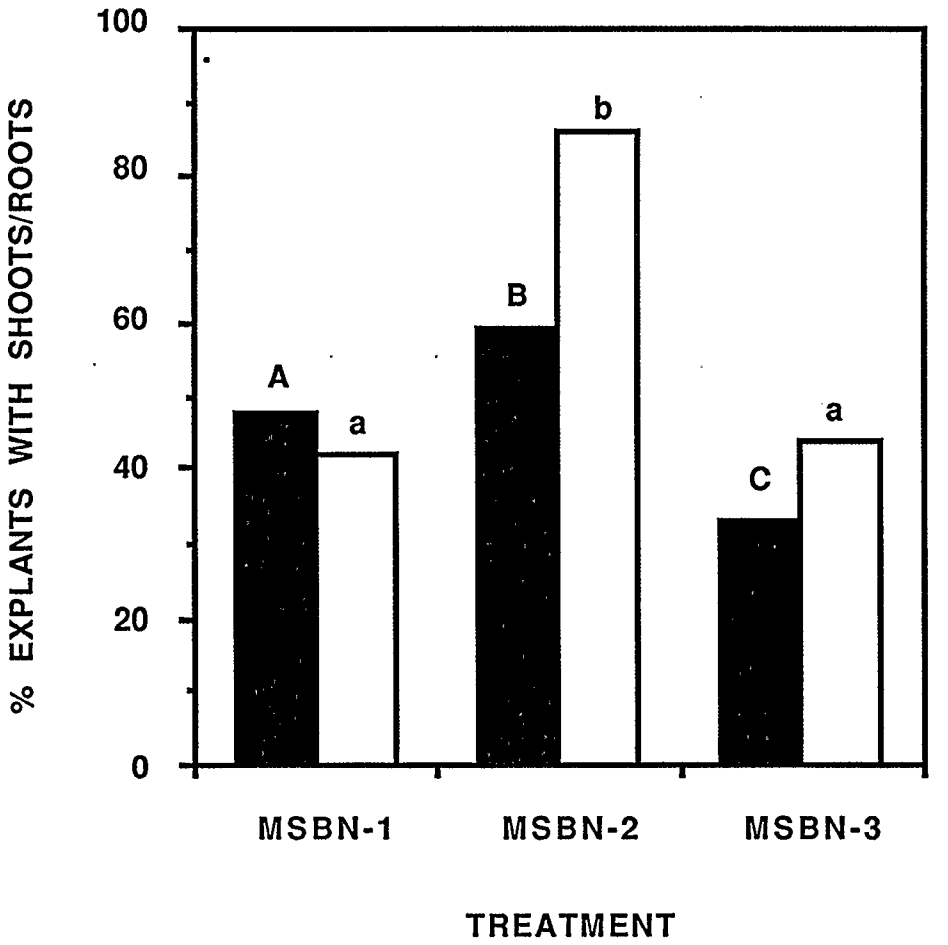
0
% EXPLANTS WITH SHOOT/ROOTS
A
a
B
a
C
a b
C
a c
4
5
6
6*
AGE OF EXPLANT (DAYS)

3.1.3 Light Intensity

It has been stated that the role of light in organogenesis is an often neglected factor (Thorpe, 1980). The effect of light intensity on morphogenesis from these explants is shown in Figure 6. Maintenance of 6-day-old explants under a high light intensity (60-80 $\mu Em^{-2}s^{-1}$) causes a decrease in shoot production relative to root formation as compared to those incubated under the low light intensity regime (30-40 $\mu Em^{-2}s^{-1}$).

In order to more fully explore the role of light in the regeneration response of *B. rapa* cotyledonary petioles, a range of illumination conditions were tested for their effect on the production of shoots and roots from these explants. Also, the level of sucrose in the media was varied in combination with light levels in order to determine if there is any interaction between these two variables with respect to their effect on regeneration. Under standard low-light conditions, optimum shoot production occurs with a sucrose concentration of 2% (Figure 7). Both shoot and root production decline when the sucrose concentration is either decreased to 1% or increased to 3%. Under high light conditions, shoot production was also highest in media containing 2% sucrose (Figure 8). However, absolute shoot production was lower under "high light" conditions than under "low light" conditions while root production was favoured by "high light". Organogenic responses were greatly depressed in the absence of light at all three sucrose levels tested (Figure 9). Rooting response increased with increasing sucrose levels while shooting response increased from 1% to 2% sucrose but remained the same from 2% to 3%. These results indicate that of the three combinations of lighting and sucrose levels tested here, the best

Figure 7. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on regeneration medium with 1, 2 or 3% sucrose under low-light conditions. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


100
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% EXPLANTS WITH SHOOTS/ROOTS
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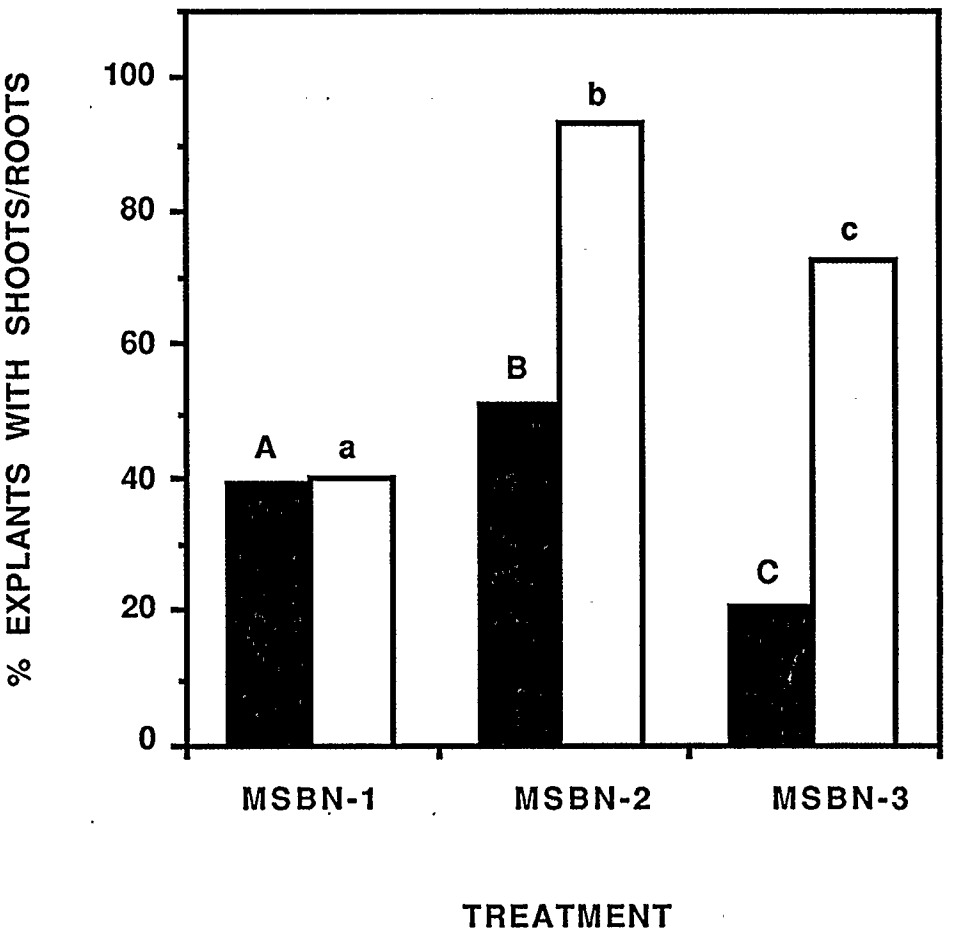
a
B
b
C
a
MSBN-1
MSBN-2
MSBN-3
TREATMENT

Figure 8. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on regeneration medium with 1,2 or 3% sucrose under high-light conditions. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


% EXPLANTS WITH SHOOTS/ROOTS
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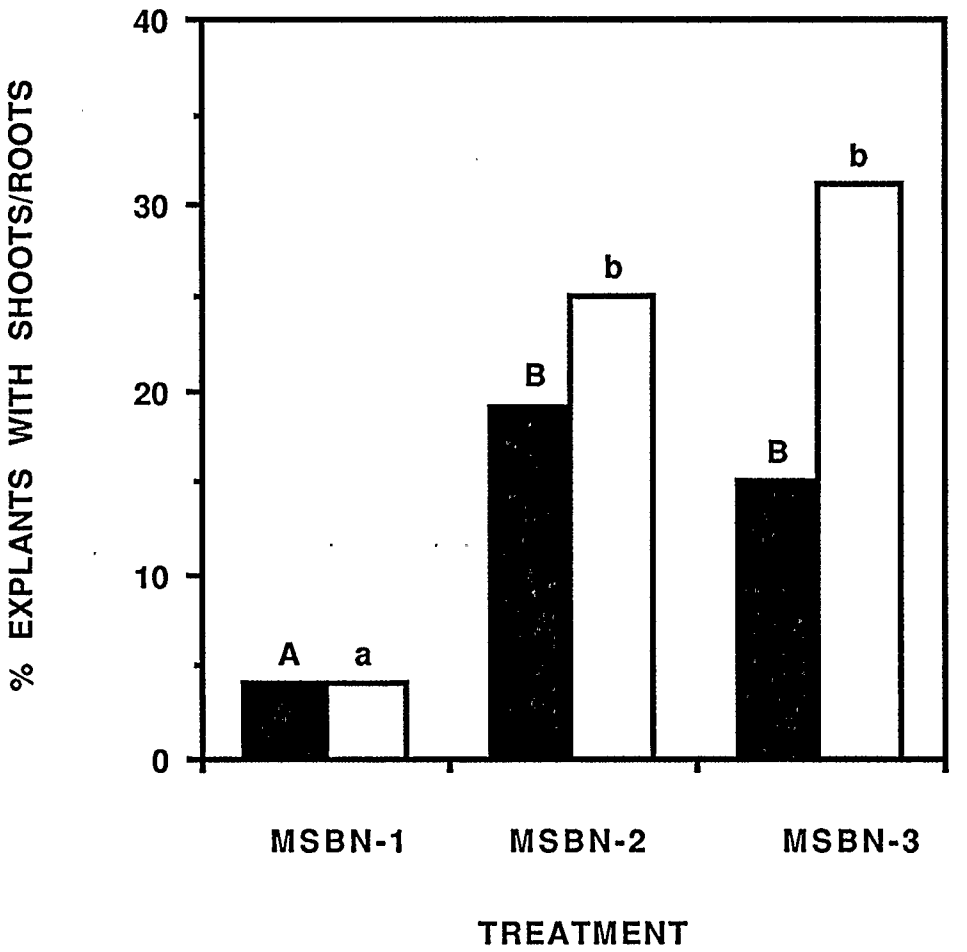
A
a
B
b
C
c
MSBN-1
MSBN-2
MSBN-3
TREATMENT

Figure 9. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on regeneration medium with 1, 2 or 3% sucrose under no - light conditions. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


% EXPLANTS WITH SHOOTS/ROOTS
40
30
20
10
0
A
a
B
b
B
b
MSBN-1
MSBN-2
MSBN-3
TREATMENT

regeneration response was obtained under "low light" conditions in media that contained sucrose at a level of 2%.

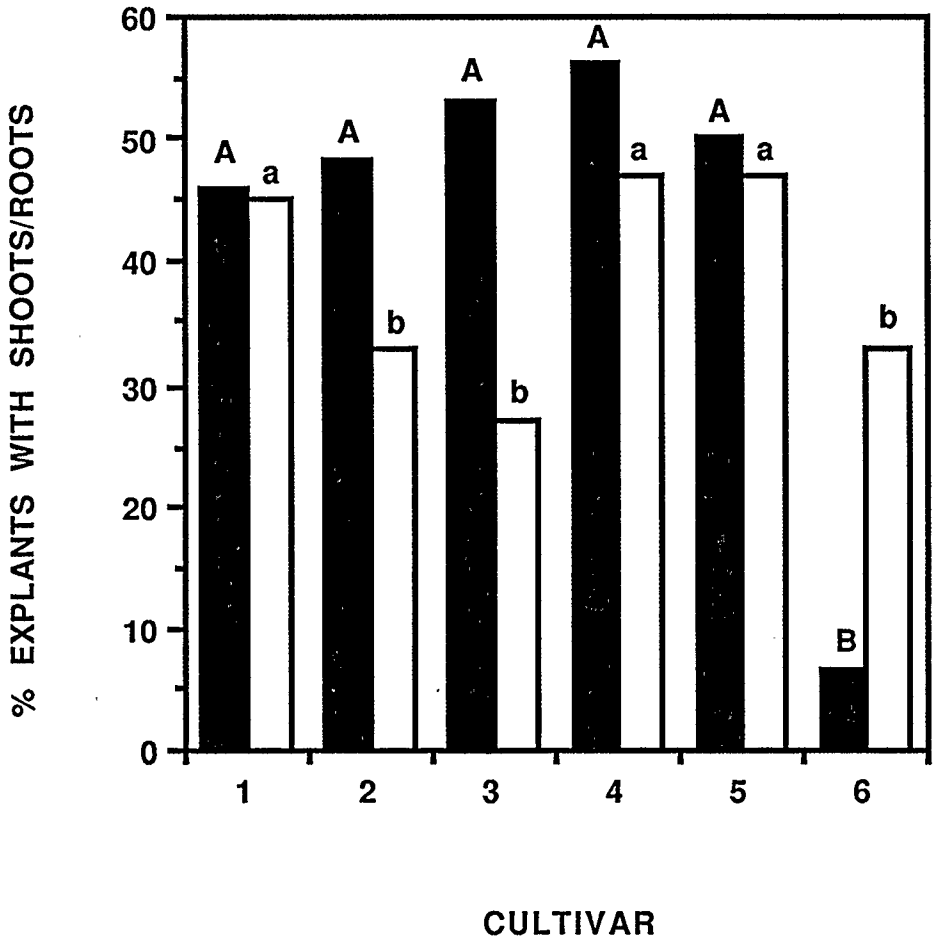
3.1.4 Genotype Specificity

To determine whether the protocol presented here is genotype specific or of general utility for the regeneration of shoots from olieferous subspecies of *B. rapa* *in vitro*, several cultivars were tested for their organogenic response (Figure 10). Of the 6 cultivars tested, 5 exhibited a shoot regeneration frequency of 40% or greater. The one recalcitrant cultivar (R-500) is a non-Canola (*i.e.* high glucosinolate) variety of *B. rapa* . Also, R-500 is an Indian Yellow Sarson cultivar (ssp. trilocularis) as distinct from most other 'Canola' quality *B. rapa* lines which are Polish types (ssp. oleifera). An experiment during which the regeneration response of R-500 was tested over a range of both BA and NAA concentrations was performed. The results of this experiment showed that R-500 is also capable of significant regeneration (up to 40%) over a range of NAA (Figure 11) and BA (Figure 12) concentrations, with an optimal response of shoot regeneration relative to root production occurring in media containing 2 mgL^{-1} BA and 0.5 mgL^{-1} NAA (Figure 12).

3.1.5 Growth of Regenerated Plants

As this regeneration system will be used for the production of transgenic *B. rapa* plants, the ability of the regenerated shoots to mature normally and set seed is an important consideration. Once regenerated shoots were placed on germination media, about 75% (41/55) displayed apical dominance and these

Figure 10. Regeneration of shoots (■) and roots (□) from cotyledon explants of different varieties of *Brassica rapa* on 2 mg/L BA and 1 mg/L NAA. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots. 1= Colt; 2= Echo; 3= Tobin; 4= Horizon; 5= Parkland; 6= R-500.


% EXPLANTS WITH SHOOTS/ROOTS
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A
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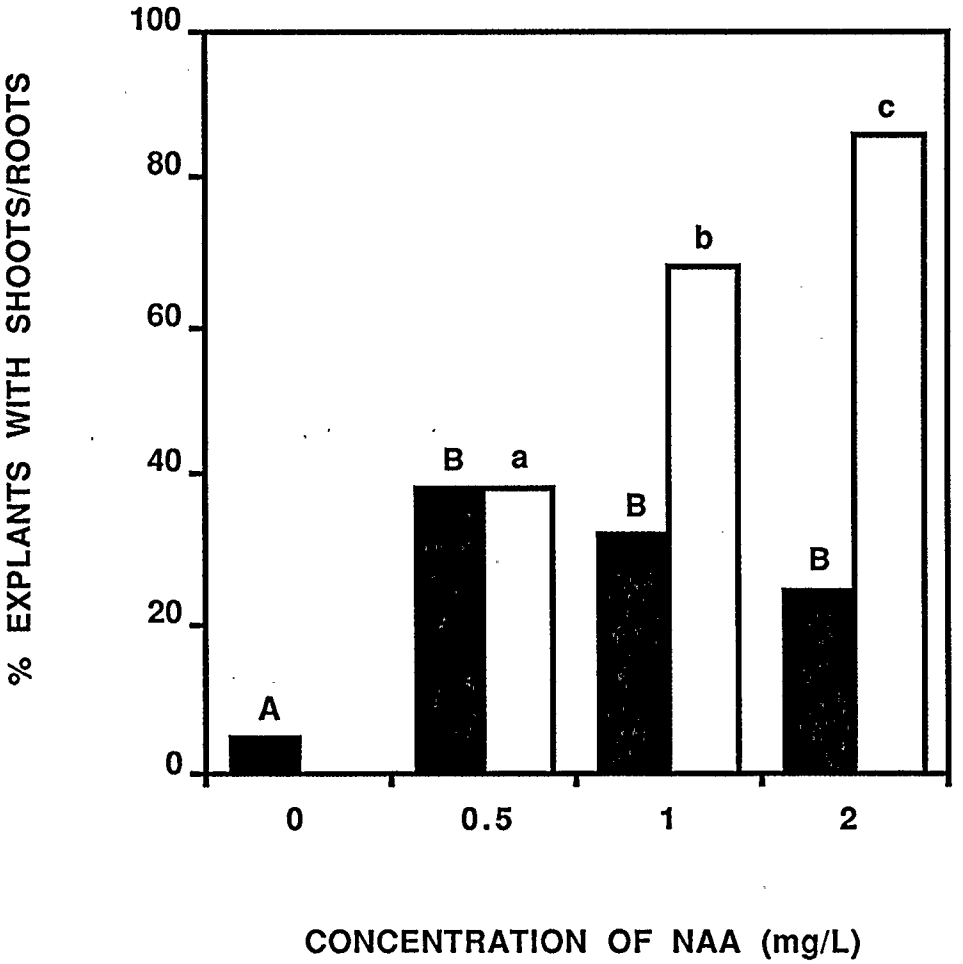
B
b
1
2
3
4
5
6
CULTIVAR

Figure 11. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. R-500 on 2 mg/L BA and various concentrations of NAA. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


100
80
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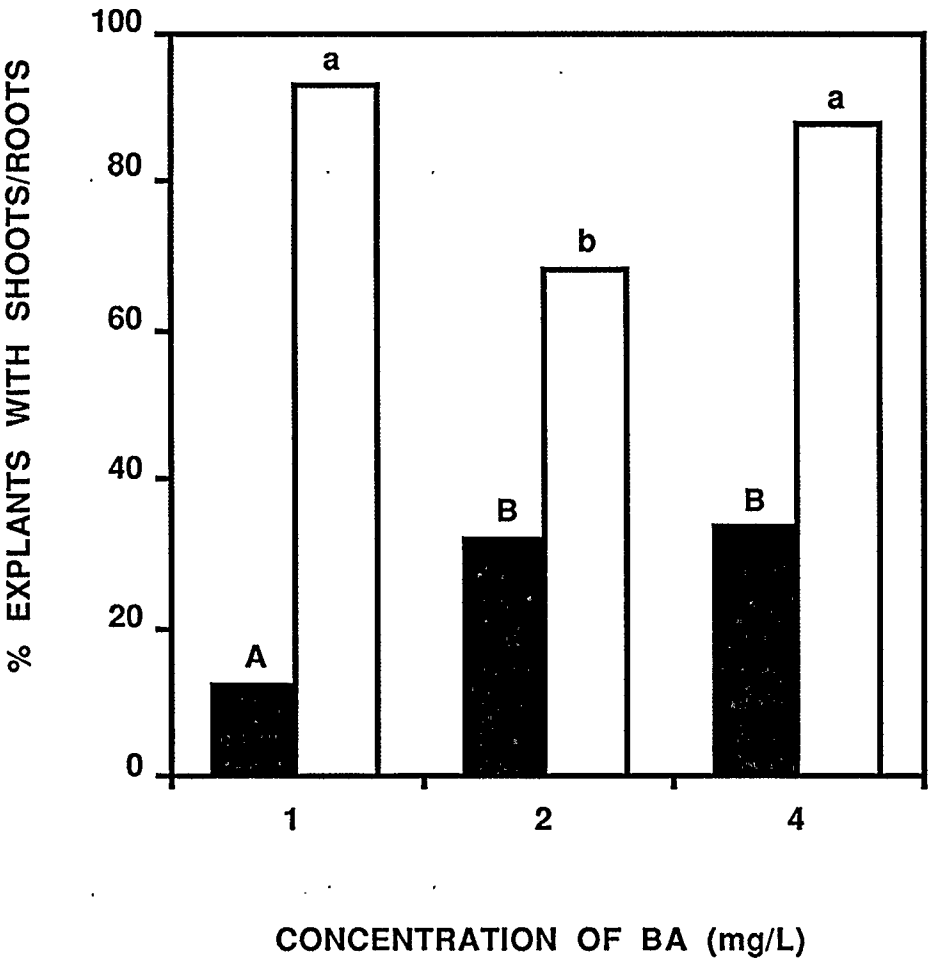
% EXPLANTS WITH SHOOTS/ROOTS
A
B
a
B
b
B
c
0
0.5
1
2
CONCENTRATION OF NAA (mg/L)

Figure 12. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. R-500 on 1 mg/L NAA and various concentrations of BA. Data for 1 mg/L NAA and 2 mg/L is from the same experiment as in Figure 11. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


100
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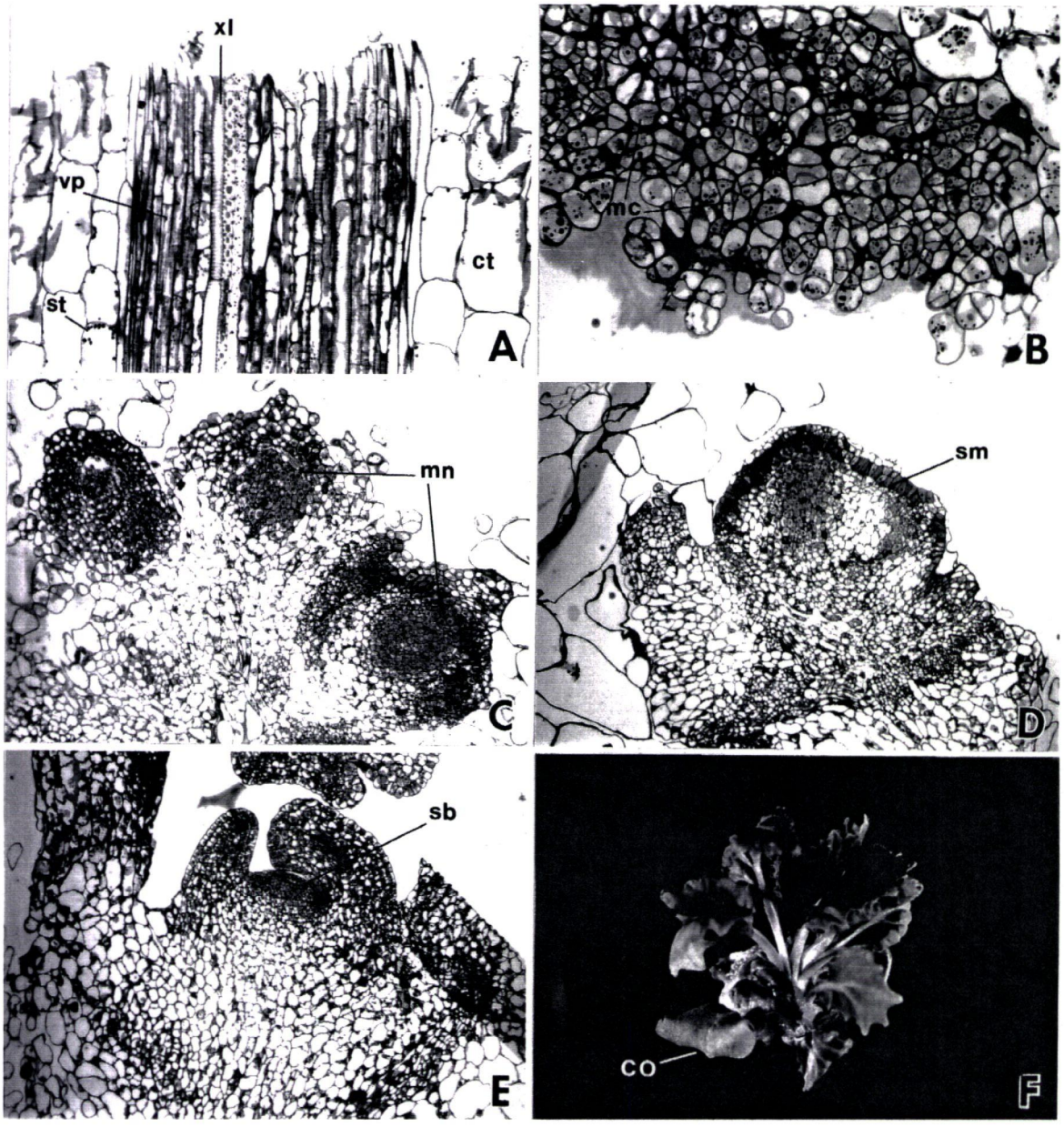
% EXPLANTS WITH SHOOTS/ROOTS
a
b
a
A
B
B
1
2
4
CONCENTRATION OF BA (mg/L)

went on to flower and mature normally (6/6 followed through to maturity). The mature plants were then induced to set seed after self-pollination through the use of a sodium chloride solution to overcome the self-incompatibility present in this species (Monteira *et al.*,1988).

3.1.6 Histogenesis of Shoot Primordia

The sequence of events that occur during shoot production from *B. rapa* cotyledonary petioles *in vitro* is shown in Figure 13. At day 0 the cortical cells of the cut end of the petioles were largely vacuolated, while the vascular parenchyma comprised highly cytoplasmic cells with a prominent nucleus. Starch grains were randomly distributed in the cortical cells (Figure 13A). By day 2 on shoot regeneration medium, random cell division activity had commenced in cells ≈ 100 µm from the cut end of the petiole (Figure 13B). By day 5, rapid cell divisions were restricted to the peripheral areas which resulted in the formation of multiple meristematic nodules (Figure 13C). These nodules gave rise to shoot bud meristems by day 7 (Figure 13D), which in turn formed leaf primordia by day 11 (Figure 13E). Shoot buds became macroscopic by day 15 and fully differentiated shoots were obtained by 21 days (Figure 13F). As can be seen in Figure 13C, each explant is capable of developing several meristematic nodules. This ultimately leads to multiple shoot proliferation from a single explant.

Figure 13. Ontogeny (A-E) and morphology (F) of shoot bud histogenesis from cotyledonary petioles of *Brassica rapa* cv. Tobin. [co-cotyledon; ct-cortex; mc-meristematic cells; mn-meristematic nodule; sb-shoot bud; sm-shoot meristem; st-starch; vp-vascular parenchyma]. A. Longitudinal section of petiolar cut end at day 0 showing the presence of highly cytoplasmic cells with a prominent nucleus in the vascular bundles. Note the presence of randomly distributed starch grains in the cortical cells (x378). B. Transverse section of the petiole about 100 μm from the cut end at day 2. The cells of the vascular parenchyma are undergoing random cell divisions (x378). C. Transverse section of the petiolar cut end at day 5 showing the formation of meristematic nodules in the peripheral regions (x189). D. Transverse section of the petiolar cut end at day 7 showing the development of an apical meristem at the surface of the meristematic nodule, indicated by the presence of organized dermal layers (x189). E. Transverse section of the petiolar cut end at day 11 showing the development of a shoot meristem along with leaf primordia (x189). F. Formation of adventitious shoots from cotyledon explants after 21 days in culture. The explant was transferred from regeneration medium to hormone free medium at day 14 (x2).


xl
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3.2 *AGROBACTERIUM*-MEDIATED TRANSFORMATION

3.2.1 Antibiotic Selection

In most DNA-mediated genetic transformation systems the proportion of cells that stably incorporate exogenous DNA is very small. For instance, the frequency at which *Brassica rapa* protoplasts are transformed by *Agrobacterium tumefaciens* under optimal conditions was found to be approximately 1 in 10^{-3} (Ohlson and Erikson, 1988). As a result, it can be difficult to distinguish the progeny of transformed cells from the progeny of untransformed cells. One way around this problem is to include in the foreign DNA a gene which will confer a selective advantage to the transformed cells relative to the untransformed cells (*i.e.* a selectable marker gene). The selective advantage is a result of the transformed cells being able to survive the presence of a normally toxic compound because of the presence of the selectable marker gene.

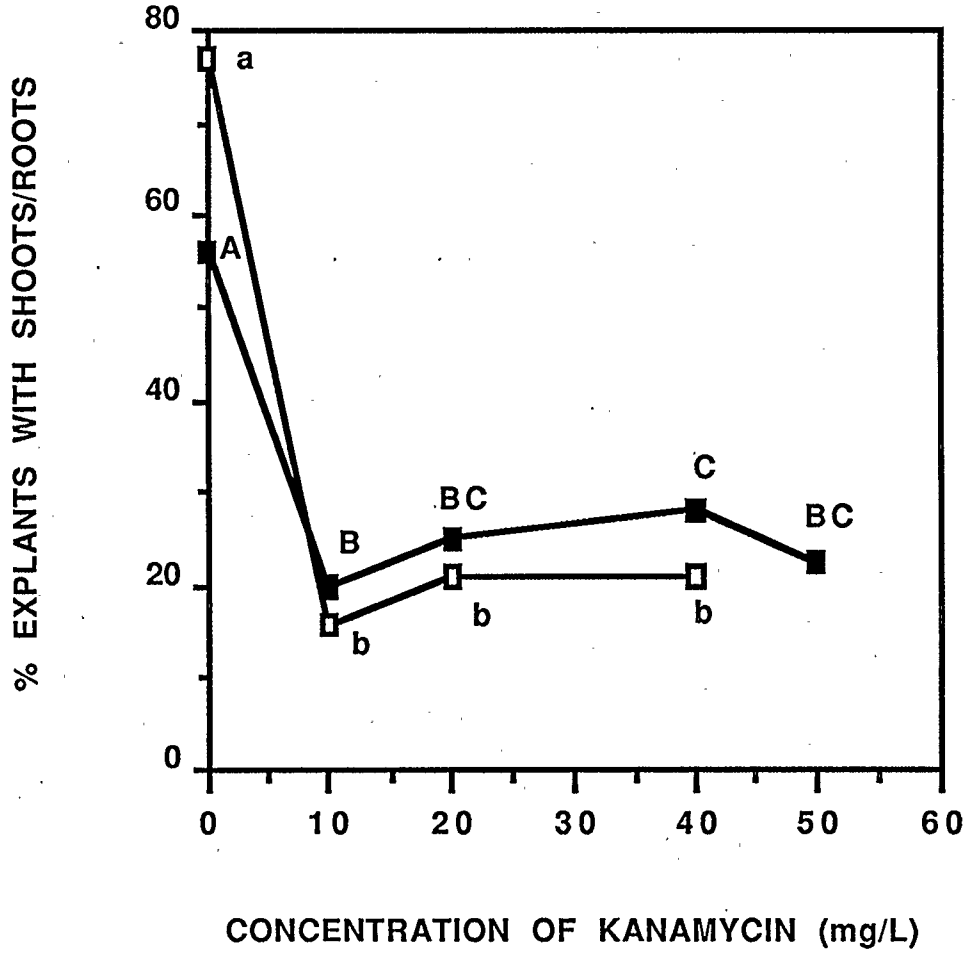
Two main classes of selectable marker genes are used in plant transformation studies. One class of gene encodes proteins that detoxify antibiotics such as kanamycin (Herrera-Estrella *et al.*, 1983B), hygromycin B (Waldron *et al.*, 1985), methotrexate (Hauptmann *et al.*, 1988), gentamycin (Hayford *et al.*, 1988), and bleomycin (Hille *et al.*, 1986). Another class of genes confer tolerance to herbicides such as phosphinotricin (DeBlock *et al.*, 1987), the sulfonylureas (Haughn *et al.*, 1988) and 2, 4-D (Streber and Willmitzer, 1989). The most common selectable marker gene in use for plant transformation studies is the gene from transposon 5 (Tn5) of *Escherichia coli* strain K12 encoding the enzyme commonly known as neomycin

phosphotransferase II (NPT II) which inactivates the antibiotics kanamycin, G418 and neomycin by phosphorylation (Flavell *et al.*, 1992). Another common selectable marker gene that is used when selection on kanamycin is inefficient (*eg.* Lloyd *et al.*, 1986) is the gene encoding the enzyme neomycin phosphotransferase IV (NPT IV) which inactivates the antibiotic hygromycin.

Prior to conducting experiments involving transformation of the plant tissue, it was necessary to ascertain what concentration of the selection agent is required for toxicity to the plant cells. Therefore, dose response experiments were carried out to ascertain the sensitivity of *Brassica rapa* cotyledonary petiole explants to the antibiotics kanamycin and hygromycin. Explants were cultured on regeneration medium for 4 days before being placed on regeneration medium supplemented with 500 mgL^{-1} carbenicillin and various concentrations of kanamycin sulfate or hygromycin B (Boehringer Mannheim). Carbenicillin was included in the selection medium to mimic conditions that would be used during transformation experiments. In transformation experiments with *Agrobacterium tumefaciens*, carbenicillin is utilized for its bactericidal activity in order to prevent bacterial growth from dominating the culture medium. Carbenicillin has been shown to be non-toxic to plant cells; a probable result of its specific action on components of the bacterial cell wall which do not exist in plant cells (Pollock *et al.*, 1983).

The production of both shoots and roots declined sharply when placed on 10 mgL^{-1} kanamycin (Figure 14). Shoot and root production did not decrease significantly from the values at 10 mgL^{-1} up to kanamycin concentrations of 50 mgL^{-1}. All expanded regenerated shoots underwent chlorosis (or bleaching)

Figure 14. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on regeneration medium supplemented with various concentrations of kanamycin. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


80
60
40
20
0
% EXPLANTS WITH SHOOTS/ROOTS
a
A
B
b
BC
b
C
b
BC
0
10
20
30
40
50
60
CONCENTRATION OF KANAMYCIN (mg/L)

on treatment with 10 mgL^{-1} kanamycin. These shoots lost all pigmentation within 10 days of the regeneration on applied selection. All subsequent selections during transformation experiments were performed at 20 mgL^{-1} kanamycin.

Hygromycin was a very potent inhibitor of shoot and root regeneration in this system (Figure 15). Shoot formation dropped dramatically at 5 mgL^{-1} hygromycin and was completely suppressed at 10 mgL^{-1}. Root formation was less sensitive to the presence of hygromycin than shoot formation but was completely suppressed on treatment with 20 mgL^{-1} of the antibiotic.

3.2.2 Transformation Experiments

Initial transformation experiments applied the successful regeneration system of *Brassica rapa* described above to the protocol of Moloney *et al.* (1989). All regenerated shoots were bleached indicating that they were not stably transformed. Standard conditions for this protocol included no preculturing of the explants, a three day co-cultivation of explants with *Agrobacterium tumefaciens* , and no use of either a feeder layer or chemical stimulators of *Agrobacterium* virulence (*eg.* acetosyringone; Stachel *et al.*, 1985). Therefore, several modifications of the procedure were tested for their effect on transformation.

Certain plant compounds that are released from cells upon wounding (*eg.* acetosyringone) have been found to induce transcription of *Agrobacterium tumefaciens* virulence genes that are essential for transformation of the plant cells (Stachel *et al.*, 1985). Also, it has been found that transformation

Figure 15. Regeneration of shoots (■) and roots (□) from cotyledon explants of *Brassica rapa* cv. Tobin on regeneration medium supplemented with various concentrations of hygromycin. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.

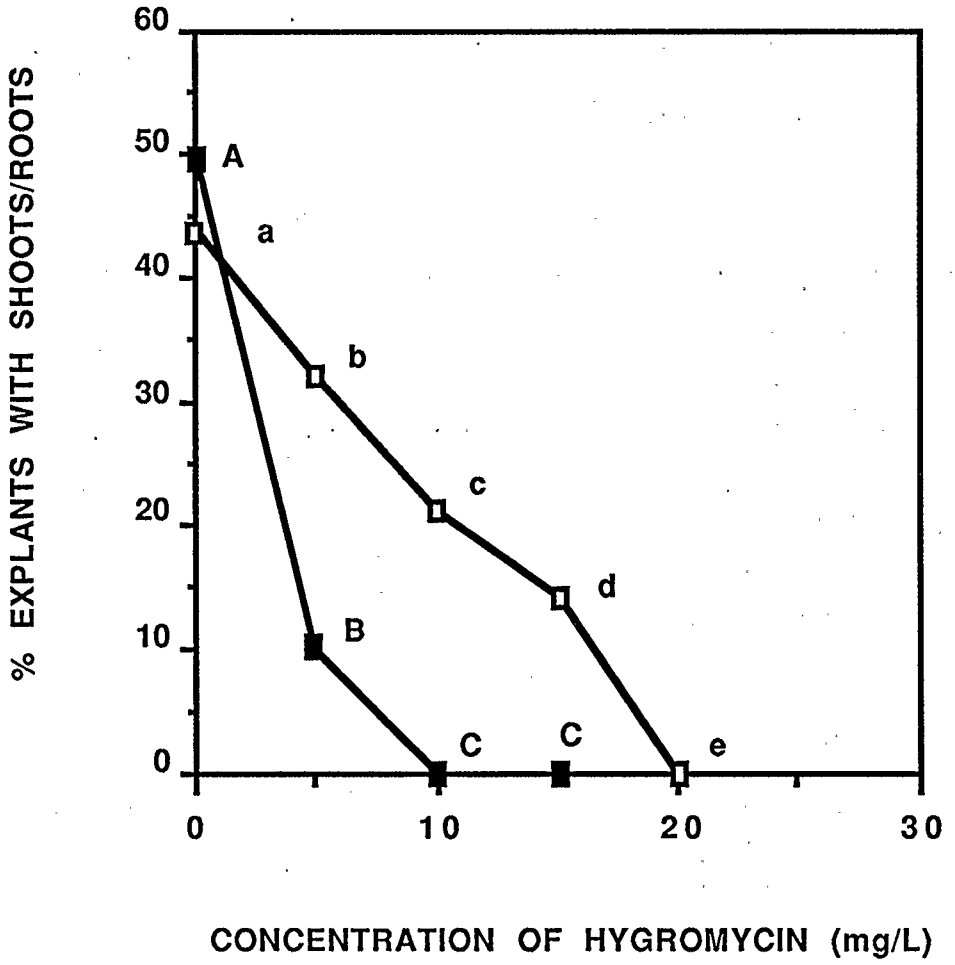
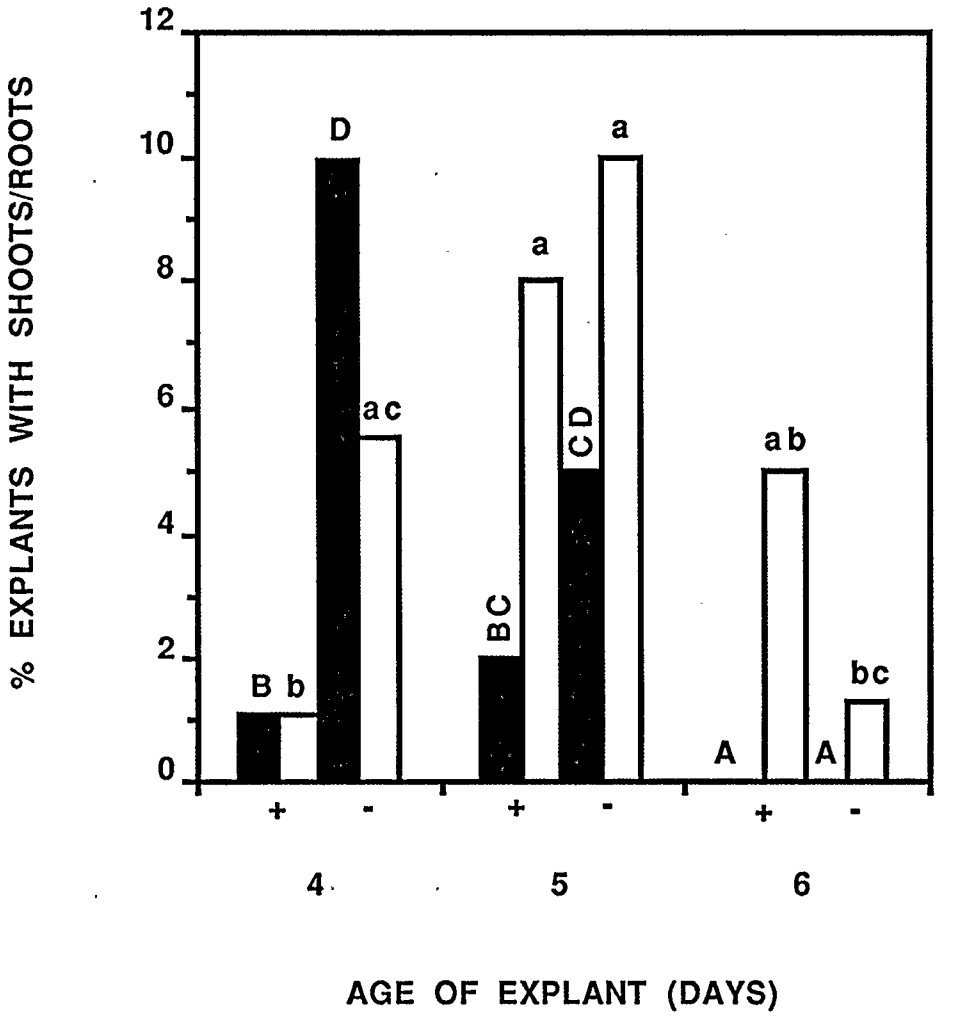

60
50
40
30
20
10
0
A
a
b
c
d
B
C
C
e
0
10
20
30
% EXPLANTS WITH SHOOTS/ROOTS
CONCENTRATION OF HYGROMYCIN (mg/L)

Figure 16. Regeneration of shoots (■) and roots (□) from 4, 5 or 6 day old cotyledon explants of *Brassica rapa* cv. Tobin treated with *Agrobacterium tumefaciens* strain EHA101 x 783 grown in the presence (+) or absence (-) of acetosyringone. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


% EXPLANTS WITH SHOOTS/ROOTS
12
10
8
6
4
2
0
B b
D
a c
BC
a
CD
a
A
a b
A
bc
+
-
+
-
+
-
4
5
6
AGE OF EXPLANT (DAYS)

efficiencies can be enhanced by the addition of acetosyringone prior to (Sheikholeslam and Weeks, 1987) or during (Owens and Smigocki, 1988) cocultivation. The use of acetosyringone (20 μM) in the *Agrobacterium* growth medium did not result in the production of transgenic shoots and actually caused a reduction in shoot production in most cases (Figure 16). This was often the result of a high incidence of bacterial growback on plates containing explants that were exposed to acetosyringone-treated *Agrobacterium*. *Agrobacterium* grown under inducing conditions may have been more proliferative resulting in a higher inoculum used for transformation. As a consequence, it would be more likely that a low frequency spontaneous mutation conferring resistance to carbenicillin could appear. This phenomenon has been observed before when *Agrobacterium* was used in concert with $AgNO_3$ (Boulter *et al.*, 1990). Perhaps a different bacteriostatic used alone or in combination with carbenicillin could overcome this problem. Figure 16 also suggests that 4-day-old explants regenerate better than 5- or 6-day old explants under transformation conditions.

In order to test whether lower concentrations of acetosyringone would reduce the growback problem while still having an effect on transformation efficiency, a range of acetosyringone concentrations were added to the *Agrobacterium* growth medium (Figure 17). An AS concentration of 5 μM had much less of a detrimental effect on regeneration than did higher concentrations. Treatments containing 10 μM or 20 μM AS in the *Agrobacterium* growth medium again resulted in much bacterial growback and a consequent reduction in regeneration. In this experiment, some shoots remained unbleached after at least 3 weeks on selection medium and were

Figure 17. Regeneration of shoots (■), green shoots (▨) and roots (□) from 4 day old cotyledon explants (with 1 day of preculture) of *Brassica rapa* cv. Tobin treated with *Agrobacterium tumefaciens* strain MP90 x ITG-15 grown in the presence of 0, 5, 10 or 20 µM acetosyringone. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots, upper case outlined letters compare antibiotic resistant shoots.

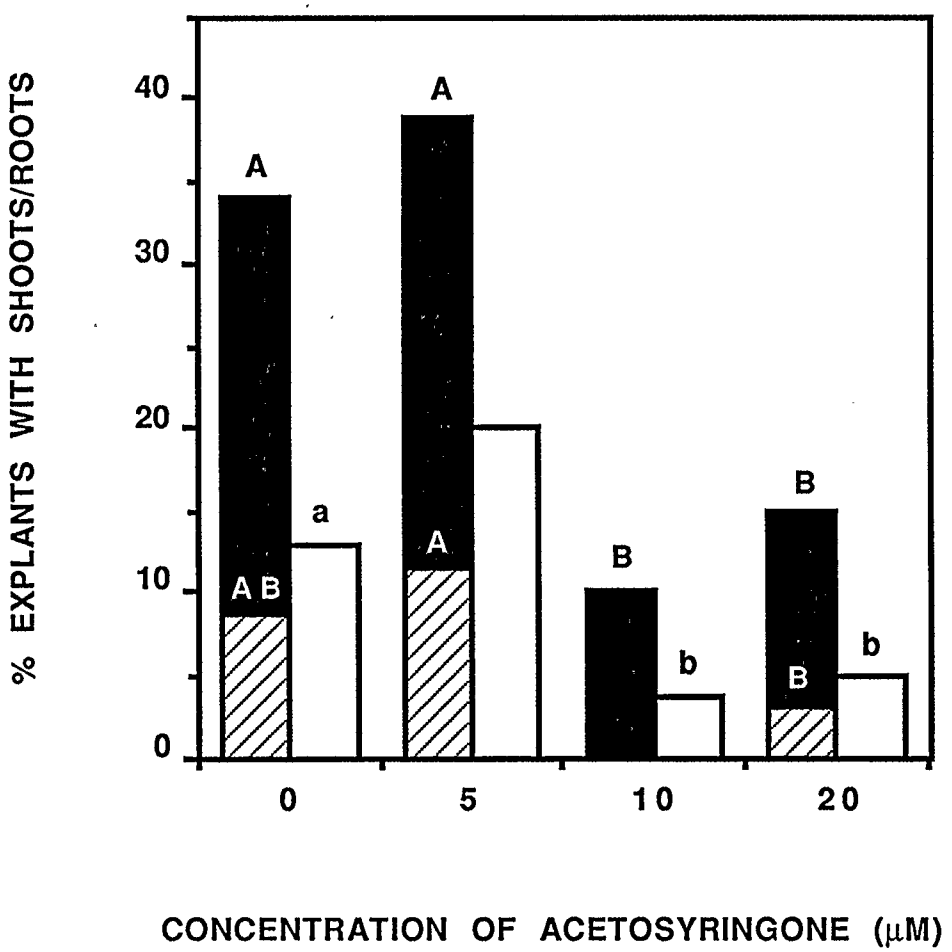
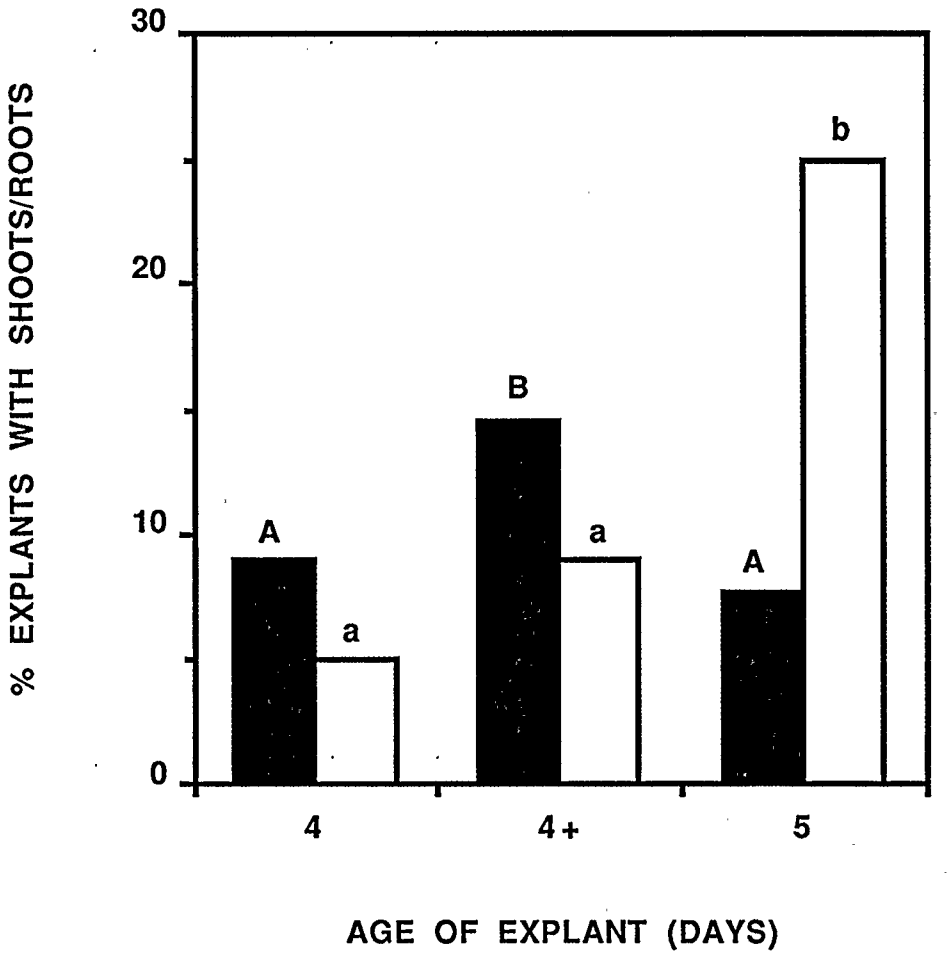

% EXPLANTS WITH SHOOTS/ROOTS
40
30
20
10
0
A
A B
a
A
A
B
b
B
B
b
0
5
10
20
CONCENTRATION OF ACETOSYRINGONE (μM)

Figure 18. Regeneration of shoots (■) and roots (□) from 4 or 5 day old cotyledon explants of *Brassica rapa* cv. Tobin treated with *Agrobacterium tumefaciens* strain EHA101 x 783. '+'= 1 day preculture. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare roots.


30
20
10
0
% EXPLANTS WITH SHOOTS/ROOTS
A
a
B
a
A
b
4
4+
5
AGE OF EXPLANT (DAYS)

retained for further analysis to determine whether they were transformed.

The effect of preculturing explants before exposure to *Agrobacterium* on the regeneration of shoots is shown in Figure 18. Precultured 4-day-old explants treated with EHA101 x 783 produced more shoots than either non-precultured 4-day-old explants or non-precultured 5-day-old explants that had undergone the same treatment. This indicates that a one day preculture period in the transformation protocol decreases the detrimental effects of *Agrobacterium* exposure on the ability of the explants to produce shoots. However, because meristematic nodules arise quickly with these explants (Figure 13), preculturing could deprive *Agrobacterium* of access to potentially regenerable cells. Also, as was seen in the regeneration experiments, the ability of the explants to produce shoots declines with the age of the explant.

To test whether longer periods of preculture would have a beneficial effect on shoot regeneration and/or transformation, explants were treated to 1, 2 or 3 days of preculture on regeneration medium before being exposed to *Agrobacterium* (Figure 19). In this case, regeneration was highest after 2 days of preculture. However, the proportion of antibiotic-resistant tissue was greatest after only one day of preculture indicating that a preculture longer than one day was detrimental to transformation of cells (the correlation between antibiotic resistance and transformation is discussed below).

Figure 19. Regeneration of shoots (■) and persistence of antibiotic resistant calli (▤) from 4 day old cotyledon explants of *Brassica rapa* treated with *Agrobacterium tumefaciens* after 1, 2 or 3 days of preculturing. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare calli.

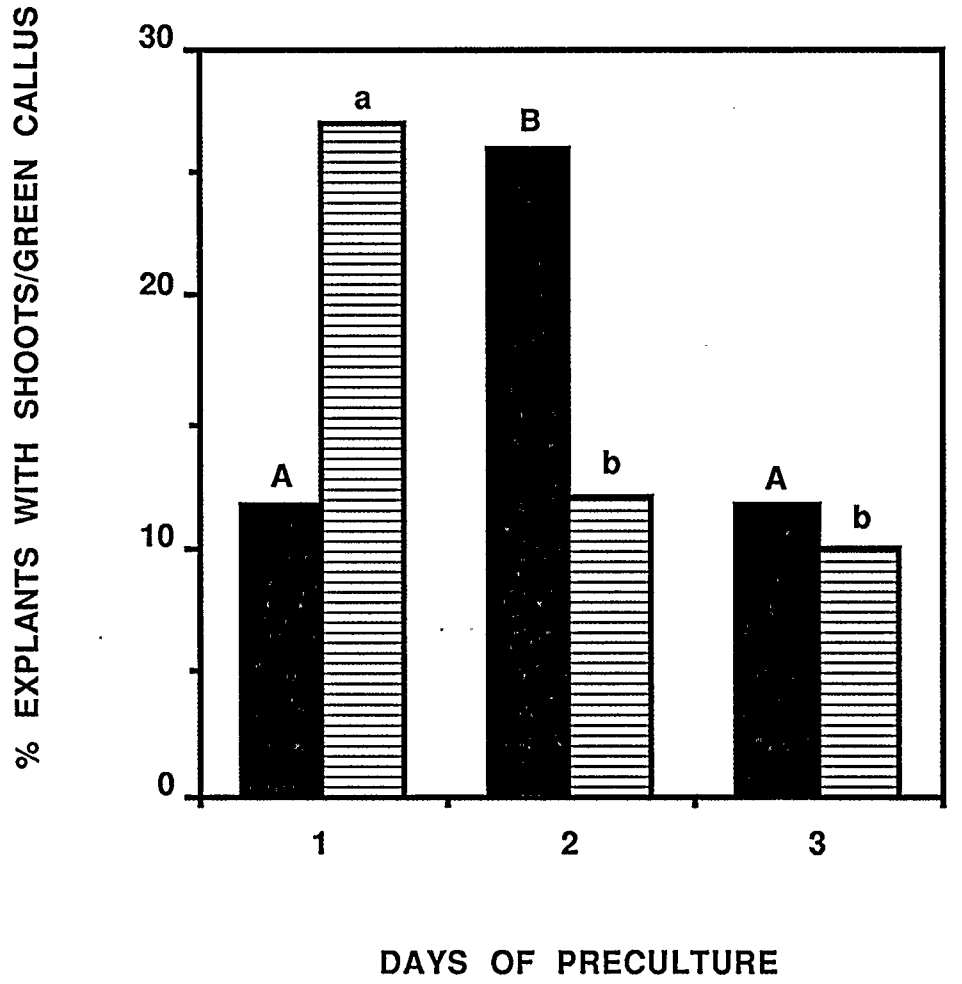
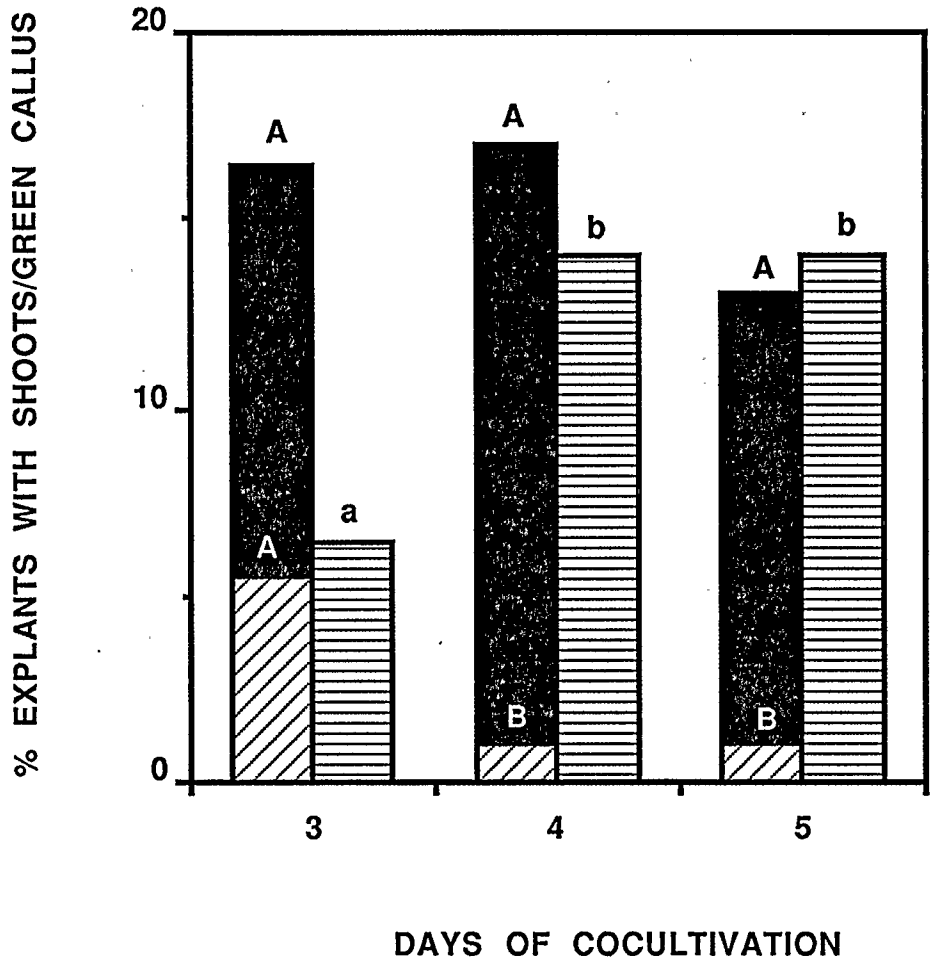

% EXPLANTS WITH SHOOTS/GREEN CALLUS
30
20
10
0
a
B
A
b
A
b
1
2
3
DAYS OF PRECULTURE

Figure 20. Regeneration of shoots (■), green shoots (▨) and persistence of antibiotic resistant calli (⊟) from 4 day old cotyledon explants of *Brassica rapa* treated with *Agrobacterium tumefaciens* strain EHA101 x 783 for 3, 4 or 5 days of cocultivation. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare calli, upper case outlined letters compare antibiotic resistant shoots.

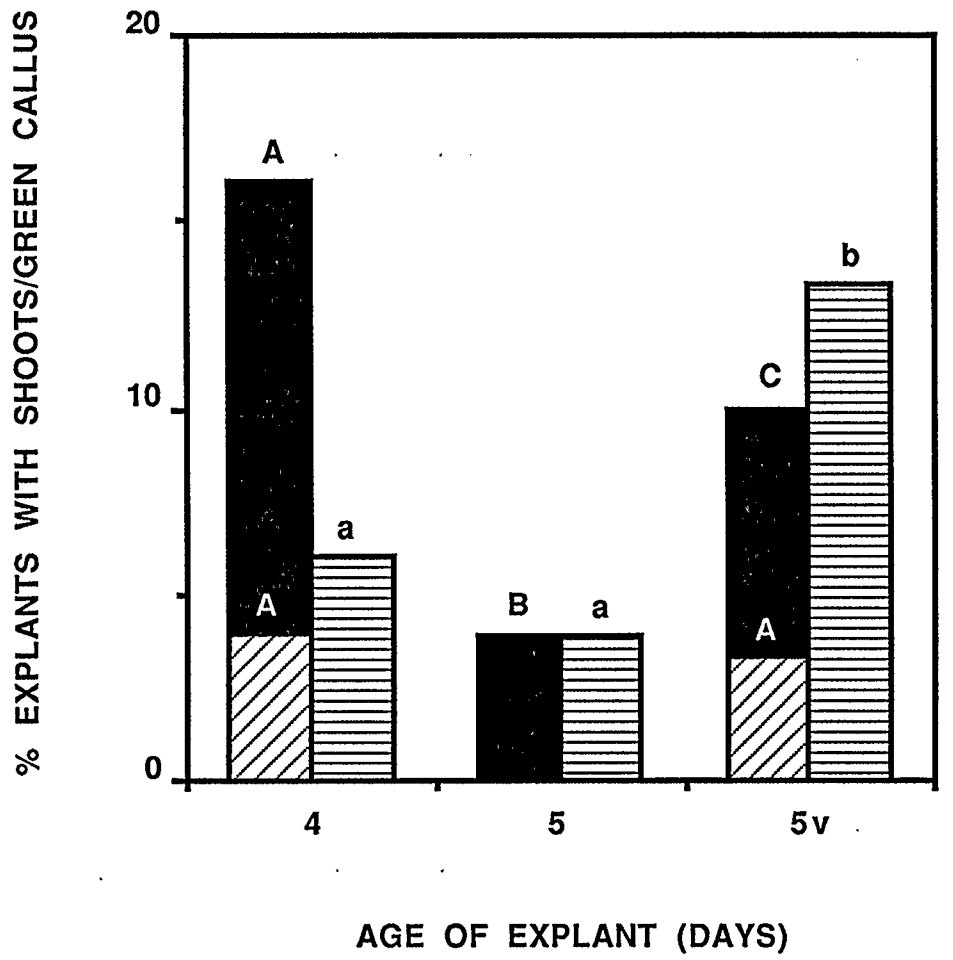

% EXPLANTS WITH SHOOTS/GREEN CALLUS
20
10
0
A
a
A
A
b
B
A
b
B
3
4
5
DAYS OF COCULTIVATION

Another factor that may affect the efficiency of transformation by *Agrobacterium* is the length of time that the explant is cocultivated with the bacteria before a bacteriostatic agent is applied. This factor was tested for its effect on regeneration and/or transformation of *Brassica rapa* (Figure 20). The duration of cocultivation did not dramatically affect regeneration from explants treated with *Agrobacterium.* However, it did have an effect on the recovery of antibiotic resistant shoots from these explants. Green shoots were more numerous from 3-day cocultivated explants than from 4- or 5-day cocultivated explants. Total production of antibiotic resistant tissue (*i.e.* the sum of shoots and callus), though, was not affected by duration of cocultivation.

If access by *Agrobacterium* to the regenerable tissue in the explant is a limiting factor in the production of transgenic shoots, then prolonging the initial exposure of the explant to the bacterium or increasing the infiltration of the tissue by the bacterium could have an effect on transformation efficiency. This was tested by submerging the cut end of the cotyledonary petiole in the *Agrobacterium* suspension for 5 minutes and performing the incubation under vacuum (Figure 21). Vacuum infiltration and subsequent co-cultivation increased the percentage of antibiotic resistant tissue and green shoots arising from explants.

In order to test whether the orientation of the marker gene within the T-DNA or the strength of the promoter driving expression of the marker gene had an effect on transformation, a series of related binary vectors (the pCGN-1500 series) were used in transformation experiments with *Brassica rapa* (Figure 22). All vectors were capable of producing green shoots. Also, there was no

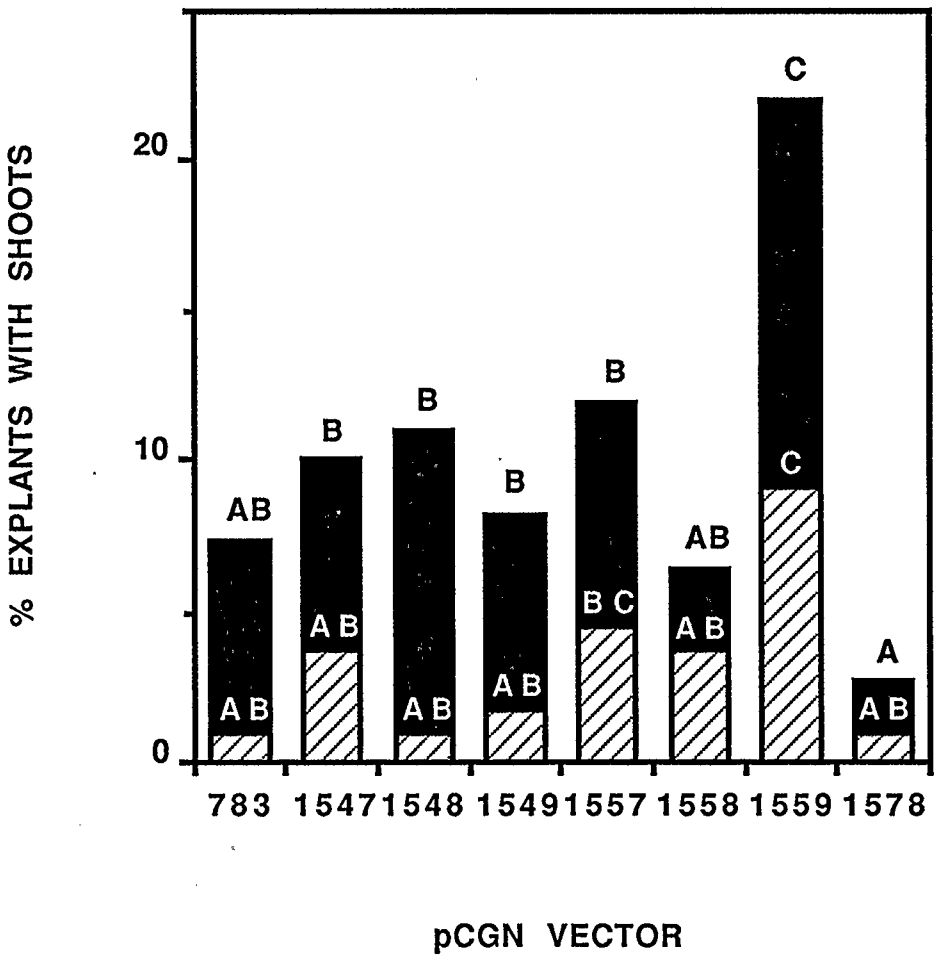
Figure 21. Regeneration of shoots (■), green shoots (▨) and persistence of antibiotic resistant calli (▤) from 4 or 5 day old cotyledon explants of *Brassica rapa* . Explants were treated with *Agrobacterium tumefaciens* strain EHA101 x Ca_2AMVGN-1548 under standard conditions (4, 5 day) or for 5 minutes under vacuum (5v). Columns with similar letters are not significantly different at P = 0.05 (Tukeys Multiple Comparison Test). Upper case letters compare shoots, lower case letters compare calli, upper case outlined letters compare antibiotic resistant shoots.


% EXPLANTS WITH SHOOTS/GREEN CALLUS
20
10
0
A
a
A
B
a
C
b
A
4
5
5v
AGE OF EXPLANT (DAYS)

significant difference between the CaMV35S promoter and MAS promoter with respect to their ability to produce green shoots (10.8 ± 2.9 S.E.vs 9.7 ± 1.2 S.E. respectively averaged over all vectors). Similar results were obtained with *B. napus* hypocotyl explants (Harpster *et al.*, 1988). While vector pCGN-1559 produced significantly more shoots than the other vectors, none of these proved to be transformed (see below). It has been shown that DNA introduced into plant cells via *Agrobacterium* is preferentially integrated into actively transcribed genomic regions (Koncz *et al.*, 1989; Herman *et al.*, 1990). It has also been shown that transcriptional readthrough actively interferes with the expression of a downstream gene in an opposite orientation in transgenic plants (Ingelbrecht *et al.*, 1991). Therefore, the orientation of the marker gene within the T-DNA could affect its expression once it is integrated into the plant genome. In addition, the 'strength' of the promoter that directs expression of the marker gene may have an effect on transformation efficiency. The CaMV 35S promoter has been shown to direct gene expression at higher levels (20-30 fold) than both the NOS promoter (Sanders *et al.*, 1987; Harpster *et al.*, 1988) and the MAS promoter (McBride and Summerfelt, 1990). This could be important if a 'position effect' on the integrated DNA is strong.

This effect, whereby the expression of foreign genes is influenced by their chromosomal location (which has been demonstrated in animal cells; Al-Shawi *et al.*, 1990) might be related to chromatin structure and/or to the location of endogenous promoters/enhancers close to the integration site (see above). The position effect has been invoked as one possible reason to explain the clonal variation (up to 200 fold) in the level of foreign gene expression usually observed between individual transformants (Weising *et al.*, 1988). If the effect is

Figure 22. Regeneration of shoots (■) and green shoots (▨) from 4 day old cotyledon explants (with 1 day preculture) of *Brassica rapa* treated with a variety of pCGN vectors. Columns with similar letters are not significantly different at $P = 0.05$ (Tukeys Multiple Comparison Test). Upper case letters compare shoots, upper case outlined letters compare antibiotic resistant shoots.


% EXPLANTS WITH SHOOTS
20
10
0
AB
B
B
B
B
AB
C
A
A B
A B
A B
A B
B C
A B
C
A B
783
1547
1548
1549
1557
1558
1559
1578
pCGN VECTOR

analogous to that seen in animal cells, then weaker promoter elements will be more susceptible to position effects than stronger promoters (Walden and Schell, 1990).

3.3 TESTING OF PUTATIVELY TRANSFORMED TISSUE

All shoots surviving selection for at least one month were tested for the presence of NPT II activity. All shoots tested were negative in this test. This could indicate that either the shoots were not transformed, the level of NPT II expression was below the detection level of this test, that chimaeric transformants had been produced, or that the shoots were transformed but the transferred genes had become inactive. A more sensitive test for NPT II activity is the leaf disc assay (De Block *et al.*, 1989). By placing leaf explants from putatively transformed tissue onto media that induces callusing (in this case, MSBN) supplemented with 50 mgL^{-1} kanamycin, even low levels of NPT II activity allow transformed tissue to survive. Leaf explants from three randomly chosen regenerants that had survived selection were tested for their callusing ability on selection medium. No callusing from these explants from any of the three plants was observed and all tissue turned yellow.

Another possible explanation for the presence of antibiotic resistant, NPT II negative shoots is that the NPT II gene is present but has stopped being expressed in the shoots surviving selection. This has been shown to occur with the nopaline synthase gene in the case of *Brassica napus* transformation (Fry *et al.*, 1987). In order to test this hypothesis, PCR was performed. The reaction amplified a band of appropriate size from DNA of many of the putatively

Figure 23. Separation of PCR products following amplification. PCR conditions are as in Materials and Methods. Lanes 1-7, 11, 12 and 14 contain PCR products from individual putatively transformed plants of *Brassica rapa* . Lane 8 contains PCR product from 1 ng of pCGN50 (positive control). Lane 9 contains 1 ng of pCGN50. Lane 10 contains PCR product from untransformed *Brassica napus* (negative control). Lane 13 contains PCR product from *B. napus* callus known to have been transformed with the oncogenic *Agrobacterium* strain A281 x 200. The expected PCR product (730 bp) is indicated with an arrow.

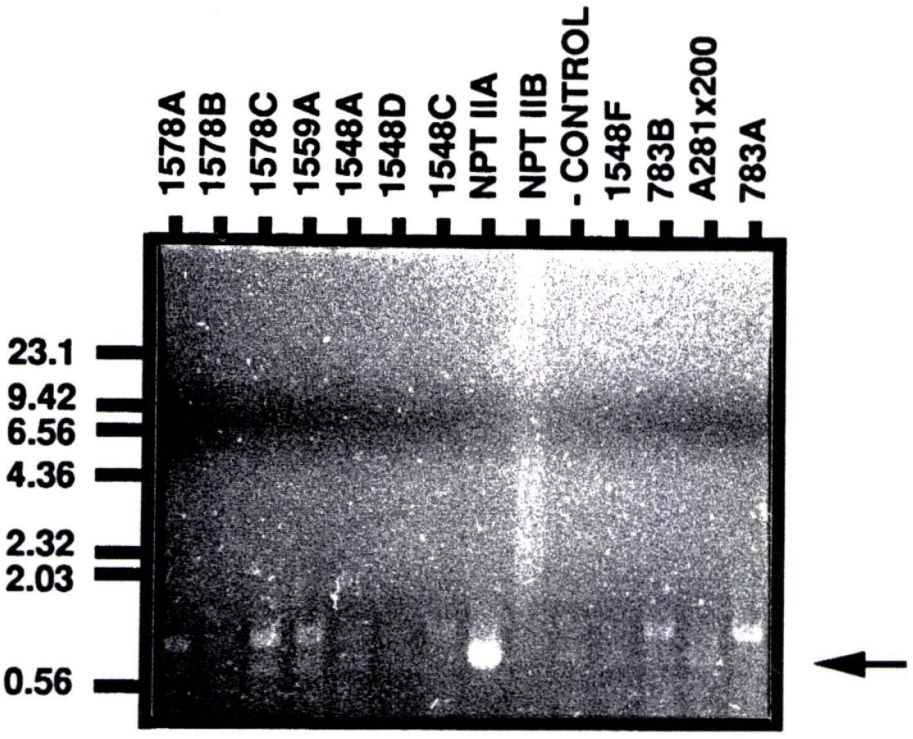
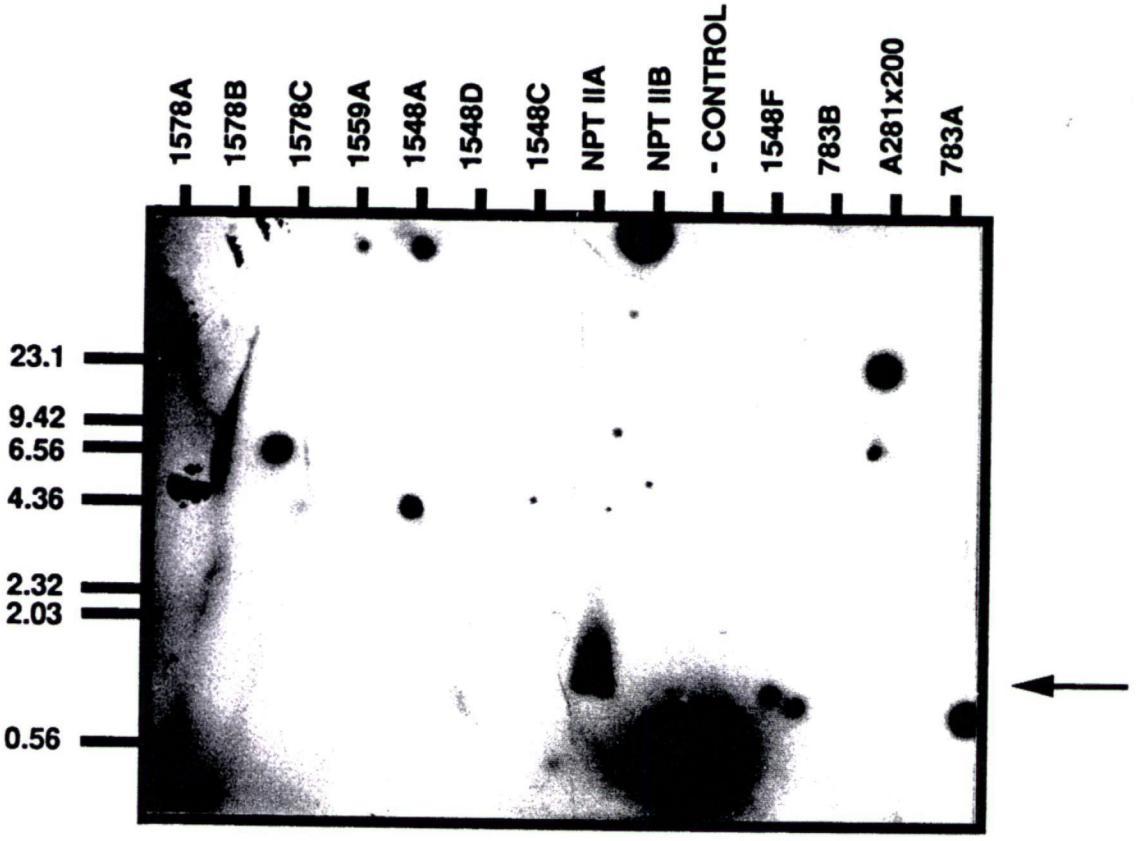

1578A
1578B
1578C
1559A
1548A
1548D
1548C
NPT IIA
NPT IIB
- CONTROL
1548F
783B
A281x200
783A
23.1
9.42
6.56
4.36
2.32
2.03
0.56

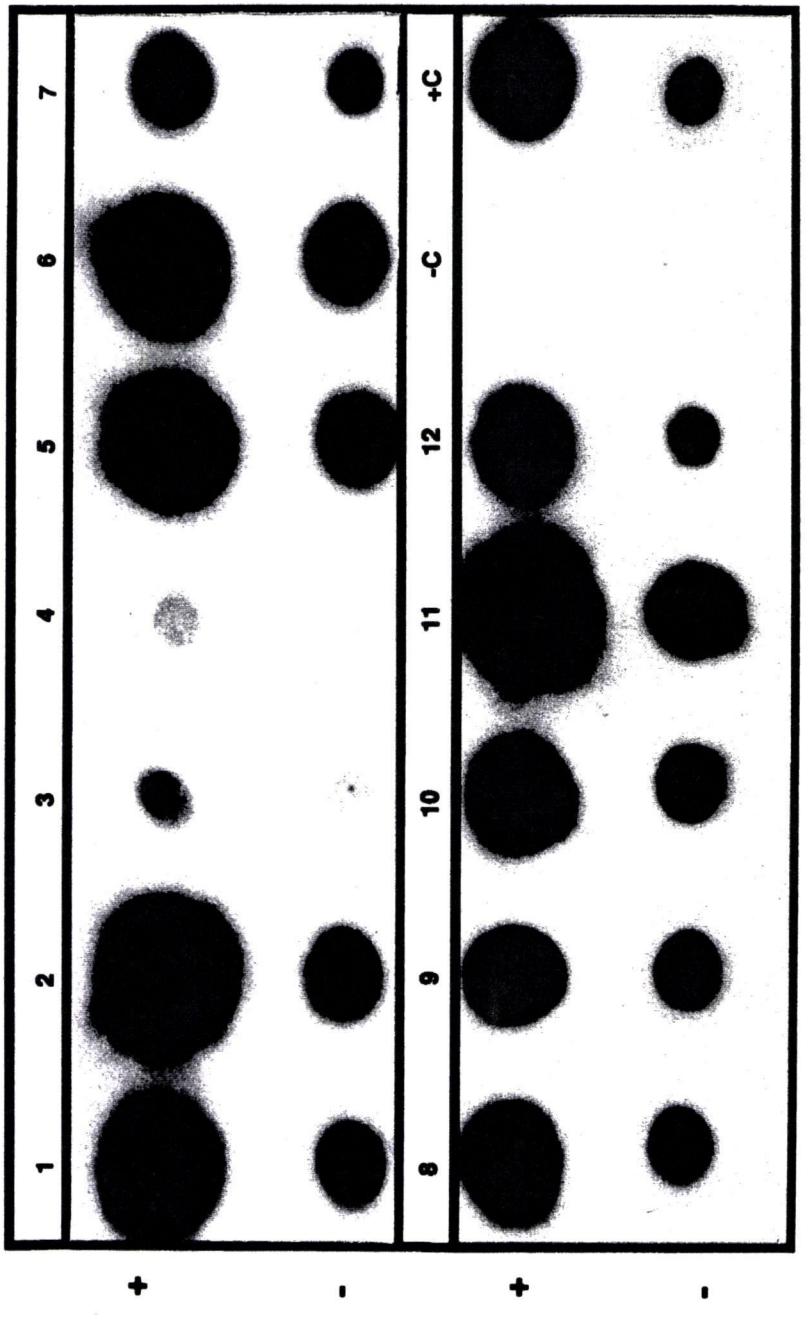
Figure 24. Southern blot analysis of separated PCR products. Lanes 1-7, 11, 12 and 14 contain PCR products from individual putatively transformed plants of *Brassica rapa* . Lane 8 contains PCR product from 1 ng of pCGN50 (positive control). Lane 9 contains 1 ng of pCGN50. Lane 10 contains PCR product from untransformed *Brassica napus* (negative control). Lane 13 contains PCR product from *B. napus* callus known to have been transformed with the oncogenic *Agrobacterium* strain A281 x 200. The expected PCR product (730 bp) is indicated with an arrow


1578A
1578B
1578C
1559A
1548A
1548D
1548C
NPT IIA
NPT IIB
- CONTROL
1548F
783B
A281x200
783A
23.1
9.42
6.56
4.36
2.32
2.03
0.56

transformed plants. However, since other bands were also present (Figure 23), the PCR amplifications were subjected to a Southern blot in order to confirm the identity of the bands. This test indicated that the amplified bands did not represent the NPT II gene (Figure 24). Therefore, this test was inconclusive for determining the presence/absence of the transgene. Based on previous results though, (Fry *et al.*, 1987; McCormick *et al.*, 1986), one would not expect all transformed regenerants to have had their transgene's expression turned off.

There are two possible explanations for the appearance of apparently kanamycin-resistant shoots from explants treated with *Agrobacterium* . Either these shoots represent 'escapes' in that they are resistant to the level of antibiotic used in these experiments without being transformed or they are able to survive selection because the antibiotic is being detoxified in the vicinity through the presence of transformed callus. As no green shoots were observed when regeneration occured from explants under selection that had not been exposed to *Agrobacterium* , it seemed the latter possibility was more likely. To test this possibility, calli originating from the cut petiolar end of the explant that remained green for at least one month under selective conditions were assayed for the presence of NPT II activity. Twelve randomly selected calli were tested (Figure 25). All the calli except two gave a strong positive signal and those two gave a weak positive signal for NPT II activity. That the plant tissue tested represented axenic material was demonstrated by a lack of bacterial growth upon placing the calli on plates containing a rich bacterial growth medium (LB).

Figure 25. NPT II dot blot assay of tissue extracts of *Brassica rapa* . *Row A* (+) represents reactions that contained kanamycin while *Row B* (-) represents reactions that did not contain kanamycin. Lanes 1-12 contain extracts from kanamycin resistant calli that had been treated with *Agrobacterium tumefaciens.* Lanes 1,2,6,9,10 and 12 were treated with strain EHA101 x pCa_2AMVGN-1548. The remainder were treated with EHA101 x pCGN-783. Lane 13 (-C) contains untransformed *Brassica napus* leaf tissue extract . Lane 14 (+C) contains extract from transformed *B. napus* crown gall tissue containing a kanamycin - resistance gene.


1
2
3
4
5
6
7
8
9
10
11
12
-C
+C
+
-
+
-

CHAPTER 4 :

DISCUSSION

4.1 REGENERATION

B. rapa has consistently proven to be one of the most recalcitrant of the *Brassica* species with respect to shoot regeneration *in vitro* (Dietert *et al.*, 1982; Dunwell, 1981; Glimelius, 1984; Jain *et al.*, 1988; Murata and Orton, 1987; Narasimhulu and Chopra, 1988). Amongst various explants tested, shoot bud regeneration from cotyledons appears to have been the most effective to date. Narasimhulu and Chopra (1988) found an optimal regeneration from one subspecies of *B. rapa* tested (ssp. *japonica* 32%) using similar media and hormone combinations as were used here. Also, Jain *et al.* (1988) found auxin in the media to be essential for regeneration of shoots from cotyledon explants of *B. rapa* (up to 18%). They noted, however, that BA was an ineffective cytokinin in this regard. The differences in regeneration reported in these two studies could be due to variations in culture conditions, preparation of the explant, or the genotype.

These results and those presented here are indicative of the need for exogenous auxins in the regeneration medium for *B. rapa*. This does not appear to be a requirement for shoot regeneration in some of the less recalcitrant members of the genus such as *B. napus* (Moloney *et al.*, 1989), *B. juncea* (Sharma *et al.*, 1990) and *B. carinata* (Jaiswal *et al.*, 1987). It was surprising to find that increasing the auxin concentrations in the media caused a reduction in the frequency of root formation while increasing the frequency of shoot formation (Figure 2). This is in contrast to what one might have expected based on the classical findings of Skoog and Miller (1957) on organogenesis in tobacco. However, as has been pointed out before (Thorpe, 1980), it is the

endogenous auxin-cytokinin balance which is important for the initiation of organogenesis.

The previous use of cotyledonary explants from *B. rapa* resulted in callus formation and shoot regeneration from all parts of the explant including non - wounded surfaces (Chi and Pua, 1989). In the material described here the regeneration occurs only at the cut end of the explant (*i.e.* at the base of the petiole; see Figure 13). The regeneration occurs with a minimum of callus formation and is very rapid (2 - 3 weeks). It is clear that the method described here may be useful for a wide variety of genotypes, although slight modifications may be necessary to optimize it for certain genotypes (*eg.* R-500). Generally, *B. rapa* regeneration has displayed a high degree of genotype specificity (Jain *et al.*, 1988), which limits the number of varieties that can be routinely manipulated.

Previous work with *B. napus* , (De Block *et al.*, 1989) *B. oleracea* (Sethi *et al.*, 1990; Williams *et al.*, 1990), *B. juncea* (Chi *et al.*, 1990) and *B. rapa* ssp. *chinensis* (Chi and Pua, 1989; Chi *et al.*, 1991) demonstrated a significant improvement in regeneration rates in a variety of explant types with inhibitors of ethylene action. In our system, the use of Ag^+ ions appeared to reduce overall yield of shoots, to result in the formation of shoots with an aberrant morphology and to increase callus production at the cut end of the explant. As no dose response experiments were done on the effect of different $AgNO_3$ concentrations on regeneration, the amount used here may have been supraoptimal. In fact, the $AgNO_3$ concentration used here was higher than that

used in most studies involving *Brassica* species. However, the use of $AgNO_3$ has also been found to inhibit shoot formation (*eg. B. oleracea* : De Block *et al.*, 1989) and in other systems ethylene has been shown to be essential for organogenesis (Kumar *et al.*, 1987; Van Aartrijk *et al.*, 1985; Cornejo-Martin *et al.*, 1979).

It was observed that the intensity of light under which explants are incubated has an effect on the regeneration frequency. Another photo-regime that has been shown to affect regeneration is the use of continuous illumination for the incubation of explants. Relative to a 16 hour photoperiod, this regime resulted in enhanced regeneration and the absence of premature flowering amongst regenerated plants (Jain *et al.*, 1988; Radke *et al.*, 1988). Premature flowering was often encountered with the *B. rapa* regenerants and was routinely discouraged by manual pruning.

As was observed previously in *B. juncea* (Sharma and Bhojwani, 1990), multiple shoot differentiation in *B. rapa* was also preceded by the formation of multiple meristematic nodules. These nodules, which formed within 2 days of culture, originated from the meristematic activity of vascular parenchyma cells within 100 μm of the cut end of the petiole. Such proximity to the cut surface would favour easy access of *Agrobacterium* to the meristematic cells during the initial, co-cultivation stage of a transformation protocol. Therefore, the system described here should lend itself readily to the transformation procedure of Moloney *et al.* (1989) which also uses cotyledonary petioles as the explant.

The cotyledonary petiole regeneration system described here has been shown to be efficient with other species of the *Brassica* genus (see above) and

has been shown to be susceptible to *Agrobacterium* mediated transformation. Therefore, this system holds promise as a generally applicable regeneration system for *Brassica* that can also be used to genetically engineer the genus. This would allow rapid dissemination of valuable agronomic traits through transformation into the many important agricultural crops included in this genus.

In addition, this regeneration system could be useful for elucidating the molecular basis of differentiation. It has been found by classical genetics and linkage analysis studies that the number of genes controlling regeneration is rather limited (Peng and Hodges, 1989; Willman *et al.*, 1989; Koorneef *et al.*, 1987) and that both nuclear and cytoplasmic genes are involved. The isolation and characterization of these genes could yield insights into important steps in plant cell development both *in vitro* and *in vivo* (Jacobsen, 1991). A regeneration system that will not regenerate under conditions where a single factor is changed (*eg.* a phytohormone) provides the opportunity to characterize these genes at the molecular level through differential screening of cDNA libraries. This approach would allow the correlation of specific and differential behaviors at the biochemical and molecular level with a morphogenic response. Insights gained in the process could change plant tissue and cell culture to a more predictive and less empirical science (Jacobsen, 1991).

4.2 TRANSFORMATION

The present study was unsuccessful in obtaining transformed, regenerated shoots from cotyledonary explants of *B. rapa*. Transformed tissue was obtained

and regeneration of shoots after cocultivation was observed. However, transformation and regeneration remain as two distinct events from this tissue and further work is required in order to integrate these events for the production of transgenic plants. A similar situation has been observed with dry bean (*Phaseolus vulgaris* ; McClean *et al.*, 1991), grapevine (*Vitus vinifera* ; Colby *et al.*, 1991), primary explants of sugarbeet (*Beta vulgaris* ; D'Halluin *et al.*, 1992) and with many monocot species (Potrykus, 1990).

Nevertheless, the production of transformed callus can be used as a system to determine factors influencing transformation (Lulsdorf *et al.* , 1991). Since it was shown that antibiotic resistant callus represented transformed cells (Figure 25) and that green regenerated shoots were always accompanied by antibiotic resistant callus, the amounts of these tissues produced during the different treatments can serve as an indicator of the relative effectiveness of each treatment in transforming *B. rapa* cotyledonary petiole cells.

The use of acetosyringone-induced *Agrobacterium* did not result in enhanced transformation frequencies (Figures 16 and 17). This was also observed with transformation of *B. napus* cotyledonary petioles (Moloney *et al.*, 1989). Owens and Smigocki (1988) observed that acetosyringone (AS) was ineffective in enhancing transformation frequencies of soybean cotyledon explants when used on the highly virulent L,L-succinamopine *Agrobacterium* strain A281 (which is the oncogenic equivalent of the strain used here, EHA101; Hood *et al.*, 1986). This was ascribed to the fact that several *vir* genes in strain

A281 have been shown to be expressed at much higher levels in response to AS than were the same genes in an octopine strain of *Agrobacterium* (Jin *et al.*, 1987). Therefore, induction of virulence in A281 type strains is very efficient and the amounts of *vir*-inducing compounds produced by the wound surface of the explant could be sufficient for maximal induction of these strains. This may also be the case for nopaline strains (Figure 17). In fact, the use of AS has been shown to reduce transformation frequencies in some cases (Godwin *et al.*, 1991).

As was observed for regeneration alone, regeneration under transformation conditions decreased with increased explant age (Figure 16). However, regeneration could be increased when the explant was cultured for 1 day before being exposed to *Agrobacterium* (Figure 18) and increased even further upon 2 days of preculture (Figure 19). As the further increase in regeneration encountered with 2 days of preculture was accompanied by a reduction in transformed callus production, a 1 day preculture was deemed optimal. A reduction in transformation frequency caused by preculturing explants before exposure to *Agrobacterium* has also been observed with pea callus (Lulsdorf *et al.*, 1991). However, some systems require longer precultures for efficient transformation (*eg.* flax (*Linum usitatissimum*) requires 9-12 days of preculture for efficient transformation, McHughen *et al.*, 1989).

The length of time that an explant is cocultivated with *Agrobacterium* has been shown to affect the efficiency of transformation. For *B. napus* hypocotyl explants the frequency of shoot regeneration decreased slightly with a cocultivation regime of 48 or 72 hours as compared to a 24 hour regime but

transformation efficiency was greatest with a 48 hour regime (Radke *et al.*, 1988). A 2 day cocultivation period was also found to be optimal for transformation of *B. napus* thin cell layer explants (Charest *et al.*, 1988). However, longer cocultivation times have resulted in increased transformation frequencies in other species (*eg.* carnation (*Dianthus caryophyllus* L.), Lu *et al.*, 1991; a highly embryogenic genotype of alfalfa (*Medicago varia*), Chabaud *et al.*, 1988; and *Kalanchoe laciniata* , Jia *et al.*, 1989).

Vacuum infiltration of explants resulted in a significant increase in shoot production, green shoot production and NPT II positive callus relative to the non-infiltrated control (Figure 21). This indicates that infiltration may increase access to wounded cells by *Agrobacterium* , resulting in a higher transformation efficiency. This higher transformation frequency is accompanied by an increase in shoot formation indicating that the presence of transformed cells may facilitate the *de novo* formation of shoots under selection conditions. However, the greater access to the target tissue did not result in the production of transformed shoots. Therefore, it would appear that access to organogenically competent cells by *Agrobacterium* is not the limiting factor for the production of transgenic shoots from this system and that the transformed cells provide 'cross-protection' from the antibiotic to nearby cells that are organogenically competent. This phenomenon has been observed with other systems. With the leaf disc procedure (Horsch *et al.*, 1985) some shoots that regenerate under selective conditions do not contain T-DNA or the inserted DNA has lost expression of the gene during development. In this case, the problem was dealt with by subjecting regenerated shoots to a second round of selection during

rooting. However, if any basal callus tissue remains on the shoots then rooting can still occur (Jordan and McHughen, 1988).

In Melon (*Cucumis melo* L.) transformation, regeneration from cotyledon explants is efficient under transformation conditions (26%-78%) but only about 4% of the explants give rise to transgenic plants (Dong *et al.*, 1991). Also, in this study it was found that the proportion of regenerated shoots that were transformed was highest when selection was applied immediately after cocultivation. This proportion became lower when when selection was applied after two weeks and was the lowest when no selection was applied at all. From these data, the authors concluded that transformed cells had to compete with non-transformed cells in differentiation, possibly due to cross-protection of the non-transformed cells.

It has been found through metabolic studies that organogenesis is an energy intensive process resulting in higher rates of respiration and a greater utilization of reducing power (NADPH) and ATP as compared to non-organogenic tissue. (Thorpe, 1980). This also seems to be the case for organogenesis from cotyledonary petioles, as evidenced by the rapid mobilization of starch from both the petiole and the cotyledon during shoot formation from these tissues of *B. juncea* (Sharma and Bhojwani, 1990). If transformed and non-transformed cells are competing for limited resources for differentiation, transformed cells may be at a disadvantage during selective conditions. Although the transformed cells are able to withstand the presence of kanamycin in the medium, this carries a high metabolic price since ATP is required to detoxify the antibiotic. This would divert ATP from other uses which

may include organogenesis. Meanwhile, neighboring organogenically competent cells may have their immediate surroundings detoxified of the antibiotic at no metabolic cost to themselves. This would leave more of their metabolic resources intact for other purposes, including organogenesis. Therefore, if selection with kanamycin was delayed until after a critical energy intensive phase of differentiation, transformed cells would be on a more even footing, with respect to energy utilization, compared with non-transformed cells.

Delayed selection has been employed in other transformation systems. It was found for the transformation of tomato stem segments that when kanamycin selection was applied at the shoot development stage of regeneration, transformation efficiency improved significantly over that obtained with immediate selection (Chyi and Phillips, 1987). Also, transformed *B. napus* shoots were obtained from transverse segments of inflorescence stalks when kanamycin selection was delayed for 2 weeks (Boulter *et al.*, 1990). Delayed selection was also employed for the transformation of *B. napus* hypocotyl explants (Radke *et al.*, 1988) and for the transformation of leaf explants from potato (Higgins *et al.*, 1992). A delay in the addition of kanamycin to the culture medium could have the additional benefit of avoiding the presence of the kanamycin breakdown product, phenylacetic acid, which is a physiologically active auxin (Holford and Newbury, 1992) and therefore could interfere with the shoot regeneration response under selective conditions.

In addition to their effect on regeneration, as discussed in section 4.1, silver compounds can also influence transformation efficiency. While the use of $AgNO_3$ did not stimulate regeneration from cotyledonary petiole explants (Table

1), this compound could have an effect under transformation conditions. DeBlock *et al.* (1989) found that for *B. oleracea* regeneration from hypocotyl explants was more efficient under non-selective conditions in the absence of $AgNO_3$. However, under selective conditions, the addition of $AgNO_3$ was a prerequisite for obtaining transformed shoots. The authors hypothesize that this compound may stimulate non-meristematic transformed cells to regenerate. It has been argued that meristematic cells may not be susceptible to integrative transformation (Potrykus, 1991). Higgins *et al.* (1992) found that the inhibition of ethylene perception by silver thiosulphate (STS) increased the transformation frequency of potato leaf explants. These workers hypothesized that the interference with ethylene response could reduce the induction of a pathogen defence response and therefore overcome resistance in the plant to *Agrobacterium* infection. Therefore, experiments with inhibitors of ethylene action under selective conditions could have an effect on the recovery of transgenic tissue from *B. rapa* cotyledon explants.

4.3 SUMMARY AND FUTURE PROSPECTS

A regeneration system has been developed for *Brassica rapa* (ssp. oleifera) that is generally applicable to many Canola varieties and, with minor modifications, to non-Canola varieties of this subspecies. The protocol requires the use of young seedling tissue (4 day old cotyledonary petioles), and a combination a cytokinin (BA) and and auxin (NAA) on MS medium. Incubation under a relatively low light intensity (30-40 $\mu Em^{-2}s^{-1}$) was beneficial and the use of ethylene inhibitors was detrimental to shoot regeneration from this system.

This system was tested for its suitability to be modified as a transformation protocol using *Agrobacterium tumefaciens* . Transformed tissue was obtained and regenerated shoots were associated with transformed callus, but transformation and regeneration remain as distinct processes from this tissue. In addition to the modifications discussed above (Section 4.2), other parameters remain to be tested with this protocol :

1) When cocultivation is performed in the presence of a nurse culture (*i.e.* an aliquot of suspension culture cells overlaying the agar medium), transformation frequencies have been shown to increase (Klee *et al.*, 1987).

2) It has been shown that the efficiency of transformation of a given host is influenced by both the genotype of the plant and the strain of *Agrobacterium* used (Hobbs *et al.*, 1989; Charest *et al.*, 1989). Therefore, genotype x strain interactions should be tested for their effect on the recovery of transgenic tissue and transgenic shoots.

3) If the recovery of transformed shoots from the cotyledonary petiole regeneration system described here remains problematic, then other explants that have been shown to be amenable to *Agrobacterium*-mediated transformation should be tested for their ability to provide transgenic *B. rapa* plants. Other members of the genus *Brassica* have been transformed using stem explants (*B. napus* : Pua *et al.*, 1987; Fry *et al.*, 1987) or thin cell layer explants (*B. napus* : Charest *et al.*, 1988). Hypocotyl explants have also provided transgenic shoots from a number of *Brassica* species (*B. napus* : Radke *et al.*, 1988; De Block *et al.*, 1989. *B. juncea* : Barfield and Pua, 1991. *B. oleracea* and *B. rapa* : De Block *et al.*, 1989).

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