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The Detection of
Glucosinolates During Development
of Brassica juncea (var. RLM 514)
Anther-Derived and Zygotic Embryos

by
Kathryn Marie Hovey, B.Sc.hon. (Carleton, 1987)

A Thesis submitted to
The Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology
Carleton University
Ottawa, Ontario
May, 1990

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acceptance of the thesis

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of Brassica juncea (var. RLM 514)
Anther-Derived and Zygotic Embryos

submitted by

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in partial fulfillment of the requirements
for the degree of Master of Science

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ABSTRACT

A developmental study of glucosinolate levels in Oriental mustard (Brassica juncea, variety RLM 514) was undertaken. The three major glucosinolates found in mature tissue were sinigrin, gluconapin and gluconasturiin at levels ranging from 3 $\mu\text{Mol/g}$ FW (roots) to 40 $\mu\text{Mol/g}$ FW (seeds). Initial experiments indicated that anther-derived embryos contained levels of glucosinolates comparable to those found in the mature plant. Subsequent experiments found anther-derived embryo glucosinolate levels to be tenfold lower. The lower levels of glucosinolate in anther-derived embryos parallel with the levels found in zygotic embryos until the 4 week stage. At the 4 week stage zygotic embryos experience a rapid increase in glucosinolate levels.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic Acid
DSGsn	Desulfoglucosinolate
FAB	Fast Atom Bombardment
FAB-MS	Fast Atom Bombardment Mass Spectrometry
Gsn	Glucosinolate
HPLC	High Performance Liquid Chromatography (or Chromatogram)
NAA	1-Naphthaleneacetic Acid
ONPGal	ortho-Nitrophenoxygalactoside
TLC	Thin Layer Chromatography (or Chromatogram)

INTRODUCTION

The Oriental brown mustard, Brassica juncea, produces a high quality protein seed and has agronomic properties which give it potential to become an oilseed food crop in addition to being a condiment. Mustard oils, the breakdown products of glucosinolates, unfortunately make the seed meal unpalatable. It would be valuable to develop a low glucosinolate variety of Brassica juncea. Although much was learned about glucosinolate metabolism during the earlier race to develop Brassica napus into an important oilseed crop, these secondary metabolites still require further investigation. A developmental study, such as has been undertaken here, can be informative.

Biotechnology, specifically anther culture, can yield large populations of Brassica juncea relatively quickly and without much of a space commitment. Plants derived from anther culture are useful for mutant selection in that their entire genetic complement is expressed. Mutations which might be of interest can thus be easily masked by replicate genes in normal amphidiploid tissue. Tissue derived from gametic tissue, such as anther-derived embryos, would have only half as many chromosomes and mutations might be more obvious.

For a selection program to work at the embryo level of development the cultures involved must produce or at least take up glucosinolates. UNDERHILL (1980) suggested that failure to find glucosinolates in Brassica tissue culture systems may have been due to insufficient sulfate levels. He has since shown that Brassica juncea cell suspension cultures can produce glucosinolates if sulfate levels are maintained (GIBLIN and UNDERHILL, 1988) but glucosinolate uptake from the maternal plant may also be a significant source of glucosinolate accumulation in zygotic embryogenesis (GIJZEN, MCGREGOR and SEGUIN-SWARTZ, 1989).

This study was initiated to provide a basis for a biotechnologically oriented breeding program for Brassica juncea. The study established that glucosinolates could be detected early in development of anther-derived embryos. A comparison of anther-derived and in situ zygotic embryos was done in order to evaluate how well in vitro development approximates in vivo embryogenesis. Experiments were also conducted to find out whether variation in media sulfate levels might amplify glucosinolate production in anther-derived embryos. An insect-based bioassay for screening large numbers of embryos is also proposed.

LITERATURE REVIEW

A. AGRONOMIC POTENTIAL OF ORIENTAL MUSTARD

When plant breeders succeeded in developing varieties of Brassica napus and B. campestris (rapeseed) which were low enough in glucosinolates to be used as animal feed and low enough in erucic acid to yield edible vegetable oils the resulting plant varieties were trade named "canola". Brassica oilseed crops, especially "canola", have been compared to soybeans in their production of vegetable oils, high protein seed meal and importance to international markets (LAMB, 1989). Their significance as a world oilseed crop grew after plant breeders modified the constituents of the seed, beginning in the late 1960's, to reduce levels of erucic acid and glucosinolates (JOSEFSSON, 1971). Canola is generally favoured over soybeans in areas which are colder or more prone to drought (LAMB, 1989).

Although currently the seed meal of Brassica juncea has too high a glucosinolate level to be suitable as a high protein meal for animal consumption, the United Nations Food and Agricultural Organization has rated it as having a better protein composition than soybeans (KIRK and ORAM, 1978). B. juncea is now generally considered a condiment crop (producing mustard seed) although in some countries young leaves are also eaten as vegetables (ibid).

In 1985, over 2.8 million hectares of canola were planted in Canada (INSECT LOSSES COMMITTEE, 1988). If a low glucosinolate Brassica juncea could be developed, it would serve to broaden the genetic base of oilseed agriculture. As well as broadening the genetic base of the oilseed industry, the development of Brassica juncea from condiment to seed meal and oil crop might expand the acreage open to oilseed cultivation. B. juncea is thought to have properties which would make it a more suitable crop than canola in some parts of Canada. In particular, B. juncea recovers more readily from drought conditions, is more cold tolerant, ripens more quickly, suffers less from silique shattering and may be more resistant to some diseases (KIRK and ORAM, 1978). Its yellow seed colour is also a valuable trait (BEVIS and KELLER, 1990) as chlorophyll is not desired in the oil.

The rapid expansion in land devoted to canola has been followed by a concomittent expansion of habitat for pests (LAMB, 1989) which may foreshadow problems to come. This is another strong argument for broadening the genetic base of Canada's oilseed industry. A more genetically diverse crop may not all be susceptible to losses due to insects or disease.

B. BIOLOGY AND CHEMISTRY OF GLUCOSINOLATES

Glucosinolates are an important group of sulfur containing glucosides which are toxic to most herbivores. They are found in all Cruciferae species examined as well as in ten other plant families (UNDERHILL, 1980). All known glucosinolates have the structure shown in Figure 1 with considerable variety demonstrated in the amino-acid derived R-side chain (SORENSEN, 1980). Over eighty different glucosinolates have been described (ROBBELEN, 1980 and SORENSEN, 1980).

Glucosinolates are accompanied within the plant cell by enzymes called myrosinases. Myrosinases are compartmentalized so that most of the glucosinolate pool is not easily accessible to them under normal conditions. These enzymes are associated with plasma membranes in the majority of Brassica embryonic cells (MAHESHWARI, STANZEY, BEVERIDGE and VAN DE VOORT, 1981). Endogenous myrosinases do lyse glucosinolates at a slow rate under normal conditions but do so much more quickly when the plant tissues are macerated (UNDERHILL, 1980). Hydrolysis products depend on reaction conditions but typically include toxic isothiocyanates (see Figure 2). It is believed that the lysis of glucosinolates by myrosinases serves to deter herbivores from consuming significant amounts of the plant.

<u>R- Group</u>	<u>Proper Prefix</u>	<u>Trivial Name</u>
$\text{CH}_2=\text{CH}-\text{CH}_2-$	Allyl	Sinigrin
$\text{CH}_2=\text{CH}-\underset{\text{OH}}{\text{CH}}-\text{CH}_2-$	2-Hydroxybut-3-enyl	Progoitrin
$\text{CH}_2=\text{CH}-\text{CH}_2-\text{CH}_2-$	But-3-enyl	Gluconapin
$\phi-\text{CH}_2-\text{CH}_2-$	2-Phenylethyl	Gluconasturtiin
---NOTE: ϕ =benzene ring		

Figure 1. General structure of a glucosinolate and some examples of variation in the R-side chain.

Glucosinolates are diffusely present in plant parenchymal tissue (ROBBELEN, 1980). The glucosinolate profile of a plant typically varies from tissue to tissue, with development and under different environmental influence.

Glucosinolate patterns vary from one species to another. For example, Brassica juncea differs from B. napus in that B. juncea typically contains no progoitrin while B. napus has no sinigrin. Glucosinolate patterns also vary within species. Within B. juncea, for example, there are varieties with and without sinigrin (ROBBELEN, 1980).

PALMER, YEUNG and SANG (1987) note that, while the tissue profiles of Brassica juncea include at least eight different glucosinolates, only two (gluconapin and sinigrin) comprise over 90% of the total in all samples. Selecting against the major glucosinolates has been accomplished without affecting how much of the minor glucosinolates remain (UNDERHILL, 1980). When the Bronowski variety of B. napus was analysed, it was discovered that levels of all three of its major glucosinolates had been simultaneously reduced (ROBBELEN, 1980).

It is important to be aware of the biological impact of glucosinolates. Concern has been expressed about the wisdom of eliminating an entire family of compounds without knowing the full biological consequences of the act. { See UNDERHILL (1980), GLAND (1980) and LAMB (1989).} One known effect of the major glucosinolates is to deter herbivory by vertebrates and generalist insects. There is evidence that isothiocyanates generally act as powerful antibacterial and antifungal agents (ROBBELEN, 1980) and may also prevent nematode hatching (BAKER and COOK, 1974). On the down side, there is solid evidence that the minor glucosinolate indole-3-methyl glucosinolate assists the economically important clubroot fungus in its infection path (RAUSCH, BUTCHER and HILGENBERG, 1983). The same glucosinolates which repel generalist insects serve as feeding and oviposition attractants for an army of specialist insects.

Figure 2. Myrosinase degradation of glucosinolates.
Degradation product shown is isothiocyanate.

It has been suggested that resource availability in the environment may be the major determinant of the amount and type of a plant's anti-herbivore arsenal (COLEY, BRYANT and CHAPIN, 1985). When resources are limited, slow-growing plants are favoured and these slow-growing plants gain more from an investment in defense.

LOUDA, FARRIS and BLUA (1987) found a strong negative correlation between concentration of methylglucosinolate and soil moisture availability. The wildflower Cleome serrata grown in drier soils produced more glucosinolates for their size than did plants grown in moist soils. These dry soil plants also had greater post-predation seed production than the larger wet soil plants, implying that the glucosinolate production did protect the stressed plants from herbivory.

Given the advantages which glucosinolates confer to plants, an ideal situation might be to reduce the glucosinolate levels in the seed without affecting the profiles in the maternal plant. This was hinted at by LAMB (1989) who observed that reduced seed glucosinolate content did not necessarily affect the rest of the tissue profile and GIJZEN, MCGREGOR and SEGUIN-SWARTZ (1989) who are investigating the potential of blocking embryo uptake to reduce seed glucosinolate level.

To this end it would be important to know what proportion of the glucosinolate content of seed is due to in situ synthesis and what proportion is due to transportation from maternal tissue. This has not yet been resolved. If transportation is the key factor, selection techniques could focus on identifying individuals with depressed transport function. On the other hand, if in situ synthesis is more significant, selection techniques should focus on identifying individuals with depressed synthetic abilities.

C. ANALYSIS OF GLUCOSINOLATES

Advances in quantitative analysis of glucosinolates have done much to improve our knowledge of these secondary plant metabolites. The major hurdles to separation and quantification of glucosinolates are the endogenous myrosinases which will degrade glucosinolates upon disruption of plant tissue and the complex variety of the R-side chains of these compounds.

Different procedures for analysis are outlined by SORENSEN (1980) in Figure 4. The volatile products of myrosinase degradation may be determined after allowing endogenous or exogenous myrosinases to react with plant tissue. If myrosinases are inactivated then intact glucosinolates or their desulfated derivatives may be determined instead.

The desulfation procedure made a major advance in glucosinolate purification. Desulfating enzymes from animal and microbial sources are commercially available. The homogenate is treated to inactivate myrosinases. The extract is then passed through an anionic and cationic exchange column to successively remove any inhibitory salts. Glucosinolates are then bound to another ion exchange column and incubated with the enzyme aryl sulfatase (THIES, 1979 and THIES, 1980).

This selective desulfation allows one to quickly obtain a fraction containing a mixture of almost pure desulfoglucosinolates (MINCHINTON, SANG, BURKE and TRUSCOTT, 1982). See Figure 3 for the desulfation reaction. Further analysis and quantification of the desulfoglucosinolates could be done by Gas Chromatography but it was found that the silylation step presented problems with some of the glucosinolate compounds, particularly when dealing with the glucosinolate profiles of low-glucosinolate varieties of Crucifers (SORENSEN, 1980). Reverse phase High Performance Liquid Chromatography is now the analytical method of choice following desulfation. (See MINCHINTON, SANG, BURKE and TRUSCOTT, 1982; SANG and TRUSCOTT, 1984; MacGREGOR, 1982; PALMER, YEUNG and SANG, 1987; BJORKQVIST and HASE, 1988.)

Figure 3. Desulfation of glucosinolates by aryl sulfatase. The breakdown product is desulfoglucosinolate.

Figure 4. Different strategies for the analysis of glucosinolates. The solid arrow pathway describes the strategy used during this project except that HPLC analysis replaces GLC[MS]. (SORENSEN, 1980).

Other analytical methods include indirect analysis by determining the total glucose content or total sulfate content by such means as a colorimetric assay (DUBOIS et al, 1956) or X-ray fluorescence (SCHNUG and HANEKLAUS, 1987). These methods have the advantage of being fairly quick and suitable for screening programs but have the disadvantage of indiscrimination. Adding the desulfation procedure to these analytical techniques reduces background from residual glucose or sulfate but makes the technique more cumbersome.

Isotachophoresis (KLEIN, 1981), thermospray Liquid Chromatography-Mass Spectrometry (MELLON, CHAPMAN and PRATT, 1987) and Fast Atom Bombardment Mass Spectrometry (FENWICK, EAGLES and SELF, 1982) have also been applied to glucosinolate analysis. These analytical techniques have not been widely used mostly because the equipment is not readily available and the cost of analysis is high.

D. TISSUE CULTURE OF BRASSICA JUNCEA

With plant tissue culture it is possible to conduct massive screening procedures using much less time and fewer resources than are required in conventional breeding.

Production of haploid lines of plants can be useful in enhancing the plant breeding process. Haploid plants can be obtained through ovule, anther or microspore culture. These plants are useful for mutant selection in that no part of their genome is masked by the other parental genome. Homozygous lines can be established quickly from the haploid plants of interest after either spontaneous doubling of chromosomes or colchicine-induced chromosome doubling. Anther culture has been accepted by Brassica breeding establishments internationally (KELLER, ARNISON and CARDY, 1987).

The taxonomic relationship between members of the Brassica family has been established. See Figure 5 below. Brassica juncea (n=18) is thought to have arisen from the combination of the B. nigra (n=8, black mustard) and B. campestris (n=10, turnip) genomes (VAUGHAN, PHELAN and DENFORD, 1976). Brassica juncea is referred to as both an amphiploid and an allotetraploid.

Figure 5. Taxonomic relationships between members of the Brassica genus. (VAUGHAN, PHELAN and DENFORD, 1976).

It is possible to resynthesize Brassica lines from the original parents. Brassica napus has been resynthesized from B. oleracea and B. campestris (BRADSHAW, HEANEY et al, 1983). Since B. juncea could be resynthesized from B. nigra and B. campestris an alternative breeding approach would be to manipulate the parental varieties.

Two geographic races of Brassica juncea exist (VAUGHAN, PHELAN and DENFORD, 1976). They are the Indian line and the Oriental line. It is thought that with regards to glucosinolate profile the Indian race resembles B. campestris more than B. nigra and that the Oriental line is more like B. nigra. The Indian race of B. juncea typically has a higher gluconapin: sinigrin ratio than does the Oriental line (ibid). The apparently low glucosinolate variety of B. juncea identified by COHEN, KNOWLES, THIES and ROBBELEN (1983) originated from an Oriental strain. Further analyses revealed that this was not a low glucosinolate type.

FAZEKAS, SEDMACH and PALMER (1986) reported that the Indian lines of Brassica juncea are more responsive to tissue culture than are the Chinese lines. GEORGE and RAO (1982) applied anther culture techniques successfully to B. juncea variety TM-4. SHARMA and BHOJWANI (1985) announced similar success with varieties Czern R1K-81-1 and R1K-80-1. All of these are Indian varieties. They noted that yields for B. juncea were lower than for other Brassicas and might improve if greenhouse donor plants were substituted for field-grown ones. By 1987, GEORGE, ABRAHAM et al reported that they were regenerating haploid and spontaneously diploid plants from anther-derived embryos of the TM-4 B. juncea. This project relies on anther culture from a Brassica juncea variety of the Indian race, RLM-514.

While Brassica juncea anther culture has been developed to a workable system, it should be noted that it is not perfect. The microspore culture system for B. napus has overcome several shortcomings still present in the B. juncea haploid system. Yields of B. juncea are still significantly lower than those of B. napus (KELLER, 1989). Brassica napus microspore systems show more synchronous development of cultures, which allows for easy sorting into stages (see McCLELLAN, KOTT and ELLIS, 1988). Brassica juncea cultures typically demonstrate a complete spectra of embryogenic development. GEORGE and RAO (1982) and SHARMA and BHOJWANI (1985) noted a lack of synchrony as they repeatedly transferred embryos to the low sucrose medium free of growth regulators which was necessary for continued development. The formation of secondary embryos has been largely eliminated in the B. napus system. This has been accomplished by providing proper nutrition to allow the primary embryos to continue development normally (KOTT 1988, personal communication). With B. juncea, SANG and CHEN (1989) studied a system which involved embryos forming, callusing, and then developing secondary embryos which grew into plantlets. GEORGE and RAO (1982) also noted callusing of their early cultures.

E. GLUCOSINOLATES AND DEVELOPMENT

Much of the art of plant tissue culture has been developed through painstaking empirical efforts. While this approach has been fruitful, there is also a recognized need for more of a basic understanding of both the biochemistry and physiology of plant development.

This project attempts to shed light on the production of the secondary metabolites, glucosinolates, during the course of in vitro and in vivo development.

The embryogenesis of ~~Oriental~~ mustard might be expected to parallel the well-studied Brassica napus system. For this study what is of most interest is how independent embryos are from the maternal plant for their glucosinolate reservoirs. It is hoped to establish whether embryos which develop in situ supplied by a maternal plant follow the same glucosinolate accumulation patterns as embryos which develop in vitro.

Obviously, a major difference between embryo development in vivo and those cultured from anthers is the embryo ploidy level. Zygotic embryos of B. juncea would be expected to have 36 chromosomes compared to half that number for the anther-derived embryos. What effect that would have on glucosinolate accumulation is not known.

Embryos in their natural state gear part of their metabolism to accumulation of storage compounds preparatory to dormancy and germination. Culture conditions may not induce the same metabolism in the embryos as would be found in embryos developing in seed. FINKELSTEIN and CROUCH (1984) found that with B. napus cultured embryos show a metabolism which is intermediate between the storage metabolism of the normally developing embryo and the growth metabolism of the germinating seedling. They were studying the metabolism of the storage protein cruciferin. It would be interesting to know if B. juncea embryos cultured from anthers exhibited a glucosinolate metabolism which resembled that of the zygotic embryo or the germinating seedling.

The other major differences between development in culture and on the maternal plant is nutrient source. On the maternal plant, the developing embryo is provided with nutrients through the triploid endosperm and maternal tissue. The endosperm and maternal tissue within the ovule initially act as sinks for nutrients coming from the maternal plant through a structure called the funiculus. When the funiculus degenerates, the ovule becomes a nutritionally closed system (DURE, 1975). In Brassica, the embryo then proceeds to continue development by absorbing the endosperm. In anther culture the nutrient source is restricted to a defined medium and the undefined influence of the somatic anther tissue.

The maternal plant has long been known to influence the glucosinolate content of the final seed (LOOF and APPELQVIST, 1976). It has been suggested that glucosinolates destined for the seed are synthesised in the silique wall and that low-glucosinolate varieties of B. napus have metabolic blocks which do not affect the glucosinolate accumulation in maternal tissue (JOSEFFSON, 1973) but possibly the phloem transportation system. GIJZEN, MCGREGOR and SEGUIN-SWARTZ (1989) also suggest that embryo uptake is an important means of accumulating glucosinolates in low-glucosinolate varieties of Brassica napus.

Some studies of glucosinolate production during Brassica in vivo embryo development have been done. RAHMAN, STOLEN and SORENSEN (1986) found glucosinolates were present in Brassica seeds early on in development before the major seed weight gain was noted. ROBBELEN (1980) noted that accumulation of glucosinolates in Brassica seeds proceeds continuously during development with maximum glucosinolate content being reached at final maturity. Final maturity would be defined as the stage at which the moisture content of the seed has declined. Brassica napus embryos continue to accumulate reserves in vivo as the seeds dry out (FINKELSTEIN and CROUCH, 1986). Again, the source of the seed glucosinolates is not known.

REST and VAUGHAN (1972) examined nine stages in the in vivo development of white mustard (Sinapis alba or Brassica hirta) embryos. They noted that there could be a time lapse of up to 4-6 days between pollination and fertilization and found a consequent range of development stages within individual pods. The embryo develops into a heart-shape within 11 days. By 20 days the cotyledons begin to fold at an angle to the hypocotyl and by 31 days the endosperm is completely absorbed so that the embryos fill the seed coat. In B. juncea, SUKHIJA, LOOMBA, AHUDA and MUNSHI (1985) glucosinolates began to accumulate between 14 and 21 days after fertilization. Accordingly in this study zygotic embryos were excised as early as was practical and stages up to 28 days were examined. Embryos derived from anther culture of similar stages to the zygotic embryos also had their glucosinolate levels assessed in this study.

It has not yet been established, then, how much of the glucosinolate content of Brassica seeds is due to uptake from maternal tissue and how much is produced by the embryo itself. It was hoped that a comparison of in vitro and in vivo embryo development could establish whether embryos actually produce glucosinolates themselves.

F. OPTIMIZING CONDITIONS FOR A SELECTION PROGRAM

It is important to have culture conditions which favour glucosinolate production in order to begin an effective selection program. Environmental influences such as water stress, nutrient status and osmotic conditions have been shown to affect glucosinolate production both in the field and in culture conditions.

ROBBELEN (1980) reported up to 15% variation in the average level of glucosinolates of rapeseed grown in 6 field stations with different soil and weather conditions. SCHUSTER and KLEIN (1978) tested mustard cultivars grown in different locations and also found that glucosinolate levels varied within the same cultivar. RAHMAN, STOLEN and SORENSEN (1986) studied glucosinolates in seeds from different silique positions on the plant. They found that the position of the seed pod on the flowering stem was correlated with the seed glucosinolate content. They suggested that, since seeds produced at the base of the flowering stem formed earlier than apical ones, the difference in glucosinolate content may reflect different environmental conditions at seed set between the base and the apex of the flowering stem. APPELQVIST (1976) also noted environmental influence in that spring-sown crops are generally lower in glucosinolates than autumn-sown crops.

Temperature and water stress may account for differences between spring and autumn-sown crops. The influence of water stress is beginning to be understood. Canola (Brassica napus) and turnip rape (Brassica rapa) were subjected to stressed and unstressed conditions by MAILER and CORNISH (1987). Plants subject to water stress roughly doubled their glucosinolate contents. The effect of water stress on glucosinolate levels of the relatively drought tolerant Brassica juncea has not yet been investigated.

Water stress could be achieved in tissue culture by the addition of agar to the media (see KLIMASZEWSKA and KELLER, 1985). This might also help a breeding program in that it has been found generally that in Brassica, embryos cultured on solid media are better able to develop into plantlets than those grown on liquid medium (KELLER, ARNISON and CARDY, 1987). Liquid media are generally used for anther culture, however, because it increases embryo yield (ibid).

Water stress may affect glucosinolate production by altering the osmotic potential. McCLELLAN, KOTT and ELLIS (1988) found that decreasing sucrose levels in Brassica napus microspore cultures triggered an increase in glucosinolate production. The drop in sucrose level triggering glucosinolate production was probably not a nutritive effect but an osmotic effect. FINKELSTEIN and CROUCH (1986) found

that a high concentration of osmoticum in the medium prevented precocious germination in Brassica napus embryos excised from seed. When McCLELLAN et al lowered the sucrose levels they may have enhanced water uptake, preventing embryos from entering the dessication phase. FINKELSTEIN and CROUCH noted this osmotic effect with sucrose as well as with sorbitol and mannitol.

Sulfur and sulfate levels seem to be the nutrients which most influence glucosinolate production in the field and in culture. KHAN, SHEIKH, HUSSAIN and KHAN (1987) found that while nitrogen and phosphorus fertilizers affect Brassica juncea seed yield they did not influence glucosinolate content. The sulfur content in soils, on the other hand, has been reported as the greatest single environmental influence on glucosinolate levels (APPELQVIST, 1976). An extensive field study on the effects of sulfur fertilizer on two Brassica crops revealed that even the newer low-glucosinolate crop varieties showed increased glucosinolate content in response to sulfur supplementation (NUTTALL, UKRAINETZ, STEWART and SPURR, 1987).

FREEMAN and MOSSADEGHI (1972) found that Brassica juncea subjected to low sulfate levels showed fewer ill effects from sulfur deficiency than other crucifers. Despite their resiliency, there was still a correlation between sulfur nutrition levels and mustard glucosinolate levels. They reported an asymptotic curve where sulfur levels more than 4 times the level required for maximum glucosinolate production actually resulted in declining glucosinolate levels.

In 1980 UNDERHILL suggested that failure to find glucosinolates in culture grown tissue might be due to a sulfate deficiency. More recently, GIBLIN and UNDERHILL (1988) examined cell suspensions of two crucifers. They found that by continually replenishing sulfate in the medium glucosinolates could continue to accumulate in culture. If sulfate levels were not maintained, glucosinolate levels in Brassica juncea cell suspensions would decline dramatically within days.

Water stress and sulfate nutrient status then, would seem to be keys to optimizing culture conditions for glucosinolate production. Other environmental conditions, such as temperature, might also be investigated. In this study the media sulfate levels were varied. An initial experiment merely halved and doubled the sulfate levels but this experiment was expanded to examine the effect of a tenfold increase and decrease in sulfate.

G. STRATEGIES FOR LOWERING SEED GLUCOSINOLATE CONTENT

Attempts to alter the glucosinolate levels in seed focus on three strategies. The first is to develop low glucosinolate varieties of the plant, either by conventional breeding or using biotechnology. The second is to manipulate the environment in which the crops are grown. The third involves treating the seed following harvest.

Conventional plant breeding led to the development of low glucosinolate varieties of canola (B. napus). A low glucosinolate variety of Brassica juncea was developed by conventional breeders in 1988 after this project had begun.

Low glucosinolate varieties may be engineered by selecting for mutants with blocks in the biochemical pathway of glucosinolate synthesis or alternately ones which have impaired glucosinolate uptake from the maternal plant. JOSEFSSON (1971 and 1973) studied the metabolic blocks in the glucosinolate pathway which were typical of the low glucosinolate Bronowski variety of B. napus. GIJZEN, MCGREGOR and SEGUIN-SWARTZ (1989) favour a selection technique which aims at the uptake mechanism but their experiments are still in preliminary stages.

Ensuring that oilseed crops receive adequate water after flowering (MAILER and CORNISH, 1987) may lower glucosinolate levels, as may growing crops on sulfur deficient soils. (See APPELQVIST 1976; NUTTALL, UKRAINETZ, STEWART and SPURR 1987; FREEMAN and MOSSADEGHI 1972; ROBBELEN 1980.)

The third strategy, treating seeds following harvest, has received more attention of late. Cooking crushed seed may inactivate the myrosinase enzymes; steaming the seeds may remove volatile components or chemical means may be used (KIRK and ORAM, 1978). Industrial treatment with heat, microorganisms or chemicals has not been favoured, however, either because of the costs involved or a resulting reduction in protein quality (ROBBELEN, 1980). Recent developments may allow for industrial removal of glucosinolates. DIOSADY, TAR, RUBIN and NACZK (1987) report that they are ready to scale-up a process for the production of glucosinolate-free canola meal.

H. POSSIBLE TECHNIQUES FOR EMBRYO SELECTION

This project, the analysis of different stages of anther-derived embryos for glucosinolates, was undertaken with the aim of finding an optimum stage of development for screening populations for glucosinolate-poor individuals. While HPLC analysis has been useful in demonstrating the presence of glucosinolates in populations of embryos it would be cumbersome to use HPLC to detect variant individuals.

Two laboratories have made progress in developing screening techniques which could be applied to this plant tissue culture system. One screening technique involves selective fluorescence and the other an enzyme-linked immunosorbent assay. UNDERHILL (1988) has demonstrated that the yellow dye 2-nitrocalanine can be formed in tissue which has taken up the synthetic 2-nitrophenylglucosinolate. GROOTWASSINK et al (1988) explain that degradation of this synthetic glucosinolate by myrosinase can release the yellow dye under specific conditions. This might be teamed with a fluorescence measuring flow cytometer as explained by PAULS (1988) to screen embryos. Even more promising, HASSAN ET AL (1988) have developed an antibody specific to the alkenyl glucosinolates.

An alternative selection technique would be to use insects which specialize on Crucifers as selection agents for (or against) glucosinolate-poor individuals in the Brassica juncea tissue culture system. The process of coevolution between glucosinolate-producing Crucifers and their specialist insects has involved the development of specific behaviours and electrophysiological responses (MA 1972, MA and SCHOONHOVEN, 1973) which are cued by glucosinolates. Two well-studied behaviours in particular which are cued by the presence of these secondary metabolites are feeding and oviposition.

Likely insect candidates for assays in which glucosinolate-rich embryos are selectively eaten include the adult cabbage aphid (Brevicoryne brassica), cabbage butterfly larvae (Pieris rapae) and adult mustard flea beetles (Phyllotreta species).

The level of phloem development in the mustard embryos would be expected to be the main determinant for success in inducing aphid feeding (see ADAMS and vanEMDEN 1972, WENSLER 1962).

Both the flea beetles and Pieris larvae could probably recognize mustard embryos as food. The trick with flea beetles would be to determine if there was a pattern to their feeding choices as they jump about quite a lot at the best of times (see FEENY, PAUWE and DEMONG 1970 and NIELSEN 1978). Pieris larvae are much more sedate and their smaller instar are also less voracious. The influence of glucosinolates on Pieris larval feeding has been the subject of numerous studies. See HOVANITZ and CHANG (1963), DAVID and GARDINER (1966), MA (1972) and MA and SCHOONHOVEN (1973). The larval instar, initial diet and hunger level of the caterpillar would be some of the important factors to manipulate in order to obtain a reasonable Pieris bioassay.

Although feeding assays should, in theory, leave glucosinolate-poor embryos undamaged there remains a risk that insects may bite the embryos before rejecting them as a food source, possibly reducing the viability of the desired embryos. An oviposition assay using the adult cabbage root fly (Delia radicum) as the selective agent would inflict even less damage to the embryos being screened. The adult female typically deposits her eggs on the host plant surface or in the surrounding soil (BROOKS, 1951). Oviposition assays, then, may involve less risk of embryo loss or damage than feeding assays would.

Although oviposition assays tend to be more complicated than feeding assays, the Delia radicum system does have potential. In particular, lab colonies of cabbage root fly have long been established (FINCH and COAKER, 1969) although currently there are no cultures being maintained in Canada. There have also been numerous studies attempting to quantitate the levels of glucosinolates which stimulate oviposition in this insect. (See TRAYNIER 1967, FINCH 1978, STAEDLER 1978, WALLBANK and WHEATLEY 1979, FINCH and SKINNER 1982, BRACKEN 1988 and TUTTLE, FERRO and IDOINE 1988.) The intent of these studies has mostly been to develop glucosinolate-baited traps but much useful information has been accumulated which could be applied to developing an oviposition selection technique.

Some very preliminary experiments that were attempts at establishing an insect-based glucosinolate selection technique are incorporated into this project.

MATERIALS AND METHODS

A. PLANT GROWTH

Donor plants for embryos were grown from seeds of Brassica juncea, variety RLM 514, provided by Eric Bevis of Dr.W.A.Keller's lab. These seeds were germinated in vermiculite. Seven days after planting, they were transferred to individual 6-inch plastic pots in soil (sandy loam: peat moss: perlite; 4:4:1; v/v/v) each with 10 ml of Nutricote slow release 14-14-14 fertilizer from Plant Products of Canada Ltd.

For the first 4 weeks, the plants were maintained in a growth chamber at 25°C with a 14 hour light period from 5 am to 7 pm. The initial hour consisted of incandescent light supplied at 200 $\mu\text{E}/\text{sec m}^2$. This was followed by 12 hours of combined incandescent and fluorescent light supplying 450 $\mu\text{E}/\text{sec m}^2$ and a final hour of light identical to the initial hour. At the end of 4 weeks the plants were transferred to a greenhouse where they received natural sunlight supplemented by fluorescent and incandescent light from 6 am to 12 pm. At this stage they also received Hoagland's solution weekly. Throughout the growing period plants were watered twice daily.

The above conditions provided donor plants from which L.Bromley cultured embryos in the summer of 1987. Excessive heat in the summer of 1988 made it necessary to keep plants in the growth chamber throughout their development. The light regime remained as before but the temperature conditions were adjusted to 16 h at 20°C and 8 h at 15°C. At the 5 week stage B. juncea experiences a rapid height gain. This brings the buds directly in contact with the lights and so to avoid scorching plants were transferred from a platform in the growth chamber to the floor at this stage.

B. MATERIALS

Sulfatase S-9626 was purchased from Sigma Chemical Company, St.Louis, Missouri and purified as per MACGREGOR 1982.

Glucosinolate standards of Progoitrin and Gluconapin were obtained from Biocatalysts Ltd., Pontypridd, Wales. Sinigrin monohydrate S330-S from Aldrich Chemical Company, Wisconsin. Desulfosinigrin standard was kindly provided by Dr.Ian MacGregor of the Saskatoon Station of Agriculture Canada's Research Branch.

DEAE-Sephadex A-25 and C-25 were obtained from Pharmacia. Aldrich HPLC grade and Fisher Optima Grade Acetonitrile was used. Deionized double distilled water was filtered through 40 μ m filters for HPLC use. All HPLC samples were passed through Nalge 45 μ m syringe filters. All other chemicals were obtained from Sigma, Aldrich or Fisher, reagent grade or better.

C. MEDIA

The standard liquid medium used for anther culture was M41 as described in Table I in Appendix. Stock solutions of 5x M41 medium containing all components except 2,4-D, NAA, KI and Sucrose could be frozen. After thawing, adding the remaining components and checking the pH, the media was autoclaved for 17 minutes. To vary the Sulfate levels of the medium, magnesium sulfate was either augmented with ammonium sulfate or replaced by magnesium chloride as seen in Table II of Appendix.

D. ANTHER CULTURE

Techniques for anther culture of B. juncea, variety RLM 514 are as described in BEVIS and KELLER (submitted). Procedures are as follows.

Optimum buds for culture were obtained from plants just after stem elongation and prior to the opening of more than one flower in each inflorescence. At this stage petals are elongated so that $1/2$ to $2/3$ of the proximal anthers are visible and, more importantly, the anther is the yellowish-green colour characteristic of the late uninucleate to early binucleate stage of pollen development.

Buds were surface sterilized in a Lipshaw basket suspended for 15 to 20 minutes in a 7% w/v calcium hypochlorite solution. The basket was then transferred to three successive five minute water rinses. Buds are used within 4 hours of being excised.

Anthers were cultured on liquid M41 medium. Each anther was positioned such that the surface which had been attached to the filament was in contact with the liquid. Each 60 x 15 mm Petri dish contains 3 ml of medium and has 18 anthers cultured on it.

High temperature pretreatment for the induction of embryogenesis is 48 hours of darkness at 35°C. Following this treatment, the plates are moved to 25°C and maintained in darkness for up to 8 weeks. More media was added as required.

Upon harvesting, anther-derived embryos were sorted according to size and morphology as described in Table I on the following page. Plates I and II on pages 64 and 65 show examples of anther-derived embryos cultured in the dark.

Table I. Description of Different Stages of Embryos Cultured from Anthers

STAGE	FRESH WEIGHT (mg)	SIZE (mm)	MORPHOLOGY
1	0.04	<1	globular
2a	0.6	1-3	small heart-shaped
2b	1.2	1-3	small torpedo-shaped
3a	4.9	>3	large heart-shaped
3b	6.5	>3	large torpedo-shaped
4	14	>3	cotyledonous

E. COLLECTION OF ZYGOTIC EMBRYOS

Donor plants were maintained in the growth conditions described above until 5 weeks and then were isolated from the younger plants. Flowers were hand-pollinated before pistil elongation had taken place and labelled with the date.

Healthy pods were harvested, split lengthwise with a razor blade and pinned onto a paraffin wax dissecting platform. The platform was not fixed to the stage of the dissecting microscope (10 x) and usually embryos from about five pods could be harvested in one pinning.

Size 5 forceps were used to hold the fertilized ovule while a Beaver knife sliced through the soft maternal tissue and teased the embryo out of the endosperm. This operation was performed using the dissecting microscope.

At one day after fertilization the pod contains 10-30 light green translucent ovules, each ovule being about 3-5 mm in diameter. Within the ovule wall, viscous endosperm is found. As the green embryo develops, the endosperm becomes less viscous. The development of the zygotic embryos resembles that of embryos cultured in vitro in light. At about 21 days, however, the cotyledons of the zygotic embryos begin to swell and the embryos fills the entire ovule. After about 35 days, the zygotic embryo loses its green colour and the drying process begins. The mature seed will have a brown seed coat and be less than 2 mm in diameter.

Upon harvesting, zygotic embryos were sorted according to size and morphology as described in Table II below. Plate III on page 66 shows zygotic embryos at three stages of development.

Table II. Description of Different Stages of
Excised Zygotic Embryos and Surrounding Endosperm

STAGE (daf)	FRESH WEIGHT (mg)	SIZE (mm)	MORPHOLOGY OF EMBRYO AND ENDOSPERM
1 (10)	---	<1	heart and torpedo-shaped gelatinous endosperm
2 (12)	.17	1-2	cotyledonous much liquid endosperm
3 (14)	---	2-3	hypocotyl starting to curl much liquid endosperm
4 (21)	---	>3	cotyledons swelling some liquid endosperm
5 (28)	8.4	>3	cotyledons fill seed little endosperm left
6 (35)	19.4	>3	yellow cotyledons

F. EXTRACTION OF GLUCOSINOLATES

Once enough embryos of one category were collected they were immersed and fast-frozen in Isopentane cooled with liquid Nitrogen. They were stored at -40° until the glucosinolates could be extracted from them. Alternatively the glucosinolates were extracted from the embryos immediately and the extracts were frozen and stored until further processing could be performed.

Glucosinolates were extracted from tissue samples and desulfated after the method of MACGREGOR 1982 as follows.

The fresh or frozen samples were plunged into boiling 80% aqueous Ethanol. They were boiled for 5 minutes, removed from the heat, ground up and then allowed to incubate for a further half hour at 35°C . The extracts were then centrifuged for 20 minutes at 20 000 rpm in a Sorvall Superspeed RC-2-B refrigerated centrifuge (4.25" diameter rotor) or filtered through Whatman 54 and Whatman 1 filter paper.

The clear liquid was then washed through a mini-column of DEAE-Sepahadex A-25 in Acetate form and rinsed with 20 volumes of 0.2 M Sodium Acetate buffer (pH 4.5). Then 0.2-0.3 ml of purified Sulfatase enzyme was lowered onto the column surface. The columns were topped with distilled deionized water, incubated at room temperature overnight and then washed with 10 mls of distilled deionized water.

Samples of embryo extract which were to be concentrated prior to HPLC analysis were first spiked with a known amount of o-Nitrophenylgalactose (ONPGAL). They were then concentrated down to approximately one-tenth the original volume.

G. ANALYSIS

Qualitative analysis was attempted using cellulose Thin Layer Chromatography and a butanol:water:acetic acid solvent system (4:4:1, v:v:v) followed by silver staining.

Total glucosinolate content for a tissue profile was done using the DUBOIS (1956) method for determination of glucose. All desulfoglucosinolates were approximated as absorbing equally to desulfosinigrin. The difference in standard curves between glucose and desulfosinigrin is shown in Figure 6.

HPLC techniques were modified from MACGREGOR (1982). Semi-preparative C-18 columns and a water-acetonitrile gradient were used for tissue profiles and initial analysis of the embryos. Quantitative analysis was done using an analytical C-18 column and an isocratic acetonitrile-water solution. Figure 7 shows standard curves for analytical HPLC chromatograms of ONPGal and desulfosinigrin.

Glucosinolates were identified using commercial standards as references. As well, eluted peaks from preparative HPLC were subjected to Fast Atom Bombardment Mass Spectrometry as per FENWICK, EAGLES and SELF (1982).

Recoveries of Desulfosinigrin from Sinigrin desulfated on column averaged 90% in seven trials done with three different batches of acetated Sephadex. When 4.0 μ Mol of Sinigrin was added to a boiling leaf extract, recovery of Desulfosinigrin was $60.5 \pm 1.5\%$ in two trials.

Figure 6. Standard curves for the Dubois assay using glucose and desulfosinigrin. Error bars represent the range of absorbance values for three trials.

Figure 7. Standard Curve for Analytical HPLC
Error bars represent the range of absorbance values for three trials.

H. ATTEMPTS TO INITIATE LAB CULTURES OF CRUCIFEROUS INSECTS

Preliminary attempts were made to initiate lab cultures of the following insects which specialize on cruciferous plants: the cabbage root fly (Delia radicum), mustard flea beetles (Phyllotreta), cabbage aphids (Brevicoryne brassicae) and the white (or cabbage) butterfly (Pieris).

In late July an informal survey was begun to find gardens with radishes infested with the Delia radicum larvae. See Appendix B for the survey format. Where an infested garden was found the afflicted radishes were harvested and a second crop of Scarlet Globe radishes was planted in the same row.

For the first harvest of infested radishes eggs were collected from these vegetables and transferred to moist filter paper. For every subsequent find, the infested cruciferous vegetables were transplanted into soil or sand in insect cages under conditions favoured for root fly culture (FINCH and COAKER, 1969).

Similarly, any other cruciferous insects were transferred with the host plant material to the lab where the host plant was transplanted and maintained.

I. PRELIMINARY INSECT BIOASSAYS

Amongst the fauna collected from infested garden plot crucifer crops were several aphid colonies, suspected of being Brevicoryne brassica, a Pieris larva, and hordes of mustard flea beetles (Phyllotreta).

(i) Aphid Feeding Experiment

Of the colonies of aphids suspected of being cabbage aphids, those which were feeding on leaves rather than stems were chosen for the feeding experiments.

Aphids were moved by using a fine paint brush dipped in water and lifting them from the rear in the manner proscribed by ADAMS and VAN EMDEN (1972). Three of them at a time were placed on an open 5.5 cm diameter Petri plate containing six 1 cm diameter filter paper disks. A cylindrical insect cage was fitted over the Petri plate and a yellow cardboard disk was placed underneath the Petri plate. The disks were moistened with water and arranged in a circle with Stage 4 anther-derived embryos placed on alternate disks. The aphid behaviour was monitored over three hours.

(ii) Butterfly Larva Feeding Experiment

The larva was subjected to the same choice test as described above for the aphids. After half an hour though it was found that the larva was crawling up the insect cage and its vertical movement was restricted. The insect cage was removed and the top of the Petri plate was covered with cheesecloth sealed with an elastic band.

(iii) Flea Beetle Feeding Experiment

Into individual baby food jars filled with water were placed young leaves of greenhouse grown Brassica juncea. The tops of the jars were covered to prevent insects from falling into the water. In this way, razor-cut leaves maintained their turgor for close to 4 days under temperature controlled conditions.

Six leaves were placed in insect cages and into each cage between four and six flea beetles was transferred. The system was left over the weekend and then the area of leaf consumed was measured.

RESULTS

A. IDENTIFICATION OF MAJOR GLUCOSINOLATES IN BRASSICA
JUNCEA VARIETY RLM 514

Initial Thin Layer Chromatography analysis of extracts from parental plant tissue indicated the presence of three major glucosinolates. Sinigrin is accompanied by at least two additional glucosinolates in RLM 514. One of these additional glucosinolates is found in all tissues examined (roots, buds, mature and young leaves, stems and anthers). The other additional glucosinolate is most strongly evident in root tissue. See Figure 8. Results for seeds are not shown but the TLC was similar to that of all tissues but roots.

Semi-preparative reverse phase High Performance Liquid Chromatographic analysis was done of the root extract in order to collect enough sample to identify the major glucosinolates. The HPLC profile of desulfoglucosinolates extracted from the whole plant is shown in Figure 9. That of desulfoglucosinolates extracted from the roots is shown in Figure 10.

Peak A appears to be an artifact which is also present in controls where no plant material was used in the desulfation procedure. It was present in desulfosinigrin desulfated from commercial sinigrin but not in the standard desulfosinigrin (see Figure 11). The desulfation rinse procedure was extended and peak A did not appear significantly when commercial standards of gluconapin and sinigrin were subsequently desulfated (Figure 11).

Figure 8 .

TLC Plate of Different Tissue Extracts from Brassica juncea variety RLM 514 Donor Plants for Anther Culture. Spot A is not a DSGsn. Spot B corresponds to DSS. Spot C corresponds to DSG and Spot D corresponds to DSP.

- a. Desulfosinigrin standard
- b. Glucose standard
- c. Whole plant extract, showing spots A, B, C and D
- d. Root extract, showing spots B, C and D
- e. Buds extract, showing spots B and C
- f. Mature leaves extract, showing spots A, B and C
- g. Young leaves extract, showing spots B and C
- h. Buds extract, showing spot C
- i. Lower stem extract, showing spots B and C
- j. Desulfosinigrin standard

Figure 9. Semi-Preparative HPLC of DS glucosinolates from Whole Plant Extracts from Brassica juncea variety RLM 514 Donor Plants for Anther Culture.

Figure 10 Semi-Preparative HPLC of DSGlucosinolates from Root Extracts from Brassica juncea variety RLM 514 Donor Plants for Anther Culture. Fractions were collected from this sample for FAB-MS analysis.

The HPLC profile of the whole plant agrees with the TLC evidence that there are three major glucosinolates present and the shoulders on peaks B and D suggest that other glucosinolates are also present in significant amounts. Nine fractions were collected during semi-preparative HPLC work. The collected fractions were then analysed using Fast Atom Bombardment Mass Spectrometry. The three fractions giving clear FAB Mass Spectra suggested that peaks B, C and D from the HPLC corresponded to the presence of desulfated sinigrin, gluconapin and gluconasturtin respectively. See Appendix C.

The identity of peak B is believed to be desulfosinigrin (DSS). The retention times correspond exactly with standards (both from Dr. MacGregor and from desulfated commercial standards); spiking samples with additional DSS resulted in no additional peaks and the FAB-Mass Spectra of the corresponding fraction collected from root extract agreed with the major ion peaks reported by FENWICK, EAGLES and SELF (1982). There appears to be a split peak in the HPLC for total plant extract but this is not evident in the root extract.

Figure 11 a. HPLC of Commercial Sinigrin Desulfated on Column showing the artifact peak A
b. HPLC of Desulfosinigrin Standard

Figure 12 . HPLC of Commercial Gsn Desulfated on Column
Following Extended Rinse to remove artifact A.
a. DS Sinigrin
b. DS Gluconapin

Peak C has been tentatively identified as desulfoglucanapin (DSG). Glucanapin is known to be the other major glucosinolate found in Indian varieties of Brassica juncea (VAUGHAN, PHELAN and DANFORD, 1976 and PALMER, YEUNG and SANG, 1987). The retention time found of 24-25 minutes is slightly earlier than the 27.8 minutes reported by SANG, MINCHINTON, JOHNSTONE and TRUSCOTT (1984) under similar conditions but the FAB-MS fragmentation pattern of the fraction agrees with the pattern expected from FENWICK, EAGLES and SELF (1982). Its retention time also corresponds to the desulfation product of commercially prepared standard glucanapin (see Figure 10b).

The retention time and FAB Mass Spectra fragmentation pattern of the fraction collected after 35 minutes (peak D) suggests that it contains desulfoglucanasturtin (desulfophenylethylglucosinolate or DSP). Glucanasturtin is not available as a commercial standard. The molecular ion weight of 344 corresponds to that of DS glucanasturtin reported by FENWICK, EAGLES and SELF (1982).

B. TISSUE PROFILE OF DONOR PLANTS FOR BRASSICA JUNCEA ANTHER CULTURE

With three major glucosinolates identified, it was possible to survey the different tissues of the donor plants. Glucosinolates were extracted from tissues from plants of an age appropriate to be anther donors and desulfated. The results of Dubois assays of the desulfoglucosinolates in the eluate are shown in Table III. Two different isolations of buds, young leaves and mature leaves reveal a similar pattern but seeds collected for the second assay showed higher glucosinolate levels than those obtained in the first extraction.

HPLC profiles of donor plant tissue using a solvent gradient (0-30% Acetonitrile) and a semi-preparative column are shown in Figure 13. HPLC analysis was later upgraded with the availability of an analytical column. Desulfoglucosinolates could then be quantitated using standard curves as shown in Figure 7. Again, all desulfoglucosinolates were approximated as responding equivalently to desulfosinigrin. Table IV shows the tissue profile obtained. It can be seen that the three identified desulfoglucosinolates comprise between 79-99% of total desulfoglucosinolates in all tissues examined.

Table III. Total DSglucosinolate Tissue Profile for Anther
Donating Plants of Brassica juncea variety RLM 514
as Determined by Dubois Assays

TISSUE	DESULFOGLUCOSINOLATE EQUIVALENTS ($\mu\text{mol/g F.W.}$)	
	Trial 1	Trial 2
Roots	3.0	--
Stem	3.7	--
Young Leaves	19.6	22.8
Mature Leaves	3.8	5.2
Buds	23.3	36.7
Mature Anthers	38.5	--
Seeds	15	46

Table IV. Desulfoglucosinolate Tissue Profile for Anther
 Donating Plants of Brassica juncea variety RLM 514
 as Determined by HPLC Analysis. Key:
 DSS=desulfosinigrin, DSG=desulfogluconapin,
 DSP=desulfogluconasturtiin

TISSUE	PROPORTION OF TOTAL AREA		
	%DSS	%DSG	%DSP
Whole Plant	16	41	28
Roots	8	37	45
Stem	12	65	2
Young Leaves	47	29	19
Mature Leaves	6	77	0
Buds	13	64	2
Mature Anthers	18	72	1
Seeds	26	73	0

Figure 13. Semi-preparative HPLC scans of a survey of different tissues from Brassica juncea variety RLM 514 anther donor plants.

Figure 13 a. Buds

Figure 13 b. Seeds

Figure 13c. Roots

Figure 13d. Lower Stem

Figure 13 e.
Mature Leaves

Figure 13 f.
Young Leaves

Figure 13 g.
Mature Anthers

Shortly after the switch was made to the use of the analytical column and computer integration of peaks, problems developed with the gradient system. Lab temperatures exceeded 30° C. Lack of air-conditioning made it very difficult to maintain the solvent gradient without constant degassing. In order to continue analysis the solvent system was changed to a single pump system using a pre-mixed solvent system and no gradient. The new HPLC was standardized and the remainder of the analyses were performed on this machine. The two major marker peaks are still adequately resolved. Figures 14 and 15 show analytical HPLCs of buds and stem tissue which can be compared to Figure 13 a and d.

Figure 14 Analytical HPLC of DSGlucosinolates from
Buds of Brassica juncea variety RLM 514

Figure 15 Analytical HPLC of DSGlucosinolates from Lower Stems of Brassica juncea variety RLM 514

C. THE HARVESTED EMBRYOS

Anther culture of Brassica juncea variety RLM 514 did not result in synchronous embryo development. Staging was done by hand-sorting according to size and morphology. Table I described the different stages into which anther-derived embryos were classified. Earliest Stage 4 embryos were found after about three weeks and harvesting was typically done the following week.

Plate I shows Scanning Electron Micrographs of typical examples of anther-derived embryos. A better idea of the range of morphology can be seen in Plate II, where different specimens of the same stage are photographed together in pairs.

Zygotic embryos excised from ovules were found to differ from anther-derived embryos. Plate III shows examples of zygotic embryos excised 14, 21 and 28 days after fertilization. Zygotic embryos consistently appear more advanced morphologically than anther-derived embryos of similar size. The 14 day old zygotic embryo (Plate III a) corresponds to a Stage 2b anther-derived embryo in size but the zygotic embryo is easily classed as cotyledonous while the anther-derived embryo more closely resembles a torpedo stage. Anther-derived embryos were cultured in the dark and, while faintly green, were not as strongly coloured as zygotic embryos.

PLATE 1.

Scanning Electron Micrographs of Typical
Examples of Different Stages of Anther-Derived
Embryos of Brassica juncea variety RLM 514

(Bar= 1 mm)

Stage 1a Globular

Stage 2a Small Heart-shaped

Stage 2b Small Torpedo-shaped

Stage 3a Large Heart-shaped

Stage 3b Large Torpedo-shaped

Stage 4 Cotyledonous

PLATE II.

Light Photographs (Back Illumination)
of Different Stages of Anther-Derived
Embryos of Brassica juncea variety RLM 514
Showing the Range of Sizes within Classifications
(Bar = 1 mm)

Stage 1a Globular

Stage 2a Small Heart-shaped

Stage 2b Small Torpedo-shaped

Stage 3a Large Heart-shaped

Stage 3b Large Torpedo-shaped

Stage 4 Cotyledonous

PLATE III.

Light Photographs (Back Illumination)
of Later Stages of Embryos Excised from Pods
of Brassica juncea variety RLM 514

a. Stage 3

b. Stage 4

c. Stage 5

(Bar= 1 mm)

Embryos are more uniform and smaller for a given stage of development than are the anther-derived embryos.

D. ANALYSIS OF GLUCOSINOLATES FROM ANTHER-DERIVED AND ZYGOTIC EMBRYOS

Anther-derived embryos cultured and harvested the summer prior to the initiation of this project were the first to be analysed. Sufficient quantities had been extracted to give reliable readings for the Dubois assay. Fresh weights were estimated on the basis of typical fresh weights for embryos of a particular stage. Table V shows that detectable amounts of glucosinolates could be extracted from large numbers of anther-derived embryos.

Qualitative HPLC analysis of the above samples were done using semi-preparative HPLC with the solvent gradient. It was found that between 600 and 2000 nMol of glucosinolates would give reasonable HPLC spectra at given conditions.

Reasonable spectra required the injection of the equivalent of anywhere between 50 and 2250 embryos (for stages 3a and 1 respectively). As can be seen, though, from Figure 15, the use of analytical HPLC allow for quantitative analysis of between 1 and 5 nMol of desulfoglucosinolates. Analytical HPLC spectra would be expected to require injections of samples containing between one and twenty embryos. See Table VI.

Table V. DSglucosinolate Content of Different Stages of Embryos Cultured from Anthers as Determined by the Dubois Assay

STAGE	# OF EMBRYOS	FRESH WEIGHT (mg)	DESULFOGLUCOSINOLATE EQUIVALENTS	
			(nMol/embryo)	(nMol/mg FW)
1	7500	11	0.25	170
2a	1040	670	3.0	4.6
2b	480	880	5.0	2.7
3a	280	300	34.6	19.0
3b	400	1150	12.8	4.5
4	1000	4530	4.7	1.0

Table VI. Sample Size Requirements for Analysis by Semi-preparative and Analytical HPLC. Amounts for Semi-preparative HPLC are based on how much was needed to give good scans. The extracts were previously analysed using the Dubois assay. The sample size requirements for Analytical HPLC calculates the number of equivalent embryos required to inject between 1-5 nMol of DSglucosinolates into the column.

STAGE	INJECTION SAMPLE SIZE REQUIREMENTS		
	Semi-Preparative HPLC		Analytical HPLC
	# Embryos	nMol DSGsn	# Embryos
1	2250	600	4-20
2a	330	1000	0.3-1.7
2b	110	600	0.2-0.9
3a	50	1700	0.03-0.15
3b	220	1300	0.17-0.85
4	190	900	0.2-1.0

A representative semi-preparative HPLC scan of Stage 3a anther-derived embryos is shown in Figure 16.

Instead of frozen, previously prepared extracts, the next sample analysed consisted of fast-frozen Stage 4 cultured embryos. The sample was divided into two groups. One group was left intact (INTACT-4); the other group was segmented into cotyledons (COT-4) and the remainder of the embryo (HYP-4). Glucosinolates were extracted from these embryos, desulfated as before, concentrated down and analysed on semi-preparative HPLC. It should be noted that these embryos were darker green in colour than was found in embryos subsequently obtained from anthers cultured in the dark.

Sample size for the scans was calculated from the results of Table VI. Figures 17 and 18 show resultant HPLC scans for the intact and segmented Stage 4 anther-derived embryos. Injection volume was adjusted in order to give the appropriate sample size for HPLC scans in this experiment. In all subsequent experiments using analytical columns the injection volume was kept constant and, where required, the amount injected was adjusted by altering the concentration of the sample.

Figure 16. Analytical HPLC scan of Stage 2a Anther-Derived Embryos from Brassica juncea variety RLM 514

Figure 17. Semi-preparative HPLC scan of Intact Stage 4 Anther-Derived Embryos from Brassica juncea variety RLM 514

Figure 18. Semi-preparative HPLC scan of Segmented Stage 4 Anther-Derived Embryos from *Brassica juncea* variety RLM 514. a. Cotyledons b. Remainder

As can be seen in Table VII, the 0.64 nMol of total glucosinolates found in each intact embryo is a lower figure than the sum of the dissected segments (1.10 nMol). Levels of the individual glucosinolate desulfosinigrin were fiftyfold lower than the approximation of those found in Stage 4 embryos according to Table V.

E. EFFECT OF VARYING SULFATE LEVELS ON ANTHER-DERIVED EMBRYOS

An attempt was made to see if an optimum sulfate level could be found for embryo glucosinolate production. It can be seen from Tables VIII and IX that varying sulfate levels (fourfold and subsequently a hundredfold) had no obvious effect on the yield of embryos from anthers. Embryos from different treatments looked alike. An analytical HPLC of desulfoglucosinolates extracted from Stage 3a embryos (cultured on media containing High sulfate levels) is shown in Figure 19. Desulfosinigrin is the most clearly evident glucosinolate.

Table VII. DSGsn Content of Intact and Segmented Stage 4 Cultured Embryos as Determined by Semi-Preparative HPLC

SAMPLE	F.W. (mg)	#	DSGsn	nMol DSGsn	
				/Embryo	/mg FW
Cot-4	136	271	DSS	0.17	0.09
			DSG	0.49	0.39
			DSP	0.56	0.28
			Sum	1.22	0.76
Hyp-4	53	271	DSS	0.51	0.10
			DSG	1.02	0.20
			DSP	0.19	0.04
			Sum	1.72	0.34
Intact-4	232	121	DSS	0.03	0.05
			DSG	0.21	0.40
			DSP	0.10	0.19
			Sum	0.34	0.64

Quantitative analysis using analytical HPLC was then performed on extracts from embryos cultured on media of varying sulfate levels. Three injections were attempted for each sample. The variation between injections was estimated as the range between high and low values for the total area integrated divided by the high value for total area integrated. It can be seen in Tables XI and XII that the variation within samples can be between 1 and 17% with the average variation being 4.4% and 4.5% within the two experiments.

Table X shows the levels of desulfoglucosinolates found in embryos harvested from media with an initial fourfold variation in sulfate levels. Overall levels of glucosinolates were found to be about tenfold lower than would be expected based on earlier results (compare to Table V on page 67). Variation of media sulfate levels further did not show any notable changes in glucosinolate levels which were again low as seen in Table XI. Table XII summarizes the levels of glucosinolates found in embryos cultured on different media for the two experiments. No pattern can be seen between media sulfate level and eventual glucosinolate content of the embryo.

Table VIII. Embryo Yield of Anthers Cultured on Media with a Fourfold Variation in Sulfate Levels

MEDIUM SULFATE LEVEL (mM SO_4^{-2})		TOTAL PLATES COUNTED	TOTAL EMBRYOS COUNTED	EMBRYO YIELD /100 ANTHERS
Lo	1.07	39	1825	260
Med	2.08	39	1615	230
Hi	4.08	39	2094	298

Table IX. Embryo Yield of Anthers Cultured on Media with a Hundredfold Variation in Sulfate Levels

MEDIUM SULFATE LEVEL [mM SO_4^{-2}]	TOTAL PLATES COUNTED	TOTAL EMBRYOS COUNTED	EMBRYO YIELD /100 ANTHERS
Def 0.20	20	1169	325
Lo 1.07	17	541	177
Med 2.08	36	2459	379
Hi 4.08	16	1267	440
Sur 20.02	20	550	153

Figure 19. Analytical HPLC Scan of desulfoglucosinolates extracted from Stage 3a embryos

Table X. DSGlucosinolate Contents of Embryos Derived from Anthers Cultured on Media with Fourfold Variation in Sulfate Levels. Neither DSGluconapin nor DSpheylethylglucosinolate were present in detectable amounts. Variation= (Range / Mean) x 100%

SAMPLE		CONCENTRATION DSS		VARIATION IN AREA (%)		
Stage	Medium Sulfate Level	nMol/ Embryo	nMol/ mg Fw	Within Sample	Within Stage	Within Experiment
1	LO MED HI	0.2 0.2 0.1	4.7 3.6 1.2	n.a. 2 17	9.5	
2a	LO MED HI	0.1 0.1 0.1	0.1 0.1 0.1	2 3 2	2.3	
2b	LO MED HI	0.7 0.1 0.4	0.6 0.1 0.4	10 3 7	6.7	
3a	LO MED HI	0.8 1.9 0.9	0.2 0.4 0.2	4 n.a. 3	3.5	
3b	LO MED HI	0.4 0.3 2.0	0.1 0.0 0.3	1 2 5	2.7	
4	LO MED HI	0.6 1.5 0.4	0.1 0.1 0.0	2 2 3	2.3	4.4

Table XI. DSGlucosinolate Contents of Embryos
Derived from Anthers Cultured on Media with
a Hundredfold Variation in Sulfate
Levels. DSpheylethylglucosinolate was
not detected. Variation= (Range / Mean) x 100%

SAMPLE		CONCENTRATION				VARIATION IN AREA			
Stage	Medium Sulfate Level	DSG nMol / Embryo	nMol / mg FW	DSG nMol / Embryo	nMol / mg FW	Within Sample	Within Stage	Within Experiment	
1	DEF	0.2	4.0	0.1	3.3	5			
	LO	4.1	100	0.2	4.8	7			
	MED	4.3	100	0.1	3.5	12			
	HI	0.1	2.5	n.d.	n.d.	10	8.5		
	SUR	4.2	110	3.4	85	n.d.			
2a	DEF	0.1	0.2	0.1	0.1	10			
	LO	0.3	0.5	0.4	0.6	n.d.			
	MED	0.1	0.2	0.1	0.2	1			
	HI	0.1	0.2	0.1	0.2	11	6.3		
	SUR	0.5	0.6	0.4	0.6	3			
2b	DEF	0.4	0.3	n.d.	n.d.	6			
	LO	0.4	0.3	0.3	0.2	3			
	MED	0.6	0.5	0.1	0.1	4			
	HI	0.1	0.1	0.0	0.0	2			
	SUR	0.4	0.3	0.4	0.3	4	3.8		
3a	DEF	0.4	0.1	0.2	0.0	2			
	LO	1.1	0.2	0.6	0.1	7			
	MED	0.2	0.0	0.1	0.0	11			
	HI	0.4	0.1	0.2	0.0	1			
	SUR	0.3	0.1	0.3	0.1	9	6.0		
3b	DEF	0.2	0.0	0.3	0.0	3			
	LO	1.1	0.2	0.8	0.1	1			
	MED	0.2	0.0	0.2	0.0	n.d.			
	HI	0.2	0.0	n.d.	n.d.	1			
	SUR	0.4	0.1	0.1	0.0	n.d.	1.0		
4	DEF	0.7	0.1	n.d.	n.d.	2			
	LO	0.3	0.0	0.1	0.0	1			
	MED	0.2	0.0	0.1	0.0	1			
	HI	0.2	0.0	0.0	0.0	1			
	SUR	0.1	0.0	0.1	0.0	2	1.4		4.5

Table XII Summary of Variation in Desulfosinigrin Levels
 From Sulfate Level to Sulfate Level of Anther-
 derived Embryos As Determined by Analytical HPLC.
 Expt.1= average of the three sulfate treatments.
 Expt.2= average of the five sulfate treatments
 Variation= (Range / Mean) x 100%

SAMPLE	SULFATE LEVEL					VARIATION
	Def	Lo	Med	Hi	Sur	(%)
Expt.1	---	0.7	1.1	1.2	---	4.4
Expt.2	0.3	0.8	0.9	0.2	1.0	4.5

F. COMPARISON OF ANTHER-DERIVED AND ZYGOTIC EMBRYOS

The first comparison of anther-derived and zygotic embryos were Dubois assays on unconcentrated desulfated extracts of cultured embryos and Stages 1-5 zygotic embryos. The extracts of all anther-derived embryos and Stages 1-4 of the zygotic embryos resulted in absorbances too low to be reliable. The level of total glucosinolates found for Stage 5 zygotic embryos, however, was 8.1 nMol/ embryo or 0.95 nMol/ mg FW. This is in the range of the previous analysis for Stage 4 anther-derived embryos. This agrees with data obtained from analytical HPLC.

Figure 20 shows representative spectra of the desulfated extracts from zygotic embryos. Samples of Stages 3 and 5 were collected and desulfated on two separate occasions. It can be seen that the glucosinolate levels rise sharply at Stage 5. This is also seen in Table III with the level continuing to be high in Stage 6 embryos excised from seed. Figure 21 graphically compares the glucosinolate contents of anther-derived embryos as determined in Experiments 1 and 2 with those of zygotic embryos.

Analyses of the ovules from which the zygotic embryos were excised shows little change in glucosinolate levels from Stage 3 to Stage 6.

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MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Table XIII. DSGlucosinolate Contents of Zygotic Embryos Excised from Pod and the Ovules from which they were Excised.
Variation= (Range / Mean) x 100%

SAMPLE		CONCENTRATION				VARIATION IN AREA (%)	
Stage	Embryo or Ovule	# Individuals	DSS		DSG		
			nMol /Embryo	nMol /mg FW	nMol /Embryo	nMol /mg FW	Within Sample
1	Embryo	153	0.1	n.a.	0.0	n.a.	2
2		87	0.3	n.a.	0.1	n.a.	6
3		81 74	0.5 0.3	n.a. n.a.	0.0 0.1	n.a. 0.9	10 1
4		136	0.2	0.8	0.1	0.4	5
5		366 148	31.4 16.5	3.7 1.9	1.0 0.2	0.1 0.0	8 3
6		45	38.2	2.0	3.8	0.2	4
3	Ovule	81	1.2	n.a.	0.0	n.a.	n.a.
6		124	2.2	n.a.	0.3	0.0	4
							4.8

a.

b.

Figure 20. Analytical HPLC scans of Desulfated extracts of Embryos excised from Seeds.

a. Stage 1

b. Stage 2

c.

d.

Figure 20 (continued)

c. Stage 3

d. Stage 4

Figure 20 (continued)
e. Stage 5
f. Stage 6

Figure 21. Summary of desulfoglucosinolate content of different stages of anther-derived and zygotic embryos *Brassica juncea* var. RLM 514 as determined by analytic HPLC.

■ DS Sinigrin
 ▨ DS Gluconapin

DISCUSSION

This section evaluates the potential for the B. juncea anther culture system to lead to a biotechnologically oriented breeding program. The glucosinolate levels in a tissue profile of anther donor plants has been established and is compared to literature values. The glucosinolate profiles of anther-derived and pod-grown zygotic embryos are compared. The attempt at amplification of glucosinolate production is discussed, as is the potential for an insect-based bioassay to screen for low glucosinolate individuals.

A. GLUCOSINOLATE TISSUE PROFILES

Analysis of a single tissue for glucosinolates does not necessarily give a complete picture of what is occurring within the secondary metabolism of the plant. Different tissues may accumulate varying ratios or amounts of glucosinolates. This may reflect different control mechanisms in glucosinolate biosynthesis located throughout the plant or else changes in substrate availability from tissue to tissue (SANG, MINCHINTON, JOHNSTONE and TRUSCOTT, 1984).

A tissue profile of glucosinolate levels can sometimes be useful in breeding programs. For instance, PALMER, YEUNG and SANG (1987) were hoping to screen B. juncea for low glucosinolate mutants by analysing single cotyledons. They first needed to establish that a correlation existed between glucosinolates synthesized in seedling cotyledons and those accumulated subsequently in developing seeds. They concluded that they did not have enough data to establish any correlation but suggested it might be feasible.

The tissue profile conducted in this study showed that there are three major glucosinolates present in the donor tissue for anther culture of B. juncea variety RLM 514. Of these, sinigrin and gluconapin are common to all tissues examined and gluconasturtiin (phenylethylglucosinolate) is characteristic of the roots. The results from HPLC and TLC found here agree on this.

The glucosinolates found in the tissue survey of B. juncea variety RLM 514 parent plants are as expected for a mustard of the Indian race. Glucosinolate profiles of mustards of the Oriental race show sinigrin and gluconasturtiin as the major glucosinolates (SANG, MINCHINTON et al 1984; KONDO, YAMAUCHI and NOZAKI 1986). Profiles of Indian varieties, however, agree with the results of this study in showing that gluconapin is also a major glucosinolate (PALMER, YEUNG and SANG, 1987). That gluconasturtin is

characteristic of root tissue agrees with the findings of SANG, MINCHINTON et al (1984).

The advantage of using HPLC as an analytical technique is its sensitivity. Furthermore comparing it to Gas Chromatography, HPLC is of particular value in that it picks up indole glucosinolates without the degradation that occurs during the silylation step required for GC analysis and there is little or no interference from non-glucosinolate substances (SANG, MINCHINTON, JOHNSTONE and TRUSCOTT, 1984). The findings of GROOTWASSINK et al (1987) seem to indicate that indole glucosinolates might be expected in tissue culture. The initial results here seem to indicate that alkenyl glucosinolates were also being produced in anther culture.

If the tissue culture system could consistently produce the levels of glucosinolates found initially it might be reasonable to use GC or GC-MS analytical techniques. The GC systems could reliably screen for levels of the alkenyl glucosinolates and once low alkenyl glucosinolate lines had been developed HPLC could be reverted to in order to screen for remaining indole glucosinolates. This would be similar to the route taken in developing low glucosinolate varieties of B.napus (UNDERHILL, 1980).

The values of 14-46 $\mu\text{mol/g}$ FW for seed glucosinolates are within the range of 0-150 $\mu\text{mol/g}$ FW found in field grown Brassica juncea (HALVA et al, 1986). SUKHIJA et al (1985) attributed 0.2% of the fresh weight of leaves and buds to glucosinolates in B. juncea. The values in Table III translate to approximately 0.6% of the fresh weight being due to glucosinolates in young leaves and buds and about 0.1% for mature leaves, roots and stems.

The range in values for seed glucosinolates may reflect different biases in the collection of seeds from the two batches of plants. In both cases, pods were selected randomly from the different plants. RAHMAN, STOLEN and SORENSEN (1986) recommend, however, that in small sampling situations seeds be collected from pods of the same age and position on the plant. Seeds were collected from plants of the same age but the seeds were not necessarily set at the same stage of development of the plant. The seeds might then reflect different plant conditions or different ages.

These results show that the analytical system used here is not in disagreement with those established in literature. Indole glucosinolates were not identified in the tissue profiles as being major components. They may have been present as some of the unidentified peaks on the HPLC.

B. COMPARISON OF ANTHER-DERIVED AND ZYGOTIC EMBRYOS

The absence of glucosinolates in tissue culture systems of B. juncea has traditionally stymied hopes of selecting for low glucosinolate individuals in tissue cultures. GROOTWASSINK et al (1987) found that in vitro cultures have much different glucosinolate profiles than do seed and vegetative tissues. The overall levels were very low and they suggested that the absence of aliphatic glucosinolates may have been due to the lack of precursors and not to differences in enzyme activities. Despite not finding glucosinolates in cell cultures they did find that at least part of the enzyme systems needed to make glucosinolates were in place. Tissue in culture could manufacture artificial glucosinolates from fed substrates (GROOTWASSINK et al, 1988).

These findings support the belief that glucosinolates can be produced in B. juncea culture if conditions are such that the natural precursors can be produced in vivo or taken up from the medium. PALMER, YEUNG and SANG (1987) found that when cotyledons were initially excised and cultured their glucosinolate levels dropped to about 2% of in vivo levels within two weeks. Transferring to fresh medium, however, brought an increase in glucosinolate levels up to the expected values. GIBLIN and UNDERHILL (1988) reported that cell suspension cultures of B. juncea manufactured glucosinolates if sulfate levels were maintained in the medium.

The levels of glucosinolates in anther-derived embryos as reported in Tables X, XI and even Table V are not unprecedented. SANG and CHEN (1988) found glucosinolate levels of almost 17 $\mu\text{mol/g}$ FW in anther-derived embryos and this rose to 35 $\mu\text{mol/g}$ when secondary embryos began to form. This would agree with the results for Stage 3a embryos in Table V. The high level of glucosinolates found in Stage 1 embryos may reflect the very low fresh weights of these small embryos. The subsequent levels of glucosinolates ranged from 0.2-1.2 $\mu\text{mol/g}$ (see Table XV) which are an order of magnitude lower than either the SANG and CHEN results or Stage 3a embryos in Table V.

On the basis of the Dubois assays and the semi-quantitative analysis of the fast-frozen Stage 4 anther-derived embryos, higher levels of glucosinolates would have been expected in Experiments 1 and 2 than were obtained. Perhaps the darker green colour of the embryos cultured previously indicated a difference in donor plant material or culture conditions which had a bearing on the ultimate glucosinolate levels of the subsequent embryos.

The levels of glucosinolates found in the previously cultured embryos indicated that with the use of analytical HPLC glucosinolates from individual embryos could be determined (see Table VI). Subsequent results would indicate that more work is needed on the culture system to maintain high levels of glucosinolates.

As mentioned before, anther-derived embryos differ from zygotic embryos in their size for a given development stage and their pigmentation. Both of these differences could be related to the fact that anther-derived embryos were cultured in the dark.

It would appear that cultured embryos parallel zygotic embryos in having low levels of glucosinolates initially. Zygotic embryos accumulate glucosinolates to significant levels at about the 4 week stage. This is in agreement with the findings of SUKHIJA, LOOMBA, AHUJA and MUNSHI (1985) who found an increase in glucosinolate levels between 21 and 28 days. Seed meal was found to contain 3.2% glucosinolates on a dry weight basis at day 7, increasing to 6.0% by day 35. This would correspond approximately to 0.6% glucosinolates at day 7 and 1.2% at day 35 on a fresh weight basis. From Table XVII by Stage 4 glucosinolate levels are 0.3% on a fresh weight basis and up to 0.7% by Stages 5 and 6. The trend seems similar then between literature values and those obtained here for zygotic embryos.

C. AMPLIFICATION OF GLUCOSINOLATES

From this study it would appear that increased sulfate levels do not amplify glucosinolate production up to the cotyledonous stage of development. Given the low levels of glucosinolates found during the sulfate experiments, however, the variation in sulfate levels may not have been the limiting factor in glucosinolate production. JOSEFSSON (1970) suggested that the photosynthetic supply might be a limiting factor in field grown B. juncea. He found that surplus nitrogen fertilizer could counter the augmenting effect that sulfate fertilizer had on glucosinolate production. He proposed that the surplus nitrogen might divert glucose into protein pathways and away from glucosinolate production.

It is suggested that an optimum stage of glucosinolate production be identified before further attempts at amplification were made. In view of the rapid accumulation of glucosinolates in zygotic embryos at Stages 4,5 and 6, later stages of anther-derived embryos should be tested.

Other means of amplification might well be tested. Altering nitrogen levels is not advised since this effect is reported as not being as significant as the sulfate effect (JOSEFSSON, 1970) but it might be interesting to vary the osmoticum levels as discussed in the Literature Review.

D. THE HARVESTED EMBRYOS

As well as being a developmental study, this work was intended to examine the feasibility of using the B.juncea anther culture system to select for low glucosinolate individuals. For the culture system to be incorporated into a breeding program it is essential that yields be consistent and that the embryos produced have a reasonable chance of regenerating into plants.

Yields in anther culture during this project dropped considerably whenever donor plant conditions deviated from the norm. For instance, at the initiation of the project there was a leaf miner infestation of the greenhouse and this caused very low yields. During the first summer of the project high temperatures in the greenhouse prior to moving plants into the temperature controlled environmental chambers also resulted in disastrously low yields. When donor plant conditions were maintained at optimum conditions, however, it was possible to get reasonably consistent yields. A protocol for regenerating plants from cotyledonous embryos also exists (BEVIS and KELLER, 1989).

The observed anther culture yields of between 2 and 4% are typical for Brassica juncea. Yields of 3% have been reported for two Indian and one Oriental variety of B.juncea (SHARMA and BHOJWANI 1985; GEO. and RAO 1985; SANG and CHEN 1988). BEVIS and

KELLER (1989) have achieved yields of close to 5% with a Russian cultivar of B. juncea and also report higher yields of variety RLM 514. The high yield of variety RLM 514 also incorporates in the system a high degree of variability with yields varying by as much as four or five orders of magnitude (ibid). Very high yields were not obtained consistently for RLM 514 in this study although daily yields did reach up to 12% (data not shown). Yields for another culture of Brassica species range between 0.9 and 11% (SHARMA and BHOJWANI, 1985).

A variation in colour was noted between previously cultured embryos and those cultured during the course of this project. The embryos cultured previously were darker green than subsequent cultures. Both sets of embryos were cultured in the dark but there may have been variation in the harvesting and culturing procedures. During the course of this project embryos were extracted or fast frozen within four hours of being harvested. Similarly when media was being added to plates after several weeks in culture, the plates were returned to the dark within four hours. The darker green of the previously cultured embryos may reflect a longer exposure to light during the periods when media was replenished or when embryos were harvested. SHARMA and BHOJWANI (1985) reported that B. juncea embryos had turned as green as zygotic embryos within 48 hours of being incubated in light. They did not report a minimum time required for the embryos to turn green.

E. POTENTIAL FOR INSECT-BASED BIOASSAYS

Given the results of the glucosinolate analyses of embryos, an insect bioassay would be best developed using embryos with high levels of glucosinolates. If the bioassay worked with zygotic embryos from Stage 4 onwards it would be reasonable to refine the system downwards to detect levels in younger anther-derived embryos. It might also be reasonable to try the system on zygotic embryos but one would lose the advantages which haploidy brings to a selection system.

Difficulties could be expected in meshing insect and plant cultures into a selection system. One is using two living systems as measures of each other. If both systems are fairly amenable to lab culture so that conditions can be controlled and individual variation minimized, then variation in the behaviour of the insect may be a reliable indicator of variation in the metabolism of the plant system.

Preliminary attempts were made to establish laboratory colonies of cruciferous insects and to conduct feeding experiments as described in the methods section. Amongst the fauna collected from infested garden plot crucifer crops were several aphid colonies, suspected of being Brevicoryne brassica, a Pieris larva, and hordes of mustard flea beetles (Phyllotreta).

Individual mustard flea beetles were found to consume between 0.8 and 2 square cm of Brassica juncea leaves over a 60 hour period.

The Pieris larva was offered a choice between Stage 4 embryos cultured on Medium Sulfate media and moist filter paper. For the first hour and a half the inch-long larva showed no interest in the embryos but by two and a half hours all three embryos were consumed.

Aphids were chosen which were eating the leaf rather than the stems of radishes. These were given the same choice as the Pieris larva was. None successfully initiated feeding of the embryos.

Of the insects surveyed, Pieris larva showed the most promise, simply because it ate the sample provided within a reasonable time frame. MA (1972) found that 5 μ Moles of glucosinolates was the threshold level for detection by Pieris. Even at the best of times, there would be expected to be only about 5 nMoles of glucosinolates in an individual Stage 4 anther-derived embryo (Table V). This might indicate that the larva was closer to the embryo than in the set-up employed by MA or that it was simply hungry. Flea beetles also certainly had an appetite for Brassica juncea but their habit of making large leaps make them really too difficult to manage in a laboratory setting.

The insect approach to selecting desired individuals from large amounts of cultured tissue merits further study. Certainly there are facilities and techniques which would be common to culturing both systems.

F. SUGGESTIONS FOR FURTHER RESEARCH

It would be reasonable to investigate the levels of glucosinolates in anther-derived embryos which have developed beyond the Stage 4 level analysed here. A protocol exists to culture embryos at this stage of development to the regenerant plant stage (BEVIS and KELLER, 1989). It would be worthwhile to trace the development of anther-derived embryos up to this stage and perhaps carry on a parallel study of zygotic embryos as they germinate and grow into seedlings.

The source of glucosinolates in seed still has not been established. When doing anther culture one must consider the possibility that the parental anthers are supplying the embryos with glucosinolates. A comparison (not shown) of freshly harvested anthers and ones which had yielded embryos did not show any substantial change in glucosinolate content. Similarly, glucosinolate analysis of freshly prepared media and media from which embryos were harvested did not indicate that glucosinolates

were released into the media. A radioactive pulse experiment might reveal the answer to this. It is interesting that the rapid accumulation of glucosinolates in zygotic embryos occurs after most of the endosperm tissue has been absorbed by the cotyledons. It would be interesting to know if the triploid endosperm supplies precursors which the embryos themselves convert into glucosinolates. While collecting zygotic embryos an attempt was made to collect the endosperm fluid but it was difficult to collect enough sample for analysis.

If an extended developmental study of anther-derived embryos showed a similar sharp rise in glucosinolate levels as is found in zygotic embryos, then it would be worthwhile to try varying media sulfate levels at the developmental stage where the rapid glucosinolate accumulation occurred.

Another avenue of study would be a comparison of light and dark grown embryos. Anther-derived embryos can be transferred into light once they are established (SHARMA and BHOJWANI, 1985). The resultant embryos in the B.napus system resemble zygotic embryos more closely than dark-grown embryos in that they are smaller for a given stage of development and the cotyledons are a darker green (POMEROY, 1990). It may be possible to initiate cultures in the light or alternatively, have zygotic embryos develop under reduced light conditions.

CONCLUSIONS

Within the tissues of anther donor plants of Brassica juncea variety RLM 514 there is about a tenfold variation in glucosinolate levels. They range from 3 to 40 $\mu\text{mol/g}$ FW in the roots and seeds respectively. The majority of these glucosinolates (upwards of 75%) consist of sinigrin, gluconapin and gluconasturtin. Gluconasturtin is characteristic of root tissue. These results agree with literature findings.

Initial experiments indicated that glucosinolate levels in anther-derived embryos were of the same order of magnitude as in older leaf and root tissue. Subsequent experiments showed levels to be tenfold lower, however.

If the glucosinolate levels of the previously cultured embryos could be obtained repeatedly, it would be feasible to employ the B. juncea anther culture system in order to select for low glucosinolate individuals.

The zygotic and anther-derived embryos both appear to have low levels of glucosinolates until about 4 weeks. At this time zygotic embryos accumulate large amounts of glucosinolates. It would be worthwhile to culture anther-derived embryos beyond the stage used here to see if they undergo a similar climb in glucosinolate levels.

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APPENDIX A

Table I. Composition of M41 Medium

Concentration (mM)		Concentration (mM)	
NaH ₂ PO ₄ *H ₂ O	1.09	Nicotinic Acid	0.008
KNO ₃	24.73	Thiamine HCl	0.030
(NH ₄) ₂ SO ₄	1.01	Pyridoxine HCl	0.005
MgSO ₄ *7H ₂ O	1.01	myo-Inositol	0.555
CaCl ₂ *2H ₂ O	5.10	Glutamine	5.474
Fe ₂ EDTA	0.10	Serine	0.952
MnSO ₄ *H ₂ O	0.053	2,4-D	0.0452
H ₃ BO ₃	0.049	NAA	0.0005
ZnSO ₄ *7H ₂ O	0.007	Sucrose	292
Na ₂ MoO ₄ *2H ₂ O	0.001	pH 5.5	
CuSO ₄	0.0002		
CoCl ₂ *6H ₂ O	0.0001		
KI	0.0050		

Table II. Variations in Sulfate Composition of M41 Medium

Final Medium [SO ₄ ⁻²]	(Def) 0.202mM	(Low) 1.07mM	(Med) 2.08mM	(High) 4.08mM	(Sur) 20.02mM
[MgSO ₄ *7H ₂ O]	0.202mM	1.01mM	1.01mM	1.01mM	1.01mM
[MgCl ₂ *6H ₂ O]	0.81 mM	----	----	----	----
[(NH ₄) ₂ SO ₄]	----	----	1.01mM	1.01mM	1.01mM
[NH ₄ Cl]	2.02 mM	2.02 mM	----	----	----
[K ₂ SO ₄]	----	----	----	2.0 mM	18.0 mM

APPENDIX B

SAVE THE WORMY RADISHES

NO, THIS IS NOT
THE LATEST GREENPEACE CAMPAIGN.

I AM A SERIOUS BIOLOGY STUDENT
ATTEMPTING TO START A CULTURE
OF CABBAGE ROOT MAGGOTS.

RADISHES ARE THE CHOICE MEAL
OF THESE INSECTS.

SO...

IF YOU HAVEN'T FINISHED HARVESTING YOUR RADISHES YET
AND ESPECIALLY IF YOU'VE NOTICED WORMS IN THOSE
YOU'VE ALREADY COLLECTED, I'D BE INTERESTED IN LOOKING
AT YOUR GARDEN.

PLEASE CALL Kathy @ 788-3652
OR 788-3650

APPENDIX C

Fast Atom Bombardment Mass Spectra used to identify the desulfoglucosinolates:

- a. Desulfoallylglucosinolate (DS Sinigrin)
(molecular ion M.W. 280)
- b. Desulfobutenylglucosinolate (DS Gluconapin)
(molecular ion M.W. 293)
- c. Desulfophenylethylglucosinolate
(DS Gluconasturtiin)
(molecular ion M.W. 343).

KEY: g.....background peaks due to glycerol (M.W.92), its polymers (M.W. 185, 277 etc.) or the sodium and potassium salts thereof (for instance the disodium glycerol dimer has a molecular weight of 207)

M.....molecular ion

M-O....molecular ion which has lost an oxygen atom

M+1....protonated molecular ion

M+Gly..glycerated molecular ion

M+Na...sodium salt of molecular ion

2M+1...protonated dimer of molecular ion

2M+Na..sodium salt of dimer of molecular ion

a. FAB Mass Spectra of Desulfosinigrin

b. FAB Mass Spectra of Desulfoglucunapin

c. FAB Mass Spectra of Desulfogluconasturtin

END

1 2 0 4 9 1

FIN