IMPROVEMENT ON THE MICROSPORE CULTURE METHODOLOGY FOR *BRASSICA RAPA* CANOLA

by

Kimberly D. Stadnyk

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements
for the Degree of

Masters of Science

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba

March 1999

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

Improvement on the Microspore Culture Methodology

for Brassica rapa Canola

by

Kimberly D. Stadnyk

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Master of Science

ACKNOWLEDGMENTS

Research and production of a thesis is a collaborative effort and involves a number of people.

First I would like to thank my supervisor Dr. Rachael Scarth for all of her patience, assistance and encouragement along the way. I also gratefully acknowledge the contributions of my committee members Drs. Don Palmer and Taing Aung for their helpful recommendations.

Financial support in the form of a University of Manitoba Fellowship, is gratefully acknowledged.

I would also like to thank Judith Nugent-Rigby and Dr. Allison Ferrie for providing their technical assistance and Holly Friesen for all the effort she put into the microspore staging component of my research.

Over the course of this research there have been many friends and fellow students who have provided support. However, special mention has to be extended to Dr. Scott Duguid, Dr. Marie Jasienieuk, Jim Blatta and Ian Brown without whom I may have never had the courage to take on such a task. Also a special thanks goes out to many friends, and colleagues at Agriculture and Agri-Food Canada.

Last but not least, warm thanks to my husband Jeff and my parents, Shirley and Robert Hamilton, for their support and encouragement throughout my education.

TABLE OF CONTENTS

LIST OF TABLES

Page

Page

Page

Page

Page

Page

Page

LIST OF FIGURES

ABSTRACT

The objective of this research is to improve the microspore culture methodology based on embryo production levels for *B. rapa*. An efficient isolated microspore culture methodology for *Brassica rapa* canola could be used by breeding programs to produce high, consistent embryo yields. The project was divided into several experiments looking at modifications to the culture media, microspore pretreatments, microspore liberation techniques and donor plant pretreatments. Four *B. rapa* genotypes were selected for the experiments: Reward, AC Parkland, AC-1 and AC-2. The standard protocol used in this research followed Baillie et al. 1992. Plant Cell Reports 11:234-237.

Experiment 1 was an evaluation of the effect of sucrose levels in the microspore culture media on embryo production of *B. rapa*. Six separate microspore culture runs were completed for this experiment, three with the media combination of NLN-17/ NLN-10 (control - which represents the standard protocol) and three with the media combination of NLN-13/NLN-13. The results of the experiment indicate that Reward responded with higher green embryo production to the standard media sucrose combination of NLN-17/NLN-10 while AC-2 responded to the media sucrose combination of NLN-13/NLN-13. Parkland and AC-1 did not respond to different media sucrose levels.

Experiment 2 was an evaluation of the effect of pH levels of the microspore culture media on embryo production of *B. rapa*. Six NLN media pH levels were tested over 18 separate microspore culture runs. Three microspore culture runs each of NLN media pH level 6.2 (control - or the standard protocol), pH 6.0, pH 5.8, pH 5.6, pH 5.4 and pH 5.2. The results of the experiment indicate that embryos can be formed under a wide range of pH levels. Reward respond positively to lower media pH levels than the standard (pH 6.2) while AC-2 responded to both lower (pH 5.2 - pH 5.4) and higher (ph 6.2) media pH levels.

Experiment 3 was an evaluation of the effect of microspore pretreatment in the form of a cold shock on embryo production in *B. rapa*. Six separate

microspore culture runs were completed for this experiment, three involving room temperature conditions (control - standard protocol), and three using cold shock procedures. The cold shock procedures resulted in the microspores being held at approximately 2-4°C for one hour during isolation. The results indicate that Reward responded to a microspore pretreatment of cold shock. There were indications of greater response after cold pretreatment for AC-1, and a greater response for Parkland and AC-2 for the room temperature pretreatment, although green embryo yields were very low.

Experiment 4 was an investigation of a large-scale microspore culture technique and its effects on green embryo production in *B. rapa*. The large-scale microspore culture technique was tested over four separate microspore culture runs. Each run was completed using the genotype Reward. The large scale microspore culture technique was successful in liberating large numbers of microspores and the procedure time of a standard microspore culture run was decreased quite substantially. However, the procedure produced consistently low embryo yields.

Experiment 5 was an evaluation of the effect of donor plant pretreatment on embryo production in *B. rapa*. Ethephon (2-chloroethyl phosphonic acid) was used to investigate the role of ethylene in microspore culture response of *B. rapa*. No embryos were produced for any of the Ethephon donor plant pretreatments for either Reward or AC-2. Cytological observations suggest that the 115 ppm treatment of Ethephon has a negative effect on the percent of microspores in the mid-late uninucleate stage of development for Reward. The percent of microspores in the mid-late uninucleate stage of development does not seem to be affected differentially for the three treatments of Ethephon on AC-2.

The two growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride) were used to investigate if a growth retardant could replace the requirement of low temperature donor plant growing conditions in the microspore

culture of *B. rapa*. The intent was to mimic retarded development achieved under low temperature growth conditions with the growth retardants. Cytological observations suggest that A-Rest has a negative effect on the percent of microspores in the mid-late uninucleate stage of development for Reward while AC-2 is unaffected. Cycocel seems to negatively affect the percent of microspores in the mid-late uninucleate stage of development for AC-2 while it has no effect on Reward.

Successful application of the isolated microspore culture technique is determined by the induction of response in many genotypes. The practical approach is to determine conditions and treatments which result in the best average embryo response across all of the genotypes being utilized rather than striving for optimal conditions for each genotype.

1.0 INTRODUCTION

The Canadian term 'canola' has achieved international recognition as a description of oilseed rape (*B. napus* ssp. *oleifera* and *Brassica rapa* ssp. *oleifera*) seed and derived products containing less than two percent erucic acid in the oil and no more than thirty micromoles of glucosinolates per gram of air-dried, oil free meal (Canola Oil and Meal Standards and Regulations, 1990). Rapeseed is generally considered to contain levels of erucic acid and glucosinolates which exceed these set limits. Canola quality oilseed rape is Canada's major oilseed crop and accounts for more than 60% of all vegetable oil produced in Canada. The seeded acreage in Canada reached 14 million acres in 1995 and has stabilized at 12 million acres in recent years.

Today utilization of the canola quality oil is widespread. Edible uses include salad oil, cooking oil, baking and frying shortening as well as table spreads. The meal produced from the seed is considered as a high protein supplement for livestock. Plant breeding programs are making continuous efforts to tailor canola quality oil and meal to meet market demands for improvements in nutrition and food processing.

Plant breeders produce crops through sexual crosses followed by selection of plants with desirable characteristics. Advances in plant cell culture and molecular biology have allowed further genetic manipulation of crops in general. A major biotechnological breeding tool available to plant breeders today is the use of the microspore culture technique to produce doubled haploids for oilseed rape improvement.

Microspore culture provides plant breeders with an efficient means of producing homozygous breeding lines in a single generation. This represents a savings of three to six generations of inbreeding, which in turn permits rapid selection for desirable characteristics. Lines with desirable traits but agronomic shortcomings can be easily crossed to productive cultivars and doubled haploid lines developed.

The efficient application of the isolated microspore culture technique in oilseed rape breeding is dependent on using genotypes which respond with high, consistent embryo yields and plant regeneration. Success lies in the ability to manipulate microspores in vitro, so large numbers of genotypes respond with high embryo production and regeneration rates. Doubled haploid production is influenced by donor plant age, donor plant pretreatment, developmental stage of the pollen, media and its constituents and culture conditions. As well, the interactions of each of the individual factors play a role in the induction of pollen embryogenesis.

There has been much success with well-established techniques of microspore culture for *B. napus* but limited work has been completed on *B. rapa*. The *B. napus* species is superior to the *B. rapa* species in yield potential, seed oil and protein content. However, the *B. rapa* species matures two to three weeks earlier and therefore is well adapted to the northern oilseed rape growing regions of western Canada. *B. rapa* oil quality is excellent, with low amounts of saturated fatty acids and low chlorophyll content. The seed is blended with higher saturate *B. napus* seed produced in central and southern areas to ensure the crop meets the quality standards for export. *B. rapa* is primarily self-incompatible and therefore the production of selfed lines is difficult and therefore expensive. A reliable microspore culture methodology to produce doubled haploid plants, to be used as pure breeding lines in oilseed rape breeding programs, would be of great importance for the production of new *B. rapa* varieties.

The most successful microspore culture protocol for *B. rapa* reported in the literature to date was published by Baillie et al. in 1992. This article outlines modifications of a *B. napus* microspore culture protocol which produced embryos from several lines of *B. rapa*. The standard protocol used in this research is based on this article and training received under the direction of Dr. A. Baillie at the National Research Council's Krisjansen Centre of Biotechnology, Saskatoon, Saskatchewan. Access to current research information was provided by the Consortium on Improvement of *B. rapa* Microspore Embryogenesis.

1.2 Objectives

The objective of this research was to improve the microspore culture methodology for *B. rapa*, by enhancing embryo yields. The research was directed at improving several aspects of a standard microspore culture methodology.

Experiment 1 was an evaluation of the effect of sucrose levels in the microspore culture media on embryo production of *B. rapa*. Many studies have been conducted on this area of microspore culture improvement, with varying results. In 1992, Baillie et al. reported that the highest number of embryos were formed for *B. rapa* genotypes when the microspores were cultured in NLN-17 media (0.1 mg BA L^{-1}) for forty-eight hours followed by a media change to NLN-10 (0BA) for the remainder of three weeks. Research had shown that Reward, a cultivar used in this project, actually responded better to the media combination of NLN-13 (0.1 mg BA L^{-1})/NLN-13 (0BA) (pers. comm.- Dr. A. Ferrie). This potential increase of embryo production with altered media sucrose levels was investigated.

Experiment 2 was an evaluation of the effect of pH of the microspore culture media on embryo production of *B. rapa*. In 1992, Baillie et al. showed that most *B. rapa* embryos were formed when microspores were cultured at pH 6.2 in NLN-17 for forty-eight hours followed by a media change to NLN-10, pH 6.2 for the remainder of three weeks. Research had shown that a lower pH culture media may actually produce more embryos (pers. comm.- Dr. A. Ferrie). The potential increase of embryo production with altered media pH levels was investigated.

Experiment 3 was an evaluation of the effect of microspore pretreatment in the form of a cold shock on embryo production in *B. rapa*. Research had indicated that a shock to the microspores would potentially increase the yield of embryos produced (pers. comm.- Dr. A. Ferrie). A potential increase of embryo production with a cold shock pretreatment to the microspores was investigated.

Experiment 4 was an investigation of a large-scale microspore culture technique and its effects on embryo production in *B. rapa*. The variable nature of the microspore culture process is reflected in the fact that no two runs under identical conditions, for the same genotype, produce the same results. *B. rapa* is an outcrossing species and genetic variability between individual plants exists. The rapid mechanical isolation of microspores from buds in a mass microspore culture technique may be able to decrease the inconsistency of response to microspore culture. By reducing interaction times between potentially detrimental factors (anther wall, pollen or bud tissue) and standardizing the treatments between buds, more consistent embryo yields may be produced (Swanson et al., 1987). Additionally, the mass microspore culture methodology would enable the efficient production of large numbers of haploid embryos to be utilized in *B. rapa* breeding programs.

Experiment 5 was an evaluation of the effect of donor plant pretreatment on embryo production in *B. rapa*. The pretreatments were A. Ethephon (2-chloroethyl phosphonic acid) and B. the two growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride).

A. Ethylene: Optimum levels of ethylene production are thought to increase the responsiveness of microspore culture in brussels sprouts (*B. oleracea var. gemmifera*) (Biddington and Robinson, 1991; Biddington et al., 1988). The differences in genotype response to microspore culture may be the result of both the capacity of a genotype to produce ethylene and sensitivity to high ethylene levels (Biddington et al., 1988). Ethephon (2-chloroethyl phosphonic acid), an ethylene releasing compound, was used to investigate the role of ethylene in microspore response to culture for *B. rapa*.

B. Growth Retardants: The change in the developmental pathway of a microspore from a highly specialized form to a embryonic form operates within a narrow period of microspore development (Palmer and Keller, 1997). If growth was slowed down, or retarded, this period of microspore development could be

lengthened to allow more microspores to be successfully induced into embryogenesis. The standard protocol used for *B. rapa* microspore culture involves the growth of the donor plants in a low temperature environment of 10/5^{0}C (day/night) temperatures, as opposed to the standard temperatures of 20/15^{0}C. The lower temperatures slow plant growth which allows a longer time period of bud collection (Baillie et al., 1992). It is also conceivable that lower temperature is lengthening the period of microspore development resulting in more microspores being induced into embryogenesis. The growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride), were used to determine if growth retardants could replace the cold temperature treatment.

2.0 LITERATURE REVIEW

2.1 Canola - Quality Oilseed Rape

2.1.1 History

The origin of oilseed rape stems from the crop known as rapeseed. It is thought that ancient civilizations used the oil of locally adapted *Brassica* weeds to fuel their lamps and as time progressed, as a cooking oil for food (Robbelen, 1991). Domestication occurred wherever the value of a locally adapted weed was recognized (Downey, 1983). However, it was not until the development of steam power that rapeseed oil's unique ability to cling to water and steam washed metal better than any other lubricant was discovered (Canola Council of Canada, 1988).

In the early 1940's, a shortage of rapeseed oil lubricant for steam powered war ships prompted the development of Canadian rapeseed production (Stefansson, 1983). Before World War II, Canadian production was limited to small research farms and an independent Saskatchewan farmer who had immigrated from Poland carrying a small amount of rapeseed. To alleviate this critical shortage of the oil, a large amount of seed was purchased from the United States. This seed was of the *B. napus* ssp. *olifera* species and had been originally secured from Argentina. The unofficial name therefore became "Argentine Rapeseed". Public release of information of the rapeseed oil shortage prompted the farmer from Saskatchewan to increase and distribute his seed. This would be the beginning of the species *B. campestris* now known as *B. rapa* ssp. *olifera* in Canada. Because of the Polish origin of the seed, the unofficial name used to describe this species became "Polish Rapeseed" (National Research Council, 1992; Canola Council of Canada, 1988; Daun, 1983).

2.1.2 Development

During World War II, rapeseed was only grown to produce oil for use as an industrial lubricant for Canada's war machines. With the end of the war, the bottom dropped out of the market for rapeseed oil as an industrial lubricant. Rapeseed oil as an edible product had only seen limited success and the meal could only be sold as fertilizer. However, early investigations demonstrated that after careful processing techniques, such as bleaching and deodorizing, the oil could be considered satisfactory for edible purposes (National Research Council, 1992).

Rapeseed slowly began to be accepted as an edible oil in the early 1950's. However, during the mid-1950's, based on animal feeding trials, the Food and Drug Directorate of the Department of National Health imposed a short-lived ban on rapeseed oil. Further review on the limited use of the oil in edible applications showed no threat to health and so the ban was lifted shortly (National Research Council, 1992; Canola Council of Canada, 1988; Daun, 1983).

The nutritional aspects of rapeseed were scrutinized. Analysis showed that the rapeseed oil was comprised of fatty acids of various chain lengths, including more than 40% in long chain fatty acids (mostly eicosenoic and erucic acid). Earlier studies had shown that erucic acid caused reduced weight gains and increased adrenal gland weights in rat feeding trials (National Research Council, 1992).

Spurred on by the detrimental effects of the long chain fatty acids, plant breeders searched for a genetic variation which decreased the eicosenoic and erucic acid content in rapeseed oil (Stefansson, 1983). Germplasm was identified with lower levels of erucic acid and this trait was introduced into agronomically suitable cultivars. The Federal Government's Health and Welfare Department along with industry, agreed to convert to production of only low erucic acid varieties of rapeseed. By December of 1973, the industry limit of erucic acid in food products was 5% (National Research Council, 1992; Daun, 1983).

The protein meal fraction of rapeseed was utilized as a protein supplement for livestock. However, animal nutritionists recommended that the sharp tasting anti-nutritive glucosinolates present in the meal fraction be lowered. Germplasm with low glucosinolate levels were identified and incorporated into agronomically acceptable cultivars (Canola Council of Canada, 1988).

The University of Manitoba professor Dr. Baldur Stefansson developed the first double-low variety which had low levels of erucic acid in the seed oil and lower levels of glucosinolates in the seed meal. This *B. napus* variety was registered as "Tower" in 1974. In 1977, the first double-low *Brassica rapa* variety, named "Candle" was released by Agriculture Canada, Saskatoon breeding program under the direction of Dr. Keith Downey (National Research Council, 1992).

These double low varieties of rapeseed adopted the commodity name of 'canola'. 'Canola' is a description of oilseed rape (*Brassica napus* and *Brassica rapa*) seed and derived products containing less than 2 percent erucic acid in the oil and no more than thirty micromoles of glucosinolates per gram of air-dried, oil free meal (Canola Oil and Meal Standards and Regulations, 1990).

2.1.3 Description

Oilseed *Brassicas* include the two rapeseed species *B. napus* and *B. rapa,* as well as the mustard species *B. juncea*. The Japanese scientist U (c.f. Downey and Rakow, 1987) demonstrated that these three species were closely related to other agronomically important *Brassicas*, namely *B. nigra* (Black Mustard), *B. oleracea* (Cabbage, Kale, Cauliflower, Broccoli) and *B. carinata* (Abyssinian Mustard). Through cytological studies, U proved that *B. nigra*, *B. rapa* and *B. oleracea* were the basic monogenomic species from which the amphidiploids *B. napus, B. juncea* and *B. carinata* are formed (c.f. Downey and Rakow, 1987). (Figure 1)

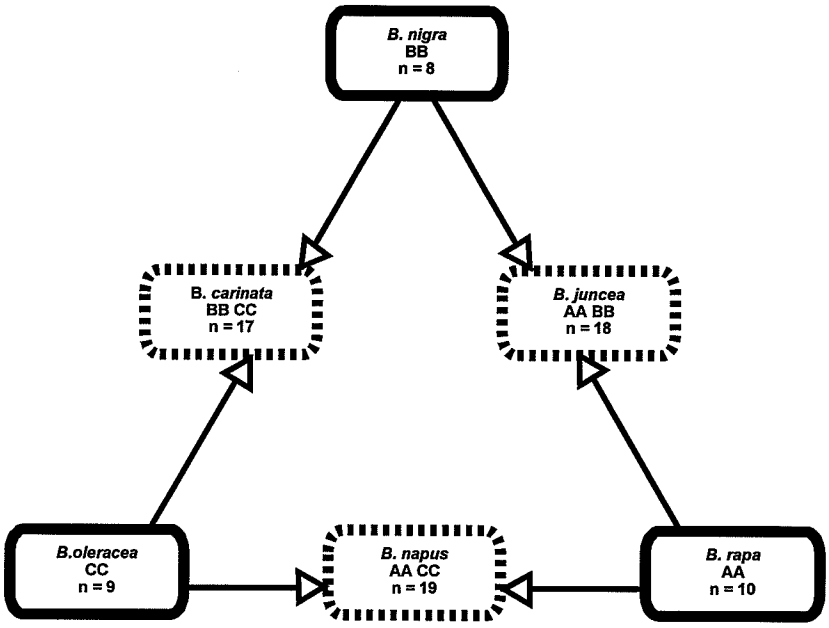

FIGURE 1. Figure commonly used to represent the relationship among the six agronomically important *Brassica* species (adapted from Downey and Robbelen, 1989).

B. napus and *B. rapa* are the two oilseed rape species of spring and winter canola which are grown in Canada. They are well adapted to the cool, moist growing conditions of the Parkland and transition zones of Canada's western prairie provinces (Canola Council of Canada, 1988). The small round seeds of canola contain more than 40% oil on a dry-weight basis and yield a high protein meal suitable for animal feed (Downey and Rakow, 1987). *B. napus* seeds are dark brown to black in colour, while *B. rapa* seeds range from yellow to black. The *B. napus* species generally matures in 95 to 110 days. It is superior to the *B. rapa* species in yield potential, oil content and protein percentage in the meal. However, the *Brassica rapa* species matures two to three weeks earlier and therefore is well adapted to the northern canola growing regions of western Canada.

2.2 Breeding Superior Canola-Quality Oilseed Rape

2.2.1 Breeding Objectives

Originally, the aims of all *Brassica* oilseed breeding was to improve agronomic performance. Then, nutritional questions prompted improvements in the nutritional shortcomings of the meal and oil. Today, breeding for agronomic traits such as seed yield, frost hardiness, disease resistance, drought resistance, early maturity, herbicide tolerance, greater resistance to lodging and shattering as well as the ability to germinate and grow at low soil temperatures is ongoing for both *B. rapa* and *B. napus*. Quality traits such as oil and protein content and oil and meal quality are constantly being improved. The alteration of fatty acid content in canola-quality oil is also a major breeding objective for specific end use markets.

2.2.2 Breeding Strategies

A plant breeding strategy, conventional or modern, involves several basic components:

1) recognizing the traits that are important,
2) designing techniques to evaluate genetic potential of the desired traits,
3) identifying sources of genes for the desired traits,
4) devising a means to combine the genetic potential of desired traits into an improved cultivar (Poehlman, 1987a).

B. rapa is primarily a self-incompatible species. Cross-pollinated species have high levels of heterozygosity, so they do not breed true. Imposed self-pollination on cross-pollinated *B. rapa* leads to inbreeding depression which is expressed as a decline in vigour and productivity. A self-incompatible cultivar is a mixture of heterozygous plants which are reasonably uniform in appearance

and performance. Instead of focussing all efforts on single superior plants which will breed true, as in self-pollinated crops, breeders concentrate on improvement on populations or gene pools of cross-pollinated species (Knowles, 1989).

Conventional *B. rapa* breeding methods generally involve selection of plants exhibiting desirable characteristics followed by a series of imposed self-pollinations to obtain highly inbred parental lines. The parents are crossed to produce the F_1 progeny. The F_1 are then subjected to sexual crosses (eg. cross-pollinations, backcrosses, or additional imposed self-pollinations). Once the desired characteristics are fixed in the population they are crossed into an agronomically acceptable background. Eventually, the frequency of genes for the desired characteristic is sufficiently high in the population to produce a superior cultivar. This process is time consuming and expensive. In attempts to overcome both inbreeding depression and the ever increasing time/financial constraints on conventional breeding programs, canola breeders have incorporated biotechnology to produce doubled haploids into their programs. Incorporation of haploid breeding practices through biotechnology offers the potential production of superior varieties economically, and within a short time span.

2.2.3 Doubled Haploid Production

2.2.3.1 Advantages. The literature documents the occurrence of haploidy in plants more than 60 years ago (Blakeslee, et al., 1922; Kostoff, 1934). Haploids can be generally described as individuals which contain chromosome numbers which have been reduced to half of the normal diploid chromosome number. Specifically, haploids are individuals whose genomic constitution is the gametic chromosome number of the species. The usefulness of haploids in plant breeding has been discussed by many authors. (Choo et al., 1985; Foroughi-Wehr and Wenzel, 1989; Nitzsche and Wenzel, 1977; Pierik, 1987; Poehlman, 1987b; Ferrie et al., 1994; Sharp et al., 1984) Haploids are utilized in plant breeding programs because once their chromosomes have been doubled

(doubled haploids), they are completely homozygous at all loci. This homozygosity is reached in the shortest possible time, a savings of three to six generations of inbreeding, which permits rapid selection for desirable genotypes. Selection pressures applied at the doubled haploid level, as opposed to the diploid level, offer more reliable and effective results. Genetic segregation is simplified because recessive genes are not being masked by dominant genes. Induced dominant and recessive genetic changes produced through mutagenesis, gametoclonal selection or transfer of genetic material are therefore immediately detected resulting in a significant reduction in the number of plants that need to be screened for selection. Fewer plants will be carried forward in a breeding program which results in a financial and time savings. Additionally, there is the potential for quantitative and qualitative inheritance studies, genotype-environment interaction studies and linkage studies. (Choo et al., 1985; Foroughi-Wehr and Wenzel, 1989; Nitzsche and Wenzel, 1977; Pierik, 1987; Poehlman, 1987b; Ferrie et al., 1994; Sharp et al., 1984)

2.2.4 Methods of Haploid Production

In order for canola-quality oilseed rape breeding programs to exploit the advantages that doubled haploids offer, haploids must first be obtained, then the chromosome numbers of the haploids must be doubled. Haploids may occur in nature or be induced experimentally. Since spontaneous haploid plants are rare, (Morrison and Evans, 1988) breeders have investigated a number of *in vitro* techniques which allow the production of haploids in greater numbers.

The most frequently used method of haploid production for the *Brassica* species is androgenesis. The basic principles of androgenesis are to culture anthers/microspores to induce cell division and embryo formation, culture the embryo to form a haploid plant, and finally double the chromosomes of the haploid plant to produce a fertile doubled haploid. Since each microspore has the potential of embryogenetic development into a plant, all genetic variation of the population of microspores is potentially available (Ferrie et al., 1994).

Androgenesis has proven to be a cost-effective procedure in *B. napus* breeding programs by the fact that large numbers of haploid embryos can be produced in less than four weeks.

The androgenetic development and production of haploid embryos can be accomplished by two different, but related culture procedures; anther culture and microspore culture. Anther culture involves the culture of whole anthers, while microspore culture involves the culture of isolated microspores.

Anther culture has proven to be quite efficient in some species, however, embryos do not only arise from microspores, but also from other parts of the anther which results in a population with a number of different ploidy levels (Pickering and Devaux, 1992). In culture, the anther wall may act as a barrier to nutrients and treatments to the microspores (Heberle-Bors, 1989; Siebel and Pauls, 1989; Bajaj, 1983; Pickering and Devaux, 1992).

Anther culture was the original method of haploid production for the species of *Brassica* (Siebel and Pauls, 1989; Bajaj, 1983). The shortcomings and low production of haploid embryos from anther culture prompted the development of microspore culture. Microspore culture requires more skill and advanced equipment than anther culture (Heberle-Bors, 1989), but it is a superior technique because of its increased efficiency of true haploid embryo production (Siebel and Pauls, 1989; Bajaj, 1983).

In microspore culture, the microspore is developed directly into an embryo and therefore the physiology and biochemistry of the process could be studied from a starting point of a single cell (Heberle-Bors, 1989; Bajaj, 1983; Palmer and Keller, 1997). Each stage of development could then be easily monitored for treatment effects (Palmer and Keller, 1997). Additional advantages of microspore culture include the ease of microspore isolation from the anther, ability to adjust for optimum microspore densities (Palmer and Keller, 1997) and to enrich the population with embryogenic microspores.

2.3 Production of *Brassica rapa* Canola-Quality Oilseed Rape Through Microspore Culture

The efficient application of isolated microspore culture in *B. rapa* breeding programs has been limited to genotypes which respond with high, consistent embryo yields and plant regeneration. Success lies in the ability to manipulate microspores in vitro, so large numbers of genotypes respond with high embryo production and regeneration rates. There has been much success with well-established techniques of microspore culture for *B. napus* but limited work has been completed on *B. rapa*.

The technique of microspore culture for doubled haploid production is influenced by many factors including donor plant genotype, donor plant physiology, donor plant pretreatment, developmental stage of the pollen, media and its constituents and culture conditions. As well, the interaction of each of the individual factors plays a role in the induction of pollen embryogenesis.

2.3.1 Genotype

Numerous studies of genotypic influences on microspore culture in *Brassica* species have shown that responsiveness of microspores in culture are strongly dependent on genotype of the donor plant (Kuginuki et al., 1997; Baillie et al., 1992; Chuong et al., 1988; Phippen and Ockendon, 1990; Ockendon, 1985; Takahata and Keller, 1991; Foisset et al., 1993). It is clear that donor plant genotype can influence not only the quantity of embryos produced but also the quality of embryos produced (Chuong et al., 1988). The genetic control of embryo production is not understood well. However, it has been demonstrated that certain cultivars of *Brassica napus*, when used as parents in producing F_1 hybrids, influence embryo production in culture (Dunwell et al., 1985). Segregation analysis of isozyme markers on isolated microspore derived embryos of *Brassica napus* have also suggested the existence of androgenetic embryogenesis genes (Foisset et al., 1993). Therefore, the ability to transfer the

trait of embryogenic responsiveness to recalcitrant genotypes may be possible. This would allow the transfer of embryogenesis genes from embryogenic, but, agronomically unacceptable germplasm, into non-embryogenic, elite germplasm (Takahata and Keller, 1991; Foisset et al., 1993; Dunwell et al., 1985).

Embryogenesis is clearly influenced by genotypes. However, Gland et al., (1988) concluded that there is always the influence of non-genetic factors, as well as the interaction with the genetic factors, to consider. By manipulating the preculture and culture conditions, differences in embryo yields from microspores of any genotype can be expected. To date there has been no identification of androgenetic embryogenesis genes which can be easily crossed into any germplasm to make it embryogenic. This means that preculture and culture conditions and the subsequent interaction with genetic factors will have to be manipulated to allow the production of embryos from recalcitrant genotypes.

2.3.2 Donor Plant Physiology

The physiological status of the donor plants is the result of the environment or the growth conditions in which the donor plant is grown. The growth conditions of the donor plant seem to have a significant effect on the behaviour of microspores and subsequent production of embryos in culture of *Brassica* species (Powell, 1990; Keller et al., 1983; Dunwell et al., 1985; Arnison et al., 1990a; Lo and Pauls, 1992; Roulund et al., 1991; Palmer and Keller, 1997).

Growth temperature of the donor plant seems to play an important role in microspore embryogenesis. Lower temperature regimes resulted in greater embryo yields for *B. napus* (Dunwell et al., 1985; Lo and Pauls, 1992) and *B. rapa* (Baillie et al., 1992). However, a cold treatment of *B. oleracea* var. *italica*, broccoli, (Arnison et al., 1990a) resulted in a reduction in embryogenesis capacity while *B. oleracea* var. *capitata*, head cabbage, showed no critical response to donor plant growth temperatures (Roulund et al., 1991).

Donor plant inflorescence and plant age also play an important role in microspore embryogenesis. These factors may be dependent on genotype and species (Palmer and Keller, 1997). It has been reported that culture of young, rather than older inflorescences of *B. napus* result in greater production of embryos (Thurling and Chay, 1984; Chuong et al., 1988). A more recent study involving *B. napus* suggest that inflorescence and plant age may not affect embryogenesis as long as the optimum developmental stage of microspores is selected (Takahata et al., 1991).

Little information on other factors such as light intensity, photoperiod and nutritional status of the donor plant and how they affect microspore embryogenesis is available. Palmer and Keller (1997) suggest that these factors may interact with growth temperature to alter embryo yield.

The reason that donor plant physiology has such a significant effect on embryogenic response is not known. Lo and Pauls (1992) suggest that microspores of donor plants grown at low temperatures may be in an altered physiological state which is independent of the developmental stage of the microspores. These altered microspores then seem to follow a different developmental pathway which results in the production of embryos.

2.3.3 Microspore Pretreatment

Microspore pretreatment refers to treatment of the microspores (for microspore culture) or anthers (for anther culture) after they have been excised from the donor plant. Pretreatment of the microspores (or anthers) seems to simulate a stress situation which may be important in induction of androgenesis (Lichter, 1982; Touraev et al., 1997). The microspore pretreatment usually is in the form of a temperature regieme.

A cold temperature pretreatment has shown to be effective for *B. napus* (Lichter, 1982) and *B. oleracea*, cabbage (Osolnik et al., 1993), but ineffective for *B. rapa* (Keller et al., 1983). A thermal temperature pretreatment of winter varieties of rape (*B. napus*) was necessary for embryo production while spring

varieties produced acceptable embryo yields without such a treatment (Dunwell et al.,1985). Thermal shock in *B. oleracea* var. *gemiferae*, brussels sprouts (Ockendon, 1984) and *B. oleracea*, broccoli (Arnison et al., 1990a) proved effective for increased embryo production at certain temperatures and durations of treatments.

A short but severe heat shock of 41°C at the onset of microspore culture has been shown to be successful in inducing embryogenesis in late bicellular pollen of *B. napus* (Binarova et al., 1997).

The response of a temperature pretreatment of microspores seems to be quite unpredictable. Considerable differences in response to varied high temperature pretreatments not only between cultivars of *B. oleracea*, brussels sprouts but also between plants of the same cultivar suggest the impossibility of predicting optimum temperature pretreatments (Biddington and Robinson, 1990).

2.3.4 Developmental Stage of Pollen

Pollen grains are produced through a complex set of cell divisions. The origin of the potential for embryogenesis in immature pollen grains is under debate. One view is that microspores are dimorphic (Horner and Street, 1978; Heberle-Bors and Reinert, 1980). That is, they have a predetermined pathway of being either gametophytic or sporophytic. If the culture conditions are conducive for embryo development, a sporophytic microspore will develop into an embryo while gametophytic microspores degenerate and never have embryogenic capacity.

A second theory is that under certain stimuli, microspores have the ability to switch from a gametophytic to a sporophytic pathway in culture (Telmer et al., 1993; Kyo and Harada, 1985; Kyo and Harada, 1986; Sunderland and Haung, 1987; Benito-Moreno et al., 1988). This switch of pathway operates within a narrow period of microspore development. Before or after this developmental stage, the microspore generally cannot respond to inductive treatment (Palmer and Keller, 1997).

Cytologically, the disruption of the asymmetrical division of the first pollen mitosis of normal gametophytic development seems to be required for initiating embryogenesis. This disruption results in a symmetrical division resulting in two roughly equal cells when the developmental pathway is embryogenic (Palmer and Keller, 1997; Zaki and Dickinson, 1990; Zaki and Dickinson, 1991; Fan et al., 1988; Iqbal et al., 1994).

The early uninucleate stage of pollen development is characterized by a typically trilobed cell with a thin exine and large central diffuse nucleus containing scattered heterochromatin particles. Mid-uninucleate microspores have a well-developed exine displaying the tricopate nature of the wall with the large nucleus moving toward one of the lobes. The late uninucleate stage displays rigid walls with a heterochromatinless nucleus forced to the periphery of the cell by extensive vacuolation. (Kott et al., 1988a)

It is important to develop a method to select microspores which have the potential to develop into embryos and exclude those which do not (Telmer et al., 1992). Numerous parameters have been examined as potential markers for embryogenic microspores. Petal length, anther length, the ratio of petal/anther length and bud length have all been investigated as selectable markers (Duijs et al., 1992; Kieffer et al., 1993; Chuong et al., 1988 and Dunwell and Cornish, 1985). Bud length seems to be the most practical character. Using simple techniques, the predominant stage of microspore development can be determined for various bud sizes. New genotypes or any change of treatment to the donor plant may affect the bud length staging of microspores. This means that for each change in experimental conditions, microspore staging is required to determine optimal bud length.

The mid-late uninucleate stage of pollen development seems to be the most efficient stage for microspore embryogenesis for *B. rapa* (Ballie et al., 1992).

2.3.5 Culture Media

A major factor on the success of haploid production through androgenesis is the composition of culture medium (Keller and Armstrong, 1977). Anther

culture was the original method of haploid production for the species of *Brassica*. When microspore culture was proven to be superior, some modifications of the original anther culture media were made. Any nutritive function which the anther wall may have provided is no longer available to the developing microspores. These missing components now have to be replaced in the culture medium (Palmer and Keller, 1997).

The carbohydrate content of the media is a crucial constituent of the culture media (Dunwell and Thurling, 1985; Ballie et al., 1992). It serves both as a carbon source and an osmoticum (Powell, 1990). Sucrose is the most commonly used carbohydrate for culture media. The optimum sucrose concentration for microspore culture is higher than for anther culture. It is thought that the higher concentration of sucrose is needed for osmotic stability (Lichter, 1982; Dunwell and Thurling, 1985).

Higher levels of sucrose have been shown to be beneficial for the initial culture stages but lower levels are required for continuation of microspore division and survival (Dunwell and Thurling, 1985). Manipulating sucrose levels in the medium by utilizing a media change can increase the number of embryos produced. Ballie et al. (1992) reported that media containing 17% sucrose for 48 hours followed by a media change to 10% sucrose increased embryo production for all *B. rapa* genotypes tested. Keller and Armstrong (1977) conclude that the post-induction transfer of *B. napus* anthers to low sucrose levels may be necessary for embryos to develop into plantlets.

Recently it has been shown that sucrose as an osmoticum can be replaced by high-molecular-weight polyethylene glycol in *B. napus* microspore culture medium (Ilic-Grubor et al., 1998).

The addition of charcoal to fresh media has been found to be beneficial for development of embryoids in culture. The charcoal may be acting to remove any inhibitors which may be accumulating in the media (Gland et al., 1988; Lichter, 1989). Caution must be used when adding charcoal to media because repeated additions may result in concentrations sufficient enough to remove not only toxic substances but essential ingredients as well (Lichter, 1989).

It has been reported for microspore culture of *B. napus* cv. Topas, that medium conditioned by culturing microspores at high culture densities for one day improved embryo yields in low density cultures. (Huang et al., 1990).

Medium pH is another key factor for induction of microspores to produce embryos (Ballie et al., 1992). The adjustment of media pH has shown that embryos could be formed throughout wide pH ranges. The pH may act as a regulator of the efflux and influx of substances between the developing pollen and nutrient medium (Gland et al., 1988). Anther culture of *B. oleracea*, broccoli, cultivars Bravo and Green Mountain, responded best to the pH range of 5.5 - 5.8 (Arnison et al., 1990a). A pH of 6.2 yielded the best embryo production for *B. rapa* microspore cultures (Ballie et al., 1992). *B. napus* microspore cultures responded best to the pH range of 5.6 - 5.8 (Gland et al., 1988).

Experiments involving *B. napus* using NLN medium have shown that cultures devoid of hormones can produce embryos (Lichter, 1989). Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D), auxins, and N^6-benzyladenine (BA), a cytokinin, are commonly used in culture media for microspore culture. The inclusion of auxins and cytokinins in media in various concentrations, depending on species and genotype, can give significantly higher embryo yields (Lichter, 1989; Gland et al., 1988). Chuong and Beversdorf (1985) showed that NAA concentrations of 1 mg/l improved embryo yields of several genotypes of *B. napus* and one genotype of *B. carinata* Braun, mustard. Charne and Beversdorf (1988) report that NAA had no effect on embryo yields with concentrations of 0.136 - 1.85 mg/l while BA in concentrations of 0.01 - 0.225 mg/l had significant influence over embryo yield of F_1 hybrids of *B. napus*.

Increased concentrations of 2,4-D (0.3 mg/l) increased embryo yields in anther culture of *B. Oleracea* var *botrytis*, cauliflower (Phippen and Ockendon, 1990). Genotype-specific responses of anther culture of *B. oleracea*, broccoli resulted from the addition of BA (Arnison et al., 1990b).

The response to the addition of growth hormones to culture media shows high specificity to species and genotype (Gland et al., 1988). This may be due to genetic differences or even physiological status of the donor plant. It is clear that the response of genotypes to growth substances should be evaluated with new experimental lines of interest to ensure optimal embryo yields (Gland et al., 1988).

The inclusion of auxins and cytokinins as growth regulatory substances in culture media are well established and considered the norm. Inclusion of other plant growth regulators in culture media has rarely been attempted. It has been suggested that ethylene may be involved in microspore embryogenesis. Biddington and Robinson (1991) demonstrated that ethylene production in the early stages of anther culture of *B. oleracea*, brussels sprouts, was higher for the non-responsive cultivar Hal, as compared to the responsive cultivars GA1 x RDF2 and Grower. This lack of responsiveness to culture could be overcome by incorporating the ethylene-action inhibitor silver nitrate ($AgNO_3$) into the media (Biddington et al., 1988). The exact role that ethylene plays in microspore development into embryos is still unclear. There may be optimum levels of ethylene required by specific genotypes for embryogenesis which, when not reached or surpassed, may be detrimental to embryo yields (Cho and Kasha, 1989; Biddington and Robinson, 1991). This concentration of ethylene also may be dependent on the sensitivity of the microspore to it (Biddington et al., 1988).

2.3.6 Culture Conditions

Evaluation of incubation time and temperatures have shown that they are important factors in microspore embryogenesis (Ballie et al., 1992). Initial elevated incubation temperatures for many *Brassica* species like *B. rapa* (Ballie et al., 1992), *B. napus* (Keller and Armstrong, 1978; Chuong and Beversdorf, 1985), *B. oleracea* (Arnison et al., 1990a; Takahata and Keller, 1991), and *B. carinata* (Chuong and Beversdorf, 1985), resulted in the production of high frequencies of embryos in culture. This elevated temperature seems critical for a brief period in culture before transfer to lower culture temperatures. Ballie et al.

(1992) reported that the optimal microspore culture temperature treatment for *B. rapa* was 32°C for 48 hours followed by 24°C for the remainder of three weeks. Gland et al. (1988) showed that 35°C for 18 hours followed by 30°C for the remainder of culture period provided the best results for *B. napus*. The split temperature regime of 35°C for 72 hours followed by 25°C incubation resulted in high embryo yields for both *B. napus* and *B. carinata* (Chuong and Beversdorf, 1985). *B. oleracea*, broccoli anther cultures responded best to temperatures of 35°C for 18 - 48 hours followed by a maintenance temperature of 25°C (Arnison et al., 1990a).

Although the importance of using elevated culture temperatures for embryogenesis has been recognized, there is little information on the mechanism of the temperature response (Palmer and Keller, 1997). The possible involvement of heat shock proteins has been investigated in cultured anthers of broccoli (Fabijanski et al., 1991). Arnison et al. (1990a) support this possibility. It seems that the elevated culture temperature brings about a switch of microspore development away from the male gametophytic pathway to the sporophytic pathway. It is unclear if the high temperature acts as a stress factor or is required for specific metabolism (Palmer and Keller, 1997).

It is thought that the arrest of development and distortion of some embryos in culture may be due to the accumulation of inhibitors in the medium (Gland et al., 1988; Kott et al., 1988b; Hansen and Svinnset, 1993). Investigations into the accumulation of toxic agents being released into the culture media in *B. napus* microspore cultures have shown that older microspores that do not develop into embryos actually appear to release anti-embryogenic agents that suppress initiation and notably distort the growth and/or development of existing embryos (Kott et al., 1988b). Exclusion of older microspores in culture is in most cases impossible, so a media change becomes necessary to dilute the anti-embryogenic agents present in the media (Kott et al., 1988b). Using *Brassica napus* ssp. *rapifera*, swede, Hansen and Svinnset (1993) have shown that not only are toxic substances produced by larger,

binucleate microspores, but also smaller, uninucleate microspores. Changing the media too often may result in the disruption of development of the microspores (Lichter, 1989) so a compromise is necessary.

Implementation of aeration to microspore cultures of different *Brassica* species have been found to be beneficial for development of embryoids in culture. The aeration may be acting to remove any inhibitors which may be accumulating in the media (Lichter, 1989).

In addition to elevated temperature treatments, media changes and aeration, microspore density in culture also plays a role in embryogenesis. Ballie et al. (1992) report using 100,000 microspores/ml for microspore culture of *B. rapa* while Hansen and Svinnset (1993) and Huang et al. (1990) describe levels up to 40,000 microspores/ml for maximum embryo production in *B. napus*.

2.3.7 Plant Regeneration

Mature embryos produced through microspore culture are generally recovered via direct germination (Palmer and Keller, 1997). The efficient regeneration of haploid plants is an important factor in production of doubled haploids. Unfortunately direct germination of mature embryos is difficult and frequency is often low (Kott and Beversdorf, 1990). Periods of low temperature treatments, partial dessication and culture agitation appeared to increase both the quality and frequency of embryo germination for the *Brassica* species (Lichter, 1989; Gland et al., 1988, Coventry et al., 1988; Kott and Beversdorf, 1990; Mathias, 1988; Kott et al., 1988a). However, protocols must be modified for specific species and genotypes within species to ensure a high frequency of recovery (Palmer and Keller, 1997).

Spontaneous doubling of chromosomes through microspore culture, of Brassica species, is rare (Chen et al., 1994; Keller and Armstrong, 1978; Charne et al., 1988). Production of doubled haploid plants from haploid embryos produced through microspore culture has been primarily achieved through use of colchicine. Colchicine has been applied to microspore-derived plants, microspore-derived embryos and microspores (Chen et al., 1994; Iqbal et al.,

1994; Mathias and Robbelen, 1991). Colchicine as a microspore treatment has even been found to stimulate embryogenesis in some cases involving *B. napus* (Chen et al., 1994; Iqbal et al., 1994).

Additionally, antimicrotubule agents such as the herbicides amiprophos-methyl, oryzalin, pronamide and trifluralin, as well as low temperature treatments of varying magnitudes and duration have been used to double chromosomes at various stages of embryo development (Charne et al., 1988; Chen and Beversdorf, 1992a; Hanning, 1993; Eikenberry, 1993).

2.4 Utilization of Microspore Derived Embryos

Once the technology of microspore culture to produce doubled haploids became available to breeders, the initial interest was in the ability to accelerate plant breeding programs. Doubled haploids are completely homozygous at all loci. This rapid development of homozygous lines saves generations of inbreeding as compared with conventional breeding procedures. Genetic segregation is simplified because recessive genes are not being masked by dominant genes. (Choo et al., 1985; Foroughi-Wehr and Wenzel, 1989; Nitzsche and Wenzel, 1977; Pierik, 1987; Poehlman, 1987b; Ferrie et al., 1994; Sharp et al., 1984)

As culture technology becomes more advanced and efficient, more canola-quality oilseed rape breeding programmes are utilizing microspore culture to produce dominant and recessive genetic changes through gametoclonal selection, mutagenesis or transfer of genetic material. Haung et al. (1991) report that microspore culture is utilized routinely to produce homozygous diploid plants which are resistant to *Sclerotinia*. Kenyon et al. (1987) use microspore culture to select for sulfonylurea herbicide tolerance in *B. napus*. Henderson and Pauls (1992) used microspore culture to introgress several recessive traits into *B. napus* canola-quality oilseed rape . They concluded that there are definite advantages to using this system in the breeding programme.

Microspore mutagenesis has produced valuable canola-quality oilseed rape mutants for herbicide tolerance (Haung et al., 1991; Swanson et al., 1988; Swanson et al., 1989), altered fatty acid profiles (Haung et al., 1991) and resistance to diseases (*Phoma lingam*) (Sacristan, 1982).

Doubled haploid lines derived from microspore culture of *B. napus* have been used in studies of physiological and biochemical control of seed degreening (Johnson-Flanagan and Singh, 1993), fatty acid and lipid biosynthesis and storage (Pomeroy et al., 1991; Wiberg et al.,1991; Chen and Beversdorf, 1991; Gruber and Robbelen, 1991; Taylor et al., 1992a; Taylor et al.,

1992b; Taylor et al., 1993; Taylor et al., 1990; Weselake et al., 1993; Holbrook et al., 1991), and vernalization (Murphy and Scarth, 1998) and pyruvate-kinase isoenzyme studies (Sangwan et al., 1992) have been completed.

Genetic transformations of *B. napus* through use of *Agrobacterium tumefaciens* (Swanson and Erickson, 1989; Oelck et al., 1991) and electroporation (Jardinaud et al., 1993) has also been accomplished with haploid embryos of *B. napus* realized through microspore culture.

Inheritance studies of fatty acids (Chen and Beversdorf, 1990) and storage proteins (Taylor et al., 1990) have also been completed using doubled haploid lines of *B. napus* canola

The development of desiccation technology of haploid embryos is being studied as a potential method of long-term storage system of germplasm via cryopreservation (Chen and Beversdorf 1992b; Charne et al., 1988; Senaratna et al., 1991).

3.0 MATERIALS AND METHODS

3.1 Genotypes

Four *B. rapa* genotypes were selected for the experiments: Reward, Parkland, Agricultural Canada Line AC-1 and AC-2.

Reward and Parkland are canola-quality oilseed rape cultivars with good seed oil, protein content and disease resistance. Reward was developed at the University of Manitoba, Winnipeg, Manitoba and was registered in 1991 (Scarth et al., 1992). Parkland was developed at Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan (GRIN, 1998). AC-1 and AC-2 are highly embryogenic *B. rapa* lines produced by Agriculture and Agri-Food Canada.

Reward and Parkland were chosen as genotypes in this research because they are both well adapted to the canola growing areas of western Canada (Scarth et al., 1992; GRIN, 1998). Additionally, the Plant Biotechnology Institute's Consortium on microspore improvement for *B. rapa* involved research with Reward, Parkland, AC-1 and AC-2. I was fortunate to be the recipient of information on all of the genotypes in this research from the Consortium.

3.2 Standard Microspore Culture Protocol

3.2.1 Plant Growth Conditions

Ten plants of each genotype were seeded and maintained in growth room conditions of 16 hour photoperiod with a light intensity of 200 $\mu E\ m^{-2}s^{-1}$ and a day/night temperature of 20/15^{0}C. Two seeds per six inch plastic pot, were planted in a soilless mix supplemented with 14-14-14 Osmicote slow release fertilizer. After approximately 26 days, just prior to bolting, the plants were transferred to a growth cabinet with the same photoperiod and approximate light intensity and a lower day/night temperature of 10/5^{0}C. 0.35g L^{-1} 15-15-18 (N,P,K) was supplied in the watering, which occurred every second day. The

plants were allowed to grow and flower for approximately one month after which they were sampled for microspore culture for approximately one month.

3.2.2 Microspore Culture

Buds selected for microspore culture were between 2 and 3 mm in length. This range of bud sizes corresponds to the mid-late uninucleate stage of pollen development. Approximately 50 buds were selected per genotype and placed in Lipshaw baskets or tea balls. The buds were surface sterilized in 6% sodium hypochlorite for 15 minutes on a shaker. The sterilization process was followed by three 5 minute washes in sterile water. The buds were then transferred to a 50 ml beaker containing 5 ml of half strength B_5-13 wash media (half-strength B_5 media supplemented with 13% sucrose and no hormones). The buds were macerated in the media with a Teflon rod. 5 ml of wash media was used to pre-moisten a 44 μm nylon filter into a sterile 50 ml centrifuge tube. The suspension was then filtered through the nylon filter into the centrifuge tube. The filter and beaker were then rinsed into the centrifuge tube with 5 ml of wash media. The suspension was centrifuged at 130-150 g for 3 minutes. The supernatant was decanted and an additional 5 ml wash media was added to re-suspend the pellet. The washing of the microspores by centrifugation, was repeated again. After the second wash, a drop of the suspension was placed on a hemacytometer to determine the density of microspores in the 5 ml of suspension. After the third centrifugation, the supernatant was decanted and the pellet was re-suspended in the appropriate amount of NLN-17 media supplemented with 0.1mg L^{-1}BA, and with a pH of 6.2, to achieve an optimum microspore density of 100,000 microspores per ml. The suspension was then distributed into 100 mm X 15 mm petriplates in 10 ml quantities and the plates were sealed with parafilm. The plates were incubated in the dark at 32^{0}C for forty-eight hours after which the microspore suspension was pipetted off and centrifuged to pellet the microspores. The NLN-17 media was decanted and replaced with an equal amount of NLN-10 media at pH 6.2 which contained no

BA. The microspore suspension was then redistributed into the original petriplates which were then transferred to 25^{0}C for the remainder of three weeks. The plates were then placed on a shaker in continuous light at 22^{0}C to green the embryos up. One week later, total embryo production was tabulated. Embryos were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

3.3 Media Preparation

Preparation of media and media stocks are outlined in Appendix A.

4.0 EXPERIMENTAL PROTOCOL

4.1 Experiment 1- Effect of Sucrose Levels in the Microspore Culture Media on Embryo Production in *B. rapa*

The objective of this experiment was to determine the optimal levels of sucrose in microspore culture media based on the level of embryo production. The sucrose levels in the standard protocol were NLN-17 media supplemented with 0.1 mg BA L^{-1} for the first forty-eight hours of culture followed by a media change to NLN-10 (no BA) for the remainder of three weeks. The modification of the standard protocol was an alteration of the media sucrose concentrations to NLN-13 supplemented with 0.1 mg BA L^{-1} for the first forty-eight hours of culture followed by a media change to NLN-13 (no BA) for the remainder of three weeks.

Preparation of NLN-17 media supplemented with 0.1 mg BA L^{-1}, NLN-10 media (no BA), NLN-13 media supplemented with 0.1 mg BA L^{-1} and NLN-13 media (no BA) is described in Appendix A.

Six separate microspore culture runs were completed for this experiment, three with the media combination of NLN-17/ NLN-10 (control) and three with the media combination of NLN-13/NLN-13. Each run was completed using the four genotypes Reward, Parkland, AC-1 and AC-2 . Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

4.2 Experiment 2 - Effect of pH Levels of the Microspore Culture Media on Embryo Production in *B. rapa*

The objective of this experiment was to determine the optimal pH level in microspore culture media for *B. rapa* genotypes based on the level of embryo production. The pH level in the standard protocol for both the NLN-17 and NLN-10 media is 6.2. The modification of the standard protocol was an alteration of media pH concentrations to pH 6.0, pH 5.8, pH 5.6, pH 5.4 and pH 5.2.

Preparation of NLN-17 and NLN-10 media is described in Appendix A. Adjustment to appropriate pH levels in the media was accomplished by using 1 N HCl and 1 N NaOH to lower and raise the pH respectively.

Six NLN media pH levels were tested over 18 separate microspore culture runs. Three microspore culture runs per NLN media pH level [pH 6.2 (control), pH 6.0, pH 5.8, pH 5.6, pH 5.4 and pH 5.2] were completed. Each run used the genotypes Reward and AC-2. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

4.3 Experiment 3 - Effect of Microspore Pretreatment (Cold Shock) on Embryo Production in *B. rapa*

The objective of this experiment was to determine if a microspore pretreatment of a cold shock would increase microspore culture response in *B. rapa* genotypes based on the level of embryo production. The modification of the standard protocol involved cold shock treatment of microspores. Cold shock pretreatment to the microspores was accomplished by: 1. chilling the surface sterilant, sodium hypochlorite to 4°C prior to bud sterilization; 2. agitation of buds in surface sterilant on a bed of ice; 3. the sterile water used to rinse off surface sterilant was chilled to 4°C; 4. half strength B5-13 used as a wash media was

chilled to 4°C; 5. maceration of microspores with a Teflon rod took place on a bed of ice; and 6. initial culture media (NLN-17 media) was chilled to 4°C. The microspores were held at approximately 2-4°C for one hour during isolation.

Six separate microspore culture runs were completed for this experiment, three involving room temperature or control conditions, and three using cold shock procedures. Each run was completed using the four genotypes Reward, Parkland, AC-1 and AC-2. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

4.4 Experiment 4 - Large-Scale Microspore Culture Technique

The objective of this experiment was to determine if a large-scale, or mass microspore culture technique would be efficient in producing large numbers of haploid embryos. The protocol for large-scale microspore culture technique is based on Polsoni, et al. (1988), and Swanson, et al. (1987), reports. Forty plants were planted according to the standard protocol. Twenty bud clusters from the upper racemes of the forty plants were collected and all buds smaller than 4 mm were used in a microspore run. The sterilization in 6% sodium hypochlorite for 15 minutes on a shaker and three 5 minute washes in sterile water followed the standard protocol with increased volumes to sufficiently cover the increased number of buds. The buds were then transferred to a sterile small capacity blender containing 70 ml of half strength B_5-13 wash media. The bud clusters were blended at high speed until a slurry was formed (approximately 10 seconds). The slurry was then filtered through 44 μm nylon filter into two sterile 50 ml centrifuge tubes, each pre-moistened with 5 ml of wash media. The blender and two filters were then rinsed with 10 ml, 5 ml and 5 ml of wash media, respectfully. The suspension was then centrifuged at 130-150 g for 3 minutes. The supernatant was decanted and an additional 40 ml of half strength wash

media was added to each tube to re-suspend the pellets. The washing of the microspores was repeated again. After the second wash and resuspension in 20 ml of wash media per tube, a drop of the suspension from each tube was placed on a hemacytometer to determine the density of microspores in the 20 ml of suspensions as described previously. After the third spin, the supernatant was decanted and the pellets were re-suspended in the appropriate amounts of NLN-17 supplemented with 0.1 mg BA L^{-1}, and with a pH of 6.2, to achieve a microspore density of 100,000 microspores per ml. The suspensions were poured into 100 mm X 15 mm petriplates in 10 ml quantities and the plates were sealed with parafilm. Procedures for the incubation of microspores and tabulation/classification of embryos followed the standard protocol.

The large-scale microspore culture technique was tested over four separate microspore culture runs. Each run was completed using the genotype Reward. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

4.5 Experiment 5 - Effect of Donor Plant Pretreatment on Embryo Production in *B. rapa*

Experiment 5 evaluated the effect of donor plant pretreatment on embryo production in *B. rapa*. Ethephon (2-chloroethyl phosphonic acid) and the two growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride) were investigated as donor plant pretreatments.

Ethephon, an ethylene releasing compound, was used to investigate the role of ethylene in microspore response to culture for *B. rapa*. The growth A-Rest and Cycocel, were used to investigate if growth retardants could replace the requirement of low temperature environments to induce microspores into embryogenesis in culture for *B. rapa*.

Thirty plants, per pretreatment, were seeded in growth room conditions of 16 hour photoperiod with a light intensity of 200 $\mu E^{-2}s^{-1}$ and a day/night temperature of 20/15^{0}C. Plants were grown under these conditions for the duration of the experiment.

Visual assessments and microspore developmental stages were determined for the three donor plant pretreatments. Procedures for the microspore staging were adapted from Coventry et al. (1988). Microspore staging of bud lengths of 0-1 mm, 1-2 mm, 2-3 mm and 3-4mm were completed. For each donor plant pretreatment, collections of two buds for each bud length per treatment for the genotypes Reward and AC-2 were fixed in a cold fixative of alcohol:glacial acetic acid (3:1 v/v) in the refrigerator, overnight. If microscopic analysis was not completed the following day, the buds were stored in 70% alcohol in the refrigerator. Collections continued for the duration of the experiment. Each bud was squashed on a microscope slide, in a drop of acetocarmine stain. After a few minutes the debris was removed with a needle and a drop of 45% acetic acid was added and the sample was covered with a cover slip. The slide was placed under a phase-contrast microscope at 10X power. One field of view for each bud was tabulated to determine the percent of each microspore stage present. The microspores were grouped into one of the following stage categories:

1. Tetrad: - very early uninucleate stage of microspore development, spores in tetrad.
2. Early Uninucleate: - thin spore walls, no colouring; central diffuse nucleus.
3. Mid- to Late-Uninucleate: - mid-uninucleate stage shows well developed exine, pale yellow with well developed lobes, central nucleus; late-uninucleate stage shows exine to be rigid and strongly yellow with define lobes, nucleous peripheral.

4.5.1 Effect of Ethylene on Microspore Response to Culture

Approximately 36 days after seeding, the first flowers began to open. At this point, foliar applications of Ethephon were applied with a hand held spray-pump bottle to the point of runoff. Three donor plant pretreatments of Ethephon (ten plants per treatment), of 0 ml/l (0 ppm), 1.5 ml/l (58 ppm) and 3.0 ml/l (115 ppm) were sprayed on three consecutive days, day 36, 37 and 38 after seeding. The pretreatment solutions were prepared using double distilled water and herbicide grade Ethephon at 39 mg a.i. per ml.

Three separate microspore culture runs on day 41, 42 and 43 after seeding were completed for the three foliar pretreatments using the genotypes Reward and AC-2. The standard protocol for the collection, isolation and culture of microspores was followed. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

Microspore staging was completed five days before the Ethephon pretreatment, day 31 to day 35 after planting, and continued on for a total of 14 days, day 44 after planting.

4.5.2 The Effect of Growth Retardants on Microspore Embryogenesis

4.5.2.1 A-Rest. Approximately 38 days after seeding, the first flowers began to open. At this point, drench applications (60 ml) of A-Rest were applied to the soil. Three donor plant pretreatments of A-Rest (ten plants per treatment), of 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm) were administered for three consecutive days. The pretreatment solutions were prepared using double distilled water and herbicide grade A-Rest at 0.264 mg a.i. per ml.

Five separate microspore culture runs of the three soil drench pretreatments were completed using the genotypes Reward and AC-2 at 43-47 days after seeding. The standard protocol for the collection, isolation and culture of microspores was followed. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

Microspore staging was completed five days before the A-rest pretreatment, day 33 to day 37 after planting, and continued on for a total of 16 days, day 48 after planting.

4.5.2.2. Cycocel. Approximately 38 days after seeding, the first flowers began to open. At this point, foliar applications of Cycocel were applied with a hand held spray-pump bottle to the point of runoff. Three donor plant pretreatments of Cycocel (ten plants per treatment), of 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm) were administered for three consecutive days. The pretreatment solutions were prepared using double distilled water and herbicide grade Cycocel at 118 mg a.i. per ml.

Three separate microspore culture runs of the three foliar pretreatments were completed using the genotypes Reward and AC-2 at 45-47 days after seeding. The standard protocol for the collection, isolation and culture of microspores was followed. Embryos produced were classified as green or albino for the following stages: globular, heart, torpedo and cotyledon.

Microspore staging was completed six days before the Cycocel pretreatment, day 32 to day 37 after planting, and continued on for a total of 17 days, day 48 after planting.

5.0 RESULTS AND DISCUSSION

5.1 Experiment 1 - Effect of Sucrose Levels in the Microspore Culture Media on Embryo Production in *B. rapa*

Experiment 1 was an evaluation of the effect of sucrose levels in the microspore culture media on embryo production of *B. rapa*. Many studies have been conducted on this area of microspore culture improvement, with varying results. In 1992, Baillie et al. reported that the highest number of embryos were formed for *B. rapa* genotypes when the microspores were cultured in NLN-17 media (0.1 mg BA L^{-1}) for forty-eight hours followed by a media change to NLN-10 (0BA) for the remainder of three weeks. Research had shown that Reward, a cultivar used in this project, actually responded better to the media combination of NLN-13 (0.1 mg BA L^{-1})/NLN-13 (0BA) (pers. comm.- Dr. A. Ferrie). The potential for increased embryo production with the altered media sucrose level was investigated.

The complete data set of embryo production is included in Appendix B, Table B-1. The total number of embryos per run for each treatment and genotype is summarized in Appendix B, Tables B2-B5.

The total number of embryos produced includes embryos classified as green or albino for the following stages: globular, heart, torpedo and cotyledon. Albino embryos were produced throughout the experiment with no predictable pattern. Because albino embryos lack the capacity to develop into haploid plants, green embryo production was used for analysis and discussion through out this research.

The total number of green embryos per run for each treatment and genotype is summarized in Tables 1-4. Total green embryo yields show that the two AC genotypes seem to respond better to the media sucrose combination of NLN-13/NLN-13, while Reward seems to respond better to the media sucrose combination of NLN-17/NLN-10. Parkland does not respond differently to the two media sucrose levels.

TABLE 1. Green Embryo Production of *B. rapa*, Reward, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	69	3
2	11	2
3	0	0
TOTAL	80	5

TABLE 2. Green Embryo Production of *B. rapa*, Parkland, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	0	0
2	3	2
3	3	3
TOTAL	6	5

TABLE 3. Green Embryo Production of *B. rapa*, AC-1, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	5	21
2	1	0
3	0	0
TOTAL	6	21

TABLE 4. Green Embryo Production of *B. rapa*, AC-2, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	3	102
2	0	8
3	0	0
TOTAL	3	110

An analysis of variance was run on the green embryo data investigating media sucrose levels, genotypes and repetitions and interactions between media sucrose level and genotypes. At the 5% significance level, there were no statistically identifiable differences for the model statement, or between the individually analysed factors and interactions (Appendix C, Table C-1).

Despite the fact the analysis did not show genotypes to be statistically different, mean green embryo yields show that two of the four genotypes (Reward and AC-2) respond to media sucrose levels (Table 5). Reward produces a higher mean number of green embryos for the standard media sucrose level of NLN-17/NLN-10 while AC-2 produces a higher mean number of green embryos for the media sucrose level of NLN-13/NLN-13. AC-1 also produces a higher mean number of green embryos for the media sucrose level of NLN-13/NLN-13, however the difference is not as great as the previous two genotypes. Parkland has a similar green embryo yield for the two media sucrose levels.

TABLE 5. Mean Green Embryo Yields for Media Sucrose Level Experiment, [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13] Using *B. rapa* Genotypes Reward, Parkland, AC-1 and AC-2.

Genotype	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 Control	NLN-13/NLN-13
	Green Embryo Yield (mean)	Green Embryo Yield (mean)
Reward	26.7	1.7
Parkland	2.0	1.7
AC-1	2.0	7.0
AC-2	1.0	36.6

When the calculation was made of embryos produced per 100 buds sampled (Table 6), the differences in genotype response to media sucrose levels is apparent for Reward and AC-2. This indicates that different genotypes may require different sucrose concentrations for optimal embryo production.

TABLE 6. Green Embryos Per 100 Buds Produced by *B. rapa*, Reward, Parkland, AC-1 and AC-2 for Media Sucrose Levels [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Genotype	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 Control	NLN-13/NLN-13
	Embryos Per 100 Buds*	
Reward	53.3	3.3
Parkland	4.0	3.3
AC-1	4.0	14.0
AC-2	2.0	73.3

*Embryos per 100 buds calculation based on 150 buds used to complete 3 microspore culture runs in the media sucrose level experiment.

Based on reports (pers. comm.- Dr. A. Ferrie), it was expected that Reward would have produced a greater mean of green embryos with the media sucrose combination of NLN-13/NLN-13. However, the results of this experiment indicate that Reward produces a greater mean of green embryos for the standard media sucrose level of NLN-17/NLN-10. Perhaps lab to lab variation in addition to different growing facilities caused this difference in results.

5.2 Experiment 2 - Effect of pH Levels of the Microspore Culture Media on Embryo Production in *B. rapa*

Experiment 2 was an evaluation of the effect of pH levels of the microspore culture media on embryo production of *B. rapa*. In 1992, Baillie et al., showed that most *B. rapa* embryos were formed when microspores were cultured at pH 6.2 in NLN-17 for forty-eight hours followed by a media change to NLN-10, pH 6.2 for the remainder of three weeks. Research had shown that a lower pH culture media may actually produce more embryos (pers. comm.- Dr. A. Ferrie). Additionally, Gland et al., (1988) found that a pH of 5.7-6.0 was the most suitable for *B. napus* embryo production in culture. The potential for increased embryo production with altered media pH levels was investigated.

The complete data set of embryo production is included in Appendix B, Table B-6. The total number of embryos per run for each treatment and genotype is summarized in Appendix B, Tables B7 - B8. Albino embryos were produced throughout this experiment with no predictable pattern.

The total number of green embryos per run for each treatment and genotype is summarized in Tables 7-8.

TABLE 7. Green Embryo Production of *B. rapa*, Reward, for Media pH Level Experiment [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

	Microspore Culture Media pH Levels					
Run	6.2 (Control)	6	5.8	5.6	5.4	5.2
1	10	1	80	32	47	107
2	14	140	2	125	40	0
3	62	0	2	0	6	0
TOTAL	86	141	84	157	93	107

TABLE 8. Green Embryo Production of *B. rapa*, AC-2, for Media pH Level Experiment [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

Run	Microspore Culture Media pH Levels					
	6.2 (Control)	6	5.8	5.6	5.4	5.2
1	0	81	23	0	0	129
2	0	11	51	5	202	18
3	199	1	0	16	0	27
TOTAL	199	93	74	21	202	174

An analysis of variance was run on the data investigating media pH levels, repetitions, genotypes and interactions between media pH levels and genotypes. At the 5% significance level, there were no statistically identifiable differences for the model statement or between the individually analysed factors and interactions (Appendix C, Table 2).

Despite the fact the analysis did not show genotypes to be statistically different, mean green embryo yields of the individual genotypes at different media pH levels showed varying results (Table 9). Reward responded best to a media pH level of 5.6 followed by pH 6.0 > pH 5.2 > pH 5.4 > pH 6.2 and > pH 5.8. AC-2 responded the best to a media pH level of 5.4 followed by ph 6.2 > pH 5.2 > pH 6.0 < pH 5.8 and >pH 5.6. The control media pH level produced the second lowest mean embryo yields for Reward, and second highest mean embryo yields for AC-2.

TABLE 9. Mean Green Embryo Yields for Media pH Level Experiment, [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2] Using *B. rapa*, Reward and AC-2.

Genotype	Microspore Culture Media pH Levels					
	6.2 Control	6.0	5.8	5.6	5.4	5.2
	Green Embryo Yield (mean)					
Reward	28.7	47.0	28.0	52.3	31.0	35.7
AC-2	66.3	31.0	24.7	7.0	67.3	58.0

Embryos produced per 100 buds sampled (Table 10) also reflect the differences in response of the media pH levels by genotype. This indicates that different genotypes may require different pH levels for optimal embryo production.

Based on reports (pers. comm.- Dr. A. Ferrie), it was expected that both genotypes would have produced more embryos with the lower pH levels. The results indicate that Reward does produce more embryos with the lower pH levels. With exception to pH 5.8 (which was very similar to the control pH 6.2), all lower pH levels tested produced more embryos than the control (pH 6.2). AC-2 seemed to have a split response to the range of pH levels tested. pH 5.4 produced the highest embryo yields for AC-2, followed very closely by pH 6.2 (control). The lowest pH level tested, pH 5.2, produced the third highest embryo yields for AC-2. The middle range of the pH levels tested for AC-2 (pH 6.0, pH 5.8, pH 5.6) did not produce as many embryos as the higher and lower ranges of the pH levels tested. The results indicate that embryos can be formed under a wide range of pH levels. Gland et al. (1988) found that several genotypes of *B. napus* produced embryoids at pH levels as high as 7.0. They advise that experiments with recalcitrant genotypes cultured at higher pH levels may be beneficial.

TABLE 10. Green Embryos Per 100 Buds Produced by *B. rapa*, Reward and AC-2 for Media pH Levels [6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

Microspore Culture Media pH Levels	Genotype	
	Reward	AC-2
	Embryos Per 100 Buds*	
6.2 - Control	57.3	132.7
6.0	94.0	62.0
5.8	56.0	49.3
5.6	104.7	14.0
5.4	62.0	134.7
5.2	71.3	116.0

*Embryos per 100 buds calculation based on 150 buds used to complete 3 microspore culture runs in the media pH level experiment.

5.3 Experiment 3 - Effect of Microspore Cold Shock Pretreatment on Embryo Production in *B. rapa* Microspore Culture

Stress resulting from bud harvest (water stress and starvation) or cold treatment may signal a microspore to begin embryogenesis (Martensson and Widell, 1993). Experiment 3 was an evaluation of the effect of microspore pretreatment in the form of a cold shock on embryo production in *B. rapa*. Research had indicated that a shock to the microspores would potentially increase the yield of embryos produced (pers. comm.- Dr. A. Ferrie). Feng and Wolyn (1993) found that a cold treatment to the flowers for *Asparagus officinalis* L. (asparagus), for three days at 5°C gave the best microspore culture response. The effect of cold shock on embryo production of the microspores was investigated.

The complete data set of total embryo production is included in Appendix B, Table B-9. The number of total embryos per run for each treatment and genotypes is summarized in Appendix B, Tables B10 - B13. Albino embryos were produced throughout this experiment with no predictable pattern.

The total number of green embryos per run for each treatment and genotype is summarized in Tables 11-14.

TABLE 11. Green Embryo Production of *B. rapa*, Reward, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	0	20
3	2	8
TOTAL	2	28

TABLE 12. Green Embryo Production of *B. rapa*, Parkland, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	1	0
3	1	1
TOTAL	2	1

TABLE 13. Green Embryo Production of *B. rapa*, AC-1, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	0	3
3	1	3
TOTAL	1	6

TABLE 14. Green Embryo Production of *B. rapa*, AC-2, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	2	0
3	5	3
TOTAL	7	3

An analysis of variance was run on the data investigating microspore pretreatments, repetitions, genotypes and interactions between microspore pretreatments and genotypes. At the 5% significance level, there were no statistically identifiable differences for the model statement or between the individually analysed factors and interactions (Appendix C, Table C3).

Despite the fact that the analysis did not show microspore pretreatments to be statistically different, mean green embryos show differences in mean values (Table 15) for the two microspore pretreatments for Reward. Parkland, AC-1 and AC-2 show similar responses to microspore pretreatments.

TABLE 15. Mean Green Embryo Production for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) or Cold Shock] Using *B. rapa* Genotypes Reward, Parkland, AC-1 and AC-2.

Genotype	Microspore Pretreatment	
	Room Temperature Control	Cold Shock
	Green Embryo Yield (mean)	Green Embryo Yield (mean)
Reward	0.67	9.34
Parkland	0.67	0.34
AC-1	0.34	2.00
AC-2	3.34	1.00

Green embryos per 100 buds sampled (Table 16) also reflected the differences in response to the two microspore pretreatments for Reward. Based on reports (pers. comm.- Dr. A. Ferrie), it was expected that a shock to the microspores could potentially increase the yield of embryos produced. Reward and AC-1 produced higher mean yields of embryos when the microspore cold shock pretreatment was used while the room temperature (control) pretreatment produced higher mean yields for Parkland and AC-2. The most dramatic increase of embryo yields were for the cold shock microspore pretreatment for Reward, which resulted in 18.6 embryos produced per 100 buds sampled. This

represents over a fourteen fold increase in embryos produced per 100 buds over the room temperature (control) pretreatment. There was an indication of higher response after cold pretreatment for AC-1, although green embryos numbers were very low. The results indicate that some genotypes may require different shock procedures to obtain optimal embryo production.

TABLE 16. Green Embryos Per 100 Buds yields by *B. rapa*, Reward, Parkland, AC-1 and AC-2 for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) or Cold Shock].

Genotype	Microspore Culture Media Sucrose Levels	
	Room Temperature Control	Cold Shock
	Embryos Per 100 Buds*	
Reward	1.3	18.6
Parkland	1.3	0.6
AC-1	0.6	4.0
AC-2	4.6	2.0

*Embryos per 100 buds calculation based on 150 buds used to complete 3 microspore culture runs in the media sucrose level experiment.

The value of the improvement in embryo yields with the cold shock pretreatment must take into account the considerable impracticality of the procedure. The modification of the room temperature microspore pretreatment (standard protocol) involved agitating buds in surface sterilant on a bed of ice as well as maceration of microspores on a bed of ice to achieve temperatures of 2-4 ^{0}C during the procedure. These procedures were cumbersome and time consuming. Increased proficiency may produce higher embryo yields. Utilization of a cold lab may alleviate the difficulty of the procedures preformed on beds of ice and eliminate exposure to the non-sterile environment caused by retrieving and replacing chilled media in the refrigerator.

5.4 Experiment 4 - Large-Scale Microspore Culture Technique

Experiment 4 was an investigation of a large-scale, or mass microspore culture technique of microspore culture and its effects on embryo production in *B. rapa*. The variable nature of the microspore culture process was reflected in the fact that no two runs under identical conditions, for the same genotype, produce the same results. This variability of response is common in *Brassica* tissue culture experimental analysis as well as for other species (Phippen and Ockendon, 1990). *B. rapa* is an outcrossing species and genetic variability between individual plants exists. The plant to plant variation may be overcome by using a large number of buds from a number of different plants in a large scale microspore culture technique.

The rapid mechanical isolation of microspores from buds in a mass microspore culture technique also may be able to decrease some of the inconsistency of response to microspore culture by reducing interaction times between potentially detrimental factors (anther wall, pollen or bud tissue) and standardizing the treatments between buds (Swanson et al., 1987). Additionally, the mass microspore culture methodology would enable the efficient and cost effective production of large numbers of haploid embryos to be utilized in *B. rapa* breeding programs (Swanson et al., 1987; Polsoni et al., 1988).

The complete data set of embryo production is included in Appendix B, Table B-14.

Table 17 shows the total number of green embryos produced in each run of the large-scale microspore culture technique.

TABLE 17. Total Green Embryo Production for Each Run in the Large-Scale Microspore Culture Technique Experiment Using *B. rapa*, Reward.

	Large Scale Microspore Culture Technique			
Genotype	Run 1	Run 2	Run 3	Run 4
Reward	1	16	1	3

Polsoni et al. (1988) and Swanson et al. (1987) report that utilizing a large-scale microspore culture system would result in very large quantities of liberated microspores which would produce a high frequency of embryo yields for *B. napus.* The large scale microspore culture technique was successful in liberating, on average, approximately 21 x 10^6 microspores per run, or approximately 21 plates (100,000 microspores per ml per plate) per run. This represents a large increase from the 3 to 4 plates per run of the standard microspore culture protocol. The efficiency of liberating large numbers of microspores was accomplished. However, it was expected that this technique would result in consistently high embryo yields. The procedure time of a standard microspore culture run was decreased quite substantially during the large scale microspore culture technique, but the procedure produced consistently low embryo yields.

Although this experiment did not produce many green embryos, no control was run for this experiment. Some factor completely unrelated to the large scale microspore culture technique may have caused the low embryo yields. A microspore culture run following the standard protocol could serve as a control.

5.5 Experiment 5 - Effect of Donor Plant Pretreatment on Embryo Production in *B. rapa*

Experiment 5 was an evaluation of the effect of donor plant pretreatment on embryo production in *B. rapa*. Ethephon (2-chloroethyl phosphonic acid) and the two growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride) were investigated as donor plant pretreatments.

5.5.1 Effect of Ethylene on Microspore Response to Culture

Optimum levels of ethylene production are thought to increase the responsiveness of microspore culture of *B. oleracea*, brussels sprouts (Biddington and Robinson, 1991; Biddington et al., 1988). The differences in genotype response to microspore culture may be the result of both the capacity of a genotype to produce ethylene (Cho and Kasha, 1989; Biddington and Robinson, 1991) and sensitivity to high ethylene levels (Biddington et al., 1988). Ethephon (2-chloroethyl phosphonic acid), an ethylene releasing compound, was used to investigate the role of ethylene in microspore culture response of *B. rapa*.

No embryos were produced for any of the donor plant pretreatments or either genotype, including the 0 ppm pretreatment. Visual observations of the Ethephon donor plant pretreatments of Reward and AC-2 were made over a 15 day period. The physical appearance of both the Reward and AC-2 plants sprayed with 58 ppm Ethephon did not significantly vary from that of the 0 ppm pretreatment which was used as a standard for comparison. However, the 115 ppm pretreatment showed very mild signs of wilting, yellowing and spotting of the leaves almost immediately after the first application of Ethephon. These symptoms continued to appear throughout the experimental period.

The complete data set of microspore developmental stages for the three donor plant pretreatments in bud lengths of 0-1 mm, 1-2 mm, 2-3 mm and 3-4mm, for Reward and AC-2, is included in Appendix D, Tables D1 and D2.

Figure 2 and 3 show the percent of microspores in mid-late uninucleate stage of development from buds 2-3 mm in length for the *B. rapa* genotypes Reward and AC-2. This bud length was chosen because this was the size of bud used in the microspore culture standard protocol.

Until day 41 the percent of microspores in the mid-late uninucleate stage of development for Reward stayed above 50% except for 0 ppm on day 32 (45%) (Figure 2). After day 41, the day of the first microspore culture run for this experiment, the percent of microspores in the mid-late uninucleate stage of development for the 115 ppm Ethephon pretreatment drops to 19%. The 0 ppm and 58 ppm Ethephon pretreatments were at 61% and 51% respectively. All three Ethephon pretreatments have over 59% of microspores in the mid-late uninucleate stage of development at day 42. Both the 58 ppm and 115 ppm Ethephon pretreatments produced 0% microspores in the mid-late uninucleate stage of development while the 0 ppm pretreatment produces 49% at day 43.

After donor plant pretreatments are applied, the highest pretreatment of Ethephon (115 ppm) always results in a lower percent of microspores in the mid-late uninucleate stage of development than the 0 ppm pretreatment (Figure 2). The 58 ppm pretreatment fluctuates above and below the level of microspores in the mid-late uninucleate stage of development for the 0 ppm pretreatment. Additionally, around day 42 there was a decline in the amount of microspores in the mid-late uninucleate stage of development for all Ethephon donor plant pretreatments, including the 0 ppm pretreatment. It was only speculation that the decline would have persisted past day 44.

Until day 42 the percent of microspores in the mid-late uninucleate stage of development for AC-2 stayed above 53% except for 0 ppm on day 39 (15%) (Figure 3). The percent of microspores in the mid-late uninucleate stage of development for all three Ethephon pretreatments is above 59% on day 41.

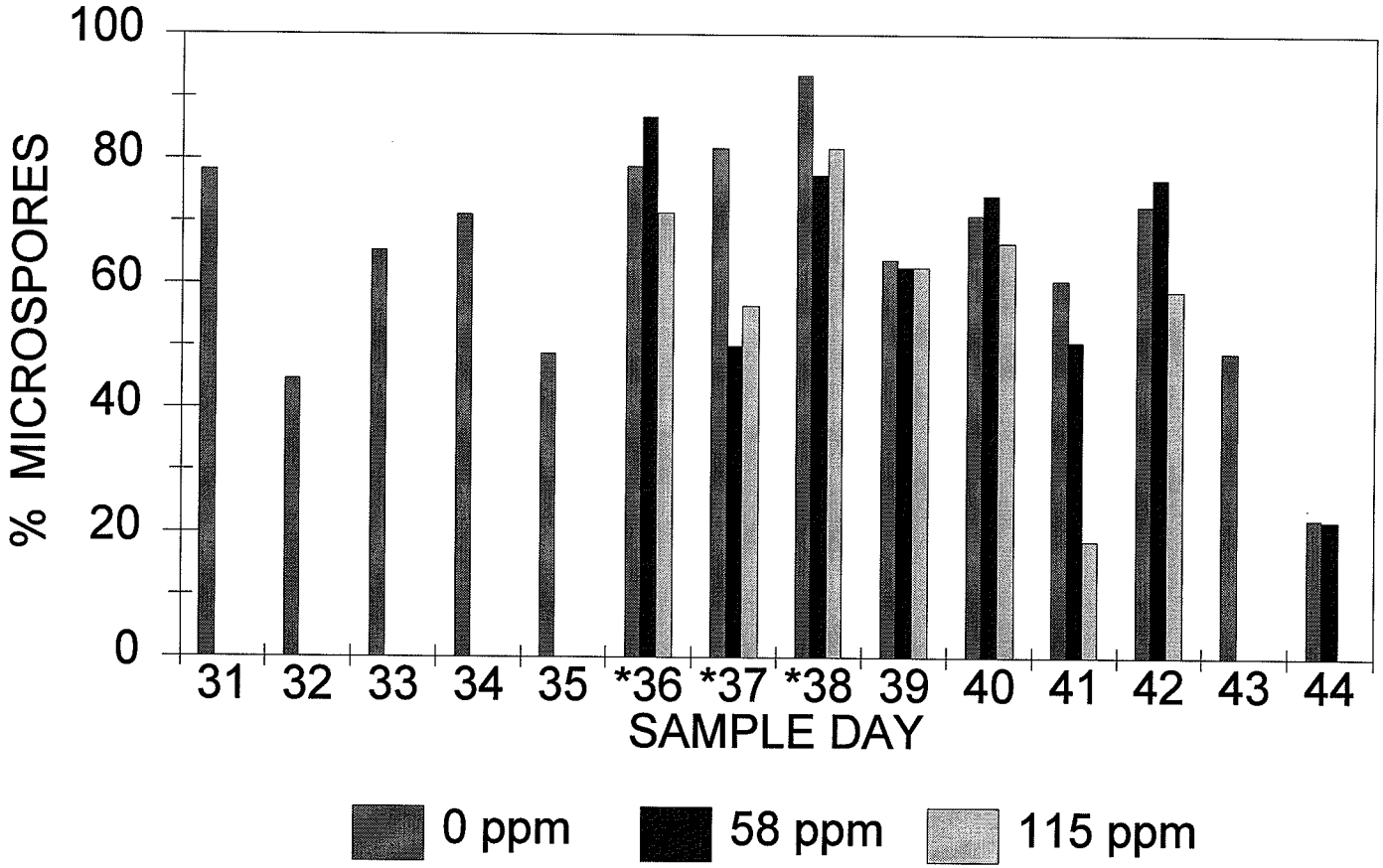

FIGURE 2. ***B. rapa*, Reward - Growth Regulator Ethephon. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

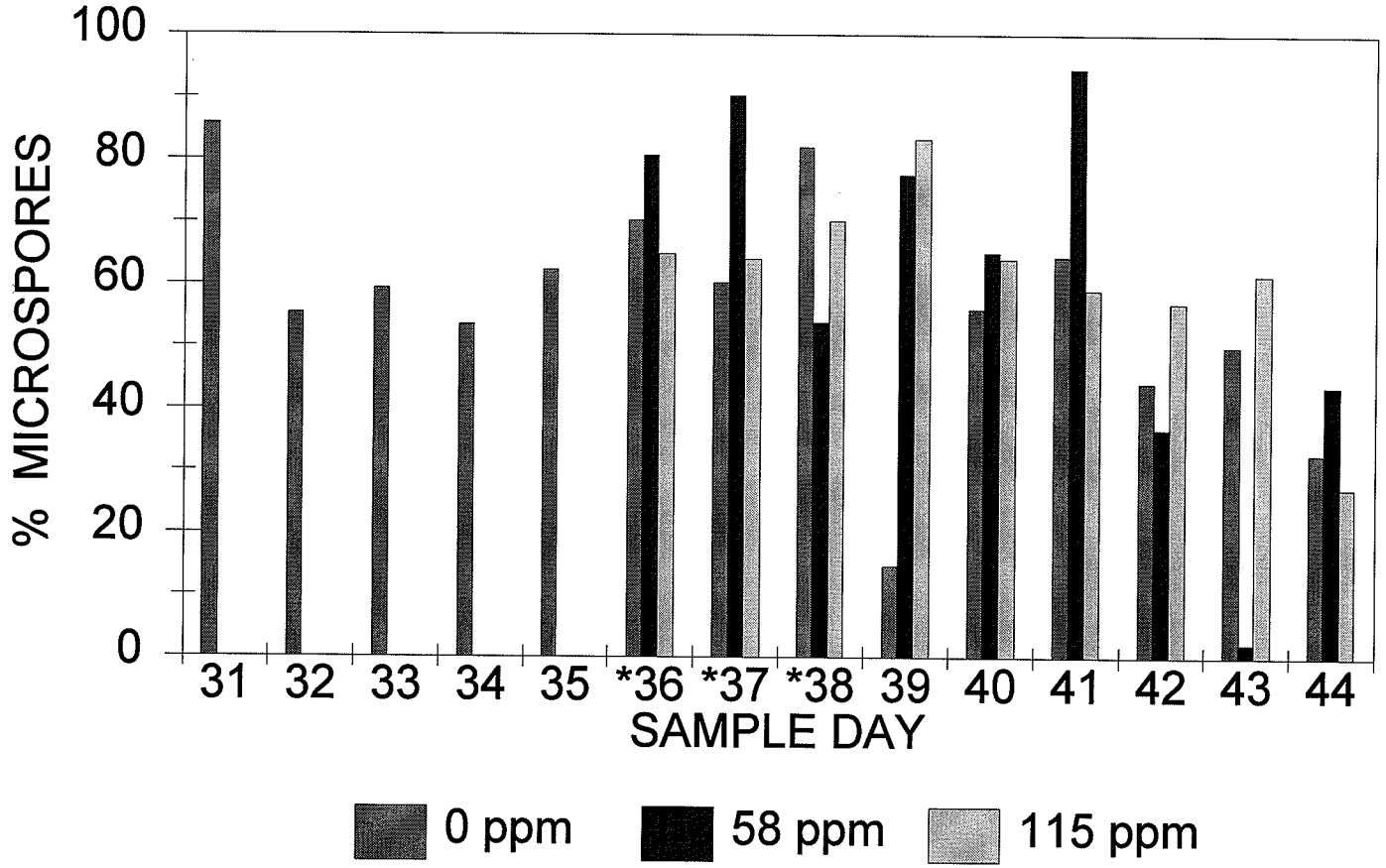

FIGURE 3. ***B. rapa*, AC-2 - Growth Regulator Ethephon. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

There is a drop of microspores in the mid-late uninucleate stage of development to 44%, 37%, and 57% for 0 ppm, 58 ppm and 115 ppm Ethephon pretreatments respectively on day 42. Both the 0 ppm and 115 ppm Ethephon pretreatments produce over 50% of microspores in the mid-late uninucleate stage of development while the 58 ppm pretreatment produces only 2% on day 43.

After donor plant pretreatments are applied, the 58 ppm and 115 ppm pretreatments of Ethephon fluctuate above and below the level of microspores in the mid-late uninucleate stage of development for the 0 ppm pretreatment (Figure 3). Additionally, around day 41 there was a decline in the amount of microspores in the mid-late uninucleate stage of development for all Ethephon donor plant pretreatments, including the 0 ppm pretreatment. It would have been interesting to see if the decline persisted past day 44.

5.5.2 The Effect of Growth Retardants on Microspore Embryogenesis

The change in the developmental pathway of a microspore from a highly specialized form to a embryonic form operates within a narrow period of microspore development (Palmer and Keller, 1997; Pechan and Keller, 1988). If growth was slowed down, or retarded, this period of microspore development could be lengthened to allow more microspores to be successfully induced into embryogenesis. The standard protocol used for *B. rapa* microspore culture involves the growth of the donor plants in a low temperature environment of 10/5^{0}C (day/night) temperatures as opposed to a standard temperature regime of 20/15^{0}C (day/night). It is conceivable that the cold environment is in fact lengthening the period of microspore development resulting in more microspores being induced into embryogenesis. The growth cabinets which could maintain these low temperature conditions are expensive and prone to breakdown. Most research facilities do not have access to reliable cold temperature cabinets large enough to support the populations of donor plants needed to run a microspore culture program. A possible alternative to the use of cold temperatures is the

use of growth retardants. The growth retardants A-Rest [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidin-5-yl)benzyl alcohol] and Cycocel (2-chloroethyl trimethyl-ammonium-chloride), were used.

5.5.2.1 A-Rest. The complete data set of embryo production is included in Appendix B, Table B-15. The total number of embryos per run for each donor plant pretreatment and genotype is summarised in Appendix B, Tables B16 - B17. Albino embryos were produced throughout the experiment with no predictable pattern.

The total number of green embryos per run for each treatment and genotype is summarized in Tables 18-19.

TABLE 18. Green Embryo Production of *B. rapa*, Reward, for Donor Plant Pretreatment Experiment - Drench Applications of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	4 ppm	8 ppm
1	0	0	2
2	0	0	0
3	0	0	1
4	0	0	1
5	0	0	0
TOTAL	0	0	4

TABLE 19. Green Embryo Production of *B. rapa*, AC-2, for Donor Plant Pretreatment Experiment - Drench Applications of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	4 ppm	8 ppm
1	1	1	0
2	0	0	0
3	0	0	0
4	1	1	0
5	0	0	0
TOTAL	2	2	0

The experiment was set up as a completely randomized design with three factors, donor plant growth retardant pretreatments, genotypes and repetitions. The statistical model statement included the terms Green Embryos = Donor Plant Growth Retardant Pretreatments Repetitions Genotypes and Donor Plant Growth Retardant Pretreatments x Genotypes. An analysis of variance was run on the data investigating these factors and interactions between donor plant growth retardant pretreatments and genotypes.

At the 1% significance level, there were statistically identifiable differences for the model statement (Table 20). When looking at the components of the statistical model statement, donor plant growth retardant pretreatments and genotypes were not shown to be significantly different. The significance of the model comes from repetitions having a significant PF>F value of 0.0214 (*significant at the 5% level), and the interactions of donor plant growth retardant pretreatments and genotypes having a PF>F value of 0.0028 (**significant at the 1% level). This shows that the mean green embryos produced for the three donor plant growth retardant pretreatments were not significantly different from one another. However, the mean green embryos produced over the five repetitions as well as the interactions between the donor plant growth retardant pretreatments and genotypes were significantly different.

TABLE 20. Analysis of Variance Results for Donor Plant Growth Retardant Pretreatment Experiment - Drench pretreatments of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm) Using *B. rapa* Genotypes Reward and AC-2.

Source	Degrees of Freedom	ANOVA MS	F-Value	PF>F
Model Statement	9	0.54	3.60	0.0081**
Error	20	0.15	R-Square = 0.618644	CV = 145.2369
Growth Retardant Pretreatments	2	0.13	0.89	0.4267
Repetitions	4	0.55	3.67	0.0214*
Genotypes	1	0.00	0.00	1.00
Growth Retardant Pretreatments X Genotype	2	1.2	8.00	0.0028**

The calculated coefficient of variance (CV) for the experiment was 145.24%. The CV value was very high and thus reflects low reliability or repeatability of the experiment. The r-squared value shows that only 61.86% of the observed variation was explained by the statistical model. A contributing factor to the high CV and unaccounted variation was the fact that the repetitions of the same donor plant pretreatments were highly variable (Tables 18-19). Some of the variability is probably due to the plant to plant differences among the ten plants being sampled per genotype.

Mean green embryo yields of Reward and AC-2 for the three A-Rest pretreatments of 0, 4 and 8 ppm in the donor plant growth retardant pretreatment experiment are presented in Table 21. Donor plant pretreatments were not statistically different from one another. Mean green embryo yields show Reward to have mean values of 0.00, 0.00 and 0.80 for 0, 4 and 8 ppm A-Rest pretreatments, respectively. AC-2 has mean values of 0.40, 0.40 and 0.00 for 0,

4 and 8 ppm A-Rest pretreatments, respectively. Genotypes were also not statistically different from one another, in fact the PF>F was 1.00.

TABLE 21. Mean Green Embryo Yields for *B. rapa* Genotypes Reward and AC-2 for the Donor Plant Growth Retardant Pretreatment Experiment - Drench pretreatments of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Genotype	ppm of A-Rest Drench		
	0 ppm Embryo Yield (mean)	4 ppm Embryo Yield (mean)	8 ppm Embryo Yield (mean)
Reward	0.00	0.00	0.80
AC-2	0.40	0.40	0.00

Green embryos per 100 buds produced by *B. rapa* genotypes Reward and AC-2 for the three A-Rest donor plant pretreatments of 0, 4 and 8 ppm in the donor plant growth retardant pretreatment experiment are presented in Table 22.

TABLE 22. Green Embryos Per 100 Buds Produced by *B. rapa*, Reward and AC-2 for the Donor Plant Growth Retardant Pretreatment Experiment - Drench pretreatments of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i.l/pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Genotype	ppm of A-Rest Drench		
	0 ppm	4 ppm	8 ppm
	Embryos Per 100 Buds*		
Reward	0.0	0.0	1.6
AC-2	0.8	0.8	0.0

*Embryos per 100 buds calculation based on 250 buds used to complete 5 microspore culture runs in the media sucrose level experiment.

The 0 ppm and 4 ppm A-Rest pretreatments produced no embryos while the 8 ppm A-Rest pretreatment produced 1.6 embryos per 100 buds sampled. AC-2 produced 0.8 embryos per 100 buds sampled for both the 0 ppm and 4 ppm A-Rest pretreatment and the 8 ppm A-Rest pretreatment produced no embryos.

Visual observations of the A-Rest donor plant pretreatments of Reward and AC-2 were made over a 15 day period. Reward plants drenched with 0 ppm A-Rest were used as a standard for comparison. The 4 ppm and 8 ppm pretreatment drenches began to wilt shortly after pretreatment (approximately 2 days). For three days the 4 ppm pretreatments showed very mild signs of wilting which developed into more severe wilting. At this point, the Reward plants began to recover and eventually regained the physical appearance of healthy plants (0 ppm pretreatment). The 8 ppm pretreatment immediately showed more severe wilting which persisted longer than the 4 ppm pretreatment. The wilting progressed for approximately 5 days before any signs of recovery were observed. The Reward plants never completely recovered from the 8 ppm pretreatment.

AC-2 plants drenched with 0 ppm A-Rest were used as a standard for comparison. AC-2 plants drenched with 4 and 8 ppm A-Rest showed the same wilting response as the Reward plants did but with greater severity. The 4 ppm pretreatment was slightly less affected than the 8 ppm pretreatment. Signs of recovery of the plants were not evident until seven days after pretreatment. Neither the 4 or 8 ppm treated AC-2 plants fully recovered from the pretreatments in the duration of this experiment.

The complete data set of microspore developmental stages for the three donor plant donor plant pretreatments in bud lengths of 0-1 mm, 1-2 mm, 2-3 mm and 3-4mm, for Reward and AC-2, are presented in Appendix D, Tables D3 and D4.

The percent of microspores in mid-late uninucleate stage of development from buds 2-3 mm in length for the *B. rapa* genotypes Reward and AC-2 is

shown in Figure 4 and 5. This bud length was chosen because this was the size of bud used in the microspore culture standard protocol.

At 38 days after planting and on, the percent of microspores in the mid-late uninucleate stage of development, for Reward, for the 0 ppm pretreatment of the growth retardant A-Rest showed a sharp increase Figure 4). Before the 38 day point, the highest recorded percent of microspores in the mid-late uninucleate stage of development was 42% on day 35. Throughout the experiment, the percent of microspores in the mid-late uninucleate stage of development for 0 ppm never drops below this percentage. Days 38, 39 and 40 after planting were the pretreatment days for the growth retardant A-Rest. With the exception of the 86% value at day 40 for the 4 ppm pretreatment, there appeared to be no effect on the percent of microspores in the mid-late uninucleate stage of development. However, after day 41, through to the end of the experiment, excluding day 47, the data showed that the 0 ppm pretreatment of A-Rest always results in a higher percent of microspores in the mid-late uninucleate stage of development than the 4 ppm and 8 ppm pretreatments. This suggests that the 4 ppm and 8 ppm pretreatments of A-Rest on Reward have a negative effect on the percent of microspores in the mid-late uninucleate stage of development. However, no differences in severity between the 8 ppm and 4 ppm pretreatment could be detected.

At 38 days after planting, the percent of microspores in the mid-late uninucleate stage of development, for AC-2, for the 0 ppm pretreatment of the growth retardant A-Rest started to decline (Figure 5). This gradual decline to the end of the experiment holds for all donor plant pretreatments of the growth retardant, including the 0 ppm pretreatment. The percent of microspores in the mid-late uninucleate stage of development does not seem to be effected differentially for the three donor plant pretreatments of the growth retardant.

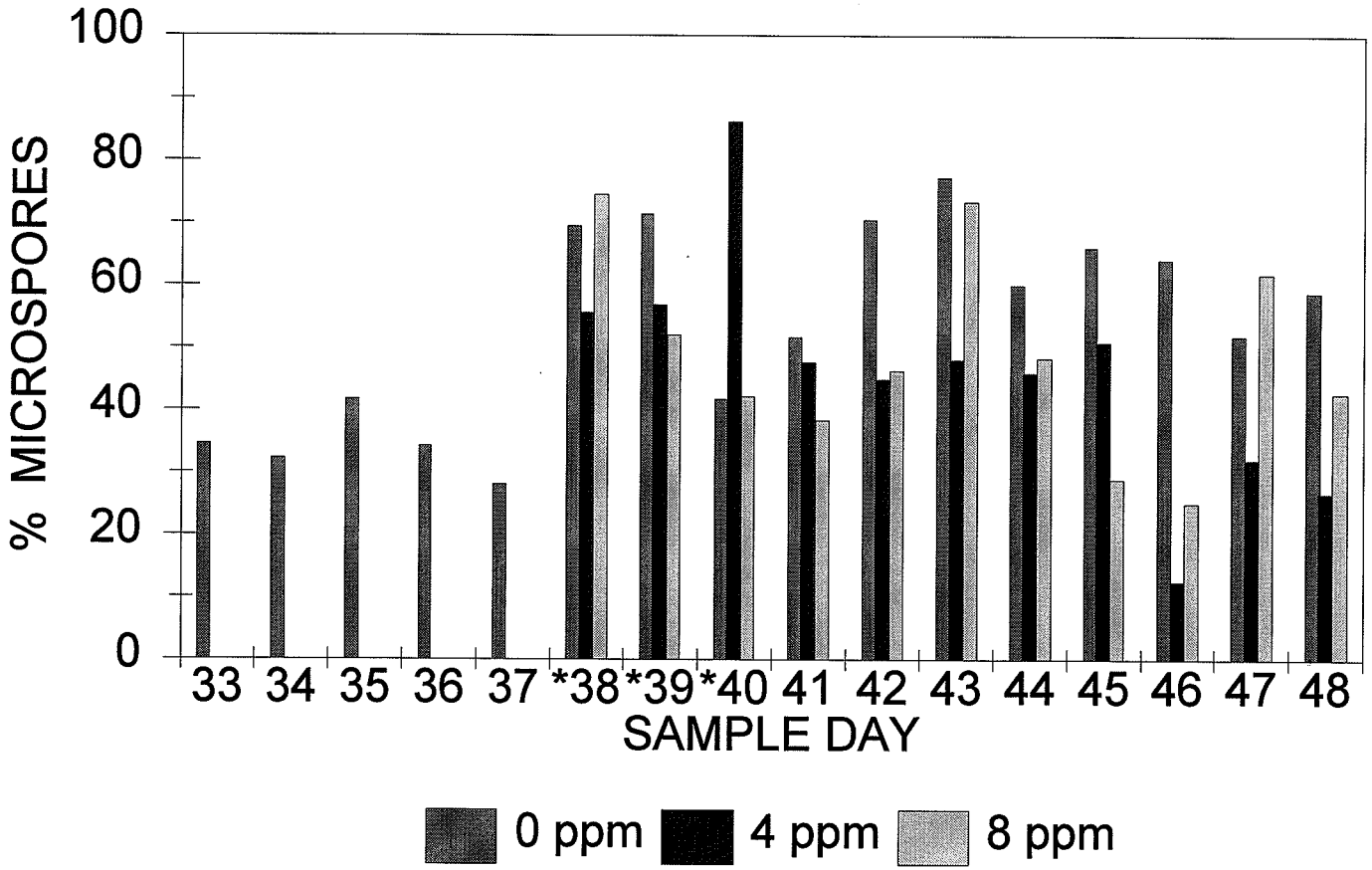

FIGURE 4. ***B. rapa*, Reward - Growth Retardant A-Rest. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

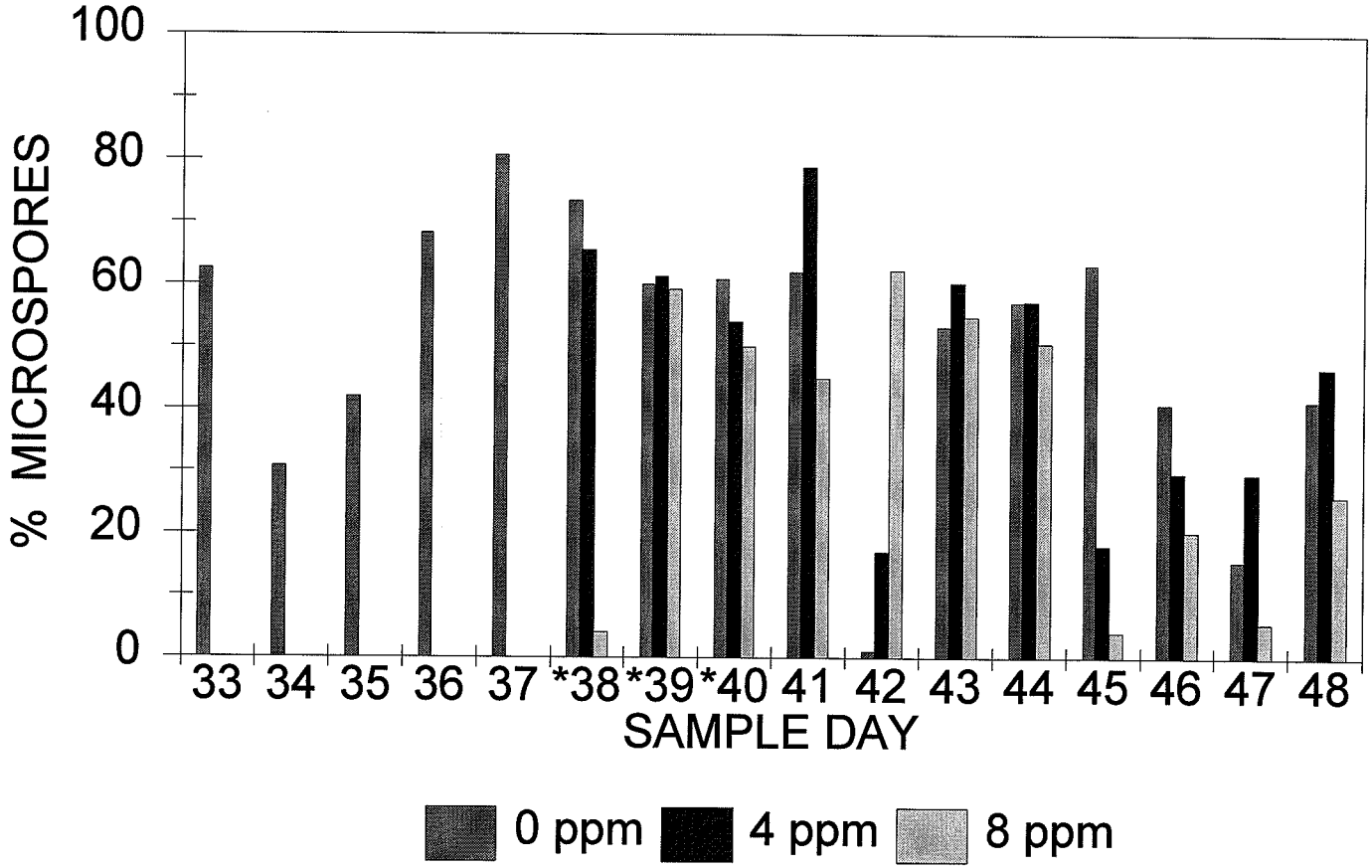

FIGURE 5. ***B. rapa*, AC-2 - Growth Retardant A-Rest. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

5.5.2.2 Cycocel. A complete data set of total embryo production is in Appendix B, Table B-18. The total number of green embryos per run for each pretreatment and genotype is summarized in Appendix B, Tables B19 - B20. Albino embryos were produced throughout the experiment with no predictable pattern. Because albino embryos lack the capacity to develop into haploid plants, green embryo production was used for analysis and discussion.

The total number of green embryos per run for each treatment and genotype is summarized in Tables 23-24.

TABLE 23. Green Embryo Production of *B. rapa*, Reward, for Donor Plant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

Run	Cycocel Spray Treatments		
	0 ppm (control)	3000 ppm	6000 ppm
1	0	2	2
2	0	0	0
3	5	0	0
TOTAL	5	2	2

TABLE 24. Green Embryo Production of *B. rapa*, AC-2, for Donor Plant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	3000 ppm	6000 ppm
1	0	0	0
2	1	0	0
3	1	2	0
TOTAL	2	2	0

An analysis of variance was run on the green embryo data investigating donor plant growth retardant pretreatments, genotypes and repetitions and interactions between donor plant growth retardant pretreatments and genotype.

At the 5% significance level, there were no statistically identifiable differences for the model statement, or between the individually analysed factors and interactions (Appendix C, Table C4).

Mean green embryo production of Reward and AC-2 and for the three Cycocel pretreatments of 0, 4 and 8 ppm in the donor plant growth retardant pretreatment experiment are presented in Table 25. Reward produced a mean value of 1.67 for the 0 ppm pretreatment of Cycocel and a mean value of 0.67 for both the 3000 and 6000 ppm Cycocel pretreatments. AC-2 produced mean values of 0.67, 0.67 and 0.00 for 0, 3000 and 6000 ppm Cycocel pretreatment, respectively.

TABLE 25. Mean Green Embryo Production for *B. rapa* Genotypes Reward and AC-2 for the Donor Plant Growth Retardant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

Genotype	ppm of Cycocel Spray		
	0 ppm Embryo Yield (mean)	3000 ppm Embryo Yield (mean)	6000 ppm Embryo Yield (mean)
Reward	1.67	0.67	0.67
AC-2	0.67	0.67	0.00

Green embryos per 100 buds produced by *B. rapa* genotypes Reward and AC-2 for the three Cycocel spray pretreatments of 0, 3000 and 6000 ppm in the donor plant growth retardant pretreatment experiment are presented in Table 26.

TABLE 26. Green Embryos Per 100 Buds Produced by *B. rapa*, Reward and AC-2 for the Donor Plant Growth Retardant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

Genotype	ppm of Cycocel Spray		
	0	3000	6000
	Embryos Per 100 Buds*		
Reward	3.3	1.3	1.3
AC-2	1.3	1.3	0

*Embryos per 100 buds calculation based on 150 buds used to complete 3 microspore culture runs in the media sucrose level experiment.

The 0 ppm Cycocel pretreatment produces 3.3 embryos per 100 buds sampled while the 3000 ppm and 6000ppm Cycocel pretreatment produce only 1.3 embryos per 100 buds sampled. AC-2 produces 1.3 embryos per 100 buds sampled for both the 0 ppm and 3000 ppm Cycocel pretreatment and the 6000 ppm Cycocel pretreatment produces no embryos.

Visual observations of the Cycocel donor plant pretreatments of Reward and AC-2 were made over a 14 day period. Reward plants sprayed with 0 ppm Cycocel were used as a standard for comparison. The 3000 ppm and 6000 ppm spray pretreatments began to show mild wilting shortly after treatment (approximately 24 hours). At the four day point, the wilting symptoms of the 3000 ppm pretreatment of Cycocel began to disappear and the plants eventually regained a more healthy appearance comparable to the 0 ppm pretreatment. In the 24 hour post-treatment, the 6000 ppm pretreatment of Cycocel began to show more severe symptoms of wilting. The wilting continued for seven days after pretreatment before showing signs of recovery. The Reward plants never completely recovered from either the 3000 ppm or 6000 ppm Cycocel pretreatments. At the end of the experiment the Reward plants still had a slightly wilted leaves.

AC-2 plants treated with 0 ppm Cycocel were used as a standard for comparison. The AC-2 plants treated with 3000 ppm and 6000 ppm Cycocel showed identical physical symptoms as the Reward plants. The AC-2 plants

never completely recovered from either the 3000 ppm or 6000 ppm Cycocel pretreatments. At the end of the experiment the plants still had a slightly wilted leaves.

A complete data set of microspore developmental stages for the three donor plant pretreatments in bud lengths of 0-1 mm, 1-2 mm, 2-3 mm and 3-4mm, for Reward and AC-2, are presented in Appendix D, Tables D5 and D6.

The percent of microspores in mid-late uninucleate stage of development from buds 2-3 mm in length for the *B. rapa* genotypes Reward and AC-2 as shown in Figure 6 and 7. This length of buds was chosen because this was the size of bud used in the microspore culture standard protocol. The three spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm) were administered on day 38, 39 and 40 and the microspore runs took place on day 45, 46 and 47.

Throughout the experiment, the percent of microspores in the mid-late uninucleate stage of development, for Reward, for the three donor plant pretreatments of the growth retardant Cycocel did not seem to be effected differentially (Figure 6).

The percent of microspores in the mid-late uninucleate stage of development, for AC-2, for the 0 ppm pretreatment of the growth retardant Cycocel appeared to be stable throughout the experiment (Figure 7). Days 38, 39 and 40 after planting were the treatment days for the growth retardant Cycocel. The initial effects of the 3000 ppm and 6000 ppm pretreatments of Cycocel seem to cause a decline in the percent of microspores in the mid-late uninucleate stage of development (days 38 and 39). This decline was followed by an increase (days 40 to 41) and another decrease (days 42 to 43). After day 44, the percent of microspores in the mid-late uninucleate stage of development, for AC-2, appeared to be stable to the end of the experiment.

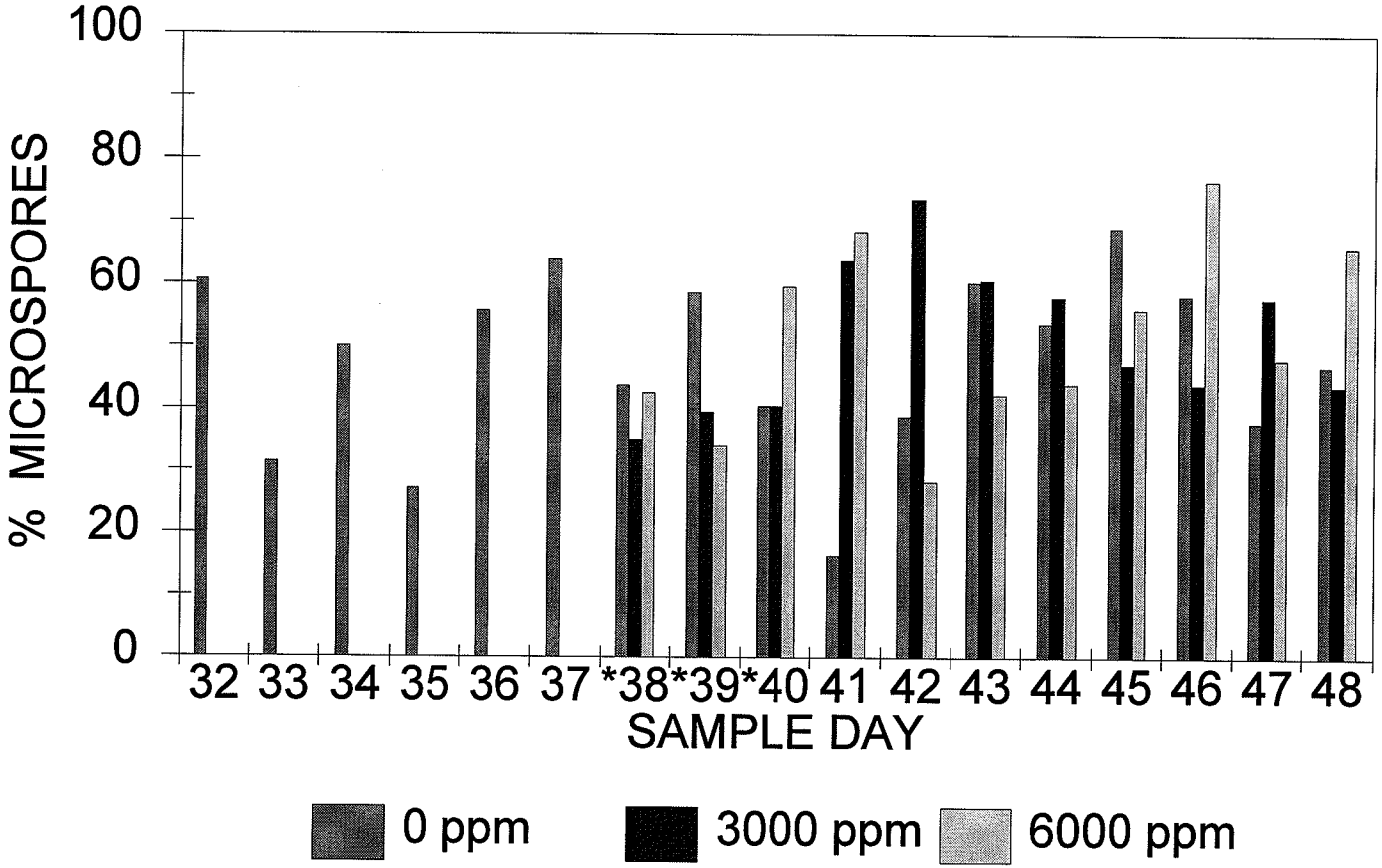

FIGURE 6. ***B. rapa*, Reward - Growth Retardant Cycocel. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

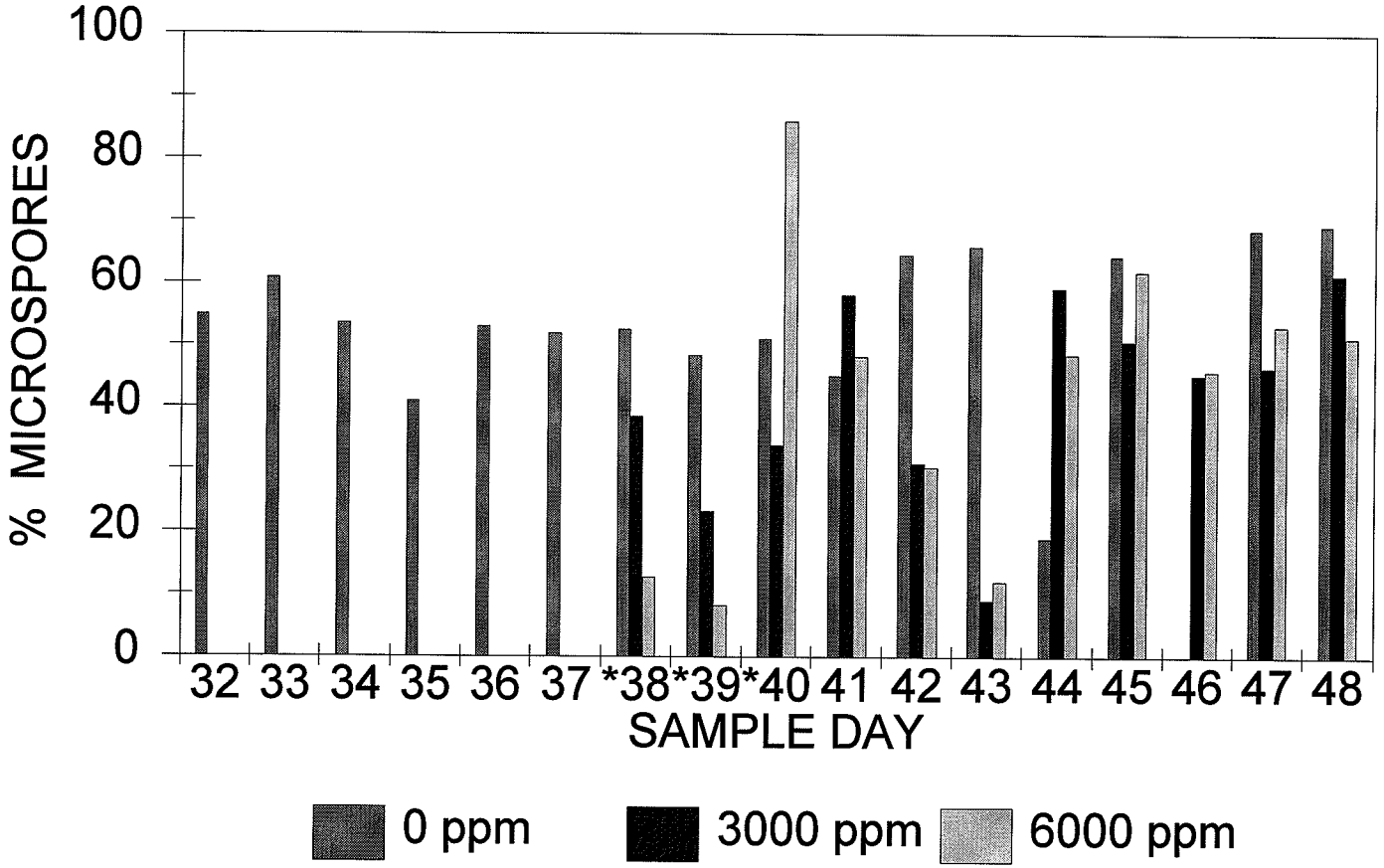

FIGURE 7. ***B. rapa*, AC-2 - Growth Retardant Cycocel. % Microspores in Mid-Late Uninucleate Stage of Development from Buds 2-3 mm in Length. *= Treatment Days.**

No embryos were produced for any of the Ethephon donor plant pretreatments and very low numbers of embryos were produced for the growth retardants A-Rest and Cycocel. However, no control was run. The experimental growing conditions did not follow the standard protocol. The growth cabinet which was able to hold these conditions was unavailable. This experiment, with a control run under standard growth conditions would have been the ideal situation. In addition to this, visual symptoms of the pretreatments showed signs of toxicity to the plants. Without a simultaneously run microspore culture following the standard protocol as a control, other factors which may have been reducing the success of the microspore culture cannot be identified.

6.0 CONCLUSIONS

Experiment 1 evaluated the potential for increased embryo production of *B. rapa* (Reward, Parkland, AC-1 and AC-2) with altered microspore media sucrose levels (NLN-17/NLN-10 (control) and NLN-13/NLN-13). The results of the experiment indicate that different genotypes require different media sucrose levels to produce optimal numbers of embryos. No statistical differences between any of the factors or interactions between factors were identified in the analysis. However, when the media sucrose level data was examined as individual means or embryos produced per 100 buds for individual genotypes, differences could be identified. Reward appeared to respond better to the standard media sucrose combination of NLN-17/NLN-10 while AC-2 responded better to the media sucrose combination of NLN-13/NLN-13. Parkland and AC-1 did not show a differential response to media sucrose levels.

Experiment 2 evaluated the potential for increased embryo production of *B. rapa* (Reward and AC-2) with altered microspore culture media pH levels (pH 6.2 (control), pH 6.0, pH 5.8, pH 5.6, pH 5.4 and pH 5.2). The results of the experiment indicate that different genotypes require different media pH levels to produce optimal numbers of embryos. No statistical differences between any of the factors or interactions between factors were identified in the analysis. However, when the media pH level data was examined as individual means or embryos produced per 100 buds for individual genotypes, differences could be identified. Based on mean green embryo yields and embryos per 100 buds, for individual genotypes, Reward may respond best to lower media pH levels than the standard (pH 6.2) while AC-2 may respond to both lower (pH 5.2 - pH 5.4) and higher (ph 6.2) media pH levels.

Experiment 3 was an evaluation of potential for increased embryo production of *B. rapa* (Reward, Parkland, AC-1 and AC-2) with a microspore pretreatment in the form of a cold shock. The results indicate that different genotypes may respond differently to cold shock pretreatments. No statistical

differences between any of the factors or interactions between factors were identified in the analysis. However, when the data was examined as individual mean green embryo yields and embryos produced per 100 buds, for individual genotypes, differences could be identified. Reward and AC-1 may respond better to the microspore cold shock pretreatment while Parkland and AC-2 may respond better to the room temperature microspore pretreatment. The value of the improvement in embryo yields with the cold shock pretreatment must take into account the considerable impracticality of the procedure.

Experiment 4 was an evaluation of a large-scale, or mass microspore culture technique of microspore culture and its effects on embryo production in *B. rapa*. The large scale microspore culture technique was successful in liberating large numbers of microspores. The procedure time of a standard microspore culture run was decreased quite substantially during the large scale microspore culture technique. However, the procedure produced consistently low embryo yields.

Although this experiment did not produce many green embryos, no control was run for this experiment. A control should have been run simultaneously to identify other factors which may have been influencing the outcome of the microspore culture process. With no control, the experimental results were inconclusive.

Experiment 5 was an evaluation of the effect of a donor plant pretreatment on embryo production in *B. rapa*. The ethylene releasing compound Ethephon was used to investigate the role of ethylene in microspore culture response of *B. rapa*. No embryos were produced for any of the Ethephon donor plant pretreatments for either genotype, including the 0 ppm pretreatment. Microspore staging data suggests that the 115 ppm treatment of Ethephon has a negative effect on Reward. The percent of microspores in the mid-late uninucleate stage of development does not seem to be affected differentially for the three treatments of Ethephon on AC-2.

Additional experiments run with the standard protocol growing conditions, additional pretreatments using ethylene inhibitors and promoters, as well as application directly to culture would shed some light on the role of ethylene in microspore response to culture.

The growth retardants A-Rest and Cycocel were used to investigate if a growth retardant could replace the requirement of low temperature growing conditions for the donor plants in the microspore culture of *B. rapa*. The analysis showed donor plant growth retardant pretreatments of A-Rest and genotypes to be not significant. However, significant differences of replications (significant at the 5% level), and the interactions of donor plant A-Rest pretreatments and genotypes (significant at the 1% level) were identified. The analysis showed no statistically identifiable differences between the individually analysed factors and interactions of the growth retardant Cycocel.

Microspore staging observations suggest that the 4 ppm and 8 ppm treatments of A-Rest on Reward have a negative effect on the percent of microspores in the mid-late uninucleate stage of development. However, no differences in severity between the 8 ppm and 4 ppm pretreatment could be detected. The percent of microspores in the mid-late uninucleate stage of development does not seem to be affected differentially for the three treatments of the growth retardant A-Rest on AC-2. Microspore staging observations suggest that the percent of microspores in the mid-late uninucleate stage of development does not seem to be affected differentially for the three pretreatments of the growth retardant Cycocel on Reward. However, the 3000 ppm and 6000 ppm treatments seem to negatively effect the stability of the microspores in the mid-late uninucleate stage of development for AC-2.

The intent was to mimic the effect of low temperature growth conditions of 10/5^{0}C (day/night) with the growth retardants. Perhaps further experiments exploring the affects of less concentrated pretreatments could revel their effects microspore culture response. These growth retardants inhibit GA synthesis, which is involved in the flowering process. It would be interesting to take a in-

depth look a the physical effects the growth retardants may be having on the entire flowering period of the genotypes.

Based on mean green embryo yields, the experimental results have shown that responsiveness of microspores in culture is dependent on the genotype of the donor plant. It is well known that genotype strongly influences the responsiveness of microspores in culture. However, no statistical differences were found between genotypes for any of the experiments. Repetitions of the same treatments were highly variable. This variability contributed to a high CV and unaccounted variation in the statistical models. Some of the variability is probably due to the plant to plant differences among the ten plants being sampled per genotype. Preliminary analysis on individual genotypes for the donor plant pretreatment was attempted but abandoned due to small numbers of repetitions resulting in an insufficient estimate of experimental error. Redesigning the experiment to include more repetitions of the treatments per genotype may have allowed analysis to identify statistical differences between the genotypes as well as other factors.

In general, the embryo yields in this research were low as compared to the published literature. Perhaps lab to lab variation in addition to different growing facilities caused this discrepancy in results as compared to the literature.

The results of the experiment indicate that different genotypes require different media sucrose levels, pH levels and shock procedures to produce optimal numbers of embryos. However, applying the optimal conditions for individual genotypes may not be practical, especially if there are a number of genotypes involved in the microspore culture program. The efficient application of the isolated microspore culture technique in oilseed rape breeding is dependent on using genotypes which respond with high, consistent embryo yields. Success then lies in the ability to manipulate microspores to induce response in many genotypes. The practical approach is to determine conditions and treatments which result in the best average embryo response across all of the genotypes being utilized instead of striving for optimal conditions for each genotype.

7.0 LITERATURE CITED

Arnison, P.G., Donaldson ,P., Ho, L.C.C. and Keller, W.A. 1990a. The influence of various physical parameters on anther culture of broccoli (*Brassica oleracea* var. *Italica*). Plant Cell, Tissue and Organ Culture **20**:147-155.

Arnison, P.G., Donaldson ,P., Jackson, A., Semple, C. and Keller, W. 1990b. Genotype-specific response of cultured broccoli (*Brassica oleracea* var. *italica*) anthers to cytokinins. Plant Cell, Tissue and organ Culture **20**:217-222.

Baillie, A.M.R., Epp, D.J., Hutcheson, D. and Keller, W.A. 1992. *In vitro* culture of isolated microspores and regeneration of plants in *Brassica campestris.* Plant Cell Reports **11**:234-237.

Bajaj, Y.P.S. 1983. In vitro production of haploids. Pages 242-243 in: Handbook of Plant Cell Culture, Volume 1. Eds. D.A. Evans, W.R. Sharp, P.V. Ammirato and Y. Yamada. Macmillan Publishing Co., New York.

Benito-Moreno, R.M, Macke, F., Hauser, M., Alwen, A. and Heberle-Bors, E. 1988. Sporophytes and male gametophytes from in vitro cultured, immature tobacco pollen. Pages 137-142 in: Sexual reproduction in higher plants. Eds. M. Cresti, P. Gori and E. Pacini. Springer-Verlag, Berlin, Heidelberg, New York.

Biddington, N.L. and Robinson, H.T. 1990. Variations in response to high temperature treatments in anther culture of brussels sprouts. Plant Cell, Tissue and Organ Culture **22**:48-54.

Biddington, N.L. and Robinson, H.T. 1991. Ethylene production during anther culture of brussels sprouts (*Brassica oleracea* var. *gemmifera*) and its relationship with factors that affect embryo production. Plant Cell, Tissue and Organ Culture **25**:169-177.

Biddington, N.L., Sutherland, R.A. and Robinson, H.T. 1988. Silver nitrate increases embryo production in anther culture of brussels sprouts. Annals of Botany **62**:181-185.

Binarova, P., Hause, G. and Cenklova, V. 1997. A short severe heat shock is required to induce embryogenesis in late bicellular pollen of Brassica napus L. Sexual Plant Reproduction. **10**:200-208.

Blakeslee, A.F., Belling, J., Farnham, M.E. and Bergner, A.D. 1922. A haploid mutant in the Jimson weed, "*Datura stramonium*". Science **55**:646-647.

Canola Council of Canada. 1988. Canada's Canola. Canola Council of Canada Publication.

Canola Oil and Meal Standards and Regulations. 1990.

Charne, D.G. and Beversdorf, W.D. 1988. Improving microspore culture as a rapeseed breeding tool: the use of auxins and cytokinins in an induction medium. Canadian Journal of Botany **66**:1671-1675.

Charne, D.G., Pukacki, P., Kott, L.S. and Beversdorf, D. 1988. Embryogenesis following cryopreservation in isolated microspores of rapeseed (*Brassica napus* L.). Plant Cell Reports **7**:407-409.

Chen, J.L. and Beversdorf, W.D. 1990. Fatty acid inheritance in microspore-derived populations of spring rapeseed (*Brassica napus* L.). Theoretical and Applied Genetics **80**:465-469.

Chen, J.L. and Beversdorf, W.D. 1991. Evaluation of microspore-derived embryos as models for studying lipid biosynthesis in seed of rapeseed (*Brassica napus* L.). Euphytica **58**:145-155.

Chen, J.L. and Beversdorf, W.D. 1992a. Production of spontaneous diploid lines from isolated microspores following cryopreservation in spring rapeseed *(Brassica napus* L.). Plant Breeding **108**:324-327.

Chen, J.L. and Beversdorf, W.D. 1992b. Cryopreservation of isolated microspores of spring rapeseed (*Brassica napus* L.) for *in vitro* embryo production. Plant Cell, Tissue and Organ Culture **31**:141-149.

Chen, Z.Z., Snyder, S., Fan, Z.G. and Loh, W.H. 1994. Efficient production of Doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breeding **113**:217-221.

Cho, U. and Kasha, K.J. 1989. Ethylene production and embryogenesis from anther cultures of barley (*Hordeum vulgare*). Plant Cell Reports **8**:415-417.

Choo, T.M., Reinbergs, E. and Kasha, K.J. 1985. Use of haploids in barley breeding. Pages 221-223 in: Plant Breeding Reviews, Volume 3. Ed. J. Janic. AVI Publishing Company, Inc., Westport, Connecticut.

Chuong, D.G. and Beversdorf, W.D. 1985. High frequency embryogenesis through isolated microspore culture in *Brassica napus* L. and *B. carinata* Braun. Plant Science **39**:219-226.

Chuong, P.V., Deslauriers, C., Kott, L.S. and Beversdorf, W.D. 1988. Effect of donor genotype and bud sampling on microspore culture of *Brassica napus*. Canadian J. of Botany **66**:1653-1657.

Coventry, J., Kott, L. and Beversdorf, W.D. 1988. Manual for microspore culture technique for *Brassica napus*. Department of Crop Science Technical Bulletin. OAC Publication 0489. University of Guelph.

Daun, J.K. 1983. The introduction of low erucic acid rapeseed varieties into Canadian production. Chapter 7 in: High and low erucic acid rapeseed oils. Eds. J.K Kramer, F.D. Sauer and W.J. Pigden. Academic Press, Ontario.

Downey, R.K. 1983. The origin and description of the *Brassica* oilseed crops. Pages 1-4 in: High and low erucic acid rapeseed oils. Eds. J.K Kramer, F.D. Sauer and W.J. Pigden. Academic Press, Ontario.

Downey, R.K. and Rakow, G.F. 1987. Rapeseed and Mustard. Pages 447-440 in: Principles of cultivar development, Volume 2. Ed. W.R. Fehr. Macmillan Publishing Company, New York.

Downey, R.K. and Robbelen, G. 1989. *Brassica* Species. Chapter 16 in: Oil Crops of the World. Eds. G. Robbleln, R.K. Downey and A. Ashri. McGraw-Hill Publishing Company, New York.

Duijs, J.G., Voorrips, R.E., Visser, D.L. and Custers, J.B.M. 1992. Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica **60**:45-55.

Dunwell, J.M. and Cornish, M. 1985. Influence of preculture variables in microspore embryo production in *Brassica napus* ssp. *oleifera* cv. Duplo. Annals of Botany **56**:281-289.

Dunwell, J.M., Cornish, M. and DeCourcel, A.G.L. 1985. Influence of genotype, plant growth temperature and anther incubation temperature on microspore embryo production in *Brassica napus* ssp. *oleifera*. J. of Experimental Botany **36**:679-689.

Dunwell, J.M. and Thurling, N. 1985. Role of sucrose in microspore embryo production *in Brassica napus* ssp. *oleifera*. J of Experimental Botany **36**:1478-1491.

Eikenberry, E. 1993. Chromosome doubling of microspore-derived canola using trifluralin. Eighth Crucifer Genetics Workshop. Abstract #54.

Fabijanski, S.F., Altosaar, I. and Arnison, P.G. 1991. Heat shock response during anther culture of broccoli (*Brassica oleracea* var. *italica*). Plant Cell, Tissue and Organ Culture **26**:203-212.

Fan, Z., Armstrong, K.C. and Keller, W.A. 1988. Development of microspores in vivo and in vitro in *Brassica napus* L. Protoplasma **147**:191-199.

Feng, X.R. and Wolyn, D.J. 1993. Recovery of haploid plants from asparagus microspore culture. Canadian Journal of Botany **72**:293-300.

Ferrie, A.M.R., Palmer, C.E. and Keller, W.A. 1994. Biotechnological applications of haploids. Pages 77-80 in: Biotechnological Applications of Plant Cultures. Eds. P.D. Shargool and T.T. Ngo. CRC Press, Florida.

Foisset, N., Delourme, R., Lucas, M.O. and Renard, M. 1993. Segregation analysis of isozyme markers on isolated microspore-derived embryos in *Brassica napus* L. Plant Breeding **110**:315-322.

Foroughi-Wehr, B. and Wenzel, G. 1989. Androgenetic haploid production. Intern. Association for Plant Tissue Culture Newsletter **58**:11-18.

Gland, A., Lichter, R. and Schweiger, H.G. 1988. Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* L. J. of Plant Physiology **132**:613-617.

GRIN (Germplasm Resources Information Network) 1998. Online Database. National Germplasm Resources Laboratory, Beltsville, Maryland. Available: www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1484347.

Gruber, S. and Robbelen, G. 1991. Fatty acid synthesis in microspore-derived embryoids of rapeseed (*Brassica napus* L.). Pages 1818-1820 in: Proceedings of the Eighth International Rapeseed Congress. Ed. D.I. McGregor. E.I.R.C. Organizing Committee, Saskatchewan.

Hanning, A. 1993. Antimicrotubule agents for diploidizing haploid tissues in *Brassica napus*. Eighth Crucifer Genetics Workshop. Abstract #57.

Hansen, M. and Svinnset, K. 1993. Microspore culture of Swede (*Brassica napus* ssp. *rapifera*) and the effects of fresh and conditioned media. Plant Cell Reports **12**:496-500.

Haung, B., Bird, S., Kemble, R., Simmonds, D., Keller, W. and Miki, B. 1990. Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. Plant Cell Reports **8**:594-597.

Haung, B., Swanson, E.B., Baszcznski, C.L., Macrae, W.D., Barbour, E., Armavil, V., Cooke, L., Arnoldo, M., Rozakis, S., Westecott, M., Keats, R.F. and Kemble, R. 1991. Application of microspore culture to canola improvement. Pages 298-303 in: Proceedings of the Eighth International Rapeseed Congress. Ed. D.I. McGregor. E.I.R.C. Organizing Committee, Saskatchewan.

Heberle-Bors, E. 1989. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sexual Plant Reproduction **2**:1-10.

Heberle-Bors, E. and Reinert, J. 1980. Isolated pollen cultures and pollen dimorphism. Naturwissenshaften **67**:311-312.

Henderson, C.A.P. and Pauls, K.P. 1992. The use of haploidy to develop plants that express several recessive traits using light seeded canola (*Brassica napus*) as an example. Theoretical and Applied Genetics **83**:476-479.

Holbrook, L.A., vanRooijen, G.J.H., Wilen, R.W. and Moloney, M.M. 1991. Oilbody proteins in microspore-derived embryos of *Brassica napus*. Plant Physiology **97**:1051-1058.

Horner, M. and Street, H.E. 1978. Pollen dimorphism - origin and significance in pollen plant formation by anther culture. Annals of Botany **42**:763-771.

Ilic-Grubor, K., Attree, S.M. and Fowke, L.C. 1998. Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. Plant Cell Reports **17**:329-333.

Iqbal, M.C.M., Mollers, C. and Robbelen, G. 1994. Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus* L. Journal of Plant Physiology **143**:222-226.

Jardinaud, M.F., Souvre, A. and Alibert, G. 1993. Transient GUS gene expression in *Brassica napus* electroporated microspores. Plant Science **93**:177-184.

Johnson-Flanagan, A.M. and Singh, J. 1993. A method to study seed degreening using haploid embryos of *Brassica napus* cv. Topas. J. of Plant Physiology **141**:487-493.

Keller, W.A. and Armstrong, K.C. 1977. Embryogenesis and plant regeneration in *Brassica napus* anther cultures. Canadian Journal of Botany **55**:1383-1388.

Keller, W.A. and Armstrong, K.C. 1978. High frequency production of microspore-derived plants from *Brassica napus* anther cultures. Z. Pflanzenzuchtg **80**:100-108.

Keller, W.A., Armstrong, K.C. and de la Roche, A.I. 1983. The production and utilization of microspore-derived haploids in *Brassica* crops. Pages 169-183 in: Plant Cell Culture in Crop Improvement. Eds. S.K. Sen and K.L Giles. Plenum Publishing Corporation, New York.

Kenyon, P.D., Marshall, G. and Morrison, I.N. 1987. Selection for sulfonylurea herbicide tolerance in oilseed rape (*Brassica napus*) using microspore culture. Pages 871-877 in: British Crop Protection Conference - Weeds. Brighton Metropole, England.

Kieffer, M., Fuller, M.P., Chauvin, J. and Schlesser, A. 1993. Anther culture of kale (*Brassica oleracea* L. convar. *acephala* (D.C.) Alef.). Plant Cell, Tissue and Organ Culture **33**:303-313.

Knowles, P.F. 1989. Genetics and breeding of oil crops. Pages 265-271 in: Oil crops of the world. Eds. G. Robbelen, R.K. Downey and A. Ashri. McGraw-Hill Publishing Company, New York.

Kostoff, D. 1934. A haploid plant of *Nicotiana sylvestris*. Nature **133**:949-950.

Kott, L.S. and Beversdorf, W.D. 1990. Enhanced plant regeneration from microspore-derived embryos of *Brassica napus* by chilling, partial desiccation and age selection. Plant Cell, Tissue and Organ Culture **23**:187-192.

Kott, L.S., Polsoni, L. and Beversdorf, W.D. 1988a. Cytological aspects of isolated microspore culture of Brassica napus. Canadian Journal of Botany **66**:1658-1664.

Kott, L.S., Polsoni, L., Ellis, B. and Beversdorf, W.D. 1988b. Autotoxicity in isolated microspore cultures of *Brassica napus*. Canadian Journal of Botany **66**:1665-1670.

Kuginuki, Y., Nakamura, K., Hida, K. and Yosikawa, H. 1997. Varietal differences in Embryogenic and Regenerative ability in microspore culture of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). Breeding Science **47**:341-346.

Kyo, M. and Harada, H. 1985. Studies on conditions for cell division and embryogenesis in isolated pollen cultures of *Nicotiana rustica*. Plant Physiology **79**:90-94.

Kyo, M. and Harada, H. 1986. Control of the developmental pathway of tobacco pollen in vitro. Planta **168**:427-432.

Lichter, R. 1982. Induction of haploid plants form isolated pollen of *Brassica napus*. Z. Pflanzenphysiol. Bd. **105**:427-434.

Lichter, R. 1989. Efficient yield of embryoids by culture of isolated microspores of different *Brassicaceae* species. Plant Breeding **103**:119-123.

Lo, K.H. and Pauls, K.P. 1992. Plant growth environment effects on rapeseed microspore development and culture. Plant Physiology **99**:468-472.

Martensson, B. and Widell, S. 1993. Pollen from cold treated *Nicotiana tabacum* buds: Embryogenic capacity, peroxidase activity and partitioning in aqueous two-phase systems. Plant Cell, Tissue and Organ Culture **35**:141-149.

Mathias, R. 1988. An improved in vitro culture procedure for embryoids derived from isolated microspores of rape (*Brassica napus* L.). Plant Breeding **100**:320-322.

Mathias, R. and Robbelen, G. 1991. Effective diploidization of microspore-derived haploids of tape (*Brassica napus* L.) by in vitro colchicine treatment. Plant Breeding **106**:82-84.

Morrison, R.A. and Evans, D.A. 1988. Haploid plants from tissue culture: new plant varieties in a shortened time frame. Biotechnology **6**:684-690.

Murphy, L. and Scarth, R. 1998. Inheritance of vernalization response determined by doubled haploid lines in spring oilseed rape (*Brassica napus* L.). Crop Science Nov.-Dec. **38**(6):in publication.

National Research Council. 1992. From rapeseed to canola - The billion dollar success story. National Research Council of Canada Publication. Pages 1-79.

Nitzsche, W. and Wenzel, G. 1977. Haploids in plant breeding. Pages 8-21 in: Supplement 8 to Journal of Plant Breeding - Advances in plant breeding. Eds. W. Horn, Hannover, G. Robbelen and Gottingen. Verlag Paul Parey, Berlin.

Ockendon, D.J. 1984. Anther culture in brussels sprouts (*Brassica oleracea* var. *gemiferae*). I. Embryo yields and plant regeneration. Ann. Appl. Biol. **105**:285-291.

Ockendon, D.J. 1985. Anther culture in brussels sprouts (*Brassica oleracea* var. *gemiferae*). II. Effect of genotype on embryo yields. Ann. Appl. Biol. **107**:101-104.

Oelck, M.M., Phan, C.V., Eckes, P., Donn, G., Rakow, G., and Keller, W.A. 1991. Field resistance of canola transformants (*Brassica napus* L.) to Ignite (phosphinotricin). Pages 292-297 in: Proceedings of the Eighth International Rapeseed Congress. Ed. D.I. McGregor. E.I.R.C. Organizing Committee, Saskatchewan.

Osolnik, B., Bohanec, B. and Jelaska, S. 1993. Simulation of androgenesis in white Cabbage *(Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection. Plant Cell, Tissue and Organ Culture **32**:241-246.

Palmer, C.D., and Keller W.A. 1997. Pollen Embryos. Chapter 20 in: Pollen Biothechnology for Crop Production and Improvement. Eds. K.R. Shivanna and V.K. Sawhney. Cambridge University Press.

Pechan, P.M., and Keller W.A. 1988. Identification of potentially embryogenic microspores in *Brassica napus*. Physiologia Plantarum **74**:377-384.

Phippen, C. and Ockendon, D.J. 1990. Genotype, plant bud size and media factors affecting anther culture of cauliflower (*Brassica oleracea* var. *botrytis*). Theoretical and Applied Genetics **79**:33-38.

Pickering, R.A. and Devaux, P. 1992. Haploid production: approaches and use in plant breeding. Pages 228-229 in: Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. Ed. P.R. Shewry. Alden Press Ltd., Oxford.

Pierik, R.L.M. 1987. The production of haploids. Pages 243-247 in: In vitro culture of higher plants. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Poehlman, J.M. 1987a. Plant breeders and their work. Pages 4-5 in: Breeding Field Crops. AVI Publishing Company, Inc., Westport, Connecticut.

Poehlman, J.M. 1987b. Plant cell and tissue culture - applications to plant breeding. Pages 160-161 in: Breeding Field Crops. AVI Publishing Company, Inc., Westport, Connecticut.

Polsoni, L., Kott, L.S. and Beversdorf, W.D. 1988. Large-scale microspore culture technique for mutation-selection studies in *Brassica napus*. Canadian Journal of Botany **66**:1681-1685.

Pomeroy, M.K., Sparace, S.A. and Keller W.A. 1991. Fatty acid and triacylglycerol biosynthesis in microspore embryos of *Brassica napus.* Pages 154-158 in: Proceedings of the Eighth International Rapeseed Congress. Ed. D.I. McGregor. E.I.R.C. Organizing Committee, Saskatchewan.

Powell, W. 1990. Environmental and genetical aspects of pollen embryogenesis. Pages 45-65 in: Haploids in Crop Improvement I - Biotechnology in Agriculture and Forestry. Ed. Y.P.S. Bajaj. Springer-Verlag, Germany.

Robbelen, G. 1991. Divergence and performance of the botanical *Brassica* species. Proc. Eighth International Rapeseed Congress **1**:29-38.

Roulund, N., Andersen, S.B. and Farestveit, B. 1991. Optimal concentration of sucrose for head cabbage (*Brassica oleracea* L. convar. *capitata* (L.) Alef.) anther culture. Euphytica **52**:125-129.

Sacristan, M.D. 1982. Resistance responses to *Phoma lingam* of plants regenerated from selected cell and embryogenic cultures of haploid *Brassica napus*. Theoretical and Applied Genetics **61**:193-200.

Sangwan, R.S., Gauthier, D.A., Turpin, D.H., Pomeroy, M.K. and Plaxton, W.C. 1992. Pyruvate-kinase isoenzymes from zygotic and microspore-derived embryos of *Brassica napus*. Plants **187**:198-202.

Scarth, R., Rimmer, S.R., and McVetty, P.B.E. 1992. Reward summer turnip rape. Canadian Journal of Plant Science **72**:839-840.

Senaratna, T., Kott, L., Beversdorf, W.D. and McKersie, B.D. 1991. Dessication of microspore derived embryos of oilseed rape (*Brassica napus* L.). Plant Cell Reports **10**:342-344.

Sharp, W.R., Reed, S.M. and Evans, D.A. 1984. Production and application of haploid plants. Pages 360-361 in: Crop Breeding: A Contemporary Basis. Eds. P.B. Vose and S.G. Blixt. Pergamon Press Ltd. Ontario, Canada.

Siebel, J. and Pauls, K.P. 1989. A comparison of anther and microspore culture as a breeding tool in *Brassica napus*. Theoretical and Applied Genetics **78**:473-479.

Stefansson, B.R. 1983. The development of improved rapeseed cultivars. Pages 144-147 in: High and low erucic acid rapeseed oils. Eds. J.K Kramer, F.D. Sauer and W.J. Pigden. Academic Press, Ontario.

Sunderland, N. and Haung, B. 1987. Ultrastructure aspects of pollen dimorphism. International Review of Cytology **107**:175-220.

Swanson, E.B. and Erickson, L.R. 1989. Haploid transformation in *Brassica napus* using an octopine-producing strain of *Agrobacterium tumefaciens.* Theoretical and Applied Genetics **78**:831-835.

Swanson, E.B., Coumans, M.P., Brown, G.L., Patel, J.D. and Beversdorf, W.D. 1988. The characterization of herbicide tolerant plants in *Brassica napus* L. after *in vitro* selection of microspores and protoplasts. Plant Cell Reports **7**:83-87.

Swanson, E.B., Coumans, M.P., Wu, S.C., Barsby, T.L. and Beversdorf, W.D. 1987. Efficient isolation of microspores and the production of microspore-derived embryos from *Brassica napus*. Plant Cell Reports **6**:94-97.

Swanson, E.B., Herrgesell, M.J., Arnoldo, M., Sippell, D.W. and Wong, R.S.C. 1989. Microspore mutagenesis and selection: Canola plants with field tolerance to the imidazolinones. Theoretical and Applied Genetics **78**:525-530.

Takahata, Y., Brown, D.C.W. and Keller, W.A. 1991. Effect of donor plant age and inflorescence age in microspore culture of *Brassica napus* L. Euphytica **58**:51-55.

Takahata, Y. and Keller, W.A. 1991. High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. Plant Science **74**:235-242.

Taylor, D.C., Barton, D.L., Rioux, K.P., MacKenzie, S.L., Reed, D.W., Underhill, E.W., Pomeroy, M.K. and Weber, N. 1992a. Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv. Reston. Plant Physiology **99**:1609-1618.

Taylor, D.C., Ferrie A.M.R., Kellerm W.A., Giblin, E.M., Pass, E.W. and MacKenzie, S.L. 1993. Bioassembly of acyl lipids in microspore-derived embryos of *Brassica campestris* L. Plant Cell Reports **12**:375-384.

Taylor, D.C., Weber, N., Hogge L.R., Underhill, E.W. and Pomeroy, M.K. 1992b. Formation of trierucoylglycerol (trierucin) from 1,2-dierucoylglycerol by a homogenate of microspore-derived embryos of *Brassica napus* L. Journal of American Oil Chemists Society **69**:355-358.

Taylor, D.C., Weber, N., Underhill, E.W., Pomeroy, M.K., Keller, W.A., Scowcroft, W.R., Wilen, R.W., Moloney, M.M. and Holbrook, L.A. 1990. Storage-protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta **181**:18-26.

Telmer, C.A., Simmonds, D.H. and Newcomb, W. 1992. Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Physiologia Plantarum **84**:417-424.

Telmer, C.A., Newcomb, W. and Simmonds, D.H. 1993. Microspore development in *Brassica napus* and the effect of high temperature on division in vivo and in vitro. Protoplasma **172**:154-165.

Thurling, M. and Chay, P.M. 1984. The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of *Brassica napus* spp. *oleifera*. Annals of Botany **54**:681-693.

Touraev, A., Vicente, O. and Heberle-Bors, E. 1997. Initiation of microspore embryogenesis by stress. Trends in plant science **2**:297-302.

Weselake, R.J., Pomeroy, M.K., Furukawa, T.L., Golden, J.L., Little, D.B. and Laroche, A. 1993. Developmental profile of diacylglycerol acyltransferase in maturing seeds of oilseed rape and safflower and microspore-derived cultures of oilseed rape. Plant Physiology **102**:565-571.

Wiberg, E., Rahlen, L., Hellman, M., Tillberg, E., Glimelius, K., and Stymne, S. 1991. The microspore-derived embryo of *Brassica napus* L. as a tool for studying embryo-specific lipid biogenesis and regulation of oil quality. Theoretical and Applied Genetics **82**:515-520.

Zaki, M.A.M. and Dickinson, H.G. 1990. Structural changes during the first divisions of embryos resulting from anthers and free microspore culture in *Brassica napus*. Protoplasma **156**:149-162.

Zaki, M.A.M. and Dickinson, H.G. 1991. Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sexual Plant Reproduction **4**:48-55.

8.0 APPENDIX A

8.1 Media Preparation

8.1.1 B_5 Media

B_5 VITAMIN STOCK (1000X)

Myo-inositol	100.0 g
Nicotinic Acid	1.0 g
Pyridoxine HCl	1.0 g
Thiamine HCl	10.0 g

Dissolve each vitamin before adding the next into double distilled water. Bring the final volume to 1 litre. Freeze in 10 ml portions.

B_5 MICRONUTRIENT STOCK (1000X)

$MnSO_4 \cdot H_2$	10.0 g
H_3BO_3	3.0 g
$ZnSO_4 \cdot 7H_2O$	2.0 g
$Na_2MoO_4 \cdot 2H_2O$	0.25 g
$CuSO_4 \cdot 5H_2O$	0.025g
$CoCl_2 \cdot 6H_2O$	0.025g

Dissolve the micronutrients into double distilled water. Bring the final volume to 1 litre. Freeze in 10 ml portions.

B_5 KI STOCK

KI	0.75 g

Dissolve the KI into double distilled water. Bring the final volume to 1 litre. Store refrigerated in brown bottle.

B_5 CONCENTRATE (10x)

KNO_3	30.0	g
$MgSO_4 \cdot 7H_2O$	5.0	g
$CaCl_2 \cdot 2H_2O$	1.5	g
$(NH_4)_2SO_4$	1.5	g
$NaH_2PO_4 \cdot H_2O$	1.5	g
Fe 330	0.28	g

Dissolve each ingredient as well as 10 ml of B_5 vitamin stock, 10 ml of B_5 micronutrient stock and 10 ml of B_5 KI stock into double distilled water and bring up final volume to 1 litre. Freeze in 100 ml portions. Note: 100 ml of B_5 10x-concentrate makes 1 litre of media.

HALF STRENGTH B_5-13 MEDIA

2 x 100 ml of B_5 10x-concentrate (frozen)

520 g sucrose (13%)

Melt the B_5 10x-concentrate and dissolve, along with the sucrose in double distilled water. Bring final volume up to 4 litres. Adjust pH to 6.0. Distribute 400 ml of media into 10, 500 ml jars. Autoclave 15 to 20 minutes at 15 PSI.

8.1.2 NLN MEDIA

NLN VITAMIN STOCK (1000X)

Myo-inositol	100.0 g
Nicotinic Acid	5.0 g
Pyridoxine HCl	0.5 g
Thiamine HCl	0.5 g
Glycine	2.0 g
Folic Acid (dissolve in HCl)	0.5 g
Biotin	0.05 g

Dissolve each vitamin before adding the next into double distilled water. Bring the final volume to 1 litre. Freeze in 10 ml portions.

NLN MICRONUTRIENT STOCK

$MnSO_4 \cdot H_2O$	22.3 g
H_3BO_3	6.2 g
$ZnSO_4 \cdot 7H_2O$	8.6 g
$Na_2MoO_4 \cdot 2H_2O$	0.025g
$CoCl_2 \cdot 6H_2O$	0.025g

Dissolve the micronutrients into double distilled water. Bring the final volume to 1 litre. Freeze in 10 ml portions.

NLN KI STOCK

KI	0.83 g

Dissolve the KI into double distilled water. Bring the final volume to 1 litre. Store refrigerated in brown bottle.

NLN CONCENTRATE (10x)

KNO_3	1.25 g
$MgSO_4 \cdot 7H_2O$	1.25 g
$Ca(NO_3)_2 \cdot 4H_2O$	5.0 g
KH_2PO_4	1.25 g
Fe 330	0.4 g

Dissolve each ingredient as well as 10 ml of NLN vitamin stock, 10 ml of NLN micronutrient stock and 10 ml of NLN KI stock into double distilled water. Add:

Glutathione	0.3 g
L-Serine	1.0 g
L-Glutamine	8.0 g

Bring up final volume to 1 litre with double distilled water. Freeze in 100 ml portions. Note: 100 ml of NLN 10x-concentrate makes 1 litre of media.

NLN-10 MEDIA (0 BA)

4 x 100 ml of NLN 10x-concentrate (frozen)

400 g sucrose (10%)

Melt the NLN 10x-concentrate and dissolve, along with the sucrose in double distilled water. Bring final volume up to 4 litres. Adjust to appropriate pH using 1 N HCl and 1 N NaOH. Media must be filter sterilized. Prefilter media using coarse filter for large particles. Follow the prefilter with a 0.65 and/or 0.22 μm filter. These filters can be completed on the bench top. The final filtering stage involves a 0.2 μm Nalgene sterile filter. This filter step must be completed in a laminar flow hood.

NLN-17 MEDIA (0.1 mg BA L^{-1})

4 x 100 ml of NLN 10x-concentrate (frozen)

680 g sucrose (17%)

0.1 mg BA L^{-1} (dissolve in HCl)

Melt the NLN 10x-concentrate and dissolve, along with the sucrose in double distilled water. Bring final volume up to 4 litres. Adjust to appropriate pH using 1 N HCl and 1 N NaOH. Media must be filter sterilized. Prefilter media using coarse filter for large particles. Follow the prefilter with a 0.65 and/or 0.22 μm filter. These filters can be completed on the bench top. The final filtering stage involves a 0.2 μm Nalgene sterile filter. This filter step must be completed in a laminar flow hood.

NLN-13 MEDIA (0 BA)

4 x 100 ml of NLN 10x-concentrate (frozen)

520 g sucrose (13%)

Melt the NLN 10x-concentrate and dissolve, along with the sucrose in double distilled water. Bring final volume up to 4 litres. Adjust to appropriate pH using 1 N HCl and 1 N NaOH. Media must be filter sterilized. Prefilter media using coarse filter for large particles. Follow the prefilter with a 0.65 and/or 0.22 μm filter. These filters can be completed on the bench top. The final filtering stage involves a 0.2 μm Nalgene sterile filter. This filter step must be completed in a laminar flow hood.

NLN-17 MEDIA (0.1 mg BA L^{-1})

4 x 100 ml of NLN 10x-concentrate (frozen)

520 g sucrose (13%)

0.1 mg BA L^{-1} (dissolve in HCl)

Melt the NLN 10x-concentrate and dissolve, along with the sucrose in double distilled water. Bring final volume up to 4 litres. Adjust to appropriate pH using 1 N HCl and 1 N NaOH. Media must be filter sterilized. Prefilter media using coarse filter for large particles. Follow the prefilter with a 0.65 and/or 0.22 μm filter. These filters can be completed on the bench top. The final filtering stage involves a 0.2 μm Nalgene sterile filter. This filter step must be completed in a laminar flow hood.

9.0 APPENDIX B

TABLE B1. Embryo Production of *B. rapa*, Reward, Parkland, AC-1 and AC-2 for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN 13/NLN-13].

Genotype	Run	Microspore Culture Media Sucrose Levels: NLN-17/NLN-10 (control) — Embryo Stage and Colour*								NLN-13/NLN-13 — Embryo Stage and Colour*							
		AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC
Reward	1	1	0	1	3	0	0	0	66	0	0	0	0	0	0	0	3
	2	1	0	1	1	0	1	0	9	0	0	0	0	0	0	0	2
	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Parkland	1	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0
	2	2	0	2	0	0	0	0	3	2	0	0	0	0	0	0	2
	3	2	0	0	0	0	0	0	3	0	0	0	1	0	0	0	2
AC-1	1	7	0	0	0	0	0	0	5	3	0	0	0	0	1	0	20
	2	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	3	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
AC-2	1	0	0	2	1	1	0	0	2	3	0	2	2	4	3	11	97
	2	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	8
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B2. Total Embryo Production of *B. rapa*, Reward, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels: NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	71	3
2	13	2
3	0	1
TOTAL	84	6

TABLE B3. Total Embryo Production of *B. rapa*, Parkland, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	0	9
2	7	4
3	5	3
TOTAL	12	16

TABLE B4. Total Embryo Production of *B. rapa*, AC-1, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	12	24
2	1	1
3	1	2
TOTAL	14	27

TABLE B5. Total Embryo Production of *B. rapa*, AC-2, for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13].

Run	Microspore Culture Media Sucrose Levels	
	NLN-17/NLN-10 (control)	NLN-13/NLN-13
1	6	122
2	6	10
3	0	0
TOTAL	6	132

TABLE B6. Embryo Production of *B. rapa*, Reward and AC-2 for Media pH Level Experiment [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

		Genotypes															
		Reward								AC-2							
Run	pH	Embryo Stage and Colour*															
		AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC
1	6.2	0	0	0	3	2	0	0	7	0	0	0	0	0	0	0	0
	6	3	0	10	1	2	0	11	18	4	0	1	1	1	0	2	80
	5.8	9	0	0	9	0	3	0	68	8	0	0	10	0	2	1	11
	5.6	5	0	25	1	4	0	1	31	0	0	0	0	0	0	0	0
	5.4	25	0	3	8	0	0	0	39	0	0	0	0	0	0	0	0
	5.2	3	0	3	9	2	1	2	97	6	0	0	24	0	2	1	103
2	6.2	3	0	1	0	0	0	0	14	0	0	0	0	0	0	0	0
	6	3	0	2	0	5	0	7	140	2	0	0	0	0	0	0	11
	5.8	1	0	3	2	0	0	0	0	0	0	5	0	3	0	2	51
	5.6	7	0	10	19	3	1	0	105	2	0	2	0	1	0	2	5
	5.4	1	0	8	3	3	0	3	37	3	0	8	0	3	3	0	199
	5.2	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	18
3	6.2	2	1	2	2	1	1	0	58	41	0	8	0	0	3	0	196
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	5.8	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
	5.6	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	16
	5.4	2	0	2	1	1	0	0	5	0	0	0	0	0	0	0	0
	5.2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	27

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B7. Total Embryo Production of *B. rapa*, Reward, for Media pH Level Experiment [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

Run	Microspore Culture Media pH Levels					
	6.2 (Control)	6	5.8	5.6	5.4	5.2
1	12	27	89	67	75	117
2	18	157	6	145	55	1
3	67	0	2	0	11	1
TOTAL	97	184	97	212	141	119

TABLE B8. Total Embryo Production of *B. rapa*, AC-2, for Media pH Level Experiment [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2].

Run	Microspore Culture Media pH Levels					
	6.2 (Control)	6	5.8	5.6	5.4	5.2
1	0	89	32	0	0	136
2	0	13	61	12	216	20
3	248	1	0	21	0	27
TOTAL	248	93	93	33	216	183

TABLE B9. Embryo Production of *B. rapa*, Reward, Parkland, AC-1 and AC-2 for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Genotype	Run	Microspore Pretreatment: Room Temperature (control) Embryo Stage and Colour*								Microspore Pretreatment: Cold Shock Embryo Stage and Colour*							
		AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC
Reward	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	1	1	2	0	17
	3	0	0	0	0	0	0	0	2	1	0	1	2	0	0	0	6
Parkland	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
AC-1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3
AC-2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	2	1	0	0	0	0	1	0	2
	3	1	0	1	2	0	1	0	2	0	0	0	0	0	0	0	0

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B10. Total Embryo Production of *B. rapa*, Reward, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels: Room Temperature (control)	Cold Shock
1	0	0
2	0	21
3	2	10
TOTAL	2	31

TABLE B11. Total Embryo Production of *B. rapa*, Parkland, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	2	0
3	1	1
TOTAL	3	1

TABLE B12. Total Embryo Production of *B. rapa*, AC-1, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	0	3
3	1	3
TOTAL	1	6

TABLE B13. Total Embryo Production of *B. rapa*, AC-2, for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock].

Run	Microspore Culture Media Sucrose Levels	
	Room Temperature (control)	Cold Shock
1	0	0
2	2	1
3	7	3
TOTAL	9	4

TABLE B14. Total Embryo Production of *B. rapa*, Reward for the Large Scale Microspore Culture Technique Experiment.

Run	Genotype Reward Embryo Stage and Colour*								
	AG	GG	AH	GH	AT	GT	AC	GC	Total
1	0	0	1	0	0	0	0	1	2
2	0	0	0	12	0	0	0	4	16
3	0	0	0	0	0	0	0	1	1
4	2	0	1	3	0	0	0	0	6

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B15. Embryo Production of *B. rapa*, Reward and AC-2 for Donor Plant Pretreatment Experiment - Drench Applications of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Genotype	Run	A-Rest Drench Treatments: 0 ppm (control) Embryo Stage and Colour*								4 ppm Embryo Stage and Colour*								8 ppm Embryo Stage and Colour*							
		AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC
Reward	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AC-2	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B16. Total Embryo Production of *B. rapa*, Reward, for Donor Plant Pretreatment Experiment - Drench Applications of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	4 ppm	8 ppm
1	0	0	3
2	0	0	0
3	3	0	1
4	0	0	1
5	0	0	0
TOTAL	3	0	5

TABLE B17. Total Embryo Production of *B. rapa*, AC-2, for Donor Plant Pretreatment Experiment - Drench Applications of A-Rest (0.264 mg a.i. per ml) at 0 mg a.i./pot (0 ppm), 0.25 mg a.i./pot (4 ppm) and 0.5 mg a.i./pot (8 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	4 ppm	8 ppm
1	2	1	0
2	0	0	0
3	0	0	0
4	1	2	0
5	0	0	0
TOTAL	3	3	0

TABLE B18. Embryo Production of *B. rapa*, Reward and AC-2 for Donor Plant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

		Cycocel Spray Treatments																									
		0 ppm (control)								3000 ppm								6000 ppm									
Genotype	Run	Embryo Stage and Colour*																									
		AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC	AG	GG	AH	GH	AT	GT	AC	GC		
Reward	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	3	0	0	0	0	0	0	2		
	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	3	0	0	0	1	0	0	0	4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0		
AC-2	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0		
	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	3	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0		

*Embryo stage and colour: AG=albino globular, GG=green globular, AH=albino heart, GH=green heart, AT=albino torpedo, GT=green torpedo AC=albino cotyledon, GC=green cotyledon

TABLE B19. Total Embryo Production of *B. rapa*, Reward, for Donor Plant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

	Cycocel Spray Treatments		
Run	0 ppm (control)	3000 ppm	6000 ppm
1	0	4	5
2	1	0	0
3	5	0	1
TOTAL	6	4	6

TABLE B20. Total Embryo Production of *B. rapa*, AC-2, for Donor Plant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm).

Run	A-Rest Drench Treatments		
	0 ppm (control)	3000 ppm	6000 ppm
1	0	2	2
2	1	0	0
3	1	2	0
TOTAL	2	4	2

10.0 APPENDIX C

TABLE C1. Analysis of Variance Results for Media Sucrose Level Experiment [NLN-17/NLN-10 (control) vs. NLN-13/NLN-13] Using *B. rapa* Genotypes Reward, Parkland, AC-1 and AC-2.

Source	Degrees of Freedom	ANOVA MS	F-Value	PF>F
Model Statement	9	773.64	1.64	0.1949
Error	14	470.47	R-Square 0.5138	CV 220.5796
Media Sucrose Levels	1	88.17	0.19	0.6717
Repetitions	2	1463.04	3.11	0.0763
Genotypes	3	384.45	0.82	0.5056
Media Sucrose Levels X Genotype	3	931.72	1.98	0.1633

TABLE C2. Analysis of Variance Results for Media pH Level Experiment, [pH 6.2 (control) vs. pH 6.0, pH 5.8, pH 5.6, pH 5.4, pH 5.2] Using B. rapa Genotypes Reward and AC-2.

Source	Degrees of Freedom	ANOVA MS	F-Value	PF>F
Model Statement	13	1152.10	0.26	0.9927
Error	22	4473.36	R-Square = 0.132174	CV = 168.2595
Media pH Levels	5	577.38	0.13	0.9841
Repetitions	2	1881.08	0.42	0.6619
Genotypes	1	250.69	0.06	0.8151
Media pH Levels X Genotypes	5	1617.83	0.36	0.8691

TABLE C3. Analysis of Variance Results for Microspore Cold Shock Pretreatment Experiment [Room Temperature (control) vs. Cold Shock] Using *B. rapa* Genotypes Reward, Parkland, AC-1 and AC-2.

Source	Degrees of Freedom	ANOVA MS	F-Value	PF>F
Model Statement	9	27.13	2.11	0.1011
Error	14	12.83	R-Square = 0.576091	CV = 171.9535
Microspore Pretreatments	1	28.17	2.19	0.1606
Repetitions	2	26.17	2.04	0.1671
Genotypes	3	24.10	1.87	0.1803
Media Sucrose Level X Genotype	3	30.50	2.38	0.1138

TABLE C4. Analysis of Variance Results for Donor Plant Growth Retardant Pretreatment Experiment - Spray applications of Cycocel (118 mg a.i./ml) at 0 ml/l (0 ppm), 25.4 ml/l (3000 ppm) and 50.8 ml/l (6000 ppm) Using *B. rapa* Genotypes Reward and AC-2.

Source	Degrees of Freedom	ANOVA MS	F-Value	PF>F
Model Statement	7	1.20	0.56	0.7693
Error	10	2.12	R-Square = 0.283302	CV = 201.7087
Growth Retardant Pretreatments	2	1.06	0.50	0.6224
Repetitions	2	2.06	0.97	0.4126
Genotypes	1	1.39	0.65	0.4374
Growth Retardant Pretreatments X Genotype	2	0.39	0.18	0.8353

TABLE D1. % Microspores in Developmental Stages for *B. rapa*, Reward - Donor Plant Pretreatment Experiment - Ethephon.

Pretreatment ETHEPHON (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	31	100	0	0	67	20	13	4	17	78	0	14	86
0	32	100	0	0	84	7	9	2	54	45	0	46	54
0	33	99	1	0	75	15	10	2	33	65	0	44	56
0	34	100	0	0	83	17	0	0	29	71	2	46	52
0	35	100	0	0	85	11	4	15	36	49	0	43	57
0	36	100	0	0	90	10	0	0	21	79	2	42	56
58	36	88	12	0	78	17	6	0	13	87	0	62	38
115	36	100	0	0	74	24	3	2	27	71	0	47	53
0	37	100	0	0	91	9	0	0	18	82	0	45	55
58	37	100	0	0	55	30	15	0	50	50	0	43	57
115	37	100	0	0	89	11	0	0	44	56	0	15	85
0	38	100	0	0	83	15	2	0	7	93	0	41	59
58	38	99	1	0	72	20	9	3	20	78	0	31	69
115	38	100	0	0	83	17	0	2	16	82	0	50	50
0	39	100	0	0	81	19	0	0	36	64	0	61	39
58	39	100	0	0	58	22	20	0	37	63	3	31	66
115	39	100	0	0	83	17	0	1	36	63	0	40	60
0	40	99	1	0	79	17	5	0	29	71	2	37	61
58	40	97	1	1	64	32	5	0	26	74	0	65	35
115	40	100	0	0	79	21	0	1	32	67	0	48	52
0	41	100	0	0	85	13	2	0	39	61	4	39	57
58	41	100	0	0	85	15	0	0	49	51	0	27	73
115	41	100	0	0	87	10	3	41	41	19	0	34	66
0	42	100	0	0	69	31	0	2	26	73	1	53	46
58	42	100	0	0	42	45	13	0	23	77	0	23	77
115	42	100	0	0	87	11	2	4	37	59	1	35	64
0	43	94	5	1	72	25	4	2	49	49	0	45	55
58	43	100	0	0	69	31	0	63	37	0	89	5	5
115	43	100	0	0	100	0	0	93	7	0	94	3	3
0	44	100	0	0	0	42	58	0	78	22	0	99	1
58	44	100	0	0	0	61	39	0	78	22	0	98	2
115	44	100	0	0	100	0	0	94	6	0	31	48	21

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE

TABLE D2. % Microspores in Developmental Stages for *B. rapa*, AC-2 - Donor Plant Pretreatment Experiment - Ethephon.

Pretreatment ETHEPHON (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	31	100	0	0	72	22	6	0	14	86	0	34	66
0	32	100	0	0	21	44	35	0	45	55	0	47	53
0	33	100	0	0	87	13	0	0	41	59	2	58	41
0	34	99	1	0	55	27	18	21	26	53	0	70	30
0	35	100	0	0	100	0	0	0	38	62	0	44	56
0	36	100	0	0	84	16	0	0	30	70	6	19	74
58	36	100	0	0	86	11	3	3	16	81	0	49	51
115	36	100	0	0	93	7	0	0	35	65	0	40	60
0	37	100	0	0	83	13	4	0	40	60	0	26	74
58	37	100	0	0	88	12	0	0	10	90	0	30	70
115	37	90	10	0	68	19	13	2	34	64	0	35	65
0	38	100	0	0	94	6	0	0	18	82	0	31	69
58	38	100	0	0	19	26	56	0	46	54	0	33	67
115	38	100	0	0	85	15	0	0	30	70	0	45	55
0	39	100	0	0	91	9	0	59	26	15	4	31	65
58	39	100	0	0	85	13	2	0	22	78	0	37	63
115	39	100	0	0	92	8	0	0	17	83	2	34	64
0	40	100	0	0	88	12	0	9	35	56	2	42	56
58	40	99	1	0	81	19	0	0	35	65	0	29	71
115	40	100	0	0	90	10	0	0	36	64	0	31	69
0	41	100	0	0	94	6	0	0	36	64	0	46	54
58	41	80	15	6	83	7	10	0	5	95	0	10	90
115	41	100	0	0	63	23	14	11	30	59	2	47	51
0	42	100	0	0	80	20	0	0	56	44	0	37	63
58	42	100	0	0	97	3	0	40	23	37	0	47	53
115	42	100	0	0	100	0	0	0	43	57	0	47	53
0	43	100	0	0	72	28	0	0	50	50	0	49	51
58	43	100	0	0	96	4	0	85	13	2	6	45	49
115	43	100	0	0	94	6	0	0	38	62	0	42	58
0	44	100	0	0	66	34	0	0	67	33	0	33	67
58	44	100	0	0	88	12	0	0	56	44	0	35	65
115	44	100	0	0	89	8	3	0	73	27	0	33	67

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE

TABLE D3. % Microspores in Developmental Stages for *B. rapa*, Reward - Donor Plant Pretreatment Experiment - A-Rest.

Pretreatment A-REST (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	33	62	38	0	40	56	4	0	65	35	0	100	0
0	34	81	19	0	62	37	2	0	68	32	0	96	4
0	35	78	20	2	72	25	3	0	58	42	0	46	54
0	36	86	14	0	43	49	8	0	66	34	0	96	4
0	37	90	10	0	67	26	7	0	72	28	0	55	45
0	38	100	0	0	88	12	0	0	31	69	0	84	16
4	38	100	0	0	59	39	2	2	42	56	3	43	54
8	38	99	1	0	90	9	1	0	26	74	0	44	56
0	39	100	0	0	95	5	0	4	24	71	0	47	53
4	39	77	21	2	27	44	29	4	39	57	0	76	24
8	39	100	0	0	59	27	14	0	48	52	0	66	34
0	40	100	0	0	25	39	36	0	58	42	0	85	15
4	40	86	14	0	66	20	14	1	13	86	3	68	29
8	40	99	1	0	33	36	31	0	58	42	0	38	62
0	41	100	0	0	77	15	8	2	47	52	1	71	27
4	41	100	0	0	46	30	24	8	44	48	5	63	33
8	41	100	0	0	67	22	11	30	32	38	0	42	58
0	42	94	6	0	23	38	38	3	26	70	7	43	51
4	42	83	16	2	63	18	52	7	48	45	2	71	27
8	42	100	0	0	100	0	0	25	29	46	1	41	58
0	43	100	0	0	95	5	0	5	18	77	0	40	60
4	43	100	0	0	47	31	23	7	45	48	1	61	38
8	43	100	0	0	91	6	3	2	24	73	0	51	49
0	44	100	0	0	84	14	1	5	35	60	0	62	38
4	44	99	1	0	32	40	28	1	53	46	0	67	33
8	44	100	0	0	25	42	33	3	48	48	14	49	37
0	45	100	0	0	82	8	11	17	17	66	0	58	42
4	45	78	21	1	38	33	29	5	44	51	2	58	40
8	45	100	0	0	78	16	6	0	71	29	3	84	13
0	46	100	0	0	78	11	10	0	36	64	0	67	33
4	46	100	0	0	78	14	9	64	23	13	37	33	31
8	46	99	1	0	35	43	22	0	75	25	2	56	42
0	47	88	7	5	26	36	38	0	48	52	0	68	32
4	47	70	18	15	51	18	31	0	68	32	1	64	35
8	47	100	0	0	85	4	11	0	38	62	1	75	24
0	48	100	0	0	99	1	0	16	25	59	0	72	28
4	48	100	0	0	100	0	0	4	69	27	0	73	27
8	48	100	0	0	93	5	1	0	57	43	2	46	52

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE

TABLE D4. % Microspores in Developmental Stages for *B. rapa*, AC-2 - Donor Plant Pretreatment Experiment - A-Rest.

Pretreatment A-REST (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	33	100	0	0	76	24	0	2	36	63	3	85	12
0	34	100	0	0	88	9	3	2	67	31	0	82	18
0	35	100	0	0	90	10	0	0	58	42	0	24	76
0	36	67	23	13	72	8	20	0	32	68	0	50	50
0	37	95	5	0	84	15	1	2	18	81	3	38	58
0	38	100	0	0	88	12	0	0	27	73	0	30	70
4	38	74	26	0	77	21	2	0	34	66	0	25	75
8	38	80	20	0	77	23	0	3	93	4	0	45	55
0	39	100	0	0	80	16	4	13	27	60	0	38	63
4	39	97	3	0	61	21	18	0	39	61	5	74	21
8	39	100	0	0	94	6	0	0	41	59	0	44	56
0	40	100	0	0	74	14	12	0	39	61	5	33	62
4	40	92	5	3	68	22	11	0	46	54	0	53	47
8	40	69	27	6	64	19	17	14	36	50	5	45	50
0	41	71	25	5	84	14	1	2	36	62	3	36	60
4	41	100	0	0	27	29	44	5	17	79	2	80	18
8	41	100	0	0	56	35	9	0	55	45	1	53	46
0	42	100	0	0	100	0	0	81	18	1	2	43	56
4	42	100	0	0	100	0	0	55	28	17	2	40	58
8	42	100	0	0	100	0	0	0	38	62	0	43	57
0	43	42	39	31	56	35	9	0	47	53	0	42	58
4	43	43	43	23	73	19	9	6	33	60	1	72	27
8	43	100	0	0	88	12	0	6	39	55	0	35	65
0	44	100	0	0	13	28	59	7	36	57	2	46	52
4	44	50	37	21	71	6	23	6	37	57	1	82	17
8	44	100	0	0	79	17	4	15	34	51	24	27	48
0	45	100	0	0	54	22	24	2	35	63	1	45	55
4	45	86	8	7	97	1	1	50	32	18	69	17	13
8	45	98	1	1	75	19	6	75	21	4	57	36	7
0	46	73	20	9	21	48	31	22	37	41	0	77	23
4	46	97	0	3	65	11	24	49	22	30	2	72	26
8	46	100	0	0	68	19	13	48	31	20	25	32	43
0	47	82	11	7	67	14	19	28	57	16	3	48	49
4	47	100	0	0	49	34	17	1	69	30	10	62	28
8	47	100	0	0	100	0	0	70	24	6	50	27	23
0	48	100	0	0	100	0	0	25	34	41	3	49	48
4	48	100	0	0	100	0	0	3	50	47	1	74	25
8	48	100	0	0	100	0	0	51	23	26	48	34	17

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE

TABLE D5. % Microspores in Developmental Stages for *B. rapa*, Reward - Donor Plant Pretreatment Experiment - Cycocel.

Pretreatment CYCOCEL (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	32	100	0	0	88	8	4	2	38	61	0	50	50
0	33	80	15	6	49	27	24	28	40	31	3	43	55
0	34	97	1	1	87	13	0	0	50	50	0	56	44
0	35	100	0	0	20	27	53	1	71	27	0	59	41
0	36	100	0	0	95	5	0	0	44	56	4	55	41
0	37	100	0	0	68	22	10	0	36	64	2	64	35
0	38	77	16	8	13	38	49	15	41	44	4	55	41
3000	38	100	0	0	40	33	27	0	65	35	0	75	25
6000	38	100	0	0	98	2	0	2	55	43	10	51	39
0	39	100	0	0	73	20	7	2	40	59	2	54	44
3000	39	100	0	0	97	3	0	5	55	39	2	30	68
6000	39	100	0	0	95	5	0	0	66	34	0	53	47
0	40	100	0	0	20	29	51	4	55	40	0	80	20
3000	40	100	0	0	20	29	51	4	55	40	0	80	20
6000	40	100	0	0	92	5	3	0	40	60	0	67	33
0	41	100	0	0	75	15	10	67	16	16	5	51	44
3000	41	100	0	0	100	0	0	0	36	64	2	45	53
6000	41	98	2	0	32	18	50	0	32	68	1	47	51
0	42	100	0	0	95	5	0	43	18	39	44	42	15
3000	42	100	0	0	77	11	11	3	24	74	8	54	38
6000	42	90	6	4	92	6	2	36	36	28	0	49	51
0	43	100	0	0	86	14	0	7	33	60	8	61	32
3000	43	100	0	0	20	30	50	0	39	61	6	72	22
6000	43	100	0	0	53	29	18	0	58	42	0	94	6
0	44	100	0	0	11	11	78	0	46	54	0	63	37
3000	44	0	0	0	63	33	6	11	31	58	3	53	44
6000	44	95	5	0	52	23	25	2	54	44	3	78	19
0	45	100	0	0	100	0	0	0	31	69	19	51	30
3000	45	100	0	0	98	2	0	4	49	47	2	78	20
6000	45	100	0	0	53	18	29	14	30	56	31	52	17
0	46	99	1	0	12	33	55	1	40	58	0	73	27
3000	46	100	0	0	100	0	0	18	38	44	0	47	53
6000	46	100	0	0	100	22	78	0	22	77	1	86	14
0	47	98	1	1	85	13	3	0	62	38	0	51	49
3000	47	98	1	1	93	6	1	10	32	58	4	55	41
6000	47	100	0	0	72	17	11	4	48	48	6	62	32
0	48	100	0	0	98	2	0	0	53	47	0	86	14
3000	48	100	0	0	88	12	0	25	31	44	20	77	3
6000	48	100	0	0	97	3	0	11	23	66	0	89	11

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE

TABLE D6. % Microspores in Developmental Stages for *B. rapa*, AC-2 - Donor Plant Pretreatment Experiment - Cycocel.

Pretreatment CYCOCEL (ppm)	Days After Planting	0-1 mm Bud Length			1-2 mm Bud Length			2-3 mm Bud Length			3-4 mm Bud Length		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	32	68	22	14	24	45	32	6	39	55	0	69	31
0	33	100	0	0	83	16	1	0	39	61	12	58	30
0	34	100	0	0	100	0	0	7	39	54	5	61	34
0	35	85	6	10	54	29	17	18	41	41	1	69	30
0	36	100	0	0	70	30	0	9	38	53	9	66	25
0	37	100	0	0	15	26	60	1	47	52	2	64	33
0	38	100	0	0	86	11	3	3	44	53	0	65	35
3000	38	96	3	1	76	15	9	19	42	38	7	33	60
6000	38	100	0	0	0	0	0	57	30	13	9	42	49
0	39	100	0	0	100	0	0	2	50	48	0	49	51
3000	39	100	0	0	99	1	0	33	44	23	0	52	48
6000	39	100	0	0	54	29	17	69	22	8	13	35	52
0	40	100	0	0	45	24	31	2	47	51	4	37	59
3000	40	100	0	0	100	0	0	41	25	34	50	18	32
6000	40	88	13	0	100	0	0	0	14	86	0	41	59
0	41	95	5	0	70	26	4	22	33	45	0	41	59
3000	41	100	0	0	74	11	15	7	35	58	22	26	52
6000	41	69	19	16	71	24	4	2	50	48	4	59	37
0	42	100	0	0	12	60	69	11	25	65	11	39	49
3000	42	91	9	0	63	29	8	40	29	31	36	42	22
6000	42	96	4	0	54	22	24	35	34	30	8	26	66
0	43	100	0	0	83	17	0	12	22	66	1	86	13
3000	43	100	0	0	81	13	6	84	7	9	14	22	64
6000	43	100	0	0	33	12	56	21	67	12	3	21	76
0	44	100	0	0	58	31	10	49	32	19	0	26	74
3000	44	100	0	0	69	14	17	0	41	59	0	39	61
6000	44	100	0	0	21	50	29	1	50	49	4	78	18
0	45	98	1	1	0	0	0	3	32	64	0	75	25
3000	45	100	0	0	100	0	0	19	30	51	45	10	45
6000	45	100	0	0	20	38	42	4	34	62	2	46	52
0	46	100	0	0	100	0	0	96	4	0	4	54	42
3000	46	100	0	0	91	7	2	24	31	45	2	34	64
6000	46	100	0	0	42	34	24	9	45	46	8	52	40
0	47	98	1	1	96	3	1	2	29	69	0	46	54
3000	47	100	0	0	20	33	47	0	53	47	8	43	49
6000	47	100	0	0	100	0	0	2	45	53	4	37	59
0	48	100	0	0	53	37	10	3	27	69	0	80	20
3000	48	100	0	0	64	21	14	8	31	62	10	36	53
6000	48	96	1	2	100	0	0	14	35	51	0	85	15

A - TETRAD

B - EARLY UNICULEATE

C - MID- TO LATE-UNINUCLEATE