Herbicides as Chromosome Doubling Agents in Microspore Cultures of Canola (*Brassica napus*).

Katrin Braasch

Supervisors: Dr. Rale Gjuric and Dr. Janice Cuthbert

A thesis submitted in partial fulfillment of

the Honours Thesis (BIOL 4111/6) Course

Department of Biology

University of Winnipeg

2008

Abstract

Doubled haploid programs are important to breeding programs for most crop species. In canola (*Brassica napus*), microspore culture is the technique used to produce doubled haploid plants using chromosome doubling agents. Since the most widely used chromosome doubling agent, colchicine, is toxic and expensive, it is important to assess less toxic and cheaper doubling agents such as the herbicides pronamide and trifluralin. To determine the efficiency of these two herbicides as antimitotic agents in doubled haploid production of canola, buds from two different lines (four genotypes per line) were collected to isolate microspores. These microspores were treated with the different antimitotic agents and ~200 plants per genotype were planted to determine the fertility rate. Additionally, the rates of embryogenesis and embryo quality were determined. By comparing these factors for each treatment to the untreated control, conclusions were made on whether the standard doubling agent, colchicine can be successfully replaced with one of the herbicides. Final results for the rate of embryogenesis and embryo quality show no significant difference between trifluralin and colchicine treatments. In addition, these results are supported by trifluralin's promising fertility results suggesting with further research, trifluralin could be an alternative to the currently used antimitotic agent, colchicine.

Acknowledgements

First, I would like to thank my supervisors Dr. Rale Gjuric and Dr. Janice Cuthbert for their patience, critique and overall support. I, especially, want to thank Janice for proofreading my writings, giving me the input I needed and teaching me proper English. Also thanks to Dr. Gjuric for helping me with the statistical analysis of my data in Agrobase.

A special thanks goes to Baoling Wang for teaching me the necessary techniques I needed to make my 'babies' as well as helping me in several processes of decision making. Thank you also for the patience to answer all the questions I had. In addition, a big thanks to Carol Scott for helping me in the lab with preparations and media changes. I also want to thank Jill Lockery for helping me with my canola plants by watering and fertilizing them on days I was not able to come in.

Many thanks also to my committee members Mrs Rittberg and Dr. Civetta, as well as Dr. Moodie for all the help along the way.

Table of Contents

Abstract ii

Acknowledgements iii

Table of Contents iv

List of Tables v

List of Figures vi

List of Appendices vii

1.0 Introduction 1

2.0 Methods 6

2.1 Donor Plant Preparation 6

2.2 Colchicine, Pronamide, and Trifluralin Stock Solutions 7

2.3 Microspore Isolation and Culture 8

2.4 Embryo Germination and Embryo Transfer 10

2.5 Embryogenesis and Embryo Analysis 10

2.6 Fertility Analysis 11

2.7 Data Treatment and Statistical Analysis 12

3.0 Results 14

3.1 Rate of Embryogenesis 14

3.2 Embryo Quality 17

3.3 Rate of Fertility 18

4.0 Discussion 22

5.0 Conclusion 27

Literature Cited 28

Appendix 31

List of Tables

Table 1: Average rate of embryogenesis for each line and treatment. 15

Table 2: Average rate of fertility for each line and treatment. 19

List of Figures

Fig. 1: Fertility restorer-system for different breeding lines. 2

Fig. 2: Timeline for doubled haploid production in canola. 6

Fig. 3: Point value system used for assessment of the quality of the embryos. 11

Fig. 4: Determination of fertile plant based on presence of anthers and their pollen production. 12

Fig. 5: Determination of sterile plant based on absence of anthers or presence of anthers but lack of pollen production. 12

Fig. 6: Observed rate of embryogenesis for experimental treatments for each genotype. 15

Fig. 7: Average rate of embryogenesis of lines for experimental treatments. 16

Fig. 8: Observed quality of embryos of lines for experimental treatments. 17

Fig. 9: Observed rate of fertility for the experimental treatments for each genotype. 19

Fig. 10: Average rate of fertility of lines for experimental treatments. 20

List of Appendices

Appendix A: Raw data for embryogenesis for genotypes and treatments. 31

Appendix B: Raw data for embryo quality for genotypes and treatments. 32

Appendix C: Raw data for fertility of individual genotypes and treatments. 33

Appendix D: Analysis of variance for rate of embryogenesis for each treatment. 34

Appendix E: Analysis of variance for rate of fertility for each treatment. 35

1.0 Introduction

Canola (*Brassica napus*) is an important crop grown world wide for food, feed and fuel. Since its introduction in 1974 to western Canada (Agriculture and Agri-Food Canada, 2005), the acreage of canola has steadily increased making canola one of the top three crops produced on the Prairies (Statistics Canada, 2007). Currently, annual production in western Canada has been estimated to be 8.8 million tonnes (Statistics Canada, 2007). This estimated production of today's Canadian canola industry generates an economic activity of $13.8 billion and canola export brings over $2.8 billion to the Canadian economy each year (Canola Council, 2008). To ensure better canola cultivars and hybrids further development is important to improve traits such as disease resistance, oil content and yield.

In hybrid canola breeding programs (Figure 1) a homozygous A line mother plant is crossed with a homozygous Rf line father plant to produce heterozygous fertile hybrid seeds resulting in plants increased in size, vigour, or productivity compared to the parental plants (Poehlman and Sleper, 1995). The A line is a cytoplasmic male-sterile (cms) line that does not contain fertility restoring genes in the nucleus (Poehlman and Sleper, 1995). The Rf (fertility restoring) line contains a fertility restoring gene from radish (*Raphanus sativus* L.) on the radish introgression introduced into the nucleus through intergeneric hybridization (Delourme *et al.*, 1998). This dominant fertility restoring gene restores fertility in the resulting hybrids (Delourme *et al.*, 1998). To maintain the male-sterile A (cms) line it is crossed with a male-fertile maintainer (B) line of identical genotype with the exception of cytoplasmic male sterility (Figure 1) meaning that the B line

has a normal cytoplasm making it fertile. These traits of the B line ensure that the A line is maintained with its cytoplasmic male sterility.

Maintenance of A-Line:	**A-Line**	**X**	**B-Line**
Cytoplasm:	**cms**		**Normal**
Fertility-restorer genes: (Nucleus)	**rf_1rf_1**		**rf_1rf_1**
Cross to produce hybrid:	**A-Line**	**X**	**R-Line**
Cytoplasm:	**cms**		**Normal**
Fertility-restorer genes: (Nucleus)	**rf_1rf_1**		**Rf_1Rf_1**
Hybrid:		**AR**	
Cytoplasm:		**cms**	
Fertility-restorer genes: (Nucleus)		**Rf_1rf_1**	

Figure 1: Fertility restorer-system for different breeding lines (adapted from Poehlman and Sleper, 1995).

In the breeding program the B and Rf lines are produced/maintained in the doubled haploid program.

Doubled haploid (DH) programs double the chromosome number of haploid plants producing homozygous diploid plants (Maluszynski *et al.*, 2003). These DH programs are important to breeding programs for most crop species because the breeding period is reduced (Dwarkesh and Sanjit, 1999; Moeller *et al.*, 1994) and 100% homozygosity in the resulting DH plants is ensured (Friedt and Zarhloul, 2005). The first DH programs included root tipping procedures in which roots were immersed in a colchicine solution, the injection of a 0.2 % colchicine solution into secondary buds, and external

application of colchicine to the buds to produce diploid seed (Hansen and Andersen, 1996; Möllers *et al.*, 1994). Although these methods were successful, there were some negative effects observed. The plants generated by root tipping were usually only partly doubled haploid (chimeric) resulting in DH plants producing only a small amount of seed (Hansen and Andersen, 1996). Therefore, DH programs using this method required extra cycles of seed multiplication to produce enough seed for field trials (Hansen and Andersen, 1996). As a result, a new *in vitro* method was developed to produce DH plants more effectively (Möllers *et al.*, 1994). For canola, microspore culture is the technique used to produce doubled haploids from the microspores derived from the canola anthers (androgenetically) (Maluszynski *et al.*, 2003). In culture, the haploid microspores are doubled using chromosome doubling agents also known as antimitotic agents (Hansen and Andersen, 1996). These agents disassemble or prevent the assembly of the spindle apparatus preventing the chromosomes' proper separation (Bartels and Hilton, 1973; Zhao and Simmonds, 1995) during mitosis causing the chromosomes to double and result in fully fertile diploid plants being produced. Although this technique is widely used, the success rate depends on a number of factors including genotype, donor plant physiology, stage of microspore development, culture conditions, culture environment, and doubling agents (Friedt and Zarhloul, 2005, Xu *et al.*, 2007).

The most widely used chromosome doubling agent in canola DH programs is colchicine. At low concentrations, colchicine binds to tubulin heterodimers during assembly of microtubules preventing their polymerization (Margolis and Wilson, 1977). However, animal and human microtubules can also be depolymerised with high concentrations of colchicine (Ravelli *et al.*, 2004). Since colchicine shows a higher affinity for animal tu-

bulin than for plant tubulin (Bartels and Hilton, 1973) higher concentrations of colchicine are necessary in microspore cultures of canola to compensate for the lower affinity. The high concentration of colchicine used and its high affinity for animal tubulin could result in toxic effects on humans. Studies dealing with the possible effects to long exposure in the work environment could not be found, but it was determined that colchicine has an oral administration LD_{50} value of 0.086 mg/kg in humans and is possibly carcinogenic (Material Safety Data Sheet). Due to the reasons mentioned above, alternative antimitotic agents are needed which have a higher affinity for plant tubulin and therefore work at lower concentrations preferably without an affinity for animal tubulin.

Two antimitotic agents with these properties are the herbicides pronamide and trifluralin. In previous studies, it was determined that the dinitroaniline trifluralin disrupts the spindle microtubules in a similar manner to colchicine (Hansen and Anderson, 1996). However, unlike colchicine, trifluralin demonstrated a higher affinity for plant tubulin producing similar results after only 30 minutes of exposure instead of 3 – 8 hours of exposure time needed for colchicine (Zhao and Simmonds, 1995). In toxicology studies of trifluralin, the herbicide was evaluated to be slightly toxic, but neither capable of causing damage to DNA (genotoxic) nor carcinogenic for lab animals tested (Ebert *et al.*, 1992). In addition, the oral administration LD_{50} value for humans is 3700 mg/kg (Material Safety Data Sheet) which is higher than the value mentioned above for colchicine. Therefore, trifluralin is a potential candidate as an alternative antimitotic agent in doubled haploid programs.

The second herbicide that could be a candidate is pronamide, which disrupts the spindle microtubule formation in a different manner to colchicine and trifluralin (Vaughn

and Vaughn, 1987). In a previous study, it was suggested that pronamide either destabilizes the microtubules or limits the supply of proteins needed for microtubule development (Vaughn and Vaughn, 1987). Like trifluralin, pronamide shows no affinity for animal microtubules/tubulin and has a high affinity for plant microtubules/tubulin (Bartels, 1973). Pronamide also shows no apparent toxicity but there are indications that it could cause cancer and also target organs like the liver, thyroid, as well as adrenal and pituitary glands (Extension Toxicology Network, 1996). The LD_{50} value for oral administration could not been found for humans but was 10,000mg/kg in dogs (Material Safety Data Sheet).

Since pronamide and trifluralin have higher LD_{50} values for animals (mammals) and show higher affinity for plant microtubules/tubulin at lower concentrations than colchicine, the possible negative effects are minimized making these two herbicides good potential doubling agents in a microspore culture program.

The objective of this thesis was to determine the efficiency of colchicine, pronamide, and trifluralin as antimitotic agents in double haploid production of canola (*Brassica napus)*. In the research the antimitotic agents were compared based on rate of embryogenesis, embryo quality, and fertility rate for the microspore cultures of 4 selected genotypes per B and Rf line maintained by the DH program. As a result, conclusions were made on whether colchicine can be replaced by one of the herbicides as a doubling agent for microspore cultures of canola.

2.0 Methods

The general flowchart in Figure 2 represents the approximate time required for the research project. Furthermore, Figure 2 illustrates the main stations during the process and the time span between these points. Each station of the research is described in detail in the next sections of the methods and was completed using the Guelph protocol (1998) as a guideline.

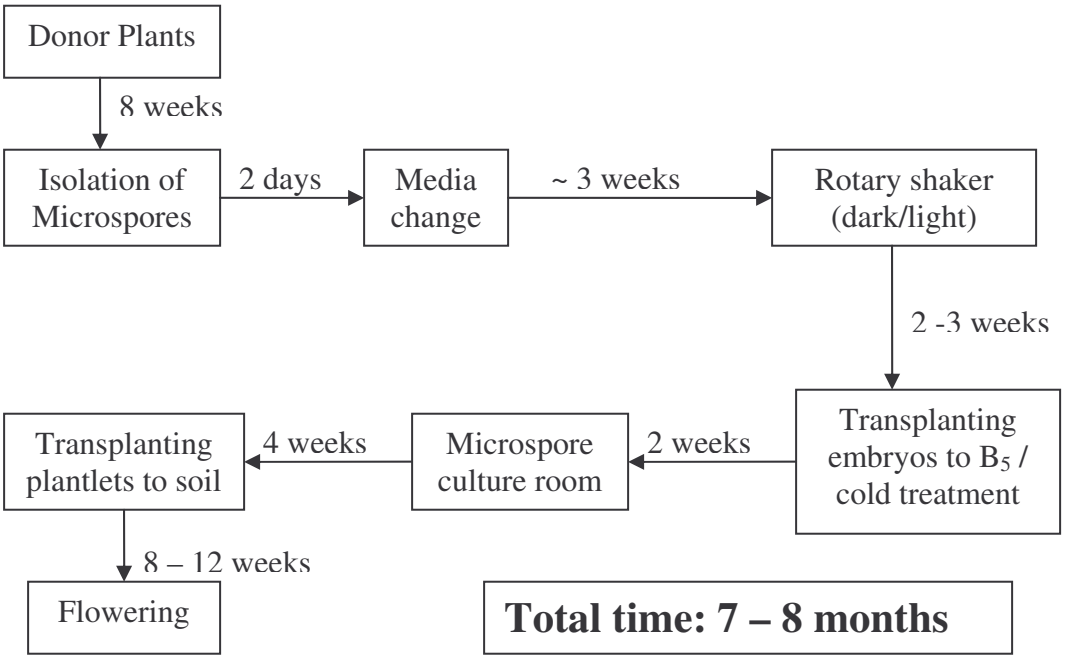

Figure 2: Timeline for doubled haploid production in canola.

2.1 Donor Plant Preparation

The donor plants of canola were grown at approximately 15ºC in a growth room with the humidity between 35 – 50% and a light cycle of 16 hours light / 8 hours dark in 60% monochromatic yellow and 40% monochromatic blue light. In the growth room, the donor plants were grown in 4 inch pots and watered as well as fertilized regularly. These controlled conditions were very important as they ensured a minimal stress influence by the environment thus guaranteeing optimal conditions for the donor plants needed to be

used in the DH program. It was also important to cut back the flowering branches to ensure the regrowth of buds with the desired size. In previous papers, the best results for microspore cultures were found using buds of about 3 - 4mm in length (Iqbal *et al.*, 1994). This size provides the best results as it corresponds with a very late uninucleate stage of the haploid microspores which are embryogenic and most responsive to chromosome doubling agents (Fletcher *et al.*, 1998). For this research project, the following genotypes of the B and Rf lines were chosen as a representative sample of genotypes used in the breeding program:

B line: 63509, 63511, 63539, 63546

Rf line: 63058, 63064, 63066, 63069

2.2 Colchicine, Pronamide, and Trifluralin Stock Solutions

Stock solutions of 0.1 M colchicine (Sigma, USA) in double-distilled water, 0.3 mM pronamide (Supelco, USA) in acetone, and 0.2 mM trifluralin (Supelco, USA) in acetone were prepared and stored in the fridge. The stock solution of colchicine and trifluralin were additionally wrapped in tin foil to prevent a decay of the chemical by light (Le Person *et al.*, 2007; Sagorin *et al.*, 1972). For the treatment of the isolated microspores, the chemicals were diluted to the appropriate concentrations in 500 mL of 13% sucrose embryo induction medium (NLN): 0.1 mM colchicine, 3.0 µM pronamide, 0.5 µM trifluralin.

2.3 Microspore Isolation and Culture

From the isolation process to the transfer of the embryos to soil aseptic technique was necessary since the nutrient rich media used presented an optimal environment for growth of microorganisms harmful to this process.

During the isolation and any further procedures the two lines (B and Rf) were treated identically. For the first step in the isolation of the microspores, buds of four different genotypes were chosen from the Rf as well as the B line donor plants ranging in size from 3 – 4 mm. After the buds were collected they were sterilized in Javex [Sodium Hypochlorite 5.25%] for 10 minutes. Following the sterilization, the buds were washed twice in double distilled water for 5 minutes each to remove any Javex residues. The buds were then transferred aseptically into a laboratory blender cup to which autoclaved sugar water was added. By blending the buds their structure was destroyed releasing the microspores into the sugar water. The blended mixture of free microspores and cell debris was poured through two sterile filters (64 and 44 µm) into a beaker funnel to collect the isolated microspores in the filtrate. The blender cup was then rinsed with sugar water and the contents again poured through the filters. Once all the fluid was drained through the funnel into the beaker, the filtrate containing the isolated microspores was poured into a sterile 50 mL centrifuge tube and the volume adjusted to approximately 50 mL with sugar water. The tubes were then centrifuged for 3 min at 1200 rpm to pellet the microspores. After the centrifugation, the supernatant was poured off so that only the spores were left behind. The spores of each line were centrifuged for a second time after they were resuspended in sugar water and separated into four 50 mL tubes to remove any toxins released from the green tissue of the buds after homogenization (Wittstock and Ger-

shenzon, 2002). Once the supernatant was poured off the microspores (pellet) were ready to be treated with the antimitotic agent. Three of the portions were cultured in media containing either colchicine, pronamide, or trifluralin and the fourth portion of microspores was cultured with pure NLN to serve as the control. The untreated control was used to observe the natural rate of embryogenesis (spontaneous diploidization expected for canola) without the influence of an antimitotic agent (Möllers *et al.*, 1994). Before plating the different microspore cultures into Petri Dishes (Fisherbrand, 100x15mm), the number of microspores was determined twice for each culture using a haemocytometer and visual estimation. The haemocytometer was used by putting one drop of microspore culture on each side of the haemocytometer. The plates were then covered with a cover slip before viewing the microspores under the microscope. The average number of microspores per culture (50 mL) was determined by counting all microspores (big and small) in all four fields of the haemocytometer and using the following equation:

$$\#microspores = \frac{\#spores_counted}{\#fields_counted(4fields)} * 10{,}000$$

In addition, a visual estimation of the number of microspores for each culture was done based on the turbidity and coloration of the culture tubes. Using the number determined with the haemocytometer and visual estimation each culture was diluted to a density of 90,000 microspores per 25 mL. Next, 15 mL of this diluted microspore culture were plated per petri dish. The cultures were then incubated at 32ºC in the dark for up to three weeks. After two days, a media change was performed for the colchicine and pronamide cultures to replace the NLN liquid containing the doubling agent with pure NLN. A media change was not done for the trifluralin treatment and the control. Once embryos were visible in the plates, the petri dishes were transferred onto a rotary shaker (in the dark at

500 rpm). When the embryos reached the desired size of approximately 4 mm, the plates were placed in the light section on the rotary shaker (500 rpm) where the embryos continued to grow and were allowed to become greener in color.

2.4 Embryo Germination and Embryo Transfer

Following the 'greening up' process, the embryos were transferred onto a solid B_5 germination medium (for recipe see Guelph protocol) containing nutrients for shoot and root development. After the transfer, the embryos underwent a cold treatment in the fridge at 4ºC for approximately two weeks to promote root development (Xu *et al.,* 2007). The embryos were then moved into the culture room at 24ºC under and a light cycle of 16 hours light / 8 hours dark where the majority of the embryos developed roots after 3 – 4 weeks. Once sufficient root development was observed the rooted plantlets were transferred to 50 cell trays and grown in a growth room at 20 - 25ºC with a humidity of 35 – 50%, and a light cycle of 16 hours light / 8 hours dark.

2.5 Embryogenesis and Embryo Analysis

The rate of embryogenesis for each treatment and genotype was determined by counting the number of embryos obtained and comparing it to the number of microspores estimated by the haemocytometer. Furthermore, the quality of the embryos was assessed based on the embryo type and root development for each line. Therefore, a number value was assigned for embryo type and root development as outlined in Figure 3. This allowed a qualitative comparison between treatments and genotypes to be made.

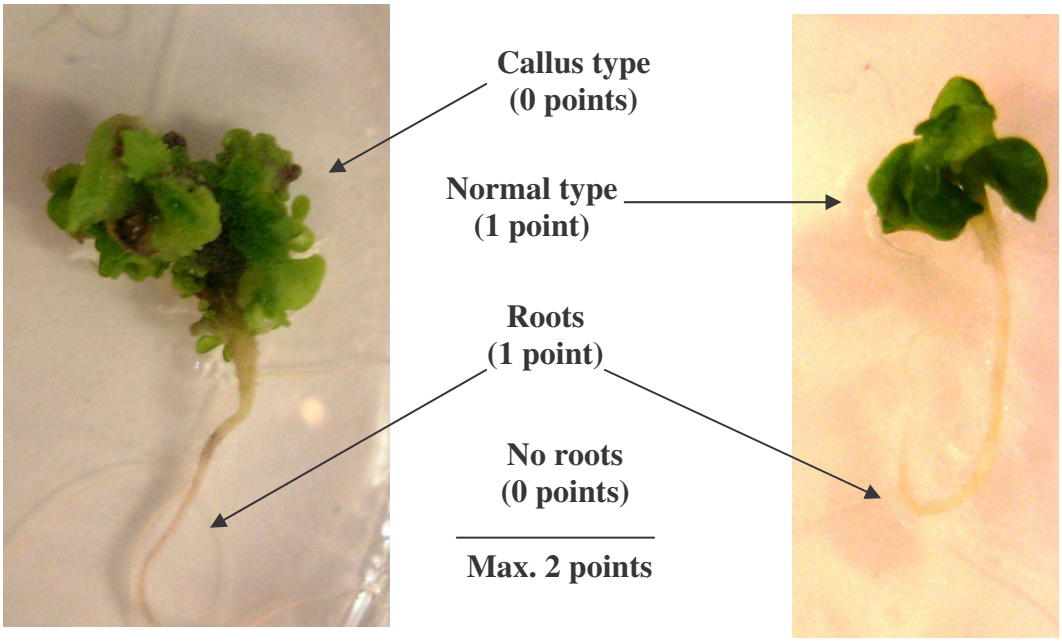

Figure 3: Point value system used for assessment of the quality of the embryos.

2.6 Fertility Analysis

The fertility of each canola plant was determined at flowering and recorded to calculate the rate of fertility for the genotypes and treatments. A plant was scored as fertile when its flowers had anthers that produced pollen (Figure 4). Pollen production was determined by tapping the anthers of the flower on the palm of the hand. If pollen was produced, a yellow residue was found on the palm. A plant was scored as sterile if the flower of the plant either had no anthers or the anthers showed no pollen production (Figure 5). Based on these scores the fertility rate for each treatment and genotype was determined.

Figure 4: Determination of fertile plant based on presence of anthers (arrow) and their pollen production.

Figure 5: Determination of sterile plant based on absence of anthers (arrow) or presence of anthers but lack of pollen production.

2.7 Data Treatment and Statistical Analysis

The raw data for embryogenesis, embryo quality, and fertility was used to calculate the rates for embryogenesis and fertility and the average embryo quality for each genotype and its particular treatment (e.g., 63058 – control, 63058 – colchicine…). In the next step, the raw data for each genotype and treatment belonging to the same line (e.g., all genotypes of the Rf line used as a control) were added to calculate the average

rate of embryogenesis, embryo quality, and rate of fertility for each line (B or Rf) and its nparticular treatment.

Results were analyzed with Agrobase (Agronomix Software Inc.) to compare the treatments and genotypes used based on an analysis of variance (ANOVA). To test for significant differences the ANOVA used [genotype*treatment], [genotype*line], and [genotype*line*treatment] as the residual factor (or error term). These combinations were used as residual factor on the assumption that they had no significant influence on the results in a small sample size as used for this experiment. To determine whether significant differences existed between the treatments and lines, Agrobase calculated the least significant difference (LSD). The LSD was then used to compare the average values between the ranks and determine significant differences.

3.0 Results

During this research, microspores were isolated and embryos were obtained for most of the treatments. The isolation and culture procedures were successfully repeated three more times. The initial results showed no embryos were produced for the pronamide treatment in the first round therefore, a change in the methods was necessary. This allowed for some embryos to be obtained, however, the numbers were low and fertility rate could only be estimated.

3.1 Rate of Embryogenesis

The rates of embryogenesis for all treatments are shown in Figure 6 for each genotype compared to the untreated control of 63058. The control of this genotype was randomly chosen to be used as an internal standard and set to 1.0 allowing an easier comparison between the genotypes and respective treatments. Figure 6 indicated that the pronamide treatment overall had a very low rate of embryogenesis. Colchicine and trifluralin, however, showed embryogenesis for all or most of the genotypes, respectively. As expected, some of the genotypes showed a lower rate of embryogenesis for the colchicine and trifluralin treatments than for their respective untreated control (e.g., 63058 – colchicine and 63546 – trifluralin). In comparison, some genotypes surprisingly showed a higher rate of embryogenesis when compared to their respective untreated control (e.g., 63509 – colchicine and 63069 – trifluralin).

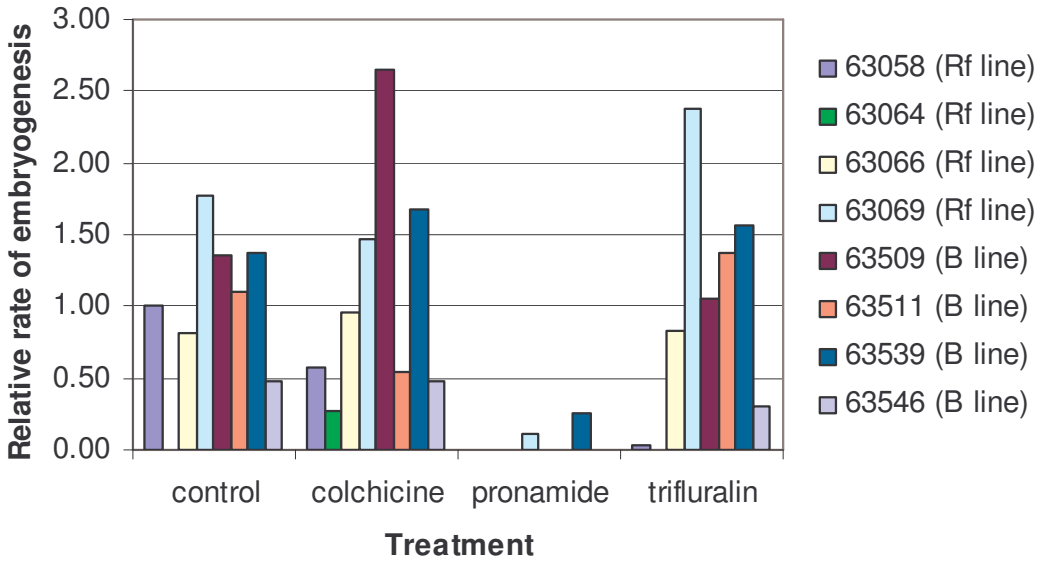

Figure 6: Observed rate of embryogenesis for experimental treatments for each genotype. The control treatment of 63058 was used as the internal standard (1.0).

To determine the average rate of embryogenesis of the B and Rf lines compared to the treatments, the number of microspores and embryos for each line and treatment were added (Table 1) and graphed (Figure 7).

Table 1: Average rate of embryogenesis for each line and treatment.

Line	Treatment	Microspores	Embryos	Embryogenesis (%)
B	Control	288750	4241	1.47
B	Colchicine	280000	4115	1.47
B	Pronamide	287500	70	0.02
B	Trifluralin	306250	4272	1.39
Rf	Control	232500	1825	0.78
Rf	Colchicine	227500	1706	0.75
Rf	Pronamide	196250	45	0.02
Rf	Trifluralin	196250	932	0.47

Figure 7 illustrates the average rate of embryogenesis for the treatments and the used the B line control as internal standard set to 1.0 to allow the rates of the antimitotic agents to be compared between the treatments and lines studied. As seen in Figure 6, the rate of

embryogenesis observed for pronamide was very low compared to the control, as well as the colchicine and trifluralin treatments (Figure 7, Table 1). However, Figure 7 and Table 1 illustrated that on average, the colchicine and control treatments, had nearly the same effect on the rate of embryogenesis for the genotypes of the Rf and B lines. While trifluralin had a similar effect on embryogenesis in the B line compared to the control, it seemed to have had a stronger effect on the genotypes of the Rf line causing a decrease in the rate of embryogenesis to about 60% of the rate observed for the untreated control (Figure 7, Table 1).

Figure 7 also illustrates the results of the statistical analysis for the rate of embryogenesis. As expected from the primary observations of Figure 7, no significant difference was observed between the colchicine and trifluralin treatments when compared to the control (see Appendix D). However, as indicated above, a significant difference was observed for the pronamide treatment (see Appendix D). When looking at the individual response of the two lines (B and Rf) for each treatment, surprisingly no significant difference was detected.

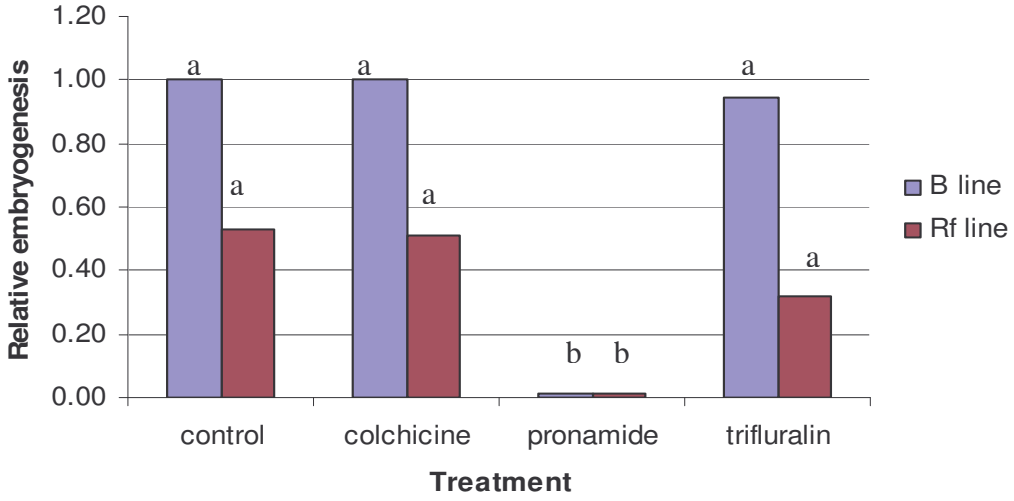

Figure 7: Average rate of embryogenesis of lines for experimental treatments.

Treatments marked with the same letter are not significantly different at $P > 0.05$. The control treatment of the B line was used as the internal standard (1.0).

3.2 Embryo Quality

The quality of the embryos was observed for each experimental treatment and is shown in Figure 8. Due to the very low rate of embryogenesis for the pronamide treatment, no quality assessment could be made for the Rf line genotypes. However, a few embryos were produced for the B line genotypes permitting a preliminary assessment of embryo quality to be completed. As illustrated in Figure 8, the B line embryos from the pronamide treatment were found to have the lowest quality among all treatments. The remainder of the treatments demonstrated that the colchicine treatment yielded slightly lower quality embryos whereas the trifluralin treatment produced slightly higher quality embryos compared to the embryos obtained in the control (Figure 8).

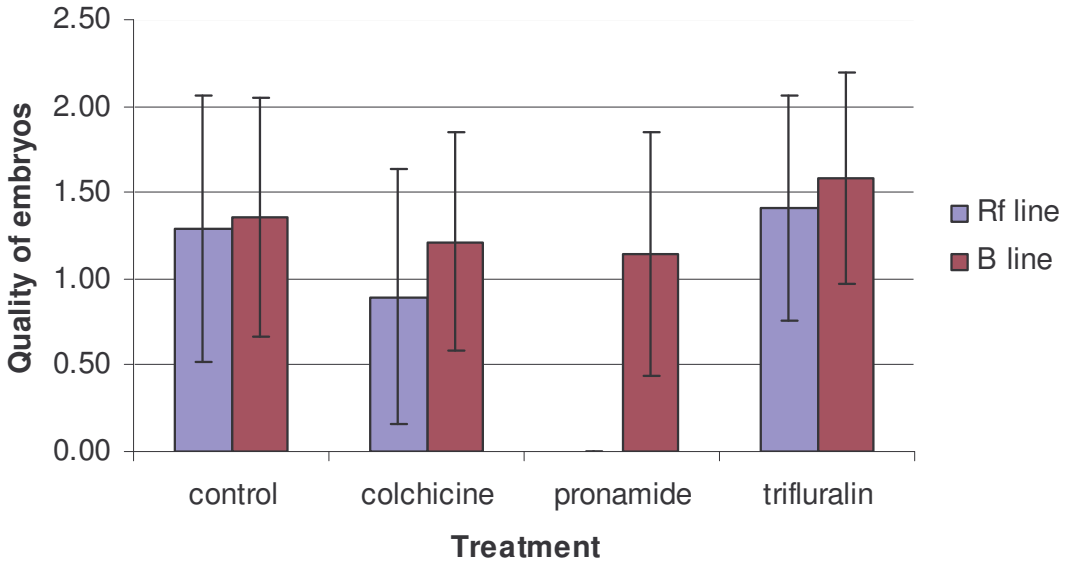

Figure 8: Observed quality of embryos of lines for experimental treatments.

3.3 Rate of Fertility

Due to the low rate of embryogenesis for the pronamide treatment not enough data was available to determine its effect on the rate of fertility for all genotypes. In addition, problems with contaminated B_5 plates caused the number of plantlets to be low for all other treatments, especially colchicine. Therefore, the colchicine derived plants produced by the DH lab were scored in addition to the ones obtained during this research project.

The effects of trifluralin, pronamide and colchicine on the rate of fertility are shown in Figure 9 for each genotype compared to the untreated control 63058. Once again the control was chosen to be used as an internal standard set to 1.0 for easier comparison between the genotypes and respective treatments. Figure 9 illustrates that the rate of fertility for the pronamide treatment could only be determined for one genotype (e.g., 63539). Yet, the rate of fertility for that genotype showed an increase to the respective control treatment. The plants derived from the colchicine and trifluralin treatment, however, showed higher fertility rates than the untreated control for all genotypes (Figure 9).

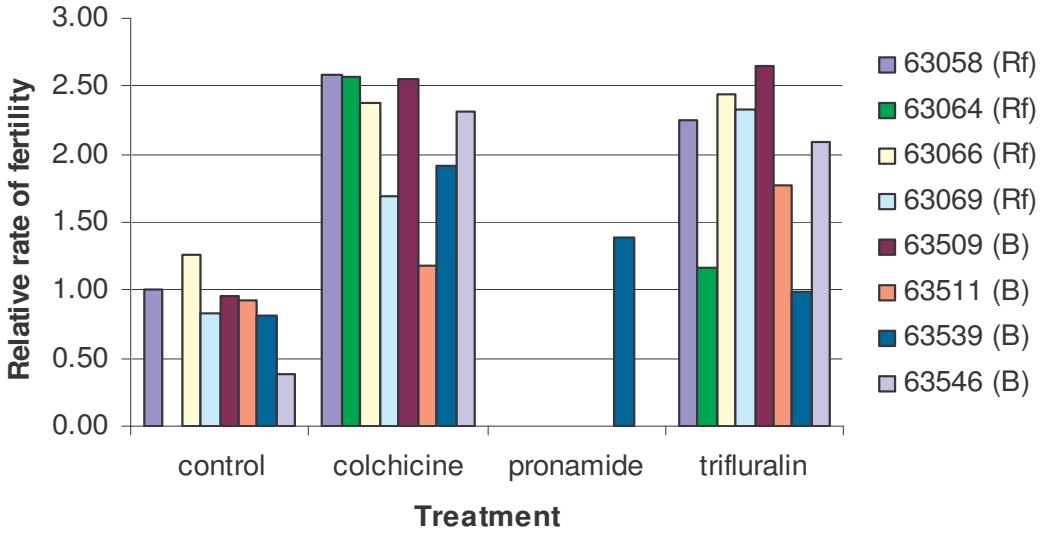

Figure 9: Observed rate of fertility for the experimental treatments for each genotype. The control treatment of 63058 was used the internal standard (1.0).

To determine trends in the rate of fertility for the B and Rf lines to the treatments, the number of fertile and sterile plants for each line and treatment were added to calculate the average rate of fertility (Table 2) and then graphed (Figure 10).

Table 2: Average rate of fertility for each line and treatment.

Line	Treatment	Fertile	Sterile	Rate of fertility
B	Control	59	183	0.24
B	Colchicine	761	320	0.70
B	Pronamide	11	12	0.48
B	Trifluralin	166	123	0.57
Rf	Control	121	223	0.35
Rf	Colchicine	775	188	0.80
Rf	Pronamide	0	0	-
Rf	Trifluralin	82	22	0.79

In Figure 10, the control of the B line was used as an internal standard set to 1.0 for an easier comparison between the lines and respective treatments. As expected, the figure shows that on average, the rate of fertility for microspores treated with colchicine and trifluralin was higher than the untreated control for both lines (B and Rf). Furthermore,

Figure 10 also illustrates that on average the rates of fertility for the colchicine and trifluralin treatments were similar for both the Rf and B line evaluated in this study. In addition, Figure 10 includes the result for the pronamide treatment indicating that the rate of fertility was doubled in the B line compared to the control. However, as seen in Figure 9 this average result was based only on the results from one genotype.

The statistical analysis of the rate of fertility illustrated in Figure 10 indicated that as expected the rates of fertility for the colchicine and trifluralin treatments were significantly different when compared to the untreated control and pronamide treatment (see Appendix E). However, no significant differences were observed between the colchicine and trifluralin treatments (see Appendix E). In addition, the B and Rf lines showed no significant differences within each treatment (see Appendix E). Pronamide was included in the analysis and showed a significant difference to the control and the colchicine and trifluralin treatments (see Appendix E). However, as mentioned above the results pronamide in Figure 10 were based only on one genotype.

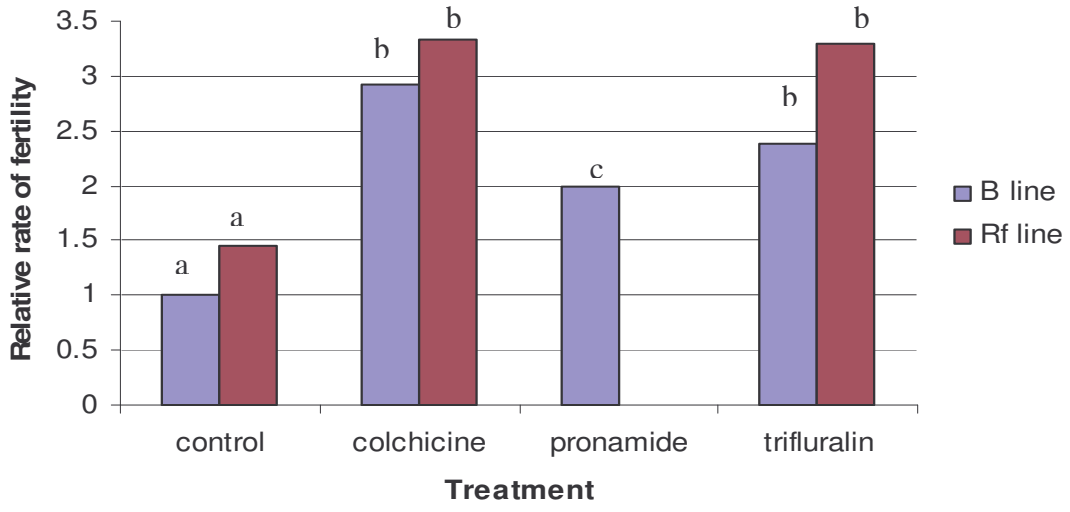

Figure 10: Average rate of fertility of lines for experimental treatments.

Treatments marked with the same letter are not significantly different at $P > 0.05$

The control treatment of the B line was used as the internal standard (1.0).

4.0 Discussion

The objective of this thesis was to determine the efficiency of the herbicides trifluralin and pronamide as chromosome doubling agents and decide whether colchicine could be replaced by one of these herbicides in DH programs of canola.

Due to the time required to obtain rates of fertility, the experiment was only completed four times (twice for each genotype) within a month. In addition, the time constraint did not allow for any major changes to the methods to be made based on the initial results. Therefore, only small sample sizes were obtained allowing some initial conclusions to be drawn from the experiment.

In the first part of the research, the rate of embryogenesis was determined for the different treatments and genotypes. It was expected that the treated cultures would show a lower rate of embryogenesis than the untreated control since it has been observed that antimitotic agents have a negative effect on embryogenesis (Zhao and Simmonds, 1995). This negative effect is based on the slow recovery rate of the microtubules after treatment with an antimitotic agent. As a result, the amount of microspores developing to embryos is reduced, therefore, lowering the rate of embryogenesis. Based on previous papers, this negative effect is linear, meaning that higher concentrations lead to a larger decrease in rate of embryogenesis (Zhao and Simmonds, 1995). In addition, higher concentrations of antimitotic agents have growth inhibitory effects on plants (Hess, 1989). Furthermore, it was expected that the Rf line genotypes would show a lower rate of embryogenesis when compared to the B line genotypes. The lower response of the Rf line was expected because of the fertility restoration gene carried on an extra piece of genomic DNA in the Rf line (Delourme *et al.*,1998). However, the results obtained for the rates of embryogenesis

did not show a lower rate for the colchicine and the trifluralin treatments compared to the untreated control as expected. Furthermore, this observation was supported by no significant differences between the untreated control and the colchicine and trifluralin treatment. In addition, the results showed that as expected the colchicine and trifluralin treatments produced better results for the B lines than for the Rf lines and agree with observations made by DSV Canada. However, no significant differences were detected between the B and Rf lines for each treatment. This indicated that the negative effects of the treatment concentrations and exposure time (48 hours before media change) used for the colchicine and no media change for the trifluralin treatments were not excessively disturbing the embryogenesis. These unexpected results do not agreed with the finding of Hansen and Andersen (1996) or Zhao and Simmonds (1995) that antimitotic agents reduce embryogenesis, were most likely due to the small sample size of this experiment or could be explained by the low concentrations of antimitotic agents used compared to previous studies. Also, although unexpected, these results indicated that the extra restorer gene in the Rf line did not negatively influence the rate of embryogenesis. Even though the results obtained are not as expected it can be concluded that the antimitotic agents colchicine and trifluralin present a good foundation for doubled haploid production of canola. Therefore, in order to make a decision on pronamide's efficiency in microspore culture of canola, further research is required to determine the concentrations and exposure time more suitable for the current protocol. Although the results might suggest that certain genotypes respond better to the trifluralin or colchicine treatments, this cannot be concluded since the use of only a haemocytometer to count numbers of microspores for each culture. These vague numbers were due to the uneven distribution of microspores

and a higher abundance of microspores outside the counting fields for most determinations. To minimize this effect, all counts were completed twice. As the uneven distribution was still present, the results only provided an indication of the number of microspores. To determine whether there were significant differences between the genotypes and the antimitotic agents, further research is necessary and should include more microspore isolations and a more accurate method of assessing the number of microspores in each culture.

The second part of the analysis was the assessment of embryo quality. Based on the results, it appeared that the trifluralin treatment provided embryos with the best quality. The colchicine treated microspore cultures in general developed embryos with lower quality when compared to the control and the trifluralin treatments. However, this assessment was a subjective rating based on a small sample size (all treatments together n = 878 embryos). Therefore, a significant difference was not observed among the treatments or the lines. To obtain a better indication of embryo quality, a larger number of embryos should be scored by several people. Although, embryo quality is not the main factor of the analysis for this research, it is important because higher quality embryos are more likely to develop into higher quality plantlets and to survive being transplanted into soil and develop into mature plants.

However, the most important part of the analysis for this experiment was the assessment of fertility for each line and genotype. The observed rate of fertility of the control treatment was based on spontaneous diploidization known to occur in canola (Möllers *et al.*, 1994). As expected, the lines treated with an antimitotic agent showed higher rates of fertility than the untreated control. This was expected as the antimitotic

agents initiate the doubling of the chromosomes in the haploid microspores (Möllers *et al.*, 1994). The results of the colchicine and trifluralin treatments for both lines (B and Rf) indicated the observed rate of fertility was more than twice the rate of the control treatment. The statistical analysis further supported this observation by indicating significant differences between the control and the colchicine and trifluralin treatments. These results and conclusions also agreed with former research done in this area (Hansen and Andersen, 1996; Zhao and Simmonds, 1995). Although, the average rate of fertility between both lines for the trifluralin treatment was close to 70% and therefore seems better than the almost 50% observed in a previous study (Zhao and Simmonds, 1995) this conclusion cannot be made due to the small sample size used. It is also observed that the B lines showed slightly lower rates of fertility when compared to the Rf line. This was surprising as the B lines were expected to have a higher rate of fertility based on the results of the DSV Canada double haploid program. However, there were no significant differences between the lines (B and Rf) in the colchicine and trifluralin treatments. Based on these results, the conclusion that the colchicine and trifluralin treatments significantly improved the rate of doubled haploid plants (= fertile plants) can be made. The results also indicate that the trifluralin treatment is equal to the colchicine treatment and agree with the findings of Hansen and Andersen (1995). In addition, no significant differences between the lines for each treatment were observed concluding no line responded better to either treatment. This was, as mentioned above, unexpected and possibly caused by the small sample size. Therefore, further work should include a larger number of microspores for each treatment to obtain more data for representative results. Due to the low rate of embryogenesis in the pronamide treated microspore cultures not

enough embryos were obtained to provide a reliable estimate for the rate of fertility. Although, the results show the pronamide treatment increased the rate of fertility for the B line this result was only based on one genotype. Therefore, the results cannot be used to make a decision about pronamide as an antimitotic agent in microspore cultures of canola. However, former research and the indications from this experiment support the possible future use of pronamide as an antimitotic agent in microspore cultures of canola.

Overall, it can be concluded there were no significant differences between the rate of embryogenesis and the rate of fertility for the colchicine and trifluralin treatments. Since there were no significant differences observed among the lines in each treatment, it can be concluded the trifluralin and colchicine treatments were successful for both the Rf and the B lines. As the quality of embryos appears to be slightly better for trifluralin, this treatment seems to be a good alternative to colchicine as a doubling agent. In addition, this conclusion can be supported by the fact that trifluralin is less toxic to humans than colchicine, especially with the very low concentrations needed. However, these results are only indications and larger sample sizes should be analyzed to support this conclusion. At this point, no conclusion can be made regarding the efficiency of pronamide as an antimitotic agent. Therefore, further research is necessary to improve the protocol for the use of pronamide to determine its efficiency as an antimitotic agent in microspore cultures of canola.

5.0 Conclusion

1. The objective of the study was met for the colchicine and trifluralin treatments. Sufficient embryos were obtained to complete a proper analysis of rate of embryogenesis, quality of embryo, and rate of fertility.
2. Since an insufficient number of embryos was obtained for the pronamide treatment, further research is necessary to assess pronamide as a replacement of the antimitotic agent colchicine. To achieve a higher rate of embryogenesis the initial concentration of pronamide should be considered as including a media change earlier in the procedure to decrease the negative effect of the antimitotic agent on the rate of embryogenesis.
3. After reviewing all the results trifluralin seems to be a good candidate to replace colchicine as an antimitotic agent in the doubled haploid production of canola. However, since the sample size was small, future research should be completed to support this conclusion.

Literature Cited

Agriculture and Agri-Food Canada. January 2005. Crop Profile for Canola in Canada. <http://www4.agr.gc.ca/resources/prod/doc/prog/prrp/pdf/canola_e.pdf> Accessed 2008 March 26.

Bartels, P.G., and J.L. Hilton. 1973. Comparison of trifluralin, oryzalin, pronamide, propham and colchicines treatments on microtubules. Pesticide Biochemistry and Physiology. 3 (4): 462-472.

Canola Council. 2008. Canada's canola industry. Innovative. Resilient. Determined to create superior value and a healthier world. <http://www.canolacouncil.org/canadian_canola_industry.aspx>. Accessed 2008 March 27.

Delourme, R., Foisset, N., Horvais, R., Barret, P., Champagne, G., Cheung, W.Y., Landry, B.S., and M. Renard. 1998. Characterisation of the radish introgression carrying the *Rfo* restorer gene for the *Ogu*-INRA cytoplasmic male sterility in rapeseed (*Brassica napus L.*). Theoretical and Applied Genetics. 97: 129-134.

Dwarkesh, S.P., and A. Sanjit. 1999. Microspore Culture for Induction of Doubled Haploidy in *B. juncea* and *B.* rapa. <http://www.regional.org.au/au/gcirc/4/246.htm> 10th International Rapeseed Congress. Accessed 2008 March 26.

Ebert, E., Leist, K.H., Hack, R., and G. Ehling. 1992. Toxicology and hazard potential of trifluralin. Food and Chemical Toxicology. 30: 1031-1044.

Extension Toxicology Network. June 1996. Pesticide Information Profile: Pronamide. <http://extoxnet.orst.edu/pips/pronamid.htm>. Accressed 2008 March 26.

Fletcher, R., Coventry, J., and L.S. Kott. 1998. Double Haploid Technology for Spring and Winter *Brassica napus* (revised ed.). Technical Bulletin p. 42. OAC Publication, University of Guelph, Ontario, Canada.

Friedt, W., and M.K. Zarhloul. 2005. Haploids in the Crop Improvement II. Biotechnology in Agriculture and Forestry. 56: 192-202.

Hansen, N.J.P., and S.B. Andersen. 1996. *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. Euphytica 88: 159-164.

Hess, F.D. 1989. Herbicide interference with cell division in plants. In: P. Boeger & G. Sandmann (Eds) Target sites of herbicide action. CRC Press, Boca Raton, p 85-103.

Iqbal, M.C.M., Möllers, C., and G. Roebbelen. 1994. Increased Embryogenesis after Colchicine Treatment of Microspore Cultures of *Brassica napus* L. Journal of Plant Physiology. 143: 222-226.

Le Person, A., Mellouki, A., Munoz, A., Borras, E., Martin-Reviejo, M., and K. Wirtz, 2007. Trifluralin: Photolysis under sunlight conditions and reaction with HO^{-} radicals. Chemosphere. 67: 376–383.

Maluszynski, M., Kasha, K.J., Forster, B.P., and I. Szarejko. 2003 *Doubled Haploid Production on Crop Plants*. Springer, p 1.

Margolis, R.L., and L. Wilson. 1977. Addition of Colchicine-Tubulin Complex to Microtubule Ends: The Mechanism of Substoichiometric Colchicine Poisoning. Proceedings of the National Academy of Sciences. 74: 3466-3470.

Möllers, C., Iqbal, M.C.M., and G. Roebbelen. 1994. Efficient production of doubled haploid *Brassica napus* plants by colchicines treatment of microspores. Euphytica. 75: 95-104.

Poehlman, J.M, and D.A. Sleper. 1995. *Breeding Field Crops*. Iowa State Press, Ames, p 209-212.

Ravelli, R.B., Gigant, B., Curmi, P.A., Jourdain, I., Lachkar, S., Sobel, A., and M. Knossow. 1994. Insight into tubulin regulation from a complex with colchicine and stathmin-like domain. Nature. 428: 198-202.

Sagorin, C., Ertel, N. H., and S.L. Wallace. 1972. Photoisomerization of colchicine. Loss of significant antimitotic activity in human lymphocytes. Arthritis & Rheumatism. 15: 213-217.

Statistics Canada. 2007. Field and specialty crops (Production and Seeded area). <http://www.40.statcan.ca/l01/cst01/>. Accessed 2008 March 26.

Vaughn, M.A., and K. Vaughn. 1987. Pronamide disrupts mitosis in a unique manner. Pesticide Biochemistry and Physiology. 28 (2): 182-193.

Wittstock, U. and J. Gershenzon. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. Current Opinion in Plant Biology. 5 (4): 300-307.

Xu, L., Najeeb, U., Tang, G.X., Gu, H.H., Zhang, G.Q., He, Y. and W.J. Zhou. 2007. *Haploid and Doubled Haploid Technology. In: S.K. Gupta (Ed) Advances in Botanical Research. Incorporating Advances in Plant Pathology. Rapeseed Breeding*. Academic Press, San Diego, p. 181-216.

Zhao, J., and D.H. Simmonds. 1995 Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. Physiologia Plantarum. 95: 304-309.

Appendix

Appendix A:

Raw data for embryogenesis for genotypes and treatments:

Geno-type	Line	Treatment	Haemocy-tometer countings	# of microspores	# of embryos	Embryo-genesis (%)
63058	Rf	Control	11.0 + 14.0	62500	819	1.31
63058	Rf	Colchicine	8.0 + 9.0	42500	324	0.76
63058	Rf	Pronamide	9.5 + 11.0	51250	0	0.00
63058	Rf	Trifluralin	6.0 + 10.0	40000	16	0.04
63064	Rf	Control	21 + 21.5	106250	0	0.00
63064	Rf	Colchicine	31.5 + 15.0	116250	410	0.35
63064	Rf	Pronamide	14.5 + 18.5	82500	0	0.00
63064	Rf	Trifluralin	22.5 + 13.0	88750	9	0.01
63066	Rf	Control	9.5 + 5.5	37500	400	1.07
63066	Rf	Colchicine	18.5 + 2.5	52500	658	1.25
63066	Rf	Pronamide	9.5 + 6.0	30000	0	0.00
63066	Rf	Trifluralin	17.5 + 2.5	58750	634	1.08
63069	Rf	Control	10.0 + 0.5	26250	606	2.31
63069	Rf	Colchicine	6.0 + 0.5	16250	314	1.93
63069	Rf	Pronamide	12.5 + 0	32500	45	0.14
63069	Rf	Trifluralin	3.5 + 0.5	8750	273	3.12
63509	B	Control	7.5 + 23.0	83750	1489	1.78
63509	B	Colchicine	6.5 + 18.5	62500	2165	3.46
63509	B	Pronamide	4.5 + 14.5	46250	0	0.00
63509	B	Trifluralin	4.5 + 14.0	47500	654	1.38
63511	B	Control	35.5 + 15.5	127500	1830	1.44
63511	B	Colchicine	33.5 + 15.5	122500	877	0.72
63511	B	Pronamide	40.5 + 18	146250	0	0.00
63511	B	Trifluralin	41.5 + 22	158750	2857	1.80
63539	B	Control	0 + 15.0	37500	674	1.80
63539	B	Colchicine	0 + 12.0	30000	661	2.20
63539	B	Pronamide	0 + 9.0	21250	70	0.33
63539	B	Trifluralin	0 + 8.5	22500	461	2.05
63546	B	Control	16 + 0	40000	248	0.62
63546	B	Colchicine	25.5 + 0.5	65000	412	0.63
63546	B	Pronamide	29 + 0.5	73750	0	0.00
63546	B	Trifluralin	30 + 1	77500	300	0.39

Appendix B:

Raw data for embryo quality for genotypes and treatments:

Genotype	Line	Treatment	2 Points	1 Point	0 Points
63066	Rf	Control	46	44	17
63066	Rf	Colchicine	-	-	-
63066	Rf	Pronamide	-	-	-
63066	Rf	Trifluralin	8	8	3
63069	Rf	Control	31	26	9
63069	Rf	Colchicine	4	9	6
63069	Rf	Pronamide	-	-	-
63069	Rf	Trifluralin	59	55	10
63509	B	Control	40	50	14
63509	B	Colchicine	11	19	8
63509	B	Pronamide	-	-	-
63509	B	Trifluralin	35	25	3
63539	B	Control	70	44	14
63539	B	Colchicine	18	30	2
63539	B	Pronamide	16	24	9
63539	B	Trifluralin	76	27	8

Summary of the embryo quality raw data for each line and treatment:

Line	Treatment	2 Points	1 Point	0 Points	Average
Rf	Control	77	70	26	1.29
Rf	Colchicine	4	9	6	0.89
Rf	Pronamide	-	-	-	-
Rf	Trifluralin	67	63	13	1.38
B	Control	110	94	28	1.35
B	Colchicine	29	49	10	1.22
B	Pronamide	16	24	9	1.14
B	Trifluralin	111	52	11	1.57

Appendix C:

Raw data for fertility of individual genotypes and treatments:

Geno-type	Line	Treatment	Fertile Plants	Sterile Plants	Rate of fertility
63058	Rf	Control	75	142	0.35
63058	Rf	Colchicine	224	27	0.89
63058	Rf	Pronamide	-	-	-
63058	Rf	Trifluralin	7	2	0.78
63064	Rf	Control	-	-	-
63064	Rf	Colchicine	250	32	0.89
63064	Rf	Pronamide	-	-	-
63064	Rf	Trifluralin	2	3	0.40
63066	Rf	Control	28	36	0.44
63066	Rf	Colchicine	169	36	0.82
63066	Rf	Pronamide	-	-	-
63066	Rf	Trifluralin	11	2	0.85
63069	Rf	Control	18	45	0.29
63069	Rf	Colchicine	132	93	0.59
63069	Rf	Pronamide	-	-	-
63069	Rf	Trifluralin	62	15	0.81
63509	B	Control	17	34	0.33
63509	B	Colchicine	258	34	0.88
63509	B	Pronamide	-	-	-
63509	B	Trifluralin	54	5	0.92
63511	B	Control	7	15	0.32
63511	B	Colchicine	93	136	0.41
63511	B	Pronamide	-	-	-
63511	B	Trifluralin	60	38	0.61
63539	B	Control	24	61	0.28
63539	B	Colchicine	179	92	0.66
63539	B	Pronamide	11	12	0.48
63539	B	Trifluralin	39	75	0.34
63546	B	Control	11	73	0.13
63546	B	Colchicine	231	58	0.80
63546	B	Pronamide	-	-	-
63546	B	Trifluralin	13	5	0.72

Appendix D:

A N A L Y S I S O F V A R I A N C E

03/20/2008

Dependent variable: EMBRYOG

Source	df	SS	MS	F-value	Pr> F
Total	31	29.368			
LINE	1	0.855	0.855	2.19	0.1575
TRT	3	9.565	3.188	8.16	0.0014
GEN	7	11.883	1.698	4.34	0.0063
LINE by TRT	3	0.422	0.141	0.36	0.7827
Residual	17	6.644	0.391		

Grand mean = 0.999 R-squared = 0.7738 C.V. = 62.57%

LSD for TRT = 0.5438 S.E.D. = 0.3126
t (1-sided a=0.050, 17 df) = 1.7396 MSE = 0.39080

TRT
Averages

Level	--- Y ---	Cv	Rank
2	1.41	74.5	1
1	1.29	56.5	2
4	1.23	87.9	3
3	0.06	204.3	4

PROC ACB: Execution Time = 0.009 seconds, Kb used = 214.03 (Memory-oriented)
C:\DOCUME~1\RALE\MYDOCU~1\DSV_SR\THESIS~1.DBF, Kb Free = 9321.0,
Thursday,
March 20 2008, 08:57:37

Appendix E:

A N A L Y S I S O F V A R I A N C E

03/20/2008

Dependent variable: FERTILITY

Source	df	SS	MS	F-value	Pr> F
Total	31	3.443			
LINE	1	0.002	0.002	0.05	0.8334
TRT	3	2.581	0.860	23.73	0.0000
GEN	7	0.185	0.026	0.73	0.6501
LINE by TRT	3	0.059	0.020	0.54	0.6585
Residual	17	0.616	0.036		

Grand mean = 0.437 R-squared = 0.8210 C.V. = 43.55%

LSD for TRT = 0.1656 S.E.D. = 0.0952
t (1-sided a=0.050, 17 df) = 1.7396 MSE = 0.03625

TRT
Averages

Level	--- Y ---	Cv	Rank
2	0.74	23.5	1
4	0.68	31.2	2
1	0.27	51.8	3
3	0.06	282.8	4

PROC ACB: Execution Time = 0.024 seconds, Kb used = 214.03 (Memory-oriented)
C:\DOCUME~1\RALE\MYDOCU~1\DSV_SR\THESIS~1.DBF, Kb Free = 9321.0,
Thursday,
March 20 2008, 08:57:37