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

by

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Improvement on the Microspore Culture Methodology for Brassica rapa Canola

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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

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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).