ISSN 1392-3196
Žemdirbystė / Zemdirbyste / Agriculture, vol. 94, No. 4 (2007), p. 129–138
UDK 633.853.494“321”:631.527:631.147

APLICATION OF BIOTECHNOLOGY METHODS IN SPRING RAPESEED (*BRASSICA NAPUS* L.) BREEDING

Natalija BURBULIS, Ramunė KUPRIENĖ, Aušra BLINSTRUBIENĖ, Rima JUOZAITYTĖ, Liuda ŽILĖNAITĖ

Lithuanian University of Agriculture
Akademija, Kaunas district
E-mail: Natalija.Burbulis@lzuu.lt

Abstract

A survey of the utilization of biotechnology methods in rapeseed breeding at the Lithuanian University of Agriculture is presented. Various factors such as age of the explant, genotype, and media composition were investigated and culture conditions for primary and secondary somatic embryos induction from immature zygotic embryos have been optimized. Our studies have determined that primary somatic embryogenesis proceeded at a lower rate than the secondary one; however, higher amount of somatic embryos per explant was obtained during the primary somatic embryogenesis. The development of techniques of anther and microspore cultures is outlined, with the focus of attention to the practical use of DH for rapeseed breeding. Special attention is given to yellow-seeded spring rapeseed development through microspore culture. Factors affecting seed coat pigmentation intensity are discussed. Evaluation results of created genotype are presented. In total, during the past few years over 2000 new *B. napus* genotypes have been created by different biotechnology methods, the best of which have been involved in spring rapeseed breeding programs.

Key words: *Brassica napus*, somatic embryogenesis, anther and microspore cultures, yellow-seeded rapeseed.

Introduction

The oilseed rapes (*Brassica napus*, *B. rapa* and *B. juncea*) are now the third most important source of edible vegetable oil in the world after palm and soybean oil. In *Brassica napus* the oil content in combination with a high seed yield is a very important selection criterion during cultivar development. The quality of oils and fats is determined by the composition of fatty acids, i.e. their chain length, degree of desaturation, kind and number of functional groups, etc. For industrial purposes either oils or fats with high amounts of a single or unique fatty acids, or vegetable oils containing unusual fatty acids or novel compositions are required /Friedt, Luhs, 1998/.

Conventional methods of plant breeding and, in present years, modern biotechnology have evolved into powerful tools for developing improved crop species and novel superior cultivars. The creation of novel germplasm through techniques of tissue culture and gene transfer holds great potential for improving the quality and agronomic traits of rapeseed. For this, *in vitro* regeneration of plants via organogenesis or embryogenesis is a prerequisite. Mature and immature zygotic embryos have been used to

initiate a regenerable culture in many plants, including *Brassica napus* /Koh, Loh, 2000/. Somatic embryogenesis has potential applications for both plant breeding practice and research. Nevertheless, there are still factors to be evaluated and optimized for different species and genotypes within species.

The production of doubled haploids via biotechnological approaches such as anther and microspore cultures offers the possibility of accelerating the breeding process, as well as facilitating basic scientific research work. Major advantages of these methods in comparison to the conventional breeding methods via repeated self-pollination include reduction of time and space for breeding and ultimately reduction of the costs for cultivars development. The value of doubled haploids in breeding is that they are diploid, fertile and homozygous for all traits, therefore are true breeding in the following generations, and each doubled haploid represents a unique combination of traits from each parent in the original cross. Complete homozygosity is achieved very rapidly since DH seed is harvested only 8–9 months after culture for spring types and 1.5 years for winter types /Kott, 1998/.

The objective of this review was to survey the utilization of biotechnology methods in spring rapeseed breeding and to present some results on somatic embryogenesis, anther culture and microspore culture obtained in the Laboratory of Agrobiotechnology at the Lithuanian University of Agriculture.

Somatic embryogenesis

Somatic embryogenesis, which has been the subject of increasing research in the genus, has become one of the most desired pathways in the regeneration of plants via tissue culture because it bypasses the necessity of time-consuming and costly manipulation of individual explants, which is a problem with organogenesis /Raemakers et al., 1995/. Mature and immature zygotic embryos have been used to initiate a regenerable culture in many plants, including *Arabidopsis thaliana* /Luo, Koop, 1997/, *Pisum sativum* /Tetu et al., 1990/ *Solanum tuberosum* /Pretova, Dedicova, 1992/, however there have been only a few reports on the induction of direct somatic embryogenesis in *Brassica napus* with zygotic embryos as the starting material /Koh, Loh, 2000/. Literature sources point out that immature and mature zygotic embryos of most plant species possess different embryogenetic potential /Raemakers et al., 1995/. Our studies have shown that mature zygotic embryos of tested *Brassica napus* genotypes did not produce somatic embryos in all of the tested media, while immature zygotic embryos formed primary somatic embryos (PSE) directly without intervening callus phase. It was ascertained that zygotic embryo age of the studied doubled haploids lines is one of the most important factors influencing the formation and development of somatic embryos. Zygotic embryos, isolated 20–21 days after pollination, formed the highest amount of primary somatic embryos from explant /Burbulis, Kupriene, 2005/.

Unlike the results of many previous experiments, we have found that exogenous growth regulators are not essential for somatic embryo induction from immature zygotic embryos of spring rapeseed. Continued secondary somatic embryogenesis also was induced on hormone-free media, thereby excluding the use of exogenous phytohormones and other complicated culture manipulation. During secondary somatic embryogenesis, new somatic embryos were developing from the lower part of PSE hypocotyl directly,

without callus formation. Our studies determined that primary somatic embryogenesis proceeded at a lower rate than the secondary one; however, higher amount of somatic embryos from explant was obtained during primary somatic embryogenesis.

The described induction of somatic embryos from immature zygotic embryos is an efficient and quick method, allowing producing normal and healthy plants. This system may be highly useful for developing effective transformation systems in order to improve important economic traits as oil and protein content and disease resistance.

Anther culture

One of the important techniques that is increasingly used in plant breeding programmes is the production of doubled haploids. The artificial production of haploid plants followed by chromosome doubling offers the quickest method for developing homozygous breeding lines from heterozygous parental genotypes in a single generation. Through anther culture, considerable progress has been achieved for a large number of economically important crop species, such as barley, wheat, maize, rapeseed and rice. For the *Brassica* species the first attempt at producing DH lines was made using the anther culture system /Keller, Armstrong, 1977/.

The first step after preparing and isolation of anthers is the treatment of the microspores within anthers in order to induce embryogenesis. This has been achieved using different methods, in most cases heat shock was applied. The temperature regime that is used to induce embryogenesis has been studied for the different *Brassica* species and for different genotypes within species.

It has been reported, that application of heat stress pre-treatment is essential factor to increase the efficiency of androgenesis in many plant species /Custers et al., 1996; Schulze, Pauls, 1998/. We have applied anther culture for several cultivars and DH lines of spring rapeseed. The influence of genotype, thermal shock pre-treatment and nutrient media on the callus induction and embryogenesis was investigated. Temperature pre-treatment of flower buds was performed at 35 °C for 24 h, 48 h and 72 h. Callus formation was observed after 12–14 days after anther isolation. Our results showed that effect of anther pre-treatment on callus formation appeared to be genotype dependent. 72-h thermal shock significantly improved the percentage of anthers producing calli for one genotype, but strongly inhibited callus formation frequency for another 5 genotypes tested. However, the callus did not develop further and did not induce shoot formation in any of tested medium. Direct embryogenesis of anther culture was also obtained on induction medium; however embryo development was often stopped after several divisions or during the globular embryo to the heart-shaped embryo stages, followed by embryo death. Further embryo development was noted only in anthers of cultivar ‘Trend’, which showed embryogenic capability without thermal pre-treatment but high temperature shock significantly improved embryo formation frequency /Burbulis et al., 2004; Kuprienė et al., 2004/.

Microspore culture

Anther culture as a method of producing DHs is widely used, but it is accompanied by the problem that regeneration from somatic cells of the anther tissue can take place leading to the formation of diploid (heterozygous) progenies. Contrary to

anther culture, in the case of microspore culture the target material is definitely derived from haploid gametic cells and is therefore homozygous. Over the last decade, researchers have made great efforts in to developing biotechnological methods to facilitate rapeseed breeding /Kott, 1998/. The microspore culture technique, which has been optimized, is used on large scale due to higher efficiency of embryo production, compared with anther culture.

Doubled haploids (DH) are presently used in breeding of number of crop species. This method enables breeders to develop completely homozygous genotypes from heterozygous parents in one single generation. Doubled haploids allow to fix recombinant gametes directly as fertile homozygous lines. Time saving is the most obvious advantage, because yield and other traits can be tested much earlier than with conventional lines.

Responsiveness to microspore culture of spring rapeseed F_1 hybrids in LUA was first investigated at the end of 90s. Our tested Lithuanian hybrids obtained from crosses between spring rapeseed cultivars 'Star', 'Bolero' and 'Cyclone' were responsive in isolated microspore culture but the yields of morphologically normal embryos were relatively low, the frequency of cotyledonous embryos varied from 0.00 % to 61.67 % depending on a genotype. Plant regeneration frequencies also varied considerably among tested hybrids, which may have been associated with genotype factors that influence synchronized embryo development and maturation /Burbulis et al., 2000/. It has been reported that *Brassica* microspore culture is strongly genotype-dependent. For example, only 30 % of tested *B. juncea* genotypes were responsive for microspore culture /Hiramatsu et al., 1995/. Genotype-dependent effects were also observed in *B. carinata* /Barro, Martin, 1999/.

Bud selection is critical for culture success. Cytological studies have shown that bud size could be used as the criterion for cytological readiness of the microspores. The very late uninucleate stage, shortly before first pollen mitosis of the microspore nuclei, was found to be optimal stage for a embryogenic response in culture /Kott et al., 1988/. A range of bud size was investigated in our study. Experiments show, that microspores of hybrids No 268 and No. 269 within a single anther at any size very well synchronized in the cell cycle, falling into only two developmental stages at any time. Therefore it is easy to select buds with spores in late uninucleate stage. Microspore development in hybrid No 274 was more asynchronous. This means that within a single anther of No 274 it was common to find spores in the miduninucleate stage and others in the trinuncleate stage as well as all intermediate types. When culturing such anthers it is impossible to avoid taking older spores along the rest. This may explain why, in our experiment, hybrid No 274 routinely produced low frequencies of embryos which were all of poor quality /Burbulis, Sliesaravičius, 2002/. In summary, it appears that the degree of synchronization of microspore development is genetically determined and depends on a genotype. The agronomic performance of 9 DH lines, developed from hybrids No 268 and No 269 were tested at Experimental Station of Lithuanian University of Agriculture and valuable recombinants have been included into the breeding programs /Burbulis et al., 2001/.

Crops used today in agriculture are the result of a long selection process. Since the early days, breeding methods have become more sophisticated and the latest inno-

vations have come from modern biotechnology. The objective of selective breeding is to optimize plants for specific purposes or conditions and to stabilize the new characteristics through the subsequent generations. Improved oil and protein content is primary objective in rapeseed breeding. These traits can be simultaneously improved by developing yellow-seeded cultivars because yellow-seeded genotypes have higher oil and protein content in comparison with dark-seeded /Rahman et al., 2001/. In our spring rapeseed breeding program a new source of yellow-seeded *Brassica napus* has been identified in doubled haploid progeny, from a cross between the two black-seeded spring cultivars 'Star' and 'Bolero'. Six yellow-seeded doubled haploid lines were extracted from the F_1 generation of this cross. Over 2 years a number of lines were produced by self pollination of plants generated from individual seeds of this DH lines. Seed colour of existing yellow-seeded rapeseed genotypes is known to be affected by ambient cultivation temperatures /Van Deynze et al., 1993/ Therefore developed yellow-seeded doubled haploid lines and some of their progeny have been tested for expression of the yellow seed trait under various temperature environments. Strong seed colour trends were observed in plants grown at different temperatures. All tested lines grown at 20 ^{o}C daily maximum temperatures resulted in seed colour consistently darker than that of the planted seed, ranging from black, brown, brown-yellow with some lighter or partially yellow seeds. In contrast, plants grown at 30 ^{o}C daily temperatures were more yellow than the planted seed, and much yellower than the seed from the cooler environment. Seed colour results from plants grown in the intermediate temperature (28 °C daily maximum) produced seeds that were intermediate in colour between the cool and the hot environments. Our results clearly show that high temperatures inhibit the accumulation of dark pigments in the seed coats of these genotypes. Line NL-350-1, which had yellow-brown and dark yellow seed development in the 20 and 28 ^{o}C conditions, was the exception, producing brown seed in 30 ^{o}C temperature. This phenomenon may be related to colour expression reversion in this mutant that is brought on by extreme heat. Oil content of new *B. napus*-derived genotypes described herein is consistent with oil enhanced yellow-seeded lines sourced from interspecific crosses. Tested doubled haploid lines show a good range of oil percent, ranging from 31.2 to 51.6 % in the cooler environment, while best performance in the hot temperature reached 48–49 % among three best lines /Burbulis, Kott, 2005/.

Isolated microspore culture technology has been utilized for the development of new double haploid yellow-seeded lines that are less sensitive to environmental conditions. Donor plants (DH268-2, NL-310-1 and NL-360) were grown in 20/16 ºC temperature to get new recombination, which possibly could be less sensitive to cooler conditions. Regenerants were grown in the same temperature to select desired genotypes. The seed colour of new DH lines ranged from brown to yellow in contrast to the donor plants which under 20/16ºC temperature formed only brown (DH268-2), black (NL-360) and brown-yellow (NL310-1) seed. Many of the new DH lines appear to be colour-stable in the brown-yellow and yellow-brown range of seed colour. Of particular interest are the 3 new yellow-seeded doubled haploids produced in cool temperature conditions. Differences in seed coat colour sensitivity to cooler temperature among tested lines indicate that there is genetic variability for this trait and new genotypes may be created by selection. The seed colour of the tested lines under field conditions varied among

lines and even within single plants. Sixteen promising DH lines, in which at last one plant produced only yellow seed, were selected.

Among yellow-seeded plants, the colour showed a continuum of variation, which means there are some modified gene affects in seed colour. Similar variation in seed colour on a given plant and even within single pods has also been noticed in the Shirzadegan investigation /Shirzadegan, 1986/. The variable expressivity further complicates breeding efforts for yellow-seeded rapeseed.

The F_1 generation of the yellow-seeded DH lines and black-seeded cultivars 'Star' and 'Bolero' reciprocal crosses was grown in the greenhouse and F_2 seed produced. Intensity of seed coat pigmentation in hybrids was evaluated at the Experimental Station of the Lithuanian University of Agriculture. A total of 400 F_2 progeny were grown in the field in a single row, 3-replicate nursery in 2004. Yellow-seeded F_2 progeny were selected and grown as F_3 lines in a double row, 2-replicate nursery in 2005. The seed colour of these lines was a mixture of yellow, brown and black seeds. Yellow seeds from each bulk seed samples were selected by hand, and only these were further tested as F_4 lines in 2006. Seven hundred plants were individually harvested from each of F_4 lines. Yellow-seeded and black–seeded plants were hand selected from each family and F_5 progeny are currently under investigation. It has been reported that seed pigmentation of interspecific hybrids is more influenced by maternal genotype, because seed coat is formed from maternal tissues /Rahman, 2001/, however, it may be influenced by the paternal genotype as well /Van Deynze, Pauls, 1994/. Having evaluated seed colour differences in different crossing combinations, it was found that in reciprocal crossings between 'Star' and 268-20, yellow-seeded genotypes comprise almost the same percentage of the population, independently of the fact whether yellow-seeded line is used as paternal or maternal form. In other studied crossing combinations, higher percentage of yellow-seeded genotypes in the population was obtained when yellow-seeded line DH 268-20 was used as paternal form. The obtained results allow to resume that in reciprocal crossings between yellow-seeded and black-seeded DH lines, seed coat pigmentation was not dependent on maternal genotype only, however, it is necessary to mention environmental factors as the main reason of such phenotypic variations.

Not only are haploids very useful in applied breeding for crop improvement but also they can efficiently be used in basic studies, for example in genetic analysis. We used doubled haploid populations to study the inheritance of seed colour in *Brassica napus.* A true breeding *Brassica napus* line DH268-2 and black-seeded cultivars 'Star' and 'Bolero' were used for reciprocal crosses from which DH populations were produced. In total 268 doubled haploid lines were produced that gave seed of sufficient quality to classify their colour. Self pollinated seeds from DH lines from four crosses were grouped into seed colour classes as follows: black, brown, yellow. Chi-square goodness-of-fit test was used to compare the observed distribution in the segregating populations to those predicted by different models for seed colour inheritance /Shirzadegan, 1986; Van Deynze, Pauls, 1994; Baetzel et al., 2003/. Depending on the source of yellow-seediness used in the genetic studies in most cases a trigenic inheritance has been proposed. The segregation data obtained for the DH populations in our study was not consistent with the corresponding ratio for DHs predicted by these models. The discrepancy between our and other studies may be observed due to

differences in the genetic material used in the crosses. The yellow seed colour character in DH268-2 is derived from black-seeded cultivars 'Star' and 'Bolero'. It is assumed that the yellow seed trait in this line results from spontaneous mutations, where mutant gene(s) block the synthesis of seed coat pigment /Burbulis, 2005/.

Numerous yellow-seeded *Brassica napus* genotypes have been developed over the past 28 years, however only one yellow-seeded cultivar has been released to date /Liu et al., 1991/. The lack of progress in fixing the genes controlling seed colour in rapeseed is probably attributed to the genetic complexity of this trait. Most of existing yellow-seeded genotypes have been derived from interspecific crosses with related yellow-seeded *Brassica* species, namely, *B.alboglabra* and *B.rapa* /Rahman, 2001/, *B.carinata* and *B.jumcea* /Rashid et al., 1994/, *B.carinata* and *B.campestris* /Meng et al., 1998/. The introgression of genes encoding seed pigmentation from related *Brassica* species and subsequent expression of yellow seed colour in *Brassica napus* can be complex due to polyploidy, multiple gene control and maternal determination /Van Deynze et al., 1993; Tang et al., 1997/.

In order to create new yellow-seeded genotypes, isolated microspore culture was used. The donor plants for microspore culture were F_1 hybrids obtained from reciprocal crosses between the yellow-seeded rapeseed lines DH268-2 and DH268-20 and black-seeded cultivars 'Star', 'Bolero' and 'Dynamite'. From each single plant progeny, yellow seeds were selected and sown in the field in double rows. In order to prevent any outcrossing, each plant was bagged before flowering. It was observed that the self-pollinated seeds produced on single plants of tested DH lines were either yellow, partially yellow or brown. A wide range of segregation for seed coat colour occurred, and the expected yellow-seeded offspring were selected. It was very difficult to catalogue the seeds according coat colour since colour varied continuously. Even in the yellow group, the seed samples could be subdivided into dark yellow, golden yellow, bright yellow or pure yellow subgroup.

The DH lines were selected due to the expression of the character "yellow seediness" in the following generation. Following a phase of seed multiplication in the field a large variation in seed characters of tested DH lines has been generated. Additionally the selection of the DH lines and hybrids progeny was continued regarding different agronomic properties. After two years of field trials, 7 DH lines, which inhibit dark pigment synthesis under Lithuanian condition, were selected. The seed of this line contains more oleic, less linolic, palmitic, stearic and erucic acids, and less glucosinolates and fibre in comparison with black-seeded cultivars /Burbulis et al., 2006/.

Since selection for yellow seediness is difficult due to strong environmental effects (e.g., temperature during seed ripening), molecular markers linked to gene loci controlling seed colour in *B.napus* had been started. Using 15 random sequence primers, DNA analysis of black-seeded cultivars 'Star', 'Bolero', F_1 hybrids and developed DH lines (with different seed colour: yellow, yellowish-brown, brownish-yellow, brown) was done. PCR profiles produced by amplification with tested primers were not polymorphic in the seeds of different colour.

Generally during past few years over 2000 new *B. napus* genotypes have been created by different biotechnology methods, favourable of which have been involved in spring rapeseed breeding programs. It can be concluded, that breeding based on the

different biotechnology methods is as effective as conventional breeding with 6–8 generations of inbreeding. But the possibility to obtain valuable genotypes with desirable agronomic performance depends on the availability of suitable initial breeding materials with genes determining favourable traits.

Received 03 08 2007
Accepted 01 10 2007

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ISSN 1392-3196
Žemdirbystė, t. 94, Nr. 4 (2007), p. 129–138
UDK 633.853.494“321”:631.527:631.147

BIOTECHNOLOGINIAI METODAI VASARINIŲ RAPSŲ (*BRASSICA NAPUS* L.) SELEKCIJOJE

N. Burbulis, R. Kuprienė, A. Blinstrubienė, R. Juozaitytė, L. Žilėnaitė

Santrauka

Straipsnyje pateikiami pagrindinių biotechnologinių metodų, taikomų vykdant vasarinių rapsų selekciją Lietuvos žemės ūkio universitete, tyrimų rezultatai. Įvertinus įvairių veiksnių (eksplanto, genotipo, maitinamosios terpės sudėties) įtaką, nustatytos optimalios sąlygos pirminių ir antrinių somatinių embrioidų indukcijai nesubrendusių zigotinių gemalų kultūroje. Nustatyta, kad pirminė somatinė embriogenezė vyksta mažesniu dažnumu nei antrinė, tačiau didesnis somatinių embrioidų kiekis iš eksplanto gaunamas pirminės somatinės embriogenezės metu. Straipsnyje nagrinėjami pagrindiniai dulkinių ir mikrosporų kultūrų aspektai, akcentuojant praktinį dvigubų haploidų panaudojimą rapsų selekcijoje. Ypatingas dėmesys skiriamas geltonsėklių vasarinių rapsų linijų kūrimui izoliuotų mikrosporų kultūroje. Straipsnyje analizuojami veiksniai, lemiantys rapsų sėklų luobelės pigmentacijos intensyvumą bei pateikiami sukurtų genotipų įvertinimo rezultatai. Nuo 1999 metų LŽŪU Agrobiotechnologijos laboratorijoje įvairiais biotechnologiniais metodais sukurta daugiau kaip 2000 naujų vasarinių rapsų genotipų, kurie įtraukti į selekcines programas.

Reikšminiai žodžiai: *Brassica napus*, somatinė embriogenezė, dulkinių ir mikrosporų kultūra, geltonsėkliai rapsai.