Synthesis of hexaploid *Brassica* from *B. napus* and *B. nigra*

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**ABSTRACT**

Canola (*B. napus*) is a tetraploid (4x) species, the highest natural ploidy in the genus *Brassica*. There are many successful hexaploids (6x) such as bread wheat, kiwifruit, triticale and oat. However, no plant in the genus *Brassica* is a natural hexaploid. Our research aims to create hexaploid *Brassica* populations through the interspecific cross between tetraploid canola (*AACC*) and diploid black mustard (*B. nigra, BB*), followed by chromosome doubling in the F$_1$ (ABC) to synthesize a fertile hexaploid *Brassica* species (6x, AABBCC).

Canola is a high-quality oilseed (35-45% oil) and black mustard has been grown as a wild or landrace oilseed species. Black mustard is hardy, widely adapted to varied climatic conditions, suitable as a rainfed crop and yields well under extreme conditions. The B genome of *B. nigra* is an important source of extremely useful genes including disease and pest resistance, other biotic and abiotic stress tolerance and oil seed quality. Our breeding target is to produce new oilseed *Brassica* varieties suitable for medium-low rainfall areas, with increased oil yield and quality, and resistance to blackleg disease.

We will attempt various methods to overcome hybridization barriers, chromosome doubling of triploid hybrids and evaluation of hybrids by molecular and cytogenetic techniques including *in situ* hybridization, flow cytometry and morphological approaches. Fertility will be assessed by examining pollen viability and seed set through selfing or backcrossing.

Preliminary crosses of 369 flowers of *B. napus* with *B. nigra* pollen resulted in 163 pods set and 116 potential hybrid seeds. The reciprocal cross of 85 flowers only resulted in 20 pods set and a single seed. Hybridity will be verified with cytogenetic, molecular and morphological methods. A diallel cross of 5 varieties of canola and 5 accessions of black mustard is underway.

**Key words**: canola, black mustard, interspecific hybridization, triploid (ABC), hexaploid (AABBCC)

**INTRODUCTION**

There are many important crop plants within the genus *Brassica*. They include oilseed, vegetable and condiment crops. *Brassica napus, B. rapa* (formerly *B. campestris*), *B. juncea* and *B. carinata* provide 12% of worldwide edible vegetable oil (Labana and Gupta, 1993). Canola (*B. napus*) is now second only to soybean as the most important source of vegetable oil in the world (Raymer, 2002; ABARE, 2006). Canola oil is considered one of the highest quality edible oils available (Porter and LeGare, 2006).

The six cultivated *Brassica* species possess three types of diploid genomes designated as A, B, and C genomes either singly or in pairs (U, 1935) (Figure 1). The three tetraploid species (*B. napus, AACC; B. juncea, AABB; B. carinata, BBCC*) are from the pair-wise genome combination of two diploid species (*B. rapa, AA; B. oleracea, CC; or B. nigra, BB*). Each genome has its own specific genes controlling agronomically important traits.

Desirable traits can be transferred or combined through interspecific hybridization between the diploid or tetraploid species. The B genome of *B. nigra* (black mustard) is an important source of useful genes including disease resistance and oil seed quality (Sacristan and Gerdemann 1986; Sjodin and Glimelius 1989; Struss et al.1991a, b; Chevre et al., 1991). Blackleg, caused by *Phoma lingam* (*Leptosphaeria maculans*), is one of the most important diseases of *B. napus* (Yu et al., 2005). Resistance genes are located on the B genome (Roy,
specifically cotyledon resistance in \textit{B. nigra} and \textit{B. juncea} (Chevre et al., 1996, 1997) and stem resistance in \textit{B. nigra}, \textit{B. juncea} and \textit{B. carinata} (Struss et al., 1996). Black mustard has been grown and used as an oilseed species and it is hardy (Koch, 2004) and widely adapted to varied climatic conditions. It is mostly grown in the temperate zone though it is suited to tropical areas and can be grown as a rainfed crop in areas of low or moderate rainfall (Duke, 1983). Thus hybrids combining the genomes of \textit{B. nigra} and \textit{B. napus} should have wider adaptation to varied environments.

In order to improve the genetic diversity available for breeding purposes, scientists have recently gone beyond the cultivated germplasm and are looking at related species. However hybridisation with these related species is very difficult, and made more complex by the presence of diploid and tetraploid genotypes. Crossing between diploids and tetraploids is more difficult than between species at the same ploidy level. So canola breeding has been at the tetraploid level (4\textit{x}) as \textit{B. napus} is a tetraploid. The other two tetraploids are \textit{B. carinata} and \textit{B. juncea}.

\textit{Meng et al. (1988)} succeeded in crossing \textit{B. napus} x \textit{B. carinata} and the hybrids were fertile. Double haploid technology was used to develop homozygous lines from interspecific hybrids of \textit{B. napus} x \textit{B. carinata} (Nelson et al., 2006). Techniques for hybridisation between \textit{B. napus} and \textit{B. juncea} have been developed (Schelfhout et al. 2004, 2006). Thus we now have technology to make crosses with all the tetraploid \textit{Brassica} species related to \textit{B. napus}. With this background, it is appropriate to take on the challenge of hybridisation between the tetraploid (4\textit{x}) \textit{B. napus} (canola) and related diploid (2\textit{x}) species.

The natural hybrid that resulted in \textit{B. napus} has evolved and been heavily selected towards current day cultivars of canola. The breeding gene pool of \textit{B. napus} has been restricted by genetic bottlenecks which occurred during the development of spring and winter habits, and cultivars with low erucic acid and low glucosinolates (canola). The canola gene pool can be widened by interspecific genome combination.

Polyploid plants have several copies of the same genes, and are generally more vigorous than diploids. The formation of stable hexaploids has been attempted across \textit{Brassica} species. The first, and currently only \textit{Brassica} hexaploids, were synthesized from interspecific crosses between \textit{B. rapa} (AA) and \textit{B. carinata} (BBCC) and from reciprocal crosses (Meng et al., 1998). However, more hexaploid populations from different sources need to be synthesised to advance breeding and gene pyramiding.

The major outcome of this project will be a detailed protocol to synthesise hexaploid \textit{Brassica} genomes from tetraploid (\textit{B. napus}) and diploid (\textit{B. nigra}) species. The hexaploid is expected to have greater genetic variation, potential resistance to a number of biotic and abiotic stresses including drought, salt, pests and diseases and increased yield in terms of seed production and higher oil content.

The main aims of this study are 1. To study the success rate on crossability between \textit{B. napus} and \textit{B. nigra}. 2. To identify the crossing barriers and means to overcome them. 3. To study the possibility of synthesizing hexaploid genotypes. 4. To evaluate hybrids by morphological, molecular and cytogenetic analysis.

The preliminary experiment was carried out on hand crossing between different genotypes of \textit{B. napus} and \textit{B. nigra}.
MATERIALS AND METHODS

Seven cultivars of *B. napus* namely Trigold, Trilogy, Rivette, ATR-Beacon, AV-Sapphire, Wester-10DH and Surpass 501TT and six lines of *B. nigra* with five 90745, 90747, 91105, 94211, 94483 from ATFCC (The Australian Temperate Field Crops Collection, Horsham, Victoria) and one 4318 from the Vavilov Institute, Russia. Plants were grown in the glasshouse from November 2006 to June 2007. Hand pollination was attempted by emasculating the buds of one species and pollination using another species where flowering was synchronous. Crossing was made in both directions. Flowers were bagged with perforated plastic bags to prevent subsequent pollination by wind/bees. The numbers of pod and seed set for each cross were recorded.

Based on the information from the preliminary experiment, five diverse genotypes of both *B. napus* and *B. nigra* were selected for interspecific hybridization. Diallel crosses were made by hand pollination with different genotype combinations. Pollen compatibility, embryo and endosperm development stages and the rate of development will be observed in the crosses to determine potential for hybrid seed development.

Triploid hybrid seedlings will be treated with chromosome doubling techniques to synthesize a hexaploid (6x, AABBCC). Progeny will be tested for valuable traits. Sterile triploid hybrid plants may double naturally or be treated with colchicine to obtain fertile hexaploids. The assumption is that the hexaploids with AABBCC genomes will be fertile. Fertility will be assessed by examining pollen viability and seed set by selfing.

Confirmation of the interspecific hybrids will be undertaken with the use of morphological traits, molecular markers (microsatellite or simple sequence repeats [SSR]), molecular cytogenetics, *in-situ* hybridization, flow cytometry and microscopic observations.

The hybrids will be evaluated based on morphological tests. The hypothesis is that hexaploids will be more vigorous than tetraploids and diploids. In this study, the morphological traits in hybrids will be compared with the parents.

RESULTS

Hybridization was possible between *B. napus* and *B. nigra*, however the success rate was higher when *B. napus* was used as the female parent. All the seven varieties of *B. napus* Trigold, Trilogy, Rivette, ATR-Beacon, AV-Sapphire, Wester-10DH and Surpass 501TT were able to hybridize with *B. nigra* lines with a varied proportion of pod set out of total pollinated flowers, except in ATR-Beacon with 94211 (Table 1). However, seed set was limited to a few combinations. Crosses of 369 flowers of *B. napus* with *B. nigra* pollen resulted in 163 pods set and 116 potential hybrid seeds. The reciprocal cross of 85 flowers only resulted in 20 pods set and a single seed in the cross 4318 x Trilogy (Table 1). Hybridity will be verified with cytogenetic, molecular and morphological methods.

DISCUSSION

Crossing between diploids and tetraploids was possible. In this study, *B. napus* (canola) was used as both the male and female parent in crosses with *B. nigra*. Crosses were more successful when canola was used as a female parent. There was poor pod set in reciprocal crosses and the number of seed set was just one. Out of the total flowers crossed, 31.4 % of crosses yielded hybrid seed from *B. napus* x *B. nigra* and 1.2 % from *B. nigra* x *B. napus*. Schelfhout et al. (2006) also found that the success rate was better in interspecific crosses when *B. napus* was used as the female parent. Although the cross between *B. napus* and *B. rapa* was successful in both directions, the use of *B. napus* as a seed parent was better to obtain more seed set. Hybrids were formed when *B. napus* pollen was used on *B. rapa* (Leckie et al., 1993). However, the cross was not successful between *B. napus* and *B. carinata*, when *B. carinata* was used as a female parent (Bechyne and Aslam, 1984).
Natural crossing between *B. napus* and *B. nigra* under field conditions is not possible (Bing et al., 1996). However, hybrid seed was obtained from 0.9% of crosses by artificial pollination when *B. napus* was used as a female parent, whereas in reciprocal crosses it was just 0.1% (Bing et al., 1996). This demonstrates the high natural barrier in crosses between *B. napus* to *B. nigra*. The barriers might be overcome by selecting suitable cross combinations involving different genotypes. Thus, in our study, a wide range of genotypes were selected to maximise success in future crossing.

There are some techniques available to overcome self incompatibility. Pre-fertilization barriers due to callose deposition in pollen tubes can be overcome by hot water treatment (Prabha et al., 1982). To overcome post-fertilization barriers, embryo culture/rescue is a popular approach to raise hybrids from incompatible crosses. Embryos are rescued by excising them from ovaries or ovules (Sharma et al., 1996) and culturing on nutrient media. In the cross between *B. nigra* x *B. napus*, an F1 was produced by in vitro culture of immature embryos. In the F2 from the open pollination of F1, two of the three plants analysed have 75% sterile pollen, the third has 14.4% sterile pollen (Rousselle and Eber, 1983). Barriers to wide hybridization vary and although many types of barriers in plants are known, we will focus on prezygotic and postzygotic barriers in our future research.

Table 1. Number of hand-crossed flowers, pod set and seed set in reciprocal interspecific hybridization between *B. napus* and *B. nigra*

<table>
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<tr>
<th>B. napus x B. nigra</th>
<th>Number of flowers crossed</th>
<th>Pod set</th>
<th>Seed set</th>
<th>B. nigra x B. napus</th>
<th>Number of flowers crossed</th>
<th>Pod set</th>
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CONCLUSIONS

The production of triploid hybrids is possible from the interspecific cross between *B. napus* and *B. nigra*. This project aims to combine desirable traits from these two *Brassica* species through the production of a hexaploid “super-Brassica” with increased yield in terms of seed production and higher oil content, and greater resistance to drought, pests and diseases.

This project investigates breeding methodologies and inheritance of important traits to develop new and improved oil-producing *Brassica* crop species through interspecific hybridization. The procedures for interspecific crossing will be examined and developed to increase breeding efficiency and to develop superior *Brassica* oilseed genotypes. The newly developed oilseed *Brassica* will produce oil for human consumption and/or industrial processing including biodiesel. Hexaploidy can have many advantages in crop plants and the outcome of this project would be very exciting both scientifically and for the oil seed industry. Our preliminary research has indicated a positive future.

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