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PRODUCING INTER-SPECIFIC HYBRIDS BETWEEN *BRASSICA JUNCEA* (L.) CZERN & COSS AND *B. OLERACEA* (L.) TO SYNTHESIZE TRIGENOMIC (ABC) *BRASSICA*

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ABSTRACT

Polyploidy is recognized as a major mechanism in plant evolution. Polyploid crops often have wider adaptation, better quality and higher yielding capacity than their diploid counterparts. Although many successful natural and man-made hexaploid crops are existing, hexaploid *Brassica* are still not available. So far, relatively a limited work has been conducted to synthesize hexaploid *Brassica* with A, B and C genomes, which will provide a very good potential to create new crops for domestication. An investigation was conducted to evaluate the possibility of synthesizing trigenomic (AABBCC) hexaploid *Brassica* by crossing *Brassica juncea* (L.) Czern & Coss and *B. oleracea* (L.). Five genotypes of *B. juncea* (AC 0747, 0790, 1099, 2180 and 7700) and five genotypes of *B. oleracea* (Chinese Broccoli, Broccoli-var. Shogun, Cauliflower-var. Snowball and var., Phenomenon Early and Cabbage-var. Sweet Eureka) were selected for the study. Hand pollination was done by emasculating buds of one species and pollination using another species in both directions. Success of pod formation of the crosses of *B. juncea* (♀) x *B. oleracea* (♂) was 25%. Totally 893 putative hybrid seeds were harvested. Although 9% pod formation was observed in reciprocal crosses, no seeds were developed. Evaluation of 80 putative hybrids by molecular markers and agro-morphological characterization confirmed four true hybrids resulting crosses between AC 0747 x Chinese Broccoli, AC 0790 x Chinese Broccoli and AC 2180 x Broccoli-var. Shogun. The present investigation confirms that hybridization of tetraploid *B. juncea* (4x, AABB) with diploid *B. oleracea* (2x, CC) is a potential approach to produce hexaploid *Brassica* (6x, AABBCC) genotypes.

Keywords: Hexaploid *Brassica*, inter-specific hybridization, polyploidy, SSR markers

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INTRODUCTION

The genus *Brassica* has been subjected to a great deal of scientific attention because it contains very important agricultural and horticultural crops. *Brassica* oilseed species together constitute the third most important oilseed crop in the world after oil palm and soybean (Ahuja *et al*., 2010). *Brassica* oilseed species are an important source of oil for human consumption, for protein rich meal for animal consumption and for bio-diesel production. Among many *Brassica* species the most common oilseed crops cultivated on a commercial scale are rapeseed (*B. napus* L. and *B. rapa* L.) and mustard (*B. juncea* [L.] Czern & Coss).

Brassica oleracea and *B. rapa* are species commonly consumed as vegetables in many countries. *Brassica* vegetables are rich in different forms of vitamins, calcium, iron, magnesium, phosphorus, potassium, zinc and soluble fiber (Zhang *et al*., 1999). They also contain multiple nutrients with potent anti-cancer properties such as diindolylmethane, sulforaphane and selenium (Lampe and Peterson, 2002; Brandi *et al*., 2005). In Sri Lanka, *B. oleracea* varieties such as cabbage, broccoli, cauliflower are widely grown as vegetables in the up-country.

Flowers in the genus *Brassica* are hypogynous, mostly actinomorphic. Sepals 4, in 2 decussate pairs and free. Petals 4, alternate with sepals, arranged in the form of a cross. Stamens 6, in 2 whorls, tetradynamous (lateral (outer) pair shorter than median (inner) 2 pairs). There are four nectar glands which are median and lateral. Anthers are dithecal, dehiscing by longitudinal slits. Pollen grains 3-colpate, trinucleate. Nectar glands receptacular and disposition around base of filaments, always present opposite bases of lateral filaments, median glands present or absent. Pistil 2-carpelled; ovary superior, sessile or borne on a distinct gynophore, mostly 2-locular and with a false septum connecting 2 placentae (Erbar and Leins, 1997).

Brassica juncea is mainly a self pollinated crop, but 8-18% out crossing is also observed (Labana and Banga, 1984). The primary pollinating mechanism is by wind. However, insect pollination substantially enhances the outcrossing (Delaplane and Mayer, 2000). *B. oleracea* is primarily a cross pollinated plant and the self fertility is prevented by a complex system of self incompatibility (Stephenson *et al*., 1997). *B. oleracea* flowers produce nectar and pollen in abundance and they are extremely attractive to the bees. It was observed that when bees were present, the plants produced more seeds for síliquas, besides the same ones were larger and viable (Mussury and Fernandes, 2000). In *B. juncea* the maximum stigma receptivity was recorded one day

before flower opening (Labana and Banga, 1984), whereas in *B. oleracea* the stigma receptivity is maximum 2-4 days before flower opening and it is poor during the evening (Delaplane and Mayer, 2000).

In *Brassica* pollen viability varies with environmental conditions, particularly temperature and humidity. Under controlled conditions in the laboratory, *Brassica* pollen can remain viable for between 24 hours and one week under natural conditions pollen viability gradually decreases over 4 - 5 days (Ranito-Lehtimäki, 1995). Therefore, as a practice *Brassica* breeders cross pollinate *Brassica* in early mornings when the environment temperature is low and the relative humidity is high to maximize successful hybridization (Meng *et al*., 1998; Li *et al*., 2005).

Polyploidy, a change whereby the entire chromosome set is multiplied, is recognized as a major mechanism in plant evolution. Allopolyploidy often occurs in association with inter-specific hybridization (Leitch & Leitch, 2008). In terms of plant breeding, induction of polyploidy could initiate new genetic combinations which provide the breeders with more variability. Further, the induction of polyploidy is a common technique to overcome the sterility of a hybrid species during plant breeding (Leitch & Leitch, 2008). Angiosperms are remarkable in their ability to tolerate the considerable genomic impact of polyploidy arising from the accommodation of divergent genomes in the same nucleus, which results in an instantaneous multiplication in DNA content (Leitch & Leitch, 2008).

There are many successful hexaploids (6x) in crops such as bread wheat, triticale, oat and kiwifruit. Common wheat (*Triticum aestivum* L.) is a fine example of hexaploid plants having wider adaptation, better quality and higher yielding capacity than its tetraploid counterpart, durum wheat (*Triticum durum* Desf.) (Gooding & Davis, 1997). Even though canola and mustard are important crops as a major source of plant-based oil, the highest ploidy level in the *Brassica* is tetraploid (4x).

The cytology and relationships between six of the *Brassica* species are well understood (U, 1935). The six species have three types of diploid genomes designated as A, B, and C either singly or in pairs (Figure 1). *Brassica napus*, *B. juncea* and *B. carinata* are the naturally occurring tetraploids. Amphidiploid species, *B. napus*, *B. juncea* and *B. carinata* have been re-synthesized by hybridizing diploid species followed by doubling the chromosomes (Song *et al*., 1995). This resulted in completely homozygous polyploid lines. So far relatively limited work has been done on the synthesis of hexaploid *Brassica* species. Hexaploids of *Brassica* (AABBCC) have been

synthesized from reciprocal inter-specific crosses between yellow-seeded *B. campestris* (AA) and *B. carinata* (BBCC) to transfer the genes for yellow seed coat from both genomes A and C to *B. napus* (AACC) (Meng *et al*., 1998). Li *et al* (2005) and Jiang *et al*. (2007) were able to produce trigenomic hexaploid *Brassicas* successfully by inter-specific crosses between *B. rapa* (AA) and *B. carinata* (BBCC). Takeda and Takahata (1996) succeeded in producing alloplasmic Chinese cabbage (*B. oleracea*) using synthesized trigenomic hexaploid *Brassica*. Extensive work on molecular mechanisms regarding the cytology in inter-generic hybrids between synthetic *Brassica* allohexaploids (2n = 54, AABBCC) and another crucifer *Orychophragmus violaceus* has also been carried out by Ge & Li (2006) and Li & Ge (2007) as a further step towards understanding the genome-specific chromosome behavior in wide hybrids. Hexaploid *Brassica* plants of the genomic constitution AABBCC were also synthesized by crosses between *B. rapa* (AA), and *B. alboglabra*/*B. oleracea* (CC) and *B. carinata* (BBCC) (Rahman, 2002). Double haploid technology was used by Nelson *et al*. (2006) and Nelson *et al*. (2009) to further advance the inter-specific hybridization of *Brassica* spp. and rapidly developed homozygous populations for cultivar development. In the genus *Brassica*, production of hexaploid somatic hybrids (AABBCC) were also reported. Yamagishi *et al*. (1989) and Arumugam *et al.* (1996) were able to produce hexaploid *Brassica* somatic hybrids respectively by protoplast fusion between *B. nigra* (BB)-*B. napus* (AACC) and *B. oleracea* (CC)-*B. juncea* (AABB).

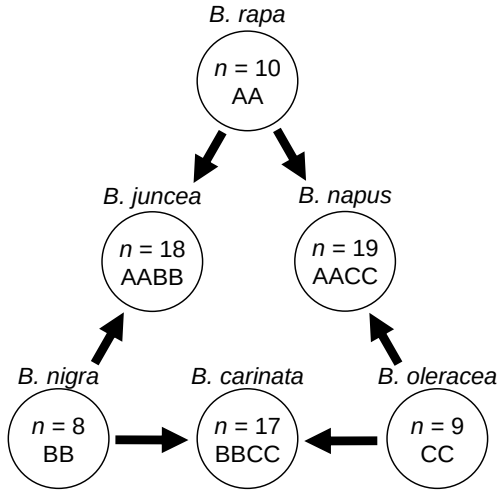

Figure 1: U's Triangle of *Brassica* species (U, 1935)

Although somatic hybridization is still an important tool in plant breeding, sexual polyploids are gaining increasing importance (Ramanna & Jacobsen, 2003). Alfalfa sexual polyploids are more productive than somatic ones (McCoy & Rowe, 1986). In red clover polyploid plants can be produced more consistently by sexual means than through somatic hybridization (Simioni *et al*., 2006). There are at least three possible approaches to produce hexaploid *Brassica* (Yan & Weerakoon, 2007). *ie*. The use of one tetraploid and one diploid as parents (4x – 2x) followed by the chromosome doubling of triploid hybrids; the use of three tetraploids as parents (4x – 4x – 4x) and the use of three diploids as parents (2x – 2x – 2x).

Hybridization between a tetraploid and a diploid species is difficult and failures occur at many stages starting from pollination incompatibility to pre/post-germination barriers. Most inter-specific crosses do not produce mature seeds due to failure of endosperm development (Nishiyama *et al*., 1991). Ovule culture was used to overcome postzygotic inter-specific incompatibility in reciprocal crosses between *B. rapa* and *B. oleracea* (Diederichsen & Sacristan, 1994). Similarly, the cross between *B. napus* and *B. oleracea* is normally unsuccessful, but the use of embryo culture techniques can produce hybrids (Gowers & Christey, 1999). *B. juncea* is a tetraploid containing AABB genomes and *B. oleracea* contains only CC genome which is a diploid (U, 1935). These different genomes have different characteristics. Inter-specific hybridition between tetraploid species is used in the *Brassica* genus to broaden the genetic diversity and to transfer valuable traits from one species to another. No records are so far available of obtaining successful trigenic hexaploid *Brassica* by conventional crossing between *B. oleracea* and *B. juncea*, except by protoplast fusion between the two species to obtain somatic trigenomic hexaploid *Brassica*s (Arumugam *et al*., 1996).

Development of hexaploid *Brassica* plants is a process of learning from nature and applying innovation to advance *Brassica* production for edible oils, bio-diesel and vegetables. The synthesis of hexaploid *Brassica* plants with all the A, B and C genomes together will have a very good potential to create new crops for domestication. The synthesized new hexaploids may provide traits for resistance to a number of biotic and abiotic stresses including drought, salt, pests and diseases, increased yield in terms of seed production, higher oil content, higher nutrition value, enhanced anti-carcinogenic, anti-oxidant and other medicinal properties as well as phytoextractive properties.

In this research the feasibility of producing a trigenomic *Brassica* population through inter-specific hybridization between *B. juncea* accessions, available in Sri Lanka and *B. oleracea* varieties widely cultivated in Sri Lanka, was examined. So far no attempt has been made to produce a hexaploid *Brassica* population *via* hybridization between *B. juncea* and *B. oleracea* using conventional breeding methods. Therefore, this study would provide a significant contribution to produce a new crop for domestication with a number of potential properties. The present study attempted to examine the success rate of crosses between tetraploid *B. juncea* and diploid *B. oleracea* and to obtain inter-specific F_1 hybrid seedlings of triploid nature (ABC).

MATERIALS AND METHODS

Genetic material

Five diverse genotypes of *B. juncea* (AC 0747, 0790, 1099, 2180 and 7700) and five diverse genotypes of *B. oleracea* (Chinese Broccoli, Broccoli-var. Shogun, Cauliflower-var. Snowball, Cauliflower-var. Phenomenon Early and Cabbage-var. Sweet Eureka) were used in the experiment. Ten replicates for each parental genotype were grown in a glasshouse at the Open University of Sri Lanka, Nawala and the crosses were made between *B. juncea* (♀) and *B. oleracea* (♂) varieties. The flowers of the female parent were opened and emasculated using a fine forcep and fresh pollen from the male parent was transferred to the stigma. There were 44 different cross combinations (Table 1). Depending on the availability of flower buds, 30-60 crosses were made for each cross combination. The pollination was carried out between 6.00 – 7.30 am to ensure successful pollination. The pollinated flowers were covered with perforated plastic bags and tagged. Reciprocal crosses (*B. oleracea* (♀) x *B. juncea* (♂)) were undertaken for each of the crosses. All hybridizations were performed without the aid of embryo rescue. The number of flowers crossed, mature pods formed and the seed set for each cross was recorded.

Agro-morphological evaluation

About 10% of the hybrids seeds were germinated and 80 F_1 hybrid seedlings were obtained. They were transplanted in pots and agro-morphological traits such as flowering time, stem height at flowering, number of leaf nodes at flowering, leaf, petiole, stem colour and hairiness, flower colour and seed coat colour were recorded for each F_1 plant.

Molecular confirmation

Genomic DNA was extracted from fresh leaf tissues of parents and 80 putative hybrid plants using MagAttract Genomic DNA Extraction Kit (Qiagen). Hybridity of 80 putative F_1 hybrid plants was tested using four SSR (Simple Sequence Repeats) markers, sN11722, OI10-D03, OI10-F09 and sN12353 (Lowe *et al*., 2004). Lyophilised oligonucleotide primers were reconstitute by addition of Mili Q water to bring the concentration to 500 µM. Then 50 µM and 10 µM dilutions were prepared from 500 µM stock solutions. Deoxyribo nucleotide mixture (dNTP), was prepared by diluting stock solutions of (100 mM) dATP, dCTP, dGTP and dTTP in an eppendrof tube to the final concentration of 2 mM. The standard PCR protocol for one sample is given in Table 2. The PCR reactions were performed in a 25 µl final volume of the reaction mixture. The different reagents were added according to the optimized protocol.

The PCR programme was optimized and is as follows: an initial step of 5 minutes at 94 ºC, followed by 30 cycles of 45 seconds at 94 ºC 45 seconds at 48 ºC, 1 minute and 30 seconds at 72 ºC, and a final extension step of 7 minutes at 72 ºC. Polymerase Chain Reaction (PCR) amplification was carried out with genomic DNA of F_1 hybrids and five parents of *B. juncea* and five parents of *B. oleracea*. The amplified PCR products from the parents and hybrids were resolved by TBE agarose gel electrophoresis (50 Volts/cm) using 4% Agarose 1000 (Invitrogen). Each DNA was amplified by using both the primers, sN11722, OI10-D03, OI10-F09 and sN12353. Based on the results, the SSR marker sN12353 was selected for confirming the true hybridity of 80 F_1 hybrid plants. Cross progeny were considered true inter-specific hybrids when they possessed sN12353 alleles from both parents.

Pollen viability

Pollen viability of the parental plants, *B. juncea* and *B. oleracea* was tested with 1% acetocarmine.

The same procedure was followed to test the pollen viability of the four confirmed true hybrids.

Table 1: The total pod and seed set for crosses and reciprocal crosses between *B. juncea* and *B. oleracea* and the pod and seed set of each cross combination.

Type of the Cross	Total pollinated buds	Total mature pods	% pod set	Total seeds
B. juncea (♀) x *B. oleracea* (♂)	1040	260	25%	793
B. juncea (♂) x *B. oleracea* (♀)	873	82	9%	0

Cross combination	Total pollinated buds	Total mature pods	% pod set	Total seeds
***B. juncea* (♀) x Chinese Broccoli (♂)**				
AC 0747	50	9	18 %	24
AC 0790	45	21	46 %	63
AC 1099	50	15	30 %	37
AC 2180	45	11	24 %	34
AC 7700	45	8	18 %	24
Chinese Broccoli (♀) x *B. juncea* (♂) – AC 0747, AC 0790, AC 1099, AC 2180, AC 7700	293	33	11 %	0
***B. juncea* (♀) x Broccoli (var. Shogun) (♂)**				
AC 0747	60	10	17 %	32
AC 0790	50	15	30 %	41
AC 1099	45	12	27 %	32
AC 2180	40	15	38 %	44
AC 7700	42	10	24 %	30
Broccoli (var. Shogun) (♀) x *B. juncea* (♂) –				

AC 0747, AC 0790, AC 1099, AC 2180, AC 7700	189	12	6 %	0
B. juncea **(♀) x Cauliflow (var. Snowball) (♂)**				
AC 2180	50	11	22 %	34
AC 7700	35	12	34 %	42
Cauliflower (var. Snowball) (♀) x *B. juncea* (♂) –				
AC 2180, AC 7700	63	4	6 %	0
B. juncea **(♀) x Caulifl. (var. Phe. Early) (♂)**				
AC 0747	55	11	20 %	35
AC 0790	45	16	35 %	51
AC 1099	50	9	18 %	24
AC 2180	40	12	30 %	31
AC 7700	45	9	20 %	30
Cauli. (var. Phe. Early) (♀) x *B. juncea* (♂) –				
AC 0747, AC 0790, AC 1099, AC 2180, AC 7700	260	18	7 %	0
B. juncea **(♀) x Cabbage (var.Sweet Eureka)(♂)**				
AC 0747	43	7	16 %	31
AC 0790	45	19	42 %	61
AC 1099	50	9	18 %	31
AC 2180	60	11	18 %	35
AC 7700	50	8	16 %	27

Table 2: Concentration and volume of reagents of the standard PCR protocol for one sample.

Reagents	Concentration of stock solutions	Working concentration of the reagents	Volume of reagents used
PCR buffer	5x	1x	5.00 µl
$MgCl_2$	25 mM/ µl	2 mM/ µl	2.00 µl
dNTP	2 mM/ µl	0.2 mM/ µl	2.50 µl
Forward primer	10 µM/ µl	0.5 µM/ µl	1.25 µl
Reverse primer	10 µM/ µl	0.5 µM/ µl	1.25 µl
Taq polymerase	5 U/ µl	1 U/ µl	0.25 µl
DNA	50 ng/ µl	50 ng/ µl	1.00 µl
Water	-	-	11.75 µl

RESULTS

Hybridization was possible between *B. juncea* and *B. oleracea*. All the five varieties of *B. juncea* were able to hybridize with *B. oleracea* varieties with a varied proportion of pod set out of total pollinated flowers. The cross (*B. juncea* (♀) x *B. oleracea* (♂)) of 1040 flowers resulted in 260 mature pod set (Table 1). The overall success of pod formation was 25%, whereas the overall seed set was 793 seeds. The % crossability (% pod set) between *B. juncea* and *B. oleracea* varied dependent on the genotype of the parent. A maximum of 38% - 46% was achieved with *B. juncea* accessions AC 0790 and AC 2180 inter-crossing with *B. oleracea* varieties as male parents. Only 3.2±0.63 seeds were present per pod and pods were very small (2.6 ± 0.34 cm) compared to that of *B. juncea* (5.24 ± 0.23 cm) and *B. oleracea* (4.21 ± 3.3 cm) parents. The average number of seeds in *B. juncea* was 12.9 ± 1.37 and in *B. oleracea* is 9.8 ± 1.14. The reciprocal cross (*B. oleracea* (♀) x *B. juncea* (♂)) of 873 flowers resulted in 82 mature pods and the success of pod formation was 9%. However, no seeds were developed in mature pods (Table 1).

About 10% of the F_1 hybrid seeds were germinated to obtain 80 F_1 seedlings. Although usually *B. juncea* and *B. oleracea* seeds take 2-3 days to germinate, the F_1 seeds took 5-20 days to germinate.

Agro-morphological evaluation

The 80 resulted F_1 hybrid plants were evaluated based on their agro-morphological characters. A range of variation in agro-morphological traits was observed in the F_1 hybrids of each cross combination. The agro-morphological characters observed were leaf colour, colour of petiole, hairiness of leaves, colour of

stem, colour of flowers, colour of stem, flowering time, stem height- and number of leaf nodes at flowering. In F_1 hybrid plants mainly the leaf morphology (shape and size), hairiness of leaves and the colour of petioles varied in different degrees compared to their respective parents. The agro-morphological variation among some selected putative F_1 hybrids with their parents were presented in Table 3. There was a wide range of morphological differences among the F_1 individuals resulted from crosses of *B. juncea* (♀) x *B. oleracea* (♂) genotypes. Figure 2A and Figure 2B show morphological differences between the leaves and pods of *B. juncea* (AC 0747) and *B. oleracea* (var. Chinese Broccoli) parents and their F_1 hybrids. True F_1 hybrid plants, which were confirmed by molecular markers, are more vigorous than none-true-hybrids (Figure 3). Compared to none-true-hybrids, true hybrids have broader and bigger leaves, taller and stronger stems, many branches, flowers and pods. However, since the pollen viability was very low, although many pods were formed, no seeds were developed in any of the true hybrid triploid (ABC) plants.

Molecular confirmation

Out of 80 putative hybrids, four F_1 hybrids, i.e. crosses of one AC 0747 x Chinese Broccoli, two AC 0790 x Chinese Broccoli and one AC 2180 x Broccoli (var. Shogun) were confirmed to be true hybrids with the SSR marker sN12353. Figure 4A

Figure 2A: Morphological differences in leaves (x 1/4) of *B. juncea* (AC 0747) (♀), *B. oleracea* (var. Chinese Broccoli) (♂) and their true F_1 hybrids.

Figure 2B: Morphological differences in pods (x 1/4) of *B. juncea* (AC 0747) (♀), *B. oleracea* (var. Chinese Broccoli) (♂) and their true F_1 hybrids.

A (x 1/5)

B (x 1/3)

Figure 3: A true F_1 hybrid (A) of the cross between *B. juncea* (AC 0790) x *B. oleracea* (Chinese Broccoli) and a no true F_1 hybrid (B) of the same cross in 8 weeks after germination.

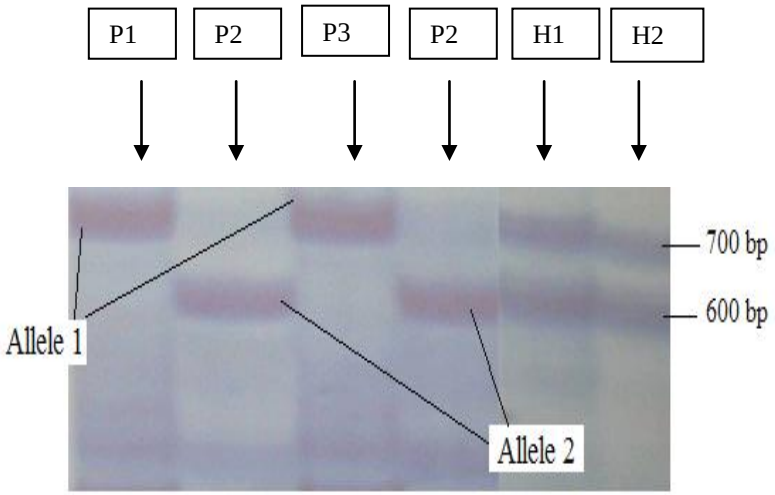

Figure 4A: DNA profile of parents and two hybrids in 4% Agarose 1000 (Invitrogen) gel using the SSR marker sN12353
[P1 – *B. juncea* (AC 0747); P2 – *B. oleracea* (Chinese Broccoli); P3 – *B. juncea* (AC 0790); H1 – Hybrid of P1 x P2 cross; H2 – Hybrid of P3 x P2 cross]

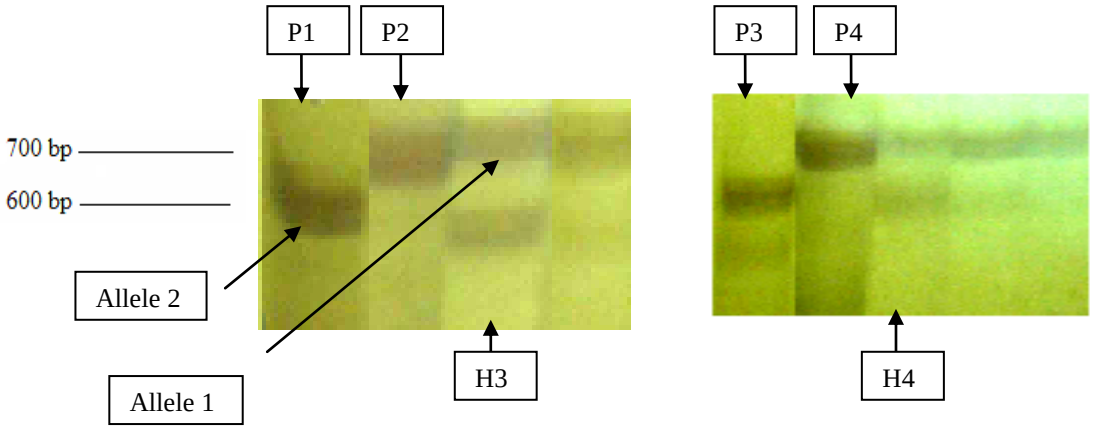

Figure 4B: DNA profile of parents and two hybrids in 4% Agarose 1000 (Invitrogen) gel using the SSR marker sN12353
[P1 – *B. oleracea* (Broccoli var. Shogun); P2 - *B. juncea* (AC 2108); P3 – *B. oleracea* (Chinese Broccoli); P4 - *B. juncea* (AC 0790); H3 – Hybrid of P1 x P2 cross; H4 – Hybrid of P3 x P4 cross]

Table 3: A comparison of morphological characters between selected F_1 hybrids and their parents

Morphological Trait	Parents and Hybrids				
	***B. juncea* (AC 0747) (♀)**	***B. oleracea* (var. Chinese Broccoli) (♂)**	**F_1 (putative hybrid 1)**	**F_1 (putative hybrid 5)**	**F_1 (putative hybrid 10)** (True hybrid)
Fl. Time (No. of days to flower)	78	88	113	106	90
Stem Height at flowering (cm)	89	35	111	68	130
No. of leaf nodes at flowering	20	12	34	35	32
Colour of leaves Colour of petiole Hairyness of leaves	Green Petiole purple Hairy	Green Petiole green Non-hairy	Green Petiole purple Non-hairy	Green Petiole purple Non-hairy	Green Petiole green Non-Hairy
Colour of stem	Green	Green	Green	Green	Green
Colour of flower	Yellow	White	Yellow	Yellow	Yellow
Colour of seeds	Light Brown	Black	Yellow	Yellow	Yellow

	***B. juncea* (AC 0790) (♀)**	***B. oleracea* (var. Chinese Broccoli) (♂)**	**F_1 (putative hybrid 2)**	**F_1 (putative hybrid 5)** (True hybrid)	**F_1 (putative hybrid 6)** (True hybrid)
Fl. Time (No. of days to flower)	73	88	87	52	80
Stem Height at flowering (cm)	75	35	80	68	98
No. of leaf nodes at flowering	22	12	25	15	23
Colour of leaves	Green	Green	Green,	Green	Green
Colour of petiole	Purple	Green	Purple	Green	Purple
Hairyness of leaves	Hairy	Non-hairy	Hairy	Non-hairy	Non-hairy
Colour of stem	Light purple	Green	Light purple	Green	Green
Colour of flower	Yellow	White	Yellow	Yellow	Yellow
Colour of seeds	Yellow	Black	Yellow	Light Yellow	Light Yellow

	***B. juncea* (AC 2180) (♀)**	***B. oleracea* (var. Broccoli - Shogun) (♂)**	**F_1 (putative hybrid 1)** (True hybrid)	**F_1 (putative hybrid 2)**
Fl. Time (No. days to flower)	71	118	42	40
Stem Height at flowering (cm)	73	14	53	36
No. of leaf nodes at flowering	15	18	12	13
Colour of leaves	Green	Green	Green	Green
Colour of petiole	Green	Green	Green	Green
Hairyness of leaves	Hairy	Non-hairy	Non-hairy	Non-hairy
Colour of stem	Green	Green	Green	Green
Colour of flower	Yellow	Yellow	Yellow	Yellow
Colour of seeds	Brown	Black	Brown	Brown

and Figure 4B shows the DNA profile of parents and two hybrids in 4% Agarose 1000 (Invitrogen) gel using the SSR marker sN12353.

Pollen viability

Brassica juncea and *B. oleracea* showed over 95% pollen stainability in 1% acetocarmine.

However, the stainability in 1% acetocarmine of four confirmed true hybrids was very poor (5%) indicating that they are sterile.

DISCUSSION

Inter-specific reproductive isolation is the main mechanism for speciation and specific maintenance (Griffiths *et al*., 2002). However, genetics leaks in the isolation system have occasionally enabled inter-specific gene flow and promoted the evolution of species (Jiang *et al*., 2007). Polyploidy often occurs in association with inter-specific hybridization. It is long been established as a key mechanism in plant evolution and adaptation (Leitch & Leitch, 2008). Increase in genome size (polyploids) of plant species is associated with an enhanced cell size and dry matter production (Arnold, 1997) which could result in agronomically superior plants. Prior studies on the cytogenetics and molecular genetics of *Brassica* species have shown that there are significant differences between the A, B, and C genomes from the three diploid species, *B. rapa*, *B. oleracea*, *B. nigra* and the three amphidiploid species, *B. napus*, *B. juncea*, *B. carinata* (McGrath & Quiros 1990). Hence, a hexaploid *Brassica* (6x, AABBCC) population generated by combining the C genome of *B. oleracea* and A and B genomes of *B. juncea* will lead to widening the genetic variability among members of the family Brasicaceae.

In the present experiment, hybridization was possible between tetraploid *B. juncea* (AABB) and diploid *B. oleracea* (CC). Crosses were only successful when *B. juncea* was used as the female parent. Although there was a 9% success in pod set in reciprocal crosses (*B. oleracea* (♀) x *B. juncea* (♂)), seeds were not developed. Out of the total flowers crossed, 25% of crosses yielded hybrid seeds from crosses of *B. juncea* (♀) x *B. oleracea* (♂). The best pod formation was achieved with *B. juncea* accessions AC 0790 (46%) and AC 2180 (38%) with *B. oleracea* varieties as male parents. Therefore, the hybrid seed set was completely genotype dependent. A number of earlier studies highlighted the importance of maternal genotype in the species

Brassica (Meng *et al*., 1992; Meng & Lu, 1993; Liu & Meng, 1995). Many *Brassica* inter-specific crosses were successful when the female parent is with a higher ploidy level than the male parent (Schelfhout *et al*., 2006). Consistent with the present results, Schelfhout *et al*. (2006) also found that the success rate was better in inter-specific crosses of *Brassica* only when tetraploids were used as female parents.

Hybridization between allotetraploid species, *B. napus*, *B. juncea*, *B. carinata* and the diploids, *B. nigra*, *B. oleracea* and *B. rapa* are naturally highly incompatible (Diederichson & Sacristan 1994). Fertilization may take place, but abortion occurs early in the development of the embryo. In the present study as well the crosses in both directions a very high rate of seed abortion occurred early in the development. Total seed set of the crosses between *B. juncea* (♀) and *B. oleracea* (♂) was 793 ie. only 3.2±0.63 seeds per pod, whereas for reciprocal crosses seed set was zero.

Natural crossing does not occur between *B. juncea* and *B. oleracea* under field condition (Bing *et al*., 1996) and also a successful artificial pollinalion has not been recorded until now except obtaining hexaploid *Brassica* somatic hybrids by protoplast fusion between *B. oleracea* (CC) and *B. juncea* (AABB) by Arumugam *et al*., (1996). In an investigation of inter-specific hybridization among several *Brassica* species, Takeda (1983) reported that hybridization between *B. juncea* and *B. oleracea* was particularly difficult. This demonstrates the high level of sexual incompatibility between *B. juncea* and *B. oleracea*. The barriers might overcome by selecting suitable cross combinations involving different genotypes. Therefore, in our study, a wide range of genotypes were selected to maximize the success rate of crossing.

To confirm the true hybrid nature of putative F_1 plants, agro-morphological traits and molecular markers (SSR) were used. The agro-morphological characterization of putative F_1 hybrids demonstrated that there was a range of agro-morphological variation compared to their respective parents. The molecular confirmation with the SSR marker sN12353 proved that four F_1 plants were true hybrids. *B. juncea* (♀) accessions 0747, 0790 and 2180 were successfully hybridized with *B. oleracea* (♂) genotypes Chinese Broccoli and Broccoli (var. Shogun) to produce true hybrids. The true F_1 hybrids which were confirmed by molecular analysis clearly showed that the agro-morphologically they are different from the respective parents. However, some of the putative F_1 hybrids were also agro-morphologically different from their respective parents, whereas some were more or less agro-morphologically similar to either of their respective parents.

In the present study, *B. juncea* accessions 0747, 0790 and 2180 appear to have allowed foreign pollen from *B. oleracea* to germinate on their stigmas and enabled pollen tubes to penetrate their styles. Only varieties with a high crossability have the mechanism(s) to enable the hybrid embryos to develop fully. Evaluation and characterization of the remaining putative hybrid seeds will be carried out and the triploid true hybrid seedlings will be treated with chromosome doubling technique to synthesize hexaploid (6x, AABBCC) population.

Besides commercially important *Brassica* species included in the "U" triangle, there is a wide variety of important *Brassica* species such as: *B. elongata* (Elongated Mustard), *B. fruticulosa* (Mediterranean Cabbage), *B. narinosa* (Broadbeaked Mustard), *B. perviridis* (Tender Green, Mustard Spinach), *B. rupestris* (Brown Mustard), *B. septiceps* (Seventop Turnip), and *B. tournefortii* (Asian Mustard). A screening of the above species for resistance to the important fungal pathogens has been carried out (Scholze & Hammer, 1998) and obtained very promising results. Thus, these species offer prospective additional sources of genes/traits for resistance to a number of biotic and abiotic stresses as well as genes/traits for yield, oil content, nutrition value and medicinal properties, when hybridized with six *Brassica* species in the "U" triangle to produce hexaploid *Brassica* cultivars.

CONCLUSIONS

Polyploidy is accepted as a key mechanism in plant evolution and adaptation (Leitch & Leitch, 2008). Many successful natural hexaploid plants such as wheat, oat and kiwifruit and man-made hexaploids such as hybrid (Triticale) of *Triticum* and *Secale* exist. However, naturally occurring hexaloid *Brassica* are not available. There are at least three possible approaches to produce hexaploid *Brassica.* In the present investigation, one tetraploid (4x) *B. juncea* and diploid (2x) *B. oleracea* were inter-crossed. Evaluation of 80 putative hybrids by molecular markers and agro-morphological characterization confirmed four true hybrids resulting crosses between AC 0747 x Chinese Broccoli, AC 0790 x Chinese Broccoli and AC 2180 x Broccoli-var. Shogun,) which is a potential approach to artificially produce hexaploid *Brassica* (6x, AABBCC) genotypes in future.

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