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Induced polyploidization in *Brassica fruticulosa* - a wild relative of Brassicas as potential source for mustard aphid tolerance

Arun Kumar , Prashant Yadav*

Directorate of Rapeseed-Mustard Research, Bharatpur -321 303, Rajasthan
*Corresponding author e-mail: aruncyto@gmail.com

Abstract

Polyploidy is an important source for acquiring new genetic recombination and creating genetic uniqueness in plants. Ploidy manipulation is generally associated with the obtainment of some increased enviable traits of the plants as well as also provides them greater adaptability to various biotic and abiotic stresses as compared to its diploids counterparts. In the present study, successful induction of autotetraploidy has been achieved through seedling treatment of colchicine in B. fruticulosa Cyr. subsp. fruticulosa (2n = 16 FF), a wild relative of cultivated brassicas. The diploid seedlings of B. fruticulosa were treated with different concentrations of aqueous colchicine using the cotton-swab method for 8 -12 hours for 2-3 days. The highest percentage of success was recorded in when the seedlings were treated with 0.2% cochicine for eight hours within two days. The synthesized plant showed remarkable enhancement in several morphological and floral characters making more robust. Induced tetraploid was cytologically distinguished from diploid by the occurrence of 32 chromosomes at diakinesis/metaphase-I with different combinations of univalent, bivalents and multivalent in the form of trivalents and quadrivalents. The anaphase I and II disjunction of bivalents/chromosomes was leading more or less regularly and equally to the formation of seeds from the synthesized plants. Significant enhancement in morphological traits as revealed in colchicine-induced plant and normal meiotic behaviour leading to a good seed set may ultimately result in providing the plant breeder with more variability, especially in mustard aphid crop improvement programmes of brassicas

Key words: Colchicine, Cytology, Meiotic behaviour, Quardivalents.

Introduction

Polyploidy is recognized as a prominent feature in the evolution of higher plants and adaptation, and polyploids have often been selected during the evolution of crop plants (Leitch and Bennet, 2008). From a plant breeder point of view, manipulation in ploidy is significant for genetic improvement in crop plants and often generates variants that may possess useful characteristics and by doubling the gene products it also provides a wider germplasm base for crop improvement studies. Indian mustard [*Brassica juncea*, (L.) Czern and Coss.] is one of most important oil seed crops of India and with yield potential of 15–30 q/ha and 38–42% oil content. This crop fulfils approximately 27% of vegetable oil requirements of our country. India is considered to be one of the secondary centres of diversity for *B. juncea* where it is well adapted to different variable agroclimatic conditions of northern India (Sharma *et al.,* 2002).

However, this crop often encounters severe biotic and abiotic stresses emerging as a result of unprecedented change in environmental conditions at local, regional and global levels (Chopra *et al*., 1996). Amongst the biotic stresses, mustard aphid, *Lipaphis erysimi* (L.) Kaltenbach is a perpetual annual threat leading to productivity losses up to 70%, depending upon the severity of outbreak (Bandopadhyay *et al*., 2013). Normally the farmers depend on chemical pesticide/ insecticides primarily based on synthetic chemical insecticides for the control of pests and diseases. The use of chemical pesticides has caused a lot of environmental hazards apart from resistance developed by the pests to the chemical. These chemicals, besides aggravating environmental pollution, can also be toxic to friendly insects. Therefore, a resistant cultivar is a more sustainable and environment-friendly option. The development of an insect-resistant cultivar requires a heritable and transferable resistance (Stoner and Shelton, 1988). However, so far cultivated *Brassica* germplasm/cultivars has failed to provide source of tolerance/resistance against mustard aphid.

Fortunately, *Brassica* coenospecies has been bestowed with nearly 100 species and genera of wild and weedy relatives, serving as rich reservoir of genes conferring many agriculturally important traits. These species can be effectively utilized to introduce economically important traits to cultivated species as well as development of potential wide hybrids (Prakash, 2001; Kumar *et al*., 2013, 2015). Notably, *B. fruticulosa*, a wild relative of cultivated brassicas, which is endemic to the Mediterranean coast and can be a potential genetic source for crop improvement because it possesses resistance to cabbage aphid (*Brevicoryne brassicae*) (Cole, 1994a; Ellis and Farrell, 1995; Ellis *et al*. 2000) and a higher concentration of lectins was suggested to be the underlying mechanism of resistance in this species (Cole, 1994b). The insecticidal properties of this species are a promising method of biological control. Through introgressive hybridization, this trait may transferred to other important vegetable cultivars of the genus *Brassica*. The insufficient within-species variability can be addressed by the effective utilization of untapped genetic diversity in wild and weedy relatives of brassicas for breeding and crop improvement (Prakash, 2001).

Attempt to improve crop brassicas, through conventional breeding/intraspecific hybridization programme cannot be expected to genetic variants, since these approaches mobilize only a limited extent of variation. The alternative strategies which have great potential in brassicas to expand gene resources are nonconventional interspecific/ intergeneric hybridization, alteration in ploidy level, structural changes in chromosomes and artificial synthesis of new as well as naturally occurring species. Therefore, the present investigations were conducted with primary objective of to obtain polyploid mutant genotypes by the implementation of colchicine in *B. fruticulosa* which were verified on basis of significant differences in various cyto-morphological traits of autotetraploid over its diploid counterparts. Thereby, resulting genome doubling or ploidy manipulation could be correlated with the enhancement of various economically important traits and utilization of synthesized autotetraploids in *Brassica* crop improvement programme for future exploitation.

Material and methods

The seeds samples were directly sown in earthen pots filled with peat, perlite and sand (1:1:1, v/v/v). Cotton swab method was employed for colchicinization. Sterilized cotton swabs immersed either in 0. 5, 0.1 or 0.2 % aqueous colchicine were placed on emerging apical tip between two cotyledonary leaves. To avoid an increase in the concentration, colchicine was added drop by drop at regular intervals on the cotton swabs with the help of sterilized syringe. Such treatment was done for 2-3 days for 3-4 hours per day.

The data on various morphological attributes of both diploid and colchicine-induced plants were recorded by physical measurements with centimetre scale or micrometre as the case may be. Length and breadth of stomata has been recorded for this young leaves were peeled off from its dorsal surface, stained with safranin for few minutes and washed thoroughly and mounted in glycerine, observed under microscope. Length and breadth of stomata was measured using ocular micrometer and their mean values were calculated.

For meiotic studies flower buds of an appropriate size were collected from selected mature plant and fixed on the spot in freshly prepared carnoy's fluid (ethanol : chloroform : acetic acid - 6 : 3 : 1), for a minimum of 24 hours at room temperature and subsequently stored in 70% alcohol at 10^{0}C. Anthers were squashed in 1% acetocarmine solution. On average 25 Pollen Mother Cells (PMC) were analysed at diplotene/diakinesis/metaphase I to estimate the range of chromosome associations and recombinational frequencies by chiasma analysis. At anaphase I/II on average15-20 cells were analysed to study the distributional pattern of chromosomes.

Results

Efficiency of colchicine treatment:

B. fruticulosa seedlings at cotyledonary leaves stage were treated with aqueous colchicine (0.5%, 0.1% and 0.2% v/v) for 2-3 days for 3-4 hours per day. The highest induction percentage (7.14%) of putative polyploidy were recovered with only in 0.2% aqueous colchicine (Counted as the number of colchicine – induced tetraploids recovered from total number of seedlings treated with aqueous colchicine) (Table 1).

Table 1. Efficiency of colchicine treatment in seedlings of *B. fruticulosa*

Colchicine Concentration (%)	Duration of treatment in hour (hr)	No. of days of treatment	No. of seedling treated	No. of seedling survived	No. of colchicine – induced tetraploids	Percentage of tetraploids
0.5	09 12	3 3	30 30	27 25	- -	- -
0.1	09 12	3 3	30 30	24 18	- -	- -
0.2	09 12	2 2	30 30	14 19	1 -	7.14 -

Morphological observations:

Colchicine-induced tetraploid plant was robost from the initial stages of development and it was maintained till maturity. Plant height, Primary branches, Secondary branches per plant, length of petiole, length and width of leaflet, corolla length and width and siliqua length showed considerable increase in comparison with the corresponding diploid. The number of seeds per siliqua decreased in the synthesized colchicine-induced tetraploid plants. Among the various morphological characters' colchicine-induced tetraploid plant, showed considerable increase in all the morphological attributes accept seeds per siliqua which were less than diploid plant *B. fruticulosa* (Fig 1, Table 2).

Table. 2. Comparative morphological characters and pollen fertility of diploid and colchicine – induced autotetraploid of *B. fruticulosa*

Characters	Diploid 2n=16	Autotetraploid 2n=4x=32
Plant height (cm)	57.3	61.7
Primary branches per plant	5	8
Secondary branches per plant	7	14
Main raceme length (cm)	29.8	31
Length of petiole (cm)	2.0	2.4
Length of odd leaflet (cm)	7.3	11.5
Width of odd leaflet (cm)	6.8	9.4
Stomatal length (µm)	28.9	53.8
Stomatal Breadth (µm)	17.7	28.4
Corolla length (cm)	1.0	1.5
Corolla width (cm)	0.5	0.7
Siliqua length (cm)	3.2	3.5
Seeds per Siliqua	15	13
Pollen diameter (µm)	26.0	51.7
Pollen fertility (%)	97.6	65.5

Stomatal and Palynological observation:

Average length of stomata in autotetrapoloid plant was 53.8 µm, although it was 28.9 µm in diploids. Similar differences were observed in breadth as well which was 28.4 µm autotetrapoloid and 17.7 diploid µm (Figure 1, Table 2). Pollen diameter in autotetraploid and diploid plants was 26.0 and 51.7 µm, respectively. That clearly reveals the difference between pollen sizes in treated as well as non-treated plants. Thus, the majority of pollen grains in autotetraploids were bigger compared to diploids. A significant reduction in the pollen fertility of autotetraploids has been observed (Fig 1, Table 2).

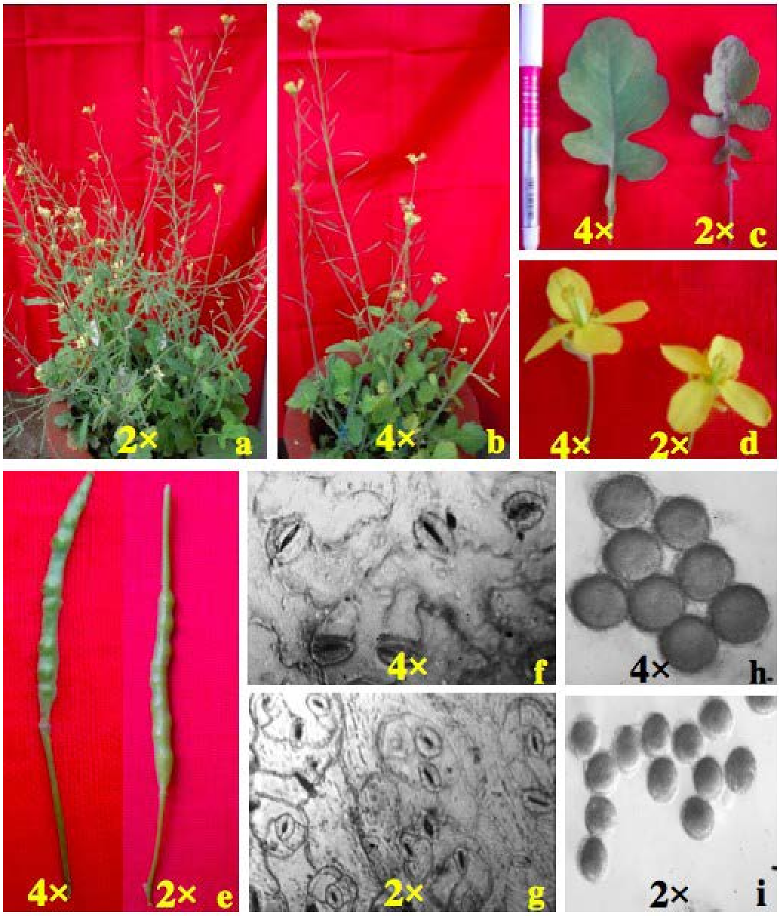

Figure 1: Comparison of morphological attributes in diploid and colchicine – induced tetraploids of *B. fruticulosa.* (a) Diploid plant; (b) Autotetraploid plant; (c) Leaf; (d) Flower; (e) Siliquae (Pods); (f)Stomata of diploid; (g) Stomata of colchcine induced autotetraploid; (h) Pollen grains of colchcine induced autotetraploid; (i) Pollen grains of diploid.

The above morphological observations clearly suggest that the induced autotetraploid plant showed robust, more compact bearing increased vegetative features and reproductive parts, and superior over its diploid counterparts.

Cytology of diploid and colchicine-induced tetraploid:

In diploid *B. fruticulosa* 25 Pollen Mother Cells (PMCs) analyzed at diakinesis/ metaphase I showed normal bivalent formation. The average number of chromosome associations was 8.50II + 0.60I. The equal distribution of chromosomes (8:8) was observed in all the 20 cells analysed at anaphase I (Figure 2 ; Table 3-4).

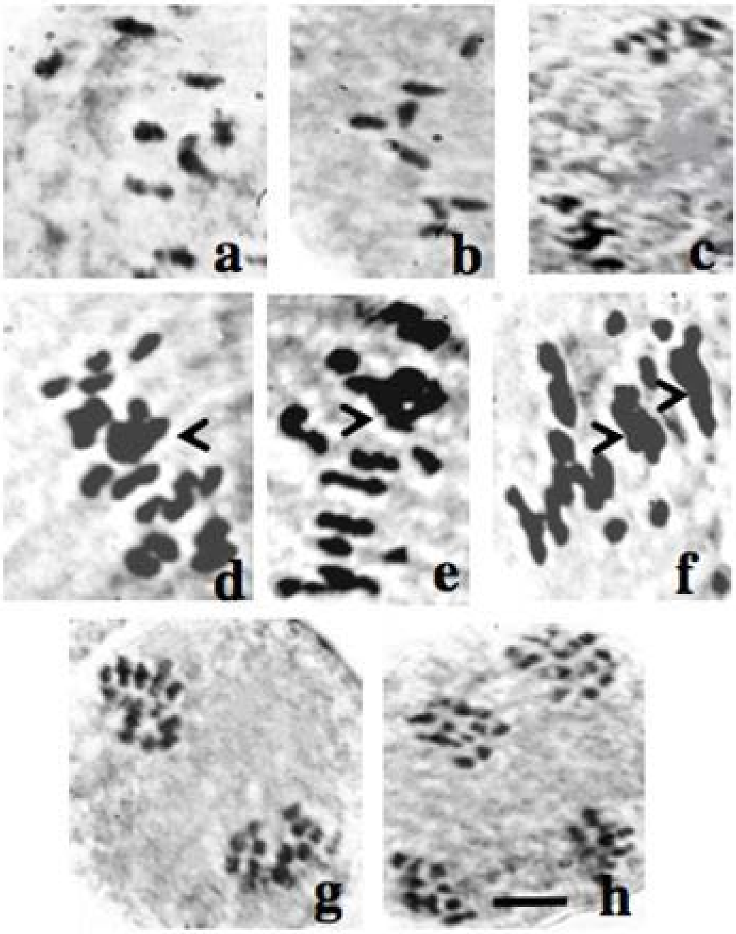

Figure 2: Documentation of cytological examinations in diploid and colchicine – induced tetraploids of *B. Fruticulosa*, Figure (a-c) *B. Fruticulosa* diploid (a) Diakinesis 8II; (b) Metaphase I 8II; (c) Anaphase I 8:8; (d-h) *B. Fruticulosa* induced autotetraploids (d) Diakinesis 1IV+9II+10I; (e) Metaphase I 2IV+10II+4I; (f) Metaphase I 2IV + 11II + 2I; (g) Anaphase I 16:16 (equal distribution); (h) Anaphase II (equal distribution); (Quadrivalents marked by arrow heads); Bar: 10 µm.

Table 3. Average number and range of chromosome associations at diakinesis/metaphase I in diploid and colchicine – induced tetraploids of *B. fruticulosa*

Ploidy level	No. of cells analyzed	Quadrivalents		Bivalents		Univalents	
		Mean	Range	Mean	Range	Mean	Range
Diploid (2x=16)	25	-	-	8.20 ± 0.59	6-8	0.70 ± 1.37	0-2
Tetraploid (4x=32)	25	2.40 ± 0.20	1-2	8.23 ± 0.25	7-10	3.30 ± 0.61	0-10

In autotetraploid plant in total 25 PMCs were analyzed. The average chromosome association determined for this plant was 2.40IV + 8.23II + 3.30I per cell, their range being 1-2, 7-10, 0-10, respectively. The maximum number of cell showed the occurrence of either one or two quadrivalents, besides other chromosome associations. 20 cells analyzed at anaphase I, the distribution of chromosomes (16:16) at anaphase I was found in 17 cells and three cells had unequal distribution of lagging two univalent (Figure 2, Table 3-4).

Table 4. Anaphase I distribution in diploid and colchicine – induced tetraploids of *B. fruticulosa*.

Ploidy level	No. of cells analysed	Chromosome distribution	No. of cells	Percentage
Diploid (2x=16)	20	8:8	20	100.00
Tetraploid (4x=32)	20	16:16 15:17 14:18	17 2 1	85.00 10.00 5.00

Discussion

Induction of polyploidy using colchicine and their cyto-morphological and genetic characterization has been a subject of immense interest among geneticists and plant breeders for a long time (Otto and Whitton, 2000, Reiseberg, 2001, Ramsey and Schemske, 2002, Mable, 2003) Polyploid plants often possess many superior agronomic traits of economic over the diploid plants. For example, ployploids may have larger leaves and flowers, thicker stems and roots, darker green leaves, an increased width to length ratio of the leaves, increased cell size, leading to larger reproductive and vegetative organs a more compact growth habit and a higher tolerance to environmental stress. On the basis of above mentioned facts, herein present study the utility of various morphological and cytological analyses in distinguishing autotetraploids from diploids has been tested.

In the present study, some morphological features of the colchicine-induced autotetraploids *viz*. plant height, primary and secondary branches, length of petiole, length and width of leaflet, corolla length and width and siliqua length etc. showed an increase in dimension while few characters viz. seed per siliqua followed a reverse trend compare to the diploid plants (Kumar and Dwivedi, 2014).

In plants stomatal analysis and pollen diameter provide an efficient mean for characterization of polyploidy as these characters generally increased in polyploids. Kumar and Dwivedi (2014) produced tetraploid in *Brassica campestris* using colchicine treatment and primarily verified the status of tetraploid via stomatal analysis and found that stomatal analyses could be the most efficient criteria for ploidy confirmation. Similarly increase in the size of pollen diameter has been documented in present case Such observations have been previously demonstrated in a large number of plant species (Srivastava and Raina, 1983; Mishra, 1986; Bewal *et al.,* 2009).

In autotetraploidy, each chromosome theoretically represents itself four times, due to which mostly the quadrivalent associations would be expected. In the present study, meiotic analysis of induced colchiploid of B. fruticulosa showed low quadrivalent frequency and high bivalent average per cell. Several reasons have been put forward to explain the low/high quadrivalent frequency in tetraploids. Sybenga (1975) suggested that the frequency of bivalents and quadrivalents in tetraploids is dependent on the points of pairing initiation. When there is a single point of pairing initiation, bivalents are formed; when there are two points of initiation, both quadrivalents and bivalents are formed in equal frequencies; when the initiation points are more than two, quadrivalents are formed in high frequency as compared to bivalents. The average chromosome association determined for this plant was 2.40IV + 8.23II + 3.30I per cell, their range being 1-2, 7-10, 0-10, respectively. The maximum number of cell showed the occurrence of either one or two quadrivalents, besides other chromosome associations

In the induced tetraploid plant had some cells at anaphase I with unequal distribution and/or lagging bivalents or univalent. Tetraploids always show irregularities and these irregularities are considered to be due to the formation of multivalent associations. It was the common feature in colchitetraploids. The cytological causes, resulting in unviable gametes, include non-disjunction of multivalents, lagging chromosomes, univalents, non-viability and other abnormalities (Verma *et al*., 2017).

Induced Polyploidization of *Brassica fruticulosa* plant obtained in the present investigations are ideal for further, utilization in the hybridization programme with *B. juncea* and other related species for introgression of traits of economic importance, especially for mustard aphid in brassica crop.

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