Distant hybridization and introgression of alien genes for improving heterotic potential and resistance to various biotic stresses in vegetable and oilseed brassicas (Brassica Sp.)

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Abstract

Family Brassicaceae comprised of diverse crop species which are highly nutritional, economically high valued and used as oilseed as well as vegetable in our daily diet. However, yield and productivity of these crops become reduced due to several biotic stresses as well as susceptibility to weeds. Additionally, to meet the growing demand of farmers, F1 hybrid development is the foremost priority to breeders with the help of any economically feasible genetically controlled system. Thus, researchers are continuously searching for stable male sterility systems for quality hybrid development and for resistant genes against various biotic stresses and concurrently, introgression of desirable gene into cultivated species through distant hybridization method by crossing with its distantly related species (Inter-specific) or genus (Inter-generic). However, to overcome pre and post-zygotic crossing barriers, various in-vitro techniques like embryo rescue, protoplast fusion, chromosome doubling or growth regulators can be used to convert sterile hybrid into fertile one.

Keywords: Brassica crops, hybrid, male sterility, resistant genes, distant hybridization, crossing barriers

1. Introduction

Brassica crops are most important as well as highly demanded crops in our country. In Brassicaceae family, six Brassica species that mentioned in U’s Triangle (Nagaharu U, 1935) consists three diploid species, namely Brassica rapa (AA genome: 2n = 2x = 20), Brassica nigra (BB: 2n = 2x = 16), and Brassica oleracea (CC: 2n = 2x = 18), and three allotetraploid species, namely Brassica juncea (AABB: 2n = 4x = 36), Brassica napus (AACCC: 2n = 4x = 38), and Brassica carinata (BBCC: 2n = 4x = 34) and Raphanus sativus are cultivated worldwide as oilseed as well as vegetable purpose (Yamagishi and Bhat 2014) [56]. All these crops are rich source vitamin A and C, several minerals such as phosphorus, potassium, calcium, sodium and iron and several glucosinolate compounds which acts as a potential inhibitor of apoptosis within cancerous cell.

In brassica, hybrid seed production is the topmost priority to the breeders to increase the yield of both vegetable and oilseed brassicas. It has long been known that brassica crops show strong heterosis (Tanaka and Niikura 2006) [48]. Oil seed crops such as B. napus and B. juncea show hybrid vigor in terms of seed yield. Vegetables such as B. oleracea (cauliflower, cabbage, etc.) also exhibit high heterosis for earliness, several curd related traits like shape, size, weight that ultimately increase the yield of the crop. However, yield as well as productivity of the crops become decreased due to several biotic stresses such as, infection of several pathogens including bacteria, fungi (Table 1) as well as susceptibility to weeds (Lv et al., 2020) [33]. Among these diseases, Turnip mosaic virus (TuMV), black rot (BR), blackleg (BL), stem rot (SR), Fusarium wilt (FW), downy mildew (DM), and clubroot cause major damage to different brassica crops. Additionally, small size of flower and complex plant architecture limits the development of F1 hybrid through hand emasculation and hand pollination method which can be performed easily. For that reason suitable genetic control systems like male sterility and self-incompatibility has been widely exploited for hybrid development since last few decades. Although, self-incompatibility-based system has not received much popularity like cytoplasmic male sterility (CMS) based system as because of its
poor stability for hybrid seed production in brassica crops (Kitashiba and Nasrallah 2014)\(^{25}\). Among sterile cytoplasms, so far only ogura based CMS has been commercially utilized in brassica crops. But, recently several new sterile cytoplasm based systems such as *Diplotaxis arvensis*, *Enarthocarpus hyratus*, *Eruca sativa*, *Erucastrum canariense*, *Brassica oxyrrhima*, *Trachystoma balli*, *Sinapis alba*, etc. that belongs to tertiary gene pool of brassica are being exploited in breeding program. As both of this systems are strongly influenced by various environmental factors like temperature, humidity, etc. or genotypic factor or any other unknown causes, it is a major challenge to crop breeders to minimize the adverse effect of global climate change.

Therefore, researchers are continuously trying to search for new male sterility systems as well as new genes that are resistant against attack of various pathogens from the secondary or tertiary gene pool of brassica and simultaneously, introgression of desirable gene into cultivated species though hybridization process to meet the negative impact of climate change. Therefore, distant hybridization (syn. wide hybridization) is one of the most useful and efficient strategy which aims to transfer desirable trait to cultivated species by crossing with genetically diverse parental line, belongs to same or different gene pool. This strategy usually used when the desirable characters are not found within the species of a crop.

### Table 1: Major Brassica crops and their important diseases

<table>
<thead>
<tr>
<th>Species Genome</th>
<th>Genotype</th>
<th>Crops</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. rapa</em> AA</td>
<td>Chinese cabbage, turnip, pak choi</td>
<td>Downy mildew, TuMV, clubroot, soft rot</td>
<td></td>
</tr>
<tr>
<td><em>B. nigra</em> BB</td>
<td>Black mustard</td>
<td>Black rot, leaf spot, blackleg, TuMV</td>
<td></td>
</tr>
<tr>
<td><em>B. oleracea</em> CC</td>
<td>Cabbage, broccoli, cauliflower, kale, brussels sprouts</td>
<td>Black rot, Fusarium wilt, clubroot, TuMV</td>
<td></td>
</tr>
<tr>
<td><em>B. napus</em> AACC</td>
<td>Oilseed rape, canola, swede (rutarbaga)</td>
<td>Clubroot, blackleg, stem rot, TuMV</td>
<td></td>
</tr>
<tr>
<td><em>B. juncea</em> AABB</td>
<td>Indian mustard, leaf mustard</td>
<td>Blackleg, white rust, stem rot, downy mildew</td>
<td></td>
</tr>
<tr>
<td><em>B. carinata</em> BBC</td>
<td>Ethiopian mustard</td>
<td>Black rot, TuMV</td>
<td></td>
</tr>
</tbody>
</table>

Source: Lv et al., 2020\(^{35}\)

Hybridization can be done between two different species of same genus (Inter-specific) or different genera (inter-generic). Introggression of genes from secondary and tertiary gene pool to primary gene pool is very difficult due to presence of several fertilization barriers which can be designated as pre-fertilization and post-fertilization barrier. Among pre-fertilization barriers, failure of pollen grain germination, slow pollen tube growth, inability of pollen tube to reach the style, arresting of growth of pollen tube in the style, ovary or ovule, varying thickness of pollen tube are the major issues that played significant role. Similarly, post fertilization barriers occurs due to embryo abortion, endosperm abortion, lethality of F\(_1\) hybrids, incompatibility of cytoplasm, elimination of some chromosomes, hybrid sterility or breakdown of hybrids occur when F\(_1\) hybrid is more vigorous and fertile compared to F\(_2\) progenies. Additionally, asynchronous flowering between two parents also possesses sometimes cross incompatibility. For these various reasons, crossing between the two parents either fully fertile, fully sterile or partially fertile (syn. partially sterile) in nature. Interspecific or inter-generic crosses are fully fertile when two species have complete chromosomal homology between them and showed normal pairing at meiosis. While, in case of fully sterile crosses, two parents doesn’t possess complete chromosomal homology and exhibits irregular meiotic pairing. Such hybrids can be made fertile only by doubling the chromosome number through colchicine treatment or other advanced breeding approaches like embryo rescue, protoplast fusion, etc. Keeping in view the above facts, in this article, we will discuss about various challenges and advantages of utilizing distant hybridization and simultaneously, introgression desirable genes for the improvement of heterotic potential through transfer of new CMS system as well as resistance against various biotic stresses of brassica crops to increase the yield and productivity of the crops.

### 2. Gene pool concept of Brassica

Gene pool consists of summation of all the genes and their alleles present in all such individual. Harlan de Wet (1971) proposed three types gene pool- Primary, secondary and tertiary. Primary gene pool (GP1) includes all the strains of the concerned crop species and crossing between this species is easy that results in increase the vigour of hybrids. Secondary gene pool (GP2) consists of members of all those species which can be hybridized with those of GP1 but with considerable difficulty and thus, hybrids are either partially fertile or partially sterile in nature. However, members of tertiary gene pool (GP3) shows considerable to great difficulty and hybrids if produced are anomalous, lethal or completely sterile. For an example, in gene pool of *Brassica oleracea* (Fig. 1), all the varietal types under species *oleracea* are come under GP1 and rest of the diploid species like *B. nigra* (BB), *B. rapa* (AA) and amphidiploid species *B. carinata* (BBCC), *B. napus* (AACC), *B. juncea* (AABB) of U’s triangle are grouped under GP2. However, member of GP2 has variable level of cross compatibility to *B. oleracea* genome (CC). *B. napus* (AACC) and *B. carinata* (BBCC) has close relationship with the *B. oleracea* and thus, they are easily crossable and widely used in most of the interspecific hybridization program. As B genome is highly ancient in nature, so crossing with *B. nigra* followed by *B. juncea* causes severe sterility and it requires tissue culture method to remove the crossability barrier. More specifically, after AACC and BBCC genome AA followed by AABB and BB genome has more probability to develop successful fertile hybrid while crossing with CC genome in distant hybridization program. Finally, GP3 includes, *Diplotaxis arvensis*, *Enarthocarpus hyratus*, *Eruca sativa*, *Erucastrum canariense*, *Brassica oxyrrhima*, *Hirschfeldia*, *Rynchosinas*, *Trachystoma balli*, *Sinapis alba*, *Sinapodendron*, *etc* which are occasionally used in breeding programme and requires special techniques (Branca and Cartea 2011)\(^{9}\).
3. Challenges in distant hybridization

Major challenges in distant hybridization is due to prevalence of several pre and post fertilization barriers.

3.1 Pre-fertilization barriers

This barriers hinders in the crossing programme before fertilization takes place. Sometimes pollen grains could not germinate on the stigmatic head due to inability of production of viable pollen grains from Pollen Mother Cell (PMC) during microsporogenesis process of sexual reproduction. In certain cases, tube nucleus that carrying two generative nucleus cannot enter into styly even after germination of pollen grain. If pollen tube enters into the styly, it may not reach to the ovule. All these factors affect the fertilization of ovules. However, differences in ploidy level also sometimes cause problem. Pollen tube of higher ploidy level is thicker compared to lower ploidy level. So, when lower ploided as a problem. Pollen tube of higher ploidy level is thicker but in reciprocal crossing compatibility can be achieved.

3.2 Post-fertilization barriers

This caused problems after fertilization of ovules takes place. Among this embryo abortion or endosperm abortion is a common phenomenon. Embryos show abortion due to differences in ploidy level. When two different species of different ploided plants are crossed, they show incompatibility. Embryos become aborted due to failure of endosperm development. Endosperm is most important for seed as because of its physical, physiological, and genetic relationships to the embryo and it act as a storage organ of food. Thus, normal endosperm development is a prerequisite for normal embryo development. Normal endosperm development depends on the Endosperm Balance Number (EBN) proposed by Johnston et al. (1980) where 2 maternal: 1 paternal ratio EBN (in the endosperm itself) is necessary for normal endosperm development. Any maternal/paternal ploidy ratio that deviates from the 2:1 ratio may affect embryo development and subsequent seed or fruit formation.

Chromosomal disharmony also acts as a pre-fertilization barrier that may arise due to three factors – i) radical differences in the chromosome number, ii) distant relationship between the parents, iii) cryptic structural hybridity (first detected by Sax 1933) that may cause normal meiosis but produce sterile hybrids. In few cases unfavourable gene interactions, either between the nuclear genomes or between nucleus and cytoplasm cause hybrid sterility. Both this cytoplasmic, genic effect or its combined effect (lack of pairing due to structural differences along with gene combination causing asynapsis or desynapsis during meiosis) results in hybrid sterility or hybrid weakness.

Zygotic lethality is also one of the barrier in distant hybridization. When a species carrying lethal genes become expressed while using in hybridization program cause death of the zygote in the early embryonic development. Sometimes, albinism of seedling also causes death of the zygote. Structural differences and spontaneous breakage in chromosome followed by reunion also tends to imbalance in zygote (Chiang et al. 1977) where 2 chromosome pairing due to structural differences along with gene cytoplasmic, genic effect or its combined effect (lack of pairing due to structural differences along with gene combination causing asynapsis or desynapsis during meiosis) results in hybrid sterility or hybrid weakness.

Chromosome elimination is another major problem in wide hybridization. After crossing of two parents, chromosomes are gradually eliminated from the zygote due to mitotic irregularities and in extreme case only one chromosome remain in the embryo and such embryos are haploid in nature. This are not true interspecific hybrid and they are sterile in nature.

4. Different breeding approaches to eliminate crossing barriers

To overcome these pre and post-fertilization crossing barriers following breeding approaches are mostly applied in brassica crops (Fig. 2).

4.1 Doubling of Chromosome

Chromosome doubling is done by applying anti-microtubule drug colchicine which is derived from Cholchicum autumnale (autumn crocus/ meadow saffron/ naked ladies. When colchicine is applied in 2-4 leaf stages, it actually inhibits the polymerization of microtubule by binding to tubulin which results in inhibition of spindle fibre formation and chromosome number become doubled. As colchicine is highly toxic in nature, other doubling agents such as oryzalin, amiprophos methyl (APM), trifluralin, promamide as an alternative become used in chromosome doubling with variable degree of success in diploidization. Doubling of chromosome can be done in two ways – i) by converting sterile amphiploids into fertile one and ii) from the haploid that produced through chromosomal elimination. Centromere of chromosomal loci attached to spindle microtubule and it is specified by CENH3 (Taylor et al., 2002) – a histone H3 variant that replaces conventional H3 in centromeric nucleosomes (Henikoff and Dalal 2005). Chromosomal
location of CENH3 is the assembly site for the kinetochore complex of active centromeres. Loss of CENH3 results in failure of centromere formation and chromosome segregation (Allshire and Karpen 2008) and as a result of it chromosomal elimination occur and F2 haploid produced. Recent works on intraspecific (Ravi and Chan 2010) and interspecific hybrids (Sanei et al., 2011) provided the experimental evidences between the loss of CENH3 and the occurrence of uniparental chromosome elimination. This F2 haploid also can be made doubled by any anti-microtubule agent.

4.2 Protoplast Fusion Technique

Protoplast is living plant of a cell devoid of call wall, contain only the Nucleus and Cytoplasm. Fusion of two protoplast can be done by either through spontaneous fusion or induced fusion. Protoplasts, during isolation, often fuse spontaneously and this phenomenon is called spontaneous fusion. Simple physical contact is sufficient to bring about the spontaneous fusion among the similar parental protoplasts. During the enzyme treatment for the isolation of protoplasts, it is found that protoplasts from adjoining cells fuse through their plasmodesmata to form a multinucleate protoplast. Normally, isolated protoplasts do not fuse with each other as because of the surface of isolated protoplast carries negative charge (-10 to -30 mV) around outside layer of plasma membrane which repels to one another. Thus, to fuse the protoplasts an external induction is needed which can be done by mechanically, chemically or through electric wave. Detail procedure of protoplast fusion technique has been discussed in Fig. 3. Mechanically, isolated protoplasts are brought into intimate physical contact under microscope using micromanipulator and perfusion micropipette. By this technique occasional fusion of protoplast has been observed. For chemical induction, Sodium nitrate (NaNO3), polyethylene glycol (PEG), Calcium ions (Ca2+), Polv-vinyl alcohol etc. are most commonly used chemical fusogens for fusion of protoplast. Chemical fusogens cause the isolated protoplasts to adhere to one another that leads to tight adhesion followed by fusion of protoplast. The adhesion of isolated protoplast takes place either due to reduction of negative charges of protoplast or due to attraction of protoplast by electrostatic forces caused by chemical fusogens. Finally, electro-fusion is the latest technique which involves the use of mild electrical fields in protoplast suspension for inducing protoplast fusion. This technique is very easy, simple and fast. It is often more efficient than chemo-fusion. Electro fusion is also applicable to those species whose protoplasts exhibit a severe toxic response to polyethylene glycol used for chemo-fusion. But before fusion, isolation of protoplast is the most tedious job which usually done in three ways - i) mechanically by simple cutting with scissors or knife, ii) by application of enzyme like cellulose or pectinase. iii) by combination of both mechanical and enzymatic method. Enzymes are generally used to dissolve the cell wall for releasing the protoplasts. Enzymatic method may be done either one or two step method. In one step method, protoplasts can be isolated directly from the tissue by using two enzymes simultaneously – cellulase and pectinase. While in two step method, cells are first isolated from callus or tissue by pectinase as it causes break down of pectin present in middle lamella. Then, cellulase is added to digest the cell wall and release the protoplasts. Protoplasts would be collected after purification using centrifugation to separate it from various cell debris.

4.3 Embryo rescue technique

Embryo rescue refers to in-vitro technique which aims to promote the development of an immature or weak embryo into viable plant. Embryo rescue has been widely used for producing plants from hybridizations in which failure of endosperm to properly develop causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. In addition, embryo rescue has been used to recover maternal haploids that have developed as a result of chromosome elimination following interspecific hybridization. For embryo rescue, three methods are usually followed - Ovary ovule culture, direct ovule culture and direct embryo culture.

Murashige and Skoog (MS) (Murashige and Skoog 1962) and Gamborg’s B-5 (Gamborg et al., 1968) media are the most commonly used basal media for embryo rescue studies (Bridgen 1994) and Raghavan (1976) identified two phases of embryo development. In the heterotropic phase, the young embryo, which is often referred to as a proembryo, is dependent on the endosperm. Embryos require amino acids like asparagine, glutamine, etc. However, often natural extracts like coconut milk, casein hydrolysate also can be used instead of amino acids. Young embryos require a medium of high osmotic potential. Sucrose often serves both as a carbon source and osmoticon. For heterotropic embryos, 232 to 352 mM (8–12%) sucrose is commonly used. The second stage of embryo development is the autotropic phase, which usually begins in the late heart-shaped embryo stage (Raghavan 1976). At this time the embryo can synthesize substances required for its growth from salts and sugar. Germination will usually occur on a simple inorganic medium, supplemented with 58 to 88 mM (2–3%) sucrose. Growth regulators like auxins facilitates normal growth, gibberellic acid caused embryo enlargement, and cytokinins inhibit growth of embryo (Sharma 1996).
4.4 Application of growth regulators (GR)

GR such as auxins, cytokinins and gibberellins applied to the pedicel or the ovary at the time of or soon after pollination may improve fruit and seed set (Al Yasiri and Coyne 1964) [2]. In few cases, application of growth regulators delay may improve fruit and seed set (Al Yasiri and Coyne 1964) [2]. In few cases, application of growth regulators delay may improve fruit and seed set (Al Yasiri and Coyne 1964) [2].

4.5 Use of bridge species

Bridge species are normally used for transfer of trait from wild species to cultivated species. When two species are cross-incompatible in nature but bridge species has cross compatibility with both the species. Fujita et al. (1983) [36] used as a bridge species to avoid incompatibility between donor and recipient parent. D. erucoides is not directly crossable with B. oleracea. So B. rapa with Diplotaxis cytoplasm crossed with B. oleracea then B. oleracea used repeatedly for recovery of B. oleracea genome. Thus, B. rapa used as bridge for crossing between D. erucoides and B. oleracea.

5. Application of distant hybridization in Brassica crops

Distant hybridization has been widely utilized in brassica crops especially for transfer of sterile cytoplasm for male sterility and S-alleles for self-incompatibility to increase heterotic potential as well as for resistance against various biotic stresses such as weeds (herbicide tolerance), major diseases in brassica crops (Table 2).

5.1 Transfer of male sterility

Till now, Ogura is the only CMS system which is commercially utilized for production of hybrid seeds, but research is going on for searching new source of CMS to meet the effect the climate change in the future and to reduce danger of epidemic diseses like southern corn leaf blight occurred in T-CMS maize (Levings 1990) [28]. Ogura CMS was first discovered within an unknown cultivar of Japanese radish (R. sativus) by Ogura 1968 [35]. Although it was not well utilized in radish breeding programme in Japan. This CMS was first introduced into European radish. Some European radish have Rf gene (Bannert et al., 1977, Bonnet 1977) [7], whereas Ogura (1968) [35] observed that Japanese radish cultivars have no such gene. European scientists introduced Ogura CMS into B. napus by intergeneric hybridization and repeated back-crossing (Bannert et al., 1974, Heyn 1976) [5, 19]. The resultant alloplasmic lines of B. napus showed male sterility, but all of them had chlorotic leaves, yellowing at low temperatures (below 15°C) (Pelletier et al., 1983) [36]. To overcome chlorosis problem in Ogura CMS B. napus, cells of an alloplasmic male sterile B. napus line and a normal B. napus variety were fused (Jarl and Bornman 1988, Menczel et al., 1987, Pelletier et al., 1983) [20, 32, 36], and regenerated plants without chlorophyll deficiency but retaining the male sterility were selected. In these lines, the alloplasmic chloroplasts derived from R. sativus were substituted with those from B. napus, and the plants grew normally even at low temperature. Further, CMS B. napus line was used to transfer the ‘nap’ CMS trait to nuclear genomic background of triazine (herbicide) resistant variety “Regent” (B. napus) for combination of both triazine tolerance and ‘nap’ CMS (Yarrow et al., 1986) [57]. Therefore, a new CMS type ‘Anand’ cytoplasm which was originally described in B. juncea but subsequently, it was identified in B. tournefortii as the most likely donor of the cytoplasm. Recently, B. tournefortii or other genotypes with tournefortii cytoplasm have been used as donors of nuclear and organelle genes to B. napus for the production of somatic hybrids and cybrids through fusion of protoplast in an experimental trial (Stiewe and Röbbelen 1994; Landgren and Glimeilius 1994; Liu et al., 1995; Liu et al., 1996) [30]. But Cardi and Earle (1997) [11] had transferred the sterile “Anand” cytoplasm from rapid cycling stock of B. rapa to B. oleracea. They have also used the protoplast fusion technique as because it is useful method for increasing cytoplasmic diversity in a population and it is possible to transfer chloroplasts and mitochondria in a single step, between two distantly related species, and to obtain novel combinations of nucleus and cytoplasm organelles (Kumar and Cocking 1987) [26]. After fusing two protoplast they found that 63% cybrids were diploid in nature and rests had ploidy level varies from 2x to 4x. Further, they have also found that 64% were male sterile with indehiscent anthers but phenotypically they are similar to cauliflower as they contain the nucleus of B. oleracea. 

Ogu-INRA cytoplasm from Raphanus sativus had been utilized for development of cauliflower line (CDT70) which subsequently utilized as a male sterile line in the improvement of three broccoli (B. o. var. italica) lines having high self-compatibility and produced a reliable number of seeds in open pollination (Kamisinki 2013) [23]. This three lines repeatedly were backcrossed to B. oleracea for three generations. In F1, floret colour was intermediate (yellowish) between parents but in further generations colour was improved and in BC3 lines floret colour was complete green with sterile flower. Very recently Chamola et al. (2013) [12] transferred the Moricandia arvensis and Erucastrum canariense cytoplasm to Brassica oleracea with the help of embryo rescue followed by back cross method. At first B. juncea with M. arvensis cytoplasm and B. napus with E. canariense cytoplasm were crossed with B. oleracea but seed set was not occurred in F1 siliqua. As B. juncea is distantly related with B. oleracea, so among them earlier senescence was observed in B. juncea. Therefore embryo was culture in a MS media and F1 was backcrossed to B. oleracea for recovery of C genome. After consecutive three years of backcrossing BC3 plants produced white colour curd similar to cauliflower with male sterile flower.
Club root of crucifers etc. are most important. Black rot among the major diseases like Powdery mildew, Black rot, by utilizing embryo rescue and protoplast fusion technique, transferred to susceptible one through hybridization followed by utilizing embryo rescue as crossing was carried out for introgression black rot resistance from B. napus to B. oleracea (CC) with the help of protoplast fusion for making fertile hybrids. Hybrid identity was also confirmed by the help of RAPD markers. Later hybrids (AACC) were again crossed to B. oleracea (F1 × CC) utilizing embryo rescue technique in BC1 generation but in BC2 no embryo rescue technique was used as field screening was carried out by reciprocal crossing between hybrids and three materials of rapid cycling of B. oleracea, white flowered broccoli and chinese kale (B. oleracea spp. alboglabra)(CC × F1) which revealed that highest pollen germination was observed in crosses with Chinese kale and high self-incompatibility was also reported even through bud pollination. These BC2 plants having genomic constitution of either CCC or ACC and plants, which contained the A genome showed the resistance against black rot. Disease screening was done with the help of artificial inoculation of X. c. var. campestris (Xcc) culture against young mature leaves through pin dip technique, where disease severity scale varies from 1 to 4 (1 = symptomless and 4 = chlorosis of entire leaves). Similar experiment was also carried out for introgression black rot resistance from B. carinata to B. oleracea through embryo rescue technique by Dey et al. (2015) [14] and Sharma et al. (2017) [41]. Experiment was carried out for resistance against the bacterial race 1 and race 4 as out of total nine pathogenic races reported throughout the world (Tonu et al., 2013) [52], these two are mostly dominated in Indian states (Singh et al., 2016). Race specific resistance to races 1 or 4 was also found in other species of the genus Brassica (Taylor et al., 2002) [69]. Source of black rot resistance reported mostly in A and B genomes of Brassica sp. (Westman et al., 1999; Taylor et al., 2002) [55, 49] and thus, pyramiding of these genes should be done to solve this problem permanently. Moreover, strong resistance to Xcc race 1 and 4 has been reported in B. nigra (B genome) and B. carinata (BC genome) accessions (Taylor et al., 2002; Vicente et al., 2002) [49, 53] and resistance was governed by single dominant gene (Tonguc et al., 2003). Gene Xca1bc recently mapped on chromosome 7 of B genome by the help

<table>
<thead>
<tr>
<th>Trait</th>
<th>Donor Parent</th>
<th>Recipient parent</th>
<th>In-vitro method used</th>
<th>References</th>
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<tr>
<td>Transfer of male sterility</td>
<td>Brassica napus</td>
<td>Brassica oleracea</td>
<td>Protoplast fusion</td>
<td>Hansen and Earle 1995 [17]</td>
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<td></td>
<td>Brassica napus</td>
<td>Brassica oleracea</td>
<td>Embryo rescue</td>
<td>Dey et al., 2015</td>
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<td></td>
<td>Brassica carinata (NPC-9; HCA-6)</td>
<td>B. o. var. botrytis</td>
<td>Embryo rescue</td>
<td>Sharma et al., 2017 [41]</td>
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<tr>
<td>Black rot</td>
<td>Brassica carinata (PI 360883)</td>
<td>B. o. var. botrytis (Pusa Sharad)</td>
<td>Embryo rescue</td>
<td>Tongue and Griffiths 2004</td>
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<td>Powdery mildew</td>
<td>Brassica carinata (NPC-9)</td>
<td>B. o. var. botrytis (Titlest &amp; Cecile)</td>
<td>Embryo rescue</td>
<td>Chiang et al., 1977 [13]</td>
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<td>Late blight</td>
<td>Brassica hirta</td>
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<td>-</td>
<td>Mohapatra and Bajaj 1985</td>
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<td>Hybridization for herbicide resistance</td>
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<td>B. o. var. botrytis</td>
<td>Protoplast fusion</td>
<td>Jourdan et al., 1989</td>
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<td>Atrazine</td>
<td>Brassica campestris</td>
<td>Brassica napus</td>
<td>Embryo rescue</td>
<td>Ayotte et al., 1987; Ayotte et al., 1988</td>
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5.2 Introgression of S-alleles for transfer of self-incompatibility

Along with CMS system, S-alleles for self-incompatibility also transferred to B. napus. Varietal development in B. napus was traditionally based on open pollinated systems. Due to lack of extent of heterosis and suitable pollination control mechanisms hybrids were not developed. For that reason, introgression of S-alleles for expression of self-incompatibility had been done from B. oleracea var. italica (having S-locus self-incompatible alleles) to B. napus with the help of ovule culture of embryo rescue as crossing was between diploid to amphidiploid species (Ripley and Beversdorf 2002).

5.3 Hybridization for resistance against various biotic stresses

Brassica crops are highly susceptible to several fungal and bacterial pathogen which severely reduce the yield of the crops. For that reason several disease resistant genes has been transferred to susceptible one through hybridization followed by utilizing embryo rescue and protoplast fusion technique. Among the major diseases like Powdery mildew, Black rot, Club root of crucifers etc. are most important. Black rot caused by the gram negative bacterium Xanthomonas campestris pv. campestris is one of the most threatening diseases in vegetable Brassicas worldwide. It has adverse effect on quality and yield losses up to 10–50% have been reported under appropriate environmental conditions in cauliflower (Singh et al., 2011). The disease spreads through vascular tissues, clogging vessels and producing V-shaped chlorotic lesions. Managing the disease is very difficult as the bacterium spreads within and between fields by water splashes, wind, insects, machinery and irrigation. Although chemicals for controlling the disease are available, but development of resistant cultivars is reliable, economical and environmentally safe approach to manage this disease. That’s why several workers are trying to incorporate genes from the different resistant sources. Hansen and Earle (1995) [17] transferred resistance from B. napus (AACC) to B. oleracea (CC) with the help of protoplast fusion for making fertile hybrids. Hybrid identity was also confirmed by the help of RAPD markers. Later hybrids (AACC) were again crossed to B. oleracea (F1 × CC) utilizing embryo rescue technique in BC1 generation but in BC2 no embryo rescue technique was used as field screening was carried out by reciprocal crossing between hybrids and three materials of rapid cycling of B. oleracea, white flowered broccoli and chinese kale (B. oleracea spp. alboglabra)(CC × F1) which revealed that highest pollen germination was observed in crosses with Chinese kale and high self-incompatibility was also reported even through bud pollination. These BC2 plants having genomic constitution of either CCC or ACC and plants, which contained the A genome showed the resistance against black rot. Disease screening was done with the help of artificial inoculation of X. c. var. campestris (Xcc) culture against young mature leaves through pin dip technique, where disease severity scale varies from 1 to 4 (1 = symptomless and 4 = chlorosis of entire leaves). Similar experiment was also carried out for introgression black rot resistance from B. carinata to B. oleracea through embryo rescue technique by Dey et al. (2015) [14] and Sharma et al. (2017) [41]. Experiment was carried out for resistance against the bacterial race 1 and race 4 as out of total nine pathogenic races reported throughout the world (Tonu et al., 2013) [52], these two are mostly dominated in Indian states (Singh et al., 2016). Race specific resistance to races 1 or 4 was also found in other species of the genus Brassica (Taylor et al., 2002) [69]. Source of black rot resistance reported mostly in A and B genomes of Brassica sp. (Westman et al., 1999; Taylor et al., 2002) [55, 49] and thus, pyramiding of these genes should be done to solve this problem permanently. Moreover, strong resistance to Xcc race 1 and 4 has been reported in B. nigra (B genome) and B. carinata (BC genome) accessions (Taylor et al., 2002; Vicente et al., 2002) [49, 53] and resistance was governed by single dominant gene (Tonguc et al., 2003). Gene Xca1bc recently mapped on chromosome 7 of B genome by the help
of intron length polymorphic markers (Sharma et al., 2016). As B genome of Brassica nigra is phylogenetically distant in nature so, B. carinata is widely used for introgression purpose for its closest similarity with B. oleracea (Zhou et al., 1997) [59]. For introgression purpose two most tolerant line of B. carinata NPC-9 (Dey et al., 2015; Sharma et al., 2017) [14, 41] and HCA-6 (Dey et al., 2015) [14] were crossed with ten different cauliflower lines [Pusa Sharad (Sharma et al., 2017) [41], Pusa Meghna, Pusa Himjiyoti, Pusa Snowball K-1, Pusa Snowball K-25, Kt-15, Kt-18, Kt-22, Kt-33, HL-SR-05 ((Dey et al., 2015) [14])] from different maturity groups. As these are the crosses between tetraploid and diploid ones normal meiosis is being not occurred. That’s why different types of embryo rescue techniques (Direct embryo, direct ovule and direct ovary or silique) were applied to compare the average success rates and among them direct ovule culture (Sharma et al., 2017) [41] are considered as most suitable method. After development of F1 they were again backcrossed to CC genome for the recovery of majority of cauliflower traits. Along with field screening, artificial screening was also done by following leaf cut and dip technique as suggested by Kapoor et al., 1985 by clipping off secondary veins and dipping in bacterial strains. However, evaluation of severity was carried out in 0-3 scale as suggested by Vicente et al. (2001) [53] by Dey et al. (2015) [14] and 0-9 scale of Vicente et al. (2002) [54] by Sharma et al. (2017) [41]. In addition to this, F1 hybridity was also confirmed with help of morphological marker such as, presence of anthocyanin pigmentation on another tip of hybrids which is also present in donor parent (NPC-9) as well as with the help of SSR markers (Sharma et al., 2017) [41]. In both the experiments, most of the BC1 plants had close similarity with B. oleracea and further, more generation of backcrossing will facilitate maximum recovery of recurrent parent.

Powdery mildew is a serious fungal disease in brassica, caused by Erisiphe polygoni. It mostly affects the crops like Brussels sprouts and late maturing cabbage under field conditions. Though several fungicides are available but it is not an permanent solution. So, breeders are trying to incorporate resistance from wild sources. Related brassica species like B. carinata, B. napus identified as novel source of resistances (Bradshaw et al., 1989; Singh et al., 1997) [44]. Brassica carinata accession PI360883 was identified as being resistant to powdery mildew in the greenhouse screening conditions (Tongue and Griffiths 2004) [31] and it was used as donor parent for introgression of PM resistance to B. oleracea. As it is a cross between tetraploid and diploid, so no seed was obtained in sexual cross but through embryo rescue. It was further backcrossed to B. carinata and in BC1, out of 24 plants (13 were intermediate like B. oleracea cv. and three were susceptible) 8 were resistant, although all F1 were resistant.

Clubroot disease of crucifers is one of most destructive disease caused by soil borne pathogen Plasmodiophora brassicaceae that survives in soil for long duration. For that reason, it is very difficult to control this disease. Chiang et al. (1977) [13] transferred resistance from B. napus to cabbage against Race-2 of Plasmodiophora brassicaceae through interspecific hybridization. Another fungal disease late blight caused by Alternaria brassicaceae sometimes reduce yield of most of the oil seed brassica crops for which development of resistance through conventional breeding is little more difficult due to cross incompatibility. Thus, Mohapatra and Bajaj (1985) found one resistant species in B. hirta and transferred the resistant genes to B. juncea through embryo rescue technique.

5.4 Hybridization for herbicide resistance

Yield of brassica crops also increased to some extent due to susceptibility to weeds and thus, to control weeds, various herbicides are used. But, these herbicides have some negative impact on the quality of crops. For that reason, distant hybridization can be applied for resistance against various herbicides especially, triazine, atrazine, etc. by transferring resistant genes from distantly related species. Jourdan et al. (1989) transferred male fertile atrazine resistant (ATR) B. napus cytoplasm to male sterile B. oleracea through protoplast fusion technique. Hybrids also contained chloroplast and mitochondria of B. napus in the nuclear genetic background of cauliflower without any effect of low temperature chlorosis problem. In addition to resistant against atrazine, this hybrid also helped in growing vegetables in rotations where soil had suffered from atrazine residuality. Similarly triazine resistance gene was transferred from B. napus through embryo culture, which was earlier found in B. campestris and from where it was transferred to B. napus (Ayotte et al., 1987). But here embryo culture technique was applied and subsequently first (Ayotte et al., 1988), second and third (Ayotte et al., 1988) backcross was done with B. oleracea to recover the genome completely.

6. Conclusion

It can be concluded that distant hybridization is one of the useful breeding strategy for introgression of desirable traits from wild relatives to the cultivated ones. In this case, different in-vitro techniques especially, embryo rescue, protoplast fusion, chromosome doubling through colchicine treatment or use of intermediate bridge species can alleviate various pre and post-fertilization crossing barrier to convert sterile hybrid to fertile one. In future, this strategy will be played significant role for maintaining the food security by eliminating negative impact of various biotic and abiotic stresses.

7. References

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