Protoplast Fusion and Brassica Improvement

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Brassica coenospecies has been bestowed with a large collection of wild and weedy relatives, which could act as donors of genes controlling agronomically important traits like disease resistance, yield attributes, modified fatty acid composition in the seed etc. Additionally, the cytoplasm of these species could be the sources for developing alloplasmic male sterility systems that are important in developing hybrid mustard varieties. The technology of protoplast culture and protoplast fusion has been very well developed for crop Brassicas and this could be effectively exploited in developing somatic hybrids and cytoplasmic hybrids, in short called as cybrids. This could pave the way for overcoming problems associated with the development of incompatibility barriers and allow for obtaining hybrids of the wild species with the cultivated species and manipulating characters that are controlled by organelle genomes. In Nicotiana species, protoplast fusion resulted in the development of novel mitochondrial genomes and plants carrying novel genomes have differing flower morphology. Protoplast fusion in Cruciferae also nearly invariably results in mitochondrial genome recombination/rearrangement. Hence, protoplast fusion can be effectively utilized in manipulating flower morphology and male sterility in crop Brassicas.

Keywords: Brassica improvement, protoplast fusion, cytoplasmic hybrids, mitochondrial genome recombination, cytoplasmic male sterility-fertility-restorer systems, manipulation of flower morphology

Introduction

Protoplast fusion technique involves the fusion of protoplasts, i.e., cells devoid of cell wall, from different partners resulting in the formation of homokaryons (if fusion occurs between protoplasts from the same genotype) or heterokaryons, when fusion occurs between protoplasts of divergent genotypes. Plants regenerated from such fusion experiments and resulting in heterokaryons are often termed as parasexual hybrids and the fusion of protoplasts is often referred to as parasexual hybridisation. The process of protoplast fusion results in the admixture of organelles (chloroplasts and mitochondria) from divergent fusion partners in the fused cell along with the corresponding nuclei to form the heterokaryon; sometimes fusion can occur between a protoplast and a cytoplast, a protoplast devoid of nucleus. After cell wall regeneration, the fused cell starts undergoing divisions. Since organelles from diverging partners cannot stay together, the segregation of organelles generally starts with the cell divisions and by the time plant regeneration occurs in the calli derived from such fused cells, the organelles segregate to homogeneity and the regenerated plant often contains organelles segregated to homogeneity. However, when organelles are brought together in the fusion cell, there is the possibility of their genomes undergoing rearrangement/recombination, particularly in the case of mitochondrial genomes and rarely in the case of chloroplast genomes. Assortment of organelles and rearrangements in the organelle genomes might result in 48 different combinations of nuclear and organelle genomes in the regenerated plants (Kumar & Cocking, 1987). These different nuclear-organelle genome combinations in the plants regenerated from protoplast fusion might have different implications in terms of their subsequent utilization in crop improvement.

The Brassica coenospecies comprises nearly 100 species and genera of wild and weedy relatives, which are a rich repository of important genes for resistance against fungal diseases and abiotic stresses. They are also a source of cytoplasmic variability for generating alloplasmic male sterile lines. When the nucleus of the cultivated species is lodged in the cytoplasm of these weedy relatives, the nucleo-cytoplasmic interactions may frequently result in alloplasmic male
sterility. These systems could be exploited for developing hybrid varieties that are important for enhanced productivity.

**Why Protoplast Fusion?**

The development of certain hybrids using different plant species as male and female partners may be hindered by the development of incompatibility barriers resulting in the failure to yield hybrids. To overcome such a situation, protoplast fusion can be effectively resorted to resulting in the development of desired hybrids. In other cases, it might be possible to develop the desired hybrids with relative ease or simply by resorting to techniques like embryo rescue, ovule and ovary culture, etc. In a situation that utilizes conventional hybridisation technique directly or the one followed by the special rescue techniques, the hybrid receives both the chloroplasts and mitochondria and their genomes from the female parent because of the uni-parental inheritance of the organelles on the female side as male cells contribute very little cytoplasm in the formation of a zygote after sexual fusion (Fig. 1). Development of cytoplasmic male sterile lines from conventionally developed inter-specific hybrids often results in moderate to severe leaf chlorosis because of the incompatibilities between crop nuclear genome and the alien chloroplast genome. This is particularly true in the case of male sterile lines developed through conventional hybridisation (Ogura, 1968; Rawat & Anand, 1979; Prakash & Chopra, 1988). To overcome chlorosis and other problems in these male sterile lines and their utilization in hybrid development programmes, one has to resort to protoplast fusion between the male sterile line and a breeding line that has got protoplast regenerability. This often results in loss of time as the development of the male sterile line itself takes five to six years of controlled pollinations (Fig. 1). This can be exemplified by the correction of chlorosis in the alloplasmic male sterile lines developed through normal sexual hybridisation by resorting to protoplast fusion (Kirti et al., 1993, 1995a, 1998). Instead, one can resort to protoplast fusion in the first instance itself to make the desirable somatic hybrids between the wild and the cultivated species, analyse them at molecular level to identify proper combination of organelle genomes in the hybrids and use selected somatic hybrids in back cross programmes to develop the male sterile and fertile plants in each generation for developing the sterile and fertile restorer lines simultaneously (Fig. 2).

Apart from reducing the duration of development of usable male sterile and restorer lines as discussed above, resorting to protoplast fusion has other advantages in terms of the qualitative characters in the male sterile line. In *Nicotiana* inter-specific somatic hybrids, flower characters are controlled by the interactions between the nuclear and mitochondrial genomes (Belliard et al., 1979). Somatic hybrid plants with differing mitochondrial genomes have differing flower morphologies. It shows that one can modify the flower morphology by suitably modifying the mitochondrial genomes and occurrence of mitochon-
Wild species (+) Cultivated species

Somatic hybrids X Cultivated species

(Through protoplast fusion)

Cytoplasmic male sterile Putative restorer line

- Analyze somatic hybrids for organelle constitution
- Select hybrid plants with proper organelle genome combinations
- Use in back cross breeding
- Select and maintain fertile and sterile plants

Fig. 2—Resorting to protoplast fusion in the first step of making desirable hybrid combinations

drial genome rearrangement/recombination is a common phenomenon in cell fusion derived plants. To exemplify this phenomenon, the cytoplasmic male sterile line of *Brassica napus* carrying *Raphanus sativus* cytoplasm (oog) that has been developed through sexual hybridisation, can be considered in Brassicas. This line has severe leaf chlorosis and a flower morphology consisting of crooked styles, petaloid anthers, significantly reduced female fertility, etc. This male sterile line has subsequently been improved by resorting to protoplast fusion thereby developing suitable cytoplasmic hybrids carrying novel mitochondrial genomes (Pelletier *et al.*, 1983).

Since easy protoplast culture and fusion technology are available for crop Brassicas, it is worth considering the technology for developing the desired somatic hybrid in the beginning itself. The efficiency of this technique can be greatly improved by developing resistance markers in fusion partners like resistance to the antibiotics kanamycin and hygromycin and herbicide like phosphinothricin by resorting to genetic transformation and introducing alien genes like neomycin phosphotransferase (*npt* II) for kanamycin resistance, hygromycin phosphotransferase (*hpt*) for hygromycin resistance, phosphinothricin acetyltransferase (*pat/bar*) for resistance to the herbicide phosphinothricin, etc. (Mukhopadhyay *et al.*, 1994).

**Protoplast Culture**

Protoplast culture is the culture of protoplasts, i.e., single cells whose cell walls have been degraded enzymatically. A repeatable and efficient protoplast regeneration system can be used for a variety of biotechnological applications including the production of somatic hybrids (otherwise called parasexual hybrids) and cytoplasmic hybrids (or cybrids) and transgenic plants by direct plasmid delivery or through *Agrobacterium*-based vectors.

The regeneration from protoplasts of *Brassica napus* (Kartha *et al.*, 1974) has resulted in very low regeneration frequency. This was followed by protoplast regeneration in *Brassica oleracea* (Vatysa & Bhaskaran, 1982) and others. However, an efficient protocol using hypocotyl protoplasts of *B. napus* resulted in high frequency regeneration (Glimelius, 1984). Studies on protoplast regeneration (Chatterjee *et al.*, 1985) in mustard started with the regeneration of mesophyll protoplasts in *Brassica juncea* (Table 1). This report, low frequency regeneration through organogensis and somatic embryogenesis from the mesophyll protoplasts of the Indian mustard, demonstrated that mustard protoplasts could be regenerated to whole plants. Subsequently, efficient regeneration protocols were developed (Kirti & Chopra, 1989, 1990) for hypocotyl protoplasts modifying the protocol developed for rapeseed hypocotyls protoplasts (Glimelius, 1984). As in rapeseed, dark grown hypocotyls were used as the source tissue for protoplast isolation and culture for improving the regeneration efficiency in mustard (Kirti & Chopra, 1989). The protoplast-derived cell colonies regenerated shoots at about 35% frequency.
either by direct crosses or even by embryo rescue, hybridisation offers an exciting possibility of conformity with the observed genomic effects on regeneration (Kirti B.

Table 1—Reports on protoplast regeneration in Indian Brassicas and related wild genera

<table>
<thead>
<tr>
<th>Species</th>
<th>Source of protoplasts</th>
<th>Reference</th>
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<tbody>
<tr>
<td>B. carinata</td>
<td>Cotyledon</td>
<td>Jaiswal et al (1990)</td>
</tr>
<tr>
<td>B. carinata</td>
<td>Etiolated hypocotyl</td>
<td>Narasimhulu et al (1992c)</td>
</tr>
<tr>
<td>B. juncea</td>
<td>Leaf mesophyll</td>
<td>Chatterjee et al (1985)</td>
</tr>
<tr>
<td>B. oleracea</td>
<td>Cotyledon</td>
<td>Vatsya &amp; Bhaskaran 1982a, b</td>
</tr>
<tr>
<td>Diplotaxis muralis</td>
<td>Leaf mesophyll</td>
<td>Sikdar et al (1990)</td>
</tr>
<tr>
<td>Eruca sativa</td>
<td>Leaf mesophyll</td>
<td>Sikdar et al (1987)</td>
</tr>
<tr>
<td>Vegetable Brassicas</td>
<td>Etiolated hypocotyl</td>
<td>Kirti et al (2001)</td>
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Subsequently modified protocol (Kirti & Chopra, 1990) increased the shoot regeneration frequency (about 45%) by resorting to liquid culture of protoplasts till they reached micro-callus stage. This has also resulted in regeneration through direct and indirect somatic embryogenesis from protoplast cultures. Later on, protoplasts of hypocotyl origin have been regenerated to plants in B. nigra (Gupta et al, 1990; Mukhopadhyay et al, 1992; Narasimhulu et al, 1993), B. carinata (Narasimhulu et al, 2001). The regeneration frequency of protoplast-derived calli of B. nigra was around 55% and those of B. carinata, 75%(Narasimhulu et al, 1992a, 1993). Vegetable Brassicas also showed high frequency protoplast regeneration (Kirti et al, 2001), which is in conformity with the observed genomic effects on regeneration (Narasimhulu & Chopra, 1988).

Protoplast Fusion

Protoplast fusion, otherwise termed as para-sexual hybridisation offers an exciting possibility of combining nuclear and organelle genomes from taxonomically divergent species that cannot be brought together through conventional hybridisation, either by direct crosses or even by embryo rescue, because of the existence of crossability barriers; when these crossability barriers are predominantly pre-zygotic in nature, hybrids could be developed only with the help of cell fusion. As mentioned earlier, a resort to cell fusion is advantageous in Brassicas when cytoplasmic genomes are to be manipulated for male sterility as it saves on time in developing a workable cytoplasmic male sterility system. Protoplast fusion is technically highly demanding, but the investment of effort is worthwhile because of the advantages it offers in selecting proper combination of organelles in the somatic hybrids and their utilization in developing male sterile and restorer lines.

Successful somatic hybridisation experiments demand that efficient regeneration protocols are available for at least one of the fusion partners. Once protocols for protoplast culture and fusion are standardized, somatic hybridisation offers the exciting possibility of creating a novel combination of organelle genomes of the cytoplasm that cannot be created by conventional sexual hybridisation and this alone paves the way for study of Somatic Cell Genetics. In sexual crosses, the female parent contributes cytoplasm through the egg cell and hybrids derive their cytoplasm from the female parent. Hence the crop nucleus gets lodged in the cytoplasm of the wild parent after subsequent back crosses of the hybrid with the cultivated species. Consequently, sexual hybrids have constraints imposed by nuclear-chloroplast and nuclear-mitochondrial interactions that may limit their use in the development of usable CMS (cytoplasmic male sterile) systems. Nuclear cytoplasmic incompatibilities often result in severe leaf chlorosis, as is seen in CMS B. napus carrying ogu (Raphanus sativus) cytoplasm (Ogura, 1968), B. juncea carrying (ogu) cytoplasm (Kirti et al, 1995a), B. juncea CMS line carrying Moricandia arvensis cytoplasm (Prakash et al, 1998). Reduced female fertility, impaired seed set, and associated floral abnormalities in CMS lines carrying ogu and B. tournefortii cytoplasts (Kirti et al, 1995a; Rawat & Anand, 1979) are similarly due to negative nuclear – mitochondrial interactions. In such circumstances, resorting to cell fusion is the only way to overcome the problems.

Protoplast Fusion Methodology

Of the many different methods of protoplast fusion available, the polyethylene glycol (PEG)-mediated fusion has been commonly used for Brassicas in India (Table 2). The PEG method has been extended to crop Brassicas with modifications to achieve high
Table 2—Protoplast fusion experiments involving Indian Brassicas

<table>
<thead>
<tr>
<th>Combination</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Diploctaxis muralis + Brassica juncea</td>
<td>Chatterjee et al (1988)</td>
</tr>
<tr>
<td>Eruca sativa + B. juncea</td>
<td>Sikdar et al (1990)</td>
</tr>
<tr>
<td>Moricandia arvensis + B. juncea</td>
<td>Kirti et al (1992b)</td>
</tr>
<tr>
<td>Trachystema bali + B. juncea</td>
<td>Kirti et al (1992a)</td>
</tr>
<tr>
<td>B. nigra + B. oleracea</td>
<td>Narasimhulu et al (1992b)</td>
</tr>
<tr>
<td>Diploctaxis catholica + B. juncea</td>
<td>Kirti et al (1995b)</td>
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frequency fusion (about 20-25%) (Kirti et al, 1992a). In only one case, fusion of protoplasts has been achieved by applying electrical stimuli, termed electro-cell fusion to produce the somatic hybrids between Sinapis alba and B. juncea (Gaikwad et al, 1996). Electrofusion involves two steps. An AC current is used initially to align protoplasts into pearl chains containing two or more protoplasts. Then, a square DC pulse of a very brief duration (approx. 30 μ sec) brings about fusion of adjacent protoplasts of the pearl chains (Zimmermann, 1982). Fusion frequencies were reported to be quite high in electro-cell fusion compared to the PEG-mediated cell fusion. The PEG-mediated protoplast fusion is economical and it has been suitably modified for Indian crop Brassicas.

Use of Markers in Identification of Somatic Hybrids

Since good level of fusion has been achieved with PEG and the regeneration efficiency of fusion derived cells is reasonably high, Kirti and his co-workers used the strategy of identifying the somatic hybrids on the basis of morphological characters, followed by confirmation of chromosome numbers and RFLP-based analyses to confirm the hybrid nature of putative hybrid plants. The strategy worked quite efficiently in deriving many inter-specific, inter-generic and inter-tribal somatic hybrids involving crop Brassicas (Kirti et al, 1991, 1992a,b, 1995b; Gaikwad et al, 1996; Narasimhulu et al, 1992b, 1994). A similar approach was followed at Bose Institute (Chatterjee et al, 1988; Sikdar et al, 1990) in developing somatic hybrids. However, a more sophisticated approach (Mukhopadhyay et al, 1994; Nagpal et al, 1996) has been undertaken by introducing selectable marker genes into parental species by Agrobacterium-based vectors and used antibiotic combinations to separate fusion products. Though, it is a good strategy, it needs approval from the Department of Biotechnology, New Delhi to make and use desired transgenic plants at field level to develop material for commercial cultivation.

For cytoplasmic hybrids, the selection generally is more comfortable since agronomically important characters e.g., male sterility and robust green plant type and so on, are to be combined in them. Plants can be easily transferred to the field and screened for the chlorosis correction at low temperature (generally in the cropping season) and male sterility. Most of the cytoplasmic hybrids in crop Brassicas are to combine these two important features (Kirti et al, 1993, 1995a, 1998; Arumugam et al, 2000). The strategy (Kirti et al, 1998) treating mustard protoplasts with iodoacetate, specifically inhibiting mitochondria of these protoplasts, and non-regenerability of the protoplasts of mustard cultivar ‘Pusa Bold’ carrying Moricandia arvensis cytoplasm as markers worked efficiently in the fusion experiment. In this case, only fusion products would continue to develop and regenerate into plants. Since, the mitochondria of normal mustard would not be contributed to the fusion products, plants regenerated from this fusion experiment would be male sterile, either green or chlorotic. Green male sterile plants are the desired types for further utilization in hybrid variety development by the breeders.

Confirmation of Somatic Hybrids

Somatic hybrid status is generally confirmed by morphological, cytogenetical, and biochemical (protein and isozyme studies) and molecular (nucleic acid) analysis. Morphologically, hybrids were observed to be intermediate with the predominance of features from the cultivated parent. Cytological analysis on some of the somatic hybrids (Kirti et al, 1991, 1992a,b, 1995b; Narasimhulu et al, 1992b) indicated that bivalent associations predominated in the pollen mother cells. Consequently, regular chromosome distribution occurred at anaphase I.
Multivalent associations were also observed, although infrequently, in the hybrids *M. arvensis* + *B. juncea*, *T. ballii* + *B. juncea*, *D. catholica* + *B. juncea*, and *S. alba* + *B. juncea*. Such associations indicate inter-genomic homeologies that are essential in introgressing desirable genes and gene blocks from the alien species to the cultivated species. Despite regular meiotic chromosome distribution, majority of the somatic hybrids were highly self sterile and hence, no seed set was obtained on selfing. However, seed could be obtained from these hybrids by allowing them to receive pollen from *B. juncea* or *B. campestris*. Plants obtained from open pollinated progenies of the somatic hybrids were generally fertile to varying extent and amenable to controlled pollinations.

**Development of Bridge Material for Gene Introgression**

Somatic hybridisation, when attempted between species differing in ploidy levels, often result in chromosome elimination during meiosis and consequently inefficient gene transfer situations in subsequent generations. This also necessitates the development of different somatic hybrids of the wild species with cultivated crop Brassica species like *B. juncea* and *B. napus* for subsequent utilization. To overcome such a difficult situation, the concept of bridge hybrids has been developed in this group of species. A bridge hybrid developed by fusing protoplasts of hygromycin-resistant *B. oleracea* (CC) to generate AABBCC hexaploid somatic hybrids (Arunugam et al., 1996). The presence of selectable marker genes facilitated the selection of hybrids in large numbers. The development of such tri-genomic hexaploid hybrids through protoplast fusion facilitates the transfer of the cytoplasm with novel organelle genomes to other amphi-diploid crop species like *B. juncea* and *B. napus* or *B. carinata*. Similarly, there was more pronounced homeologous chromosome pairing in the tri-genomic hybrids that have been produced as bridge materials compared to the allodiploids (Nagpal et al., 1996). Such hybrids have some fertility too showing that the strategy of bridge material for gene introgression is a viable one that needs consideration in future studies involving Brassicas.

**Mitochondrial Genome Recombination and Novel Male Sterility Systems**

In inter-specific *Nicotiana* hybrids, nuclear-mitochondrial interactions were shown to be involved in controlling flower morphology which can be manipulated through protoplast fusion as two different mitochondrial genomes come together in the cell fusion product. The occurrence of unacceptable flower morphology has been very clearly documented in the male sterile systems developed through conventional sexual hybridisation, as in the case of *B. juncea*, where alloplasmic male sterile line carrying 'ogu' (Kirti et al., 1995a) and 'tour' (Rawat & Anand, 1979) cytoplasms. Both these lines have petaloid anthers, reduced female fertility (which is responsible for poor seed yield), crooked styles and under-developed nectaries. The development of proper nectaries in Brassicas is of considerable importance as nectar production attracts bees for cross pollination. Hence, male sterile lines should show well-developed nectaries for efficient seed production in Brassicas.

The first mitochondrial genome recombination in the cytoplasmic hybrids in crop Brassicas was in the development of cybrids (Pelletier et al., 1983), in which atrazine resistant *B. campestris* chloroplasts were combined with male sterility inducing *Raphanus sativus* mitochondria. Incidentally, the chlorosis corrected cybrids were shown to possess rearranged/recombined mitochondrial genomes and vastly improved flower characters. Thereafter, mitochondrial genome recombination has been shown to occur quite frequently in cell fusion derived plants. An improved mustard CMS line (Kirti et al., 1995a), which is green fertile, have been developed carrying ogu cytoplasms through protoplast fusion between the CMS *B. juncea* line and a mustard cultivar RLM 198. In the identified cybrids, chloroplasts of *Raphanus sativus* were replaced by the chloroplasts of RLM 198, which has resulted in the correction of chlorosis. The cybrids had plants with different Southern hybridisation patterns when probed with mitochondrial gene probes that were indicative of changes in the mitochondrial genome organization. These changes were associated with differing flower morphologies indicating that flower morphologies are controlled by nuclear-mitochondrial genome interaction and can be manipulated. Arumugam et al. (2000) used the cell fusion approach to derive novel mitochondrial genomes that are of significance in producing viable male sterility systems in Brassicas. Similar rearranged/recombined mitochondrial genome organization has been observed in the somatic hybrids involving *B. juncea* (Kirti et al., 1995b; Mohapatra et al., 1998) and the male sterility systems of *Brassica*
juncea carrying Trachystoma cytoplasm (Kirti et al., 1995c) and cybrids derived from the fusion of CMS oxy B. juncea with B. oleracea protoplasts (Arumugam et al., 2000).

**Cytoplasmic Hybrids in Indian Brassicas**

With the production of cytoplasmic hybrids in a commercially important crop like Brassica napus (Pelletier et al., 1983), protoplast fusion has been resorted to in the context of Indian crop Brassicas like mustard, B. juncea. Unlike in the Western countries, where work has concentrated on the rapeseed B. napus and combining herbicide resistance and chilling tolerance with male sterility, protoplast fusion work in India has dealt mainly with correction of leaf chlorosis that prevailed in the alloplasmic male sterile lines that were developed through conventional sexual hybridisation. Starting with the correction of leaf chlorosis in a mustard line carrying B. oxyrrhina (oxy) cytoplasm (Kirti et al., 1993), many cytoplasmic hybrids were produced. These included the improvement of mustard male sterile line carrying ogu cytoplasm including correction of chlorosis (Kirti et al., 1995a), correction of severe leaf chlorosis in the male sterile line carrying Moricandia arvensis cytoplasm. (Kirti et al., 1998) and the novel male sterile mustard lines from somatic hybrids of CMS mustard line carrying oxy cytoplasm with B. oleracea (Arumugam et al., 2000).

**Protoplast Fusion and Re-synthesis of Crop Brassicas**

To widen the narrow genetic base of the oilseed Brassicas such as B. juncea, B napus and B carinata, it is advisable to re-synthesize them by combining the progenitor species, Viz., B. campestris and B. nigra in the case of B. juncea, B campestris and B. oleracea in the case of B. napus, and B. oleracea and B. nigra in the case of B. carinata using different accessaries of the progenitor species. This is particularly true when hybrid cultivar development becomes a breeding priority. In natural amphidiploids, only specific progenitor species acted as the female parent contributing cytoplasm. In the case of B juncea and B napus, it is presumed that B. campestris acted as the female parent and contributed the cytoplasm. However, the effect of cytoplasm is quite pronounced in crop Brassicas (Narasimhulu & Chopra, 1988; Narasimhulu et al., 1988) indicating that lines with different cytoplasmic backgrounds would have potential in crop improvement. Narasimhulu et al (1992b) have re-synthesized B. carinata by fusing the protoplasts of the progenitor species B. oleracea and B. nigra. They have obtained certain organelle genome combinations not realizable in nature. Similarly, B. juncea has been re-synthesized by resorting to protoplast fusion using a wide array of accessions and the variability generated is being ascertained (Bhat et al, unpublished data).

**Protoplast Fusion and Male Sterility Systems**

Apart from the chlorosis correction in the sexually derived alloplasmic male sterility systems as described earlier, protoplast fusion has been utilized in developing male sterile and restorer lines from some somatic hybrids. Probably the first male sterile line developed directly from the somatic hybrids involving B. juncea is the CMS (Trachystoma) B. juncea from the somatic hybrid T. ballii + B. juncea (Kirti et al, 1992a). Restorer gene for this cytoplasm has also been introgressed into B. juncea background from the corresponding somatic hybrid (Kirti et al., 1997). Similarly, Prakash et al (1998) have developed the male sterile and restorer lines in B. juncea through cyto-genetic manipulation from the somatic hybrid Moricandia arvensis, (+) Brassica juncea (Kirti et al., 1992b). For the male sterility system in mustard developed through conventional hybridisation between Diplotaxis catholica and B. juncea, a restorer has been obtained in the back cross generations of the somatic hybrid between D. catholica and B. juncea (Pathania et al, 2003).

**Conclusions and Future Perspective**

Already considerable work has been done in India in the field of protoplast culture and fusion technology with respect to cultivated Brassicas and their wild and weedy relatives. Protoplast culture protocols have been standardized for crop Brassicas and some wild species with a view to utilizing them in the production of somatic hybrids and cybrids, and for introducing foreign DNA into protoplasts for developing transgenic plants. Many somatic hybrids were developed and efficiently utilized in producing CMS systems and the corresponding restorer lines for developing hybrid mustard varieties.

Since yield levels are reaching a plateau in mustard breeding because of the narrow genetic base, alien germplasm plays a significant role in mustard improvement for developing suitable genetic stacks. It is imperative to look into the possibility of the utilization of wild species to widen the genetic base.
for the effective development of proper combiner lines in hybrid variety development programs also. It has been shown recently that the wild species of the family Cruciferae are very good sources for resistance to the most vexing problem of Alternaria leaf spot (Sharma et al., 2002). Hence, disease resistant genetic stocks could also be developed simultaneously from the hybrids between wild and the cultivated species being developed for tackling disease problems in mustard cultivation.

References


