International Journal of African and Asian Studies www.iiste.org
ISSN 2409-6938 An International Peer-reviewed Journal
Vol.43, 2018

A Review on Somatic Hybridization and Its Utilization in Crop Improvement

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Abstract
Plant breeding throughout the 21th century has been successful because of the incorporation of resistance from exotic germplasm into modern cultivars. Somatic hybridization broadens the base of accessible germplasm and offers additional opportunities for introgression of desirable traits into cultivars. Even though measurable success of somatic hybridization in terms of cultivar release may still be limited, the potential for their use in plant breeding remains great. In general, somatic hybridization provides excellent opportunities for research on plant improvement, first by exploring genetic variations among the existing crops and then by attempting to transfer the available genetic information from one species to another through fusion of protoplasts isolated from somatic tissues of these crops.
Keywords: Somatic hybridization, Protoplast fusion, Protoplast

1. INTRODUCTION

Somatic hybridization, also called somatic cell fusion or protoplast fusion, refers to the fusion of plant protoplasts from somatic cells of different species and the subsequent regeneration of hybrid plants from the fused protoplasts. Somatic hybridization of plants by protoplast fusion is a technique that has captivated the imagination of plant breeders for three decades (Hoffmann *et al*., 1995). It offers the possibility of accessing sexually incompatible germplasm between crop species and distant relatives, merging genomes of sexually dysfunctional cultivars or breeding lines, and substituting one cytoplasm for another with little effect on the nuclear genome. Since Carlison *et al*., (1972) first reported successes with parasexual hybridization of tobacco (*Nicotiana tabacum L.*), hundreds of reports have been published to extend the procedures to additional plant genera and to evaluate the potential of somatic hybrids in many crops. Somatic hybridization has even been conducted under microgravity as part of a space laboratory experiment (Hoffmann *et al*., 1995).

Somatic hybridization is one of the most important uses of protoplast culture. This is particularly significant for hybridization between species or genera, which can not be made to cross by conventional method of sexual hybridization. Although somatic hybridization was successfully achieved first in animals and only later in plants, its significance has been realized fully in plants because the hybrid cells can be induced to regenerate into whole plants (Evans & Bravo, 1983).

Therefore, somatic hybridization provides excellent opportunities for research on plant improvement, first by exploring genetic variations among the existing crops and then by attempting to transfer the available genetic information from one species to another through fusion of protoplasts isolated from somatic tissues of these crops.

2. SOMATIC HYBRIDIZATION AS A PROTOPLAST FUSION

Protoplast fusion has often been suggested as a means of developing unique hybrid plants which cannot be produced by conventional sexual hybridization. Protoplasts can be produced from many plants, including most crop species (Feher and Dudits 1994). However, while any two plant protoplasts can be fused by chemical or physical means, production of unique somatic hybrid plants is limited by the ability to regenerate the fused product and sterility in the interspecific hybrids (Gleddie *et al*., 1986) rather than the production of protoplasts. Perhaps the best example of the use of protoplasts to improve crop production is that of *Nicofiana,* where the somatic hybrid products of a chemical fusion of protoplasts have been used to modify the alkaloid and disease-resistant traits of commercial tobacco cultivars (Pandeya and White, 1994). Somatic hybrids were produced by fusing protoplasts, using a calcium-polyethylene glycol treatment, from a cell suspension of chlorophyll-deficient *N. rusfica* with an albino mutant of *N. tabacum* (Douglas *et al.,* 1981a,). The wild *N. rusficaparent* possessed the desirable traits of high alkaloid levels and resistance to black root rot. Fusion products were selected as bright green cell colonies, the colour being due to the genetic complemention for chlorophyll synthesis the hybrid cells. Plants recovered by shoot organogenesis showed a wide range of leaf alkaloid content but had a high level of sterility. However, after three backcross generations to the cultivated *N. tabacum* parent, plant fertility was restored in the hybrid lines, although their alkaloid content and resistance to blue mould and black root rot were highly variable. Interestingly, neither parent was known to possess significant resistance to blue mould.

Where mutant cell lines of donor plants are not available for use in a genetic complementation selection

system, it has been demonstrated that mesophyll protoplasts from donor parents carrying transgenic antibiotic resistance can be used to produce fertile somatic hybrids selected by dual antibiotic resistance (Sproule *et al.,* 1991). The fusion of protoplasts from 6-azauracil-resistant cell lines of *Solanum melongena* (aubergine) with protoplasts from the wild species yielded hybrid, purple-pigmented cell colonies that underwent regeneration via organogenesis (Gleddie *et al.,* 1986). As protoplasts from the parental cell suspension cultures could not be regenerated, hybrids could be screened by their 6-azauracil resistance, capacity to synthesize anthocyanins (purple pigment) and ability to undergo shoot organogenesis. The restoration of regeneration ability through complementation has also been observed in *Nicofiana* cell-fusion products (Douglas *et al*., 1981a; Gleddie *et al.,* 1986). The hybrids resulting from the study were found to be resistant to root knot nematodes and spider mites, important agricultural traits. However, they were also completely sterile and could not be incorporated into an aubergine-breeding programme. Two possible ways of solving this sterility problem, 'back' fusions of somatic hybrids with the cultivated parents and initiation of suspension cultures of the hybrid cells so that more of the wildspecies chromosomes can be eliminated, have so far been unsuccessful with these hybrids (*S. Gleddie*). Selection of hybrids and use of protoplast fusion for hybridization in crop plants has been reported in Brassicas, citrus, rice, carrot, canola, tomato, and the forage legumes alfalfa and clover (Akagi *et al.,* 1989; Bajaj, 1989; Tanno- Suenaga *et al.,* 1988; Vardi *et al.,* 1989, Kao *et al.,* 1991).

Evans & Bravo (1988) have recommended that production of novel hybrids through protoplast fusion should focus on four areas: (1) agriculturally important traits; (2) achieving combinations that can only be accomplished by protoplast fusion; (3) somatic hybrids integrated into a conventional breeding programme; and (4) the extension of protoplast regeneration to a wider range of crop species. In the case of the above-mentioned example of *Nicotiana,* all of these criteria were met although this took 12 years from the isolation of the fusion product in 1978 to the release of the first variety in 1990; this underlines the often overlooked fact that it takes 10 to 20 years to take initial research results to the stage of a recognized cultivar (Kuckucketal, 1991).

3. TYPES OF SOMATIC HYBRIDS

If the complete genome of two different species are combined parasexually, then an amphiploid somatic hybrid results. This is the most common occurrence in Somatic hybridization experiments. If one fusion partner is an unadapted species with some desirable trait, then the resulting somatic hybrids can be expected to carry many of the undesirable traits of the unadapted species along with the trait of interest. Therefore plant breeders would indeed be foolishly optimistic to expect table ready germplasm from such effort; considerable back crossing and ploidy reduction are required to introgress the desirable trait into germplasm suitable for cultivar release. Indeed much of the recent effort in Somatic hybridization has been directed at development of somatic hybrids and/or verification that the trait of interest has been transmitted. Subsequent utilization of somatic hybrid germplasm in plant breeding has been less frequent (Evans and Bravo, 1988).

In an effort to limit the genetic contribution of an unadapted ‘parent’ to the product of protoplast fusion, some geneticists have promoted asymmetric Somatic hybridization, where by the genome of the donor species if fractionated by irradiation prior to fusion. The resulting asymmetric hybrids retain the complete genome of the recipient (adapted) species and only fragments of the genome of the donor (unadapted) species. Irradiation is imprecise and damage to the donor genome is random. Therefore transmission of the trait of interest is not guaranteed and the amount of the donor genome transmitted is highly variable, depending upon the irradiation dosage and the tolerance of the recipient genome to chromosome fragments and rearrangements (Evans and Bravo, 1988).

Cybrids or cytopasmic hybrids result from protoplast fusion between a cultivated species and enucleated or nucleus inactivated protoplasts bearing a different plastome. For plant breeding purposes, cybridization offers the possibility of developing lines for hybrid breeding via cytoplasmic male sterility systems in a single step. Such systems are the mainstay of hybrid cultivar production in many crops and availability of suitable plastome variation has been a limitation in breeding (Gleddieet al., 1983).

4. METHODS FOR ISOLATION AND FUSION OF PLANT PROTOPLAST

Successful Somatic hybridization was originally obtained through the use of PEG-mediated (polyethylene glycol) fusion. More recently, electro fusion techniques have become available.

4.1. Direct One Step Method

In one step method, the leaf segments are incubated overnight (15-18h) with enzyme mixture at 25°C and teased gently to liberate the protoplasts. The mixture is filtered through fine wire gauze to remove leaf debris, transferred to 13 X 1000 mm screw capped tubes and centrifuged at 100g for 1 min. The protoplasts form a pellet and supernatant removed. The process is repeated three times and protoplasts washed with 13% sorbitol solution, which is later replaced by 20% sucrose solution and centrifuged at a speed of 200g for 1 min. The cleaned protoplasts, which will now float (debris settles do), can be pipetted out and bulked (Gamborg et al.,

1981).

4.2. Enzymatic Method of Isolation of Protoplast

The enzymatic method is almost invariably used now for the isolation of protoplasts, since it gives large quantities of protoplasts, where cells are not broken and osmotic shrinkage is minimum. However, sometimes mechanical and enzymatic methods are combined, where cells are first separated mechanically and later used for isolation of protoplasts through enzymatic treatment. (Gamborg et al., 1981).

4.3. Mechanical Method of Isolation of Protoplast

In mechanical method, cells are kept in a suitable plasmolyticum (in plasmolysed cells, protoplasts shrink away from cell wall) and cut with a fine knife, so that protoplasts are released from cells cut through the cell wall, when the tissue is again deplasmolysed.This method is suitable for isolation of protoplasts from vacuolated cells (e.g. onion bulbs, scales, radish roots). However, this method gives poor yield of protoplasts and is not suitable for isolating protoplast from meristematic and less vacuolated cells. The mechanical method, though, was used as early as 1892, is now only rarely used for isolation of protoplasts (Gamborg et al., 1981).

5. SELECTION OF FUSION PRODUCTS AND VERIFICATION OF HYBRIDS

5.1. Selection Schemes

When protoplast of two different sources are mixed in the hope of obtaining somatic hybrids, a range of possible products may regenerate, resulting from unfused protoplasts of either"parent**"** (somaclones produced), homofusion between genetically similar protoplast of either parent (polyploidy somaclones produced), heterofusions between single protoplast of each parent (amphidiploids somatic hybrids produced), or multiple fusions among several protoplasts (highly polyploid somatic hybrids produced). In addition, aneuploids often arise due to chromosome loss during cell culture or aberrant mitotic cycles within the hybrid nucleus. Without any selection against the undesirable products, researchers are faced with distinguishing the desired amphidiploids somatic hybrids from the undesired variable ploidy somaclones of each parent and highly polyploidy hybrids resulting from multiple protoplast fusions (Evans *et al.,* 1984).

The simplest selection has been to analyze whatever plants may regenerate by morphological or molecular markers to determine their possible genomic constitution. When heterokaryons regenerate more readily than homokaryons, this can be effective. Otherwise, many more sophisticated selection schemes have been developed to encourage regeneration of only the somatic hybrids, but all generally carry some inherent cost. Selection media can be used if sufficient tissue culture research has been done on the prospective fusion partners. If each protoplast source can reliably be predicted to regenerate only in the presence of a distinct unique media component, elimination of both components in the medium used for regeneration following protoplast fusion should result exclusively in somatic hybrids. If one parent is known to be non-regenerable, then this procedure can be simplified by omitting only the medium requirement of the regenerable "parent". This particular complementation scheme requires the conduct of rigorous plant tissue culture experimentation on both fusion parents prior to their utilization in Somatic hybridization schemes in order to predict the outcome with any reliability. In addition, absolute medium requirements in plant tissue culture are difficult to identify (Evans *et al*., 1984).

The use of metabolic inhibitors such as iodoacetate (IOA) to prevent regeneration of unfused protoplasts of one fusion partner has greatly enhanced complementation schemes. Because it is common to find that one protoplast source is unregenerable, protoplasts from such a source can be fused with IOA-treated protoplasts of an ordinarily regenerable source with the expectation that only fusions will regenerate. Introduction of transgenic antibiotic resistance into one or both fusion partners has also been used to facilitate complementation schemes. This provides an easy selection scheme by which protoplasts lacking antibiotic resistance will not regenerate on medium containing the antibiotic. Antibiotic resistance in an unregenerable protoplast source combined with antibiotic sensitivity in a regenerable protoplast source then provides a suitable selection scheme for exclusive regeneration of somatic hybrids. Ishige (1995) used transgenic resistance to kanamycin in one potato dihaploid and hygromycin in another to regenerate exclusively somatic hybrids that varied for ploidy. However, the restrictions of this system are the extensive pre-breeding required to introduce transgenic resistance into the protoplast fusion partner(s) and the limitation of the procedure only to transgenic plants.

In order to eliminate the necessity of pre-breeding one of the fusion partners, Dorr *et al*., (1994) developed a selection method to label protoplasts with superparamagnetic beads mediated by biotinylated lectins. Electrofusion of these labeled protoplasts with transgenic kanamycin resistant protoplasts was followed by sorting using a magnetic cell sorter to retain protoplasts labeled with the microbeads. Subsequent culture of selected cells on kanamycin containing medium resulted in a significant enrichment for somatic hybrids among the regenerants. Surface labeling of prospective fusion partners with biotin and avidin has been used to facilitate heterologous aggregation of fusion partners during electrofusion (Waara *et al*., 1998)

Hoffmann-Tsay *et al*., (1994) identified several surface active chemicals that could be used as adjuvant to treat protoplasts prior to electrofusion to increase the fusion rate. They concentrated on adjuvants that did not need to be removed from the culture medium in order to retain one of the advantages of electrofusion over chemical fusion i.e., the direct culture of fusion products. Mollers *et al.,* (1994) likewise studied the possibility of increasing the frequency of somatic hybrid formation during electrofusion by selectively inactivating one protoplast source with the mitochondrial inhibitor, nonly-acridine orange (NAO). NAO was considered to be an alternative to IOA that might have the specific result of predetermining the mitochondrial composition of somatic hybrids. Neither of these two techniques, i.e. chemical adjuvants or NAO, has been used extensively in somatic hybridization subsequent to the original reports. Funatsuki *eat al*., (1994) described a modification of chemical fusion where the fusion was conducted on a Millicell (Millipore) membrane on a puddle of PEG, this facilitated heterofusions between protoplasts that differed considerably in size.

5.1.1. Biochemical selection of somatic hybrid

Gamborg *et al.,* (1981) demonstrated the value of biochemically based selection. This selection procedure was based on a prior knowledge of the differential growth characteristics and nutritional requirements of unfused and hybrid mesophyll protoplasts isolated from the genetically different Nicotiana glauca and N. langsdorffii. Protoplasts of the hybrid were able to grow on a defined medium in culture to form calli, whereas parental types failed to develop into calli. This selection system has an advantage in that the requirement of a mutant as one of the fusion partners is totally eliminated.

Schieder and Kohn (1986) utilized the differential sensitivity of protoplasts isolated from Petunia parodii and P. hybrida to the drug actinomycin D. In an MS medium the mesophyll protoplasts of *Petunia hybrida* developed up to a macroscopic callus stage and those of *P. parodii* divided to form only small cell colonies. The addition of actinomycin D to the culture medium apparently had a slight effect on the regeneration potential of *P. parodii* protoplasts while those of *P. hybrida* failed to divide.

Pandeya *et al., (*1986) dopted a similar procedure for somatic hybrids between *Nicotiana sylvestris* and *N. knightiana*. This selection system makes use of two dominant drug resistant cell lines. Selection of somatic hybrids following protoplast fusion of two such cells, lines is carried out on, media containing both drugs.

5. 1.2. Complementary selection of somatic hybrid

The selection of somatic hybrids as a result of complementation by auxotrophic mutants may be useful as only the hybrid lines are expected to survive in the minimal medium. Auxotrophs are mutants requiring specific compounds for their growth. Pandeya *et al.,* (1986) succeeded in selection of numerous somatic hybrids by utilizing protoplasts of nitrate reductase deficient and chlorate resistant mutant lines of tobacco.

Protoplasts of two genetically different mutants were fused and cultured in a, medium containing nitrate as the sole nitrogen source. Parental protoplasts did not grow in the presence of nitrate, whereas fusion products regenerated. The complementation selection based on auxotrophic mutants, even though desirable and efficient, is very limited because of the limitation due to the paucity of higher plant auxotroph. Nonallelic albino mutants were used for selection by Melchers (1992). He fused haploid chlorophyll deficient and light-sensitive protoplasts of *Nicotiana tabacum* and cultured them under high intensities of light. After two months green colonies were observed in culture dishes as a consequence of complementation between the two albino mutants. On further culturing these green colonies regenerated somatic hybrid plants.

5.1.3. Visual selection of somatic hybrids

In most of the somatic hybridization experiments selection procedures involve fusion of chlorophyll deficient (non-green) protoplasts of one parent with the green protoplasts of the other parent (wild type) since this facilitates visual identification of heterokaryons at the light microscope level. Non-green protoplasts are isolated from cultured cells, epidermal cells, or antibiotic induced albino plantlets. The visual selection procedure is coupled with complementary natural differences in the sensitivity of parental protoplasts to media constituents which enable only the hybrid cells to develop in cultures and regenerate plants. Wild type protoplasts of *Petunia parodii* were fused with albino protoplasts isolated from cell suspension cultures of *P. hybrida, P. inflata*, and *P. parviflora* in separate experiments (Atanassov et al., 1995).

In all these combinations *P. parodii* green protoplasts were eliminated at the small colony stage, while the albino protoplasts of the other parent developed colorless colonies. Hybrid components proliferated into green calli and subsequently regenerated somatic hybrid plants. Using this method Evans and Bravo (1983), recovered somatic hybrids in Datura. In these systems the protoplasts of a chlorophyll deficient mutant regenerated into shoots on a defined medium while the wild type protoplasts did not. After fusion of these two types of protoplasts somatic hybrids were recovered and the intermediate nature of the hybrid could be confirmed. The hybrid thus recovered demonstrated the potential for shoot production of the chlorophyll deficient mutant plus the potential for chlorophyll synthesis of the wild type.

In experiments on intergeneric somatic hybridization, Schieder and Kohn (1986) used the scheme in which the parental protoplasts and heterokaryons were allowed to develop calli in cultures. The morphological differences in the resultant three types of calli permitted identification of the hybrid tissue, which could then be

selected out to regenerate somatic hybrid plants. Individual heterokaryons can be identified visually under a light microscope, be isolated mechanically by means of Drummond pipette, and can be cloned in microdrop cultures.

This approach suffers from the fact that it requires special culture media for each particular hybrid cell type to divide and form clusters. This is also called the fishing method. Somatic hybrid callus has been similarly obtained in fusion between colorless protoplasts of *Glycine max* derived from cell cultures with the green mesophyll protoplasts of *Nicotiana glauca*. The fusion products can be identified by the presence of chloroplasts in one half of the cell and starch granules in the other half. Diffusion of chloroplasts throughout the cell occurs shortly after fusion. Even though the mechanical method of isolation of somatic fusion products is the most tedious method, it may be the most likely method for recovering osmotic hybrids in a variety of different plants, especially legumes, cereals, and tree species (Gamborg *et al.,* 1981).

5.1.4. Flow cytometry and sorting selection of somatic hybrids

Various laboratories are using techniques of flow cytometry and fluorescent activated cell sorting for analysis of plant protoplasts while maintaining their viability. These techniques have also been applied for sorting and selection of heterokaryons. Galbraith *et al.,* (1989) have described a universally applicable method for electronic sorting of heterokaryons formed by fusing the protoplasts of two parents labeled with different vital fluorescent dyes, such as rhodomine isothiocyanate and floreceine isothiocyanate. The fused and unfused products are sorted in a "cell sorter" machine based on the presence or absence of fluorescence of both dyes in the fusion products (Gamborg *et al.,* 1981).

6. METHODS OF DETECTION

Once putative hybrids have been regenerated, an array of techniques is now available for proof of their hybrid nature. Although intermediate morphology and heteroallelic isozyme patterns are still frequently employed in recent reports, most authors are now using molecular analysis to demonstrate hybridity. Even if specific probes for restriction fragment length polymorphisms (RFLPs) have not been developed for the species under investigation, probes that anneal to universal genes, such as rDNA from other species, will often reveal differences between the two protoplast sources following restriction enzyme digest and southern blotting (Harding and Millam, 1999).

As Johnson *et al*., (2000) reported there in no phylogenetic limitation to protoplast fusion. Even fusions between plant and animal protoplasts have been conducted. However, regeneration of somatic hybrids has only been possible when fusion partners are somewhat related. Somatic hybridization has allowed us to exceed the limits of sexual compatibility and evidence of partial genome transfer has even been presented after fusion between a monocot (*Hordeum vulgare L.*) and a dicot (*Daucus carota L.*) (Kisaka *et al*., 1997). However it has been far more common to obtain only unregenerable callus if the fusion partners are too phylogenetically distant, because such callus has little potential in plant breeding.

7. APPLICATION OF SOMATIC HYBRIDIZATION

One of the most significant developments in the field of plant tissue culture, Witnessed during the last few decades, is the isolation, culture and fusion of protoplasts (Gamborg *et al.,* 1981). A more recent achievement is the manipulation and regeneration of these cultured or fused protoplasts into whole plants. Since in plant cells, the plasma membrane is bound by a rigid cellulose wall (unlike animal cells), it has been relatively difficult to handle plant cells. In 1960, it was demonstrated by Cocking at the University of, Nottingham (U.K.), that naked cells called protoplasts can be obtained through enzymatic degradation of cell walls. This led to significant developments in the field of somatic cell genetics in higher plants. Cultured protoplasts can be used not only for somatic cell fusions, but also for taking up foreign DNA, cell organelles, bacteria and virus particles. In view of this, the isolation and culture of protoplasts has become a very important area of research, within the realm of plant biotechnology (Bajaj, 1990). The essential ingredients of the technique include isolation of protoplasts, culture of protoplasts, introduction of foreign DNA into protoplasts, raising whole plants from cultured protoplasts and fusion of protoplasts leading to somatic hybridization.

Somatic hybridization involves fusion of two distantly related, to closely related plant protoplasts at intraspecific, interspecific, intergeneric, and interfamily levels, with sub sequent regeneration of hybrid cells into hybrid plants. The term protoplast was first used by Manstein in 1880. Plastids and mitochondrial genomes (cytoplasmically encoded traits) are inherited maternally in sexual crossings. Through the fusion process the nucleus and cytoplasm of both parents are mixed in the hybrid cell (heterokaryon). This results in various nucleocytoplasmic combinations. Sometimes interactions in the plastome and genome contribute to the formation of cybrids (cytoplasmid hybrids).

Cybrids, in contrast to conventional hybrids, possess a nuclear genome from only one parent but cytoplasmic genes from both parents. The process of protoplast fusion resulting in the development of cybrids is known as cybridization. In cybridization, heterozygosity of extrachromosomal material can be obtained, which has direct application in plant breeding.

According Gamborg *et al.,* (1981) to somatic hybridization involves four major steps:

i. Isolation of protoplasts from different species
ii. Culture of protoplasts
iii. Fusion of protoplasts, and
iv. Identification and selection of hybrid cells and their subsequent regeneration into whole plants

Somatic cell fusion appears to be the only means through which two different parental genomes can be recombined among plants that cannot reproduce sexually (asexual or sterile). Protoplasts of sexually sterile (haploid, triploid, and aneuploid) plants can be fused to produce fertile diploids and polyploidy. Somatic cell fusion overcomes sexual incompatibility barriers. In some cases somatic hybrids between two incompatible plants have also found application in industry or agriculture. Somatic cell fusion is useful in the study of cytoplasmic genes and their activities and this information can be applied in plant-breeding experiments.

7.1. Somatic Hybrids for Cytoplasmic Male Sterility

Methods have been developed to substitute the nucleus of one species into the cytoplasm of another species, whose mitochondria are inactivated. This type of substitution in some cases, led to generation of cytoplasmic male sterility. For this purpose, a report by Melchers (1992); and his coworkers, the two types of protoplasts, used for the production of, somatic hybrids, and were treated differently, as follows:

- ❖ mesophyll protoplasts of tomato (*Lycopersicon esculentum*) were treated with iodoacetamide (IDA) to inactivate mitochondria and
- ❖ mesophyll protoplasts of *Solanum acaule* (or *S. tuberosum* = potato) were irradiated with γ or x-rays to inactivate nuclei. The protoplasts were mixed in 1: 1 ratio and induced to fuse using Ca_2+ and PEG, leading to the production of heterologous hybrids. Among the fusion products, some hybrid tomato plants were indistinguishable from the original cultivars, with respect to morphology, physiology and chromosome number (2n=24), but exhibited various degrees of male sterility. The variation included (Melchers,1992):
  - ✓ Complete lack of pollen or malformation of anthers;
  - ✓ shrunken pollen,
  - ✓ Normal looking stainable pollen that could not germinate. In five tomato cultivars, male sterility induced in this manner was inherited maternally over several generations. Therefore, it was obviously cytoplasmic male sterility.

    The mitochondrial DNA of these CMS hybrids did not resemble mtDNA of either parent, and was instead recombinant type, representing a hybrid mitochondrial genome. Therefore, protoplast fusion can be effectively used for production of CMS lines and has the following advantages:

- Only one step is required;
- The nuclear genotype of the cultivar remains unaffected,
- There are prospects that 100% of the progenies of somatic hybrids will be CMS. The restorer lines for these CMS lines have also been shown to be available in tomato, so that hybrid seed can be produced without manual emasculation.

7.2. Somatic Hybrids for Gene Transfer

The family Solanaceae contains the most commonly used species for somatic hybridization. The genera from this family that have been often used for somatic hybridization include Nicotiana, Datura, Petunia, Solanum, Lycopersicon, etc. In Rutaceae, Citrus has been combined with species from other genera. In Leguminoseae also, several genera including Medicago, Trifolium and Lotus have been used for somatic hybridization. Even among cereals, hybrids between rice (*Oryza sativa*) and *Echinochloa oryzicola* (barnyard grass) have been obtained.

However, one of the most extensive programmes has been in progress in the family Brassicaceae at Uppsala, Sweden. A collaborative programme in Brassicaceae is also underway at, New Delhi, the callaborative institutes being IARI, TERI and Delhi University. Some of the interspecific somatic hybrids produced. Similarly intergeneric somatic hybrids and intertribal hybrids within Brassicaceae. The hybrids within Brassicaceae are also diagrammatically represented.Intergeneric fertile hybrids were obtained in several cases particularly within the tribe Brassiceae. These fertile hybrids were back crossed to cultivated species followed by screening for economic traits like drought and insect resistance (transferred from Eruca), pathogen resistance (transferred from Sinapis), cytoplasmic male sterility or CMS (transferred from Diplotaxis), cold tolerance (from Barborea), and high concentration of nervonic acid, a lubricant (from Thalspi). Symmetric hybrids between tomato and potato, produced by protoplast fusion, have been shown to exhibit intermediate cold tolerance. In., another example, substitution of *Solanum acaule* genome into *S. tuberosum* resulted in an appreciable increase of frost resistance.

7.3. Somatic Hybridization - An Alternative to Sex

Plant breeders through classical sexual hybridization have contributed significantly to agriculture. High-lysine

corn, high-protein rust-tolerant wheat, and new varieties of fruits are all examples of successful breeding and selection. Generally, any single genus contains an adequate gene pool which, if in the right combination(s), could produce nearly any desired quality (qualities). Often however, a combination of "qualities" may be found only in species which are not sexually compatible, i.e., neither seeds nor embryos form (Melchers, 1992).

Historically, plant breeding has been restricted to either pollination of one plant with another or the reciprocal cross, producing seeds which, when sown, germinated and produced plants. Evaluation of the seedlings could require more than 10 years at considerable expense to the grower. Occasionally, no seeds resulted from failure of ovule development. Incompatibility sometimes resulted from ploidy (chromosome number) where the pollen haploid number was not the same as the ovule number. Because the gametes were of different ploidy, chromosomes did not pair off during meiosis and no embryos formed. In other instances pollen tubes (which penetrate the stigma) did not form sufficiently to travel the distance from the style to the ovary (Melchers, 1992).

Investigators have determined by scanning electron microscopy (SEM) that pollens which are incompatible with another plant simply lay on the stigma and do not enter the style. It appears that the stigma may recognize given pollen on a chemical basis, accepting what is "compatible" and rejecting what is "incompatible."

When incompatibility occurs by failure of the pollen tubes to grow to sufficient length, the plant breeder has several alternatives. One choice is to induce the stigma to "recognize" pollen by treating the stigma with mixed pollen (including one that is desired in the cross) or by modifying the stigma surface using paraffin oil (power *et al*., 1989). Another choice is to practice style amputations close to the ovary, apply stigmatic fluid, and pollinate. The latter procedure can be applied to any flowering plant with varying degrees of difficulty. Often, however, seeds produced are devoid ofendosperm, requiring then the application of what is termed " embryo culture." Embryos are cultivated on sterile nutrient agar which acts as the "endosperm" for the growing embryo. This technique has been noted as a means of generating interspecific hybrid crosses of the genus Lilium (power *et al*., 1989). Before alternative techniques are attempted in plant breeding, various modifications of sexual propagation should be used. They still remain the easiest and the most certain to yield results.

8. PRINCIPLE OF SOMATIC HYBRIDIZATION

Obtaining hybrids from widely differing species within a genus may be difficult if not impossible by sexual means. An "alternative to sex" is now being acclaimed as a potential solution to disease tolerance, decreased crop yields, climatic maladjustment, etc. This technique is somatic hybridization. Somatic cells are non-sex cells. Cells from any organ of the plant theoretically can be used to form somatic hybrids (power *et al*., 1989).

Somatic hybridization is conducted in a sterile environment. All reagents are either sterilized in an autoclave or by filtration with a Millipore filter. In theory the procedures are simple to a plant or animal biochemist. For example, one could attempt to somatically hybridize a *Martagon lily* with *L. brownii*, using either leaf mesophyll cells or cells from scale sections. Leaves or scales from both species would be completely disinfected using hypochlorite solutions (Chlorox) followed by thorough washing with sterile distilled water. The organ materials are then treated separately with an enzyme mixture consisting of pectinase, cellulase, and hemicellulase to disperse the tissue and remove cell walls. This treatment would be given separately to both species. Under gentle agitation for 12 hours, the reaction mass becomes a viscous mixture of cell wall debris, cell organelles, and the desired protoplasts. Under the microscope, protoplasts appear as spheres devoid of their cell walls. The fragile protoplasts are purified by a series of centrifugations in media-sugar solutions to remove cell debris and the degradative enzymes (power *et al*., 1989).

The principle of somatic hybridization is to combine the protoplasts of two species and "fuse" the two protoplasts to produce a heterokaryon (a cell containing genetically different nuclei). The process of fusion is carried out in the presence of calcium ions either at high pH (10.5) or with the aid of polyethylene glycol (PEG) at a concentration of 25070-30%. Often a high loss of viable cells occurs, depending upon the genus, species, age of plant materials, and laboratory procedures, the principle problem being microbial contamination. However, with cell concentrations (density) at 2 x 105 cells/milliliter, a number of heterokaryons are still expected (Power *et al*., 1989).

Following removal of the fusion media, the cells are transferred to a new medium for growth, and the cells can be observed microscopically for fusion of the two species, viability, and for rate of growth. If the fused cells are viable, the first mitotic division can be observed within 4 days. If this event does not occur, death of the heterokaryon ensues. Because plants vary widely in their need for hormones, light, and temperature, every somatic hybrid cross must entail a thorough study of growth requirements for success. Quite often the new somatic hybrid will develop a callus tissue (undifferentiated tissue) which must be transferred to a suitable medium for bulb growth, root development, and subsequent growth (power *et al*., 1989).

8.1. Intraspecific somatic hybrids

Although the greatest efforts in somatic hybridization have been for introgression of alien germplasm into crop

plants through interspecific protoplast fusion, there has also been considerable effort at intraspecific somatic hybridization for various purposes. Intraspecific somatic hybridization has been applied to potato, for genetic reconstruction of tetraploids by fusion of selected dihaploids. Chase in 1963 proposed an analytical breeding schemes for potato and other polyploidy crops. The tetraploid genome is first reduced to the dihaploid level by haplodization schemes, then breeding is conducted among dihaploids where segregation ratios and inheritance of desirable quantitative traits is simpler. Finally, the tetraploid condition is restored through sexual polyploidization, colchicines doubling or some other means. Wenzel *et al*., (1979) were quick to realize that the newly developed technique would facilitate analytic breeding in potato, especially because experience with dihaploids had revealed them to be reluctant to flower and frequently sterile, there by prohibiting sexually combining their genomes. Another purported advantage of this breeding strategy is possibility of eliminating deleterious alleles harbored in the tetraploid, but revealed at the diploid level through partial breeding.

Rasmussen (1995) electrofused two potato dihaploids that exhibited resistance to the cyst nematode, *Globodera pallid*. Although one dihaploid never flowered and the other was male and female fertile. The somatic hybrids also exhibited a range of resistance to *G.pallida*, some combining the resistance to separate pathotypes from each dihaploid fusion partner (Rasmussen *et al*., 1996). Employing a similarity strategy, Cooper-Bland *et al*., (1994) found variable resistance to *G.pallida* among tetraploid somatic hybrids between selected dihaploid clones. Considerably variation has been found among intraspecific somatic hybrids regenerated from a single fusion; however, there appears to be little cytoplasmic influence on these phenotypes (Frei *et al*., 1998).

In other crop genera, intraspecific somatic hybridization has been used to over come various breeding barriers. Koyama *et al.,* (1995) used protoplast fusion to regenerate plants from a carrot (Daucus carota L.) cell line, selected for utilization of Al-phosphate that had been maintained in vitro for over 10 years and had lost regenerative capacity. Protoplast fusion of this cell line with IOA-inactivated wild-type carrot resulted in regenerable somatic calluses that were tolerant of Al-phosphate. Tamura *et al.,* (1998) fused protoplasts of two hexaploid (2n=6x=90) cultivars of Japanese persimmon that produced only female flowers to obtain dodecaploid (2n=12x=180) somatic hybrids. Sexually incompatible cultivars of hexaploid sweet potato [*Ipomoea batatas* (L.) Lam.] Were also combined into dodecaploid (2n=12x=180) somatic hybrids eliminated to have limited pollen fertility (Wang *et al*., 1997). Protoplasts of two cultivars of taro (*Colocasia esculenta schott*) have been electrofused to develop autotetraploid somatic hybrids (Murakami *et al.,* 1998). The utility of these highly polyploid intraspecific somatic hybrids has yet to be demonstrated. It may well depend on the efficacy of chromosome reduction procedures such as anther culture or pseudogamy in these species to generate useful variation for breeding programs.

8.2. Interspecfic somatic hybrids

The majority of somatic hybridization has been conducted to obtain interspecfic somatic hybrids. Occasionally sexual hybrids are possible, but generally the objective is to transcend breeding barriers (Guo *et al.,* 1998)

8.3. Intergeneric Somatic Hybrids

Intergeneric somatic hybrids generally bridge a much wider taxonomic gap between fusion partners than interspecific somatic hybrids. In many cases, regeneration of hybrid callus has been impossible or only weak sterile plants have resulted. However, in other cases, viable hybrids with evidence of at least partial genome transfer resulted (Guo and Deng, 1999

9. ADVANTAGES, LIMITATIONS, AND CONSTRAINTS OF SOMATIC HYBRIDIZATION

While the concept of somatic hybridization (protoplast fusion) is over 80 years old, the method still has not found widespread application in the development of new plant hybrids. However, Government, university, and corporate research units are examining the potentials of such methods. Somatic hybridization is not "genetic engineering." While nuclei fuse, as do the cell walls, the method itself usually does not involve the infusion of outside genes or genomes into the chromosomes (recombinant DNA). The method involves changes at the cellular level where chromosomes are either compatible at nuclear fusion or incompatible (Bajaj, 1989).

The method of somatic hybridization is not intended to replace sexual propagation; it is intended for use when sexual hybrids cannot be made because of the incompatibility of widely divergent species. Indeed, somatic hybrids would not be expected to be any more fertile than interspecific hybrid crosses obtained by sexual means. Moreover, the hybrids may prove to be lacking in utility as garden or commercial plants. Nevertheless, the need for plants with specific qualities, heretofore unavailable from classical breeding, may be achieved through somatic hybridization (Bajaj, 1989).

It is tempting to speculate what diverse crosses could arise from heterokaryon. Each genus and each species offers a challenge in basic research to determine specific growth requirements for the fused cells. The technique itself, no doubt, depletes the system nutrients and plant hormones necessary for meiosis and subsequent mitosis.

It may further damage cell components, called organelles, such that the cells cannot divide normally. As scientific investigation determines the best possible methods for cell fusion, nutrition, and subsequent growth, somatic hybridization will take off as a method. In the meantime, the plant breeder is admonished to rely on standard breeding methods, with some modification, and develop plants which have useful qualities—disease tolerance, flower colour and form, glasshouse forcing capability, etc.

10. CONCLUSION

Most of somatic hybrids must be backcrossed to the adapted parent in order to contribute cultivar development. Of critical importance, therefore, is the elucidation of somatic hybrid chromosomal behavior during sexual crosses.

Due to rapid elimination of chromosomes through backcrossing, it is important that homologous paring occurs in somatic hybrids so that opportunities for intergenomic recombination arise. Such paring is likely to happen in interspecific and even intergeneric somatic hybrids. However, combinations of more phylogenetically distant plant species (intertribal somatic hybrids) may not be useful for transferring nuclear traits due to a lack of homologous paring. Such fusion may be more useful for creating novel nuclear-cytoplasmic combinations often important for the development of CMS systems.

Transgenic plant production has certainly overwhelmed somatic hybridization as a technique heavily utilized by plant breeders and geneticists over the past decade. This is understandable considering that transgenic modification theoretically affects only a single trait of interest without introducing innumerable extraneous genes of uncertain agronomic influence. The opportunity of genetic revision of existing cultivars is afforded by genetic engineering. Conversely, somatic hybridization involves introgressing traits of interest into crop plants without any prior knowledge of their genetic control. Resulting somatic hybrids are generally not expected to be ready for cultivar release without additional breeding effort to acquire the trait of interest in a more acceptable genetic background resembling current cultivars. However, an advantage of somatic hybridization compared to genetic engineering concerns the controversy surrounding genetically modified organisms, a stigma that does not apply somatic hybrids. Current restrictions that hinder the release of transgenic crops are avoided using somatic hybrid plant material.

The impact of somatic hybridization can be expected to be much quieter than that of genetic engineering because traditional breeding must intervene between the biotechnological events (protoplast fusion) and the release of a product for market. Valuable resistances to various pathogens or environmental stresses have been identified in direct somatic hybrids and many have been entered into current plant breeding programs.

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