Asian Journal of Research in Crop Science

4(1): 1-11, 2019; Article no.AJRCS.49096
ISSN: 2581-7167

Plant Hybridization as an Alternative Technique in Plant Breeding Improvement

Immaculate Mueni Mwangangi[1], Joshua Kiilu Muli[2] and Johnstone Omukhulu Neondo[3*]

[1]Department of Biochemistry, Microbiology and Biotechnology, Kenyatta University, P.O.Box 43844-00100, Nairobi, Kenya.
[2]Department of Biological Sciences, University of Embu, P.O.Box 6-60100, Embu, Kenya.
[3]Institute of Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, P.O.Box 62000-00200, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRCS/2019/v4i130059
Editor(s):
(1) Dr. Bojan Stipesevic, Professor, Department of Plant production, Faculty of Agriculture in Osijek, University of J.J. Strossmayer in Osijek, Croatia.
Reviewers:
(1) O. Grace Tona, Ladoke Akintola University of Technology, Nigeria.
(2) Schirley Costalonga, Universidade Federal do Espírito Santo, Brazil.
(3) Junaid Iqbal, University of Agriculture Fasisalabad, Pakistan.
Complete Peer review History: http://www.sdiarticle3.com/review-history/49096

Review Article

Received 03 March 2019
Accepted 15 May 2019
Published 22 May 2019

ABSTRACT

For ages, plant breeders have relied on the genetic variability that results from sexually crossing plants within the same species. However, the variability that exists within species populations is inadequate, hence the need to exploit desirable traits of interest in distantly related or even unrelated plants through hybridization techniques. Hybridization can be categorized into two; sexual and somatic. Sexual hybridization, also referred to as wide or distant hybridization involves combining two genomes from different parental taxa through pollination, either naturally or by induction. Somatic hybridization involves the fusion of somatic cells instead of gametes, which highly depends on the ability to obtain viable protoplasts and eventually differentiate them to whole plants *in vitro.* The impacts of hybrids can either be positive or negative. Among the positive attributes of hybrids that have been exploited is heterosis, which results either from dominance, over-dominance or epistasis. Negative ones include sterility, arrested growth of the pollen tube and

**Corresponding author: E-mail: jneondo@jkuat.ac.ke;*

embryo abortion. To overcome these problems, chromosome doubling, the use of hormones such as 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and embryo rescue have been employed to overcome sterility, arrested growth of pollen tubes and embryo abortion respectively. After the development of hybrids, different hybrid identification techniques have been used to test them such as the use of molecular and morphological markers, cytogenetic analysis and fluorescent *in situ* hybridization. The use of hybridization techniques in plant improvement remains a vital tool to cross species barriers and utilization of important attributes in unrelated crop plants which could not have been achieved through conventional techniques of plant breeding.

Keywords: Hybridization; reproductive barriers; molecular markers.

1. INTRODUCTION

Genetic variability within the species has been efficiently utilized by breeders in their efforts to improve crops [1]. However, the existing variability in any given plant breeding population is not sufficient for modern plant breeding purposes, and hence the need to broaden the existing gene pool of crops [2,3]. Introduction of new traits in plants largely relied on sexual crosses between different genotypes within or between closely related species [1]. However, due to the presence of various reproductive barriers, gene transfer has been restricted to sexually-compatible species, thus limiting the possibilities of modifying and improving crop plants [4]. Many desirable and agronomically-interesting traits may only be found in distantly related species or even in unrelated plants [5]. Since they constitute a genetic resource potential, an array of techniques identifies and isolates these genes and transfer them into crops [2,5]. Therefore, in cases in which genetic variation is limited, then the most feasible approaches involve the application of transgenic and hybridization approaches to exploit the desirable traits genes from different species [6,7]. Hybridization between distant plant genera is a driver of genome evolution and new species formation. Distant hybridization generates novel germplasm by causing genetic recombination [8]. Where interesting genes have been identified and isolated, they have been transferred by transformation, however in cases where genes coding for certain traits have not been identified, wide hybridization has been the technique of preference.

Hybridization is the natural or artificial process of producing hybrids through crossing two individuals from different populations that are genetically different [9]. This process does not change the genetic contents of organisms but rather produces new combinations of genes which could have certain desirable characteristics or phenotypes. This technique also circumvents problems such as sexual incompatibility, polyembryony, and male or female sterility encountered in conventional sexual crossing [10]. In crop improvement, hybridization is done for one of the following reasons. Firstly, to create a variable plant population for selecting hybrids within these populations with certain desirable combination of characteristics. Secondly, to combine certain desirable characteristics in certain crops into a single individual or thirdly, to exploit and utilize hybrid varieties. Whatever the intension of the breeder, the overall aim of hybridization is always to create genetic variation when two genetically different plants are brought together in the first filial generation.

There are two main categories of hybridization techniques; sexual and somatic. Sexual hybridization, commonly known as wide or distant hybridization, hybrid combinations are produced within specific taxonomic distances. Sexual hybridization techniques have been used over time to produce better as well as new crops such as triticale, which is a crop species produced from the sexual cross between wheat (*Triticum vulgar*) and rye (*Secale cereale*) in 1875 [11]. However wide/distant hybridizations of individuals in different species and even genera have been achieved. When two species in the same genera are crossed, this is referred to as inter-specific hybridization, while crossing of two individuals in different genera is referred to as inter-generic hybridization. These kinds of crossing are important because they break species barriers for transfer of genes and therefore, make it possible to transfer genomes of one species to another which results in phenotypic or genotypic changes in the progeny [12].

Somatic hybridization on the other hand results when somatic cells are fused instead of gametes. This technique unlike sexual hybridization is

done *in vitro* and requires specific handling of the materials to be fused [6]. Precisely, somatic hybridization is done via protoplast fusion and it has become an important tool for ploidy manipulation in plant improvement schemes, allowing researchers to combine somatic cells from different cultivars, species, or genera, resulting in novel allotetraploid and autotetraploid genetic combinations [13]. After the successful establishment of plant protoplast isolation and fusion techniques, this hybridization strategy was realized, first by fusing the protoplasts of *Nicotiana tabacum* and *Nicotiana glauca* [14]. In the gramineae family, the first ever somatic hybrid plantlet was a protoplast fusion of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola*), which was done in 1987 [15].

This technique can facilitate conventional breeding, transfer of genes such as disease resistance genes, rapid growth rate genes, more product formation rate genes, drought resistance genes and heat or cold resistance genes, from one species to another, and cultivar development by bypassing some problems associated with conventional sexual hybridization including sexual incompatibility, nucellar embryogenesis, and male or female sterility [13,16].

This write-up provides an overview regarding the utilization of sexual and somatic hybridization as a method of transferring alien genes to crop species. The potential of somatic hybridization for restoring ploidy level in polyploid species after breeding at reduced ploidy level, as well as the challenge of resynthesizing allopolyploid species, will also be discussed. Focus on documented work in crops belonging to Gramineae family, methodologies used and the fate of the transferred alien DNA in the specific hybrids and their progeny will be highlighted.

2. SOMATIC HYBRIDIZATION

Plant protoplasts can be prepared by treatment of plant cells with specific lytic enzymes which remove the cell wall [16]. Protoplast fusion is a physical process during which two or more protoplasts come into contact with each other in the presence of fusion-inducing agents like polyethylene glycol (PEG) [16,17]. This is an inexpensive and rapid mechanism whereby two genetically different protoplasts, isolated from somatic cells, are fused to obtain parasexual hybrid protoplasts containing heteroplasmic cytoplasm and two fused parent nuclei [16]. Protoplasts of a variety of plants can be fused using PEG, and the hybrid products will regenerate cell walls and divide [16,18].

3. CLASSIFICATION OF SOMATIC HYBRIDS

Somatic hybrids can be classified into three types: symmetric somatic hybrids, asymmetric somatic hybrids, and cytoplasmic hybrids (cybrids) based on how they are developed [19]. Symmetric somatic hybridization refers to the combination of nuclear and cytoplasmic genetic information of both parents [20]. Asymmetric somatic hybridization is incomplete, with the loss of some cytoplasmic or nuclear DNA, and this type of hybridization has been used to introduce fragments of the nuclear genome from one parent (donor) into the intact genome of another one (recipient) [21]. Cybrids harbor only one parental nuclear genome and either the cytoplasmic genome of the other (non-nuclear) parent or a combination of both parents [22]. Both symmetric and asymmetric fusion experiments can generate these three types of somatic hybrids [23]. With the development of somatic hybridization technology, many new avenues have been adopted to create somatic hybrids. The evolution of such techniques is continuing, as [24] recently obtained asymmetric hybrids in sunflower via microprotoplast fusion with partial chromosome transfer from the micronuclear parent.

4. METHODS TO PRODUCE CYBRIDS

Symmetric hybrids often have no economic value because of the associated increase in ploidy level, and the combining of all nuclear encoded traits of both parents. Cybridization is a more attractive alternative for crop improvement because one or more traits can be added while maintaining cultivar integrity (just as with genetic transformation). Three methods are routinely used to create cybrids.

4.1 Asymmetric Fusion Treatment

Cybrids can be obtained by asymmetric fusion between irradiated donor protoplasts whose nuclei have been destroyed, and recipient protoplasts whose organelle genomes usually have been metabolically inhibited by iodoacetate (IOA). As a result, the heterokaryons combine vital cytoplasm from the donor parent with the intact nucleus from the recipient parent, resulting in the creation of asymmetric hybrids or

cybrids [25]. In addition to donor-recipient asymmetric hybridization, IOA treatment of one parent (or irradiation of one parent) and keeping the other parent intact can also be applied to create cybrids. Some previous researchers [26] once obtained cybrids via protoplast fusion between mesophyll protoplasts of a chlorophyll deficiency mutant *Lycopersicon peruvianum var. dentatum* and gamma-irradiated mesophyll protoplasts of *L. esculentum*.

4.2 Cytoplast Isolation and Fusion

Cytoplast-protoplast fusion was introduced first between protoplasts of *Nicotiana tabacum* and *Nicotiana plumbaginifolia* [27]. Presently, two procedures for eliminating the nuclear DNA are used, one is by cytochalasin B treatment [28], and the other is by a discontinuous percoll/mannitol gradient ultracentrifugation [29]. This method can also realize transfer of organelle-encoded traits to obtain cybrids [30]. For example, [31] used this method to isolate cytoplasts. Because many nucleated protoplasts were present, the cytoplast/protoplast fraction was then subjected to gamma- irradiation, and finally they successfully transferred a desirable male-sterile cytoplasm into cabbage.

4.3 Cybrids Produced by Symmetric Fusion

Besides asymmetric fusion and cytoplast-protoplast fusion, intraspecific, interspecific or intergeneric symmetric hybridization can spontaneously produce cybrids in higher plants. This is a common phenomenon in some species, especially tobacco and citrus. In interspecific symmetric somatic hybridization in tobacco (*Nicotiana tabacum* and *N. suaveolens*), cybrids with carpelloid stamens were obtained [32]. Citrus cybrids can sometimes be produced as a byproduct from the application of standard symmetric somatic hybridization procedures. To date, more than 40 of 250 parental combinations produced cybrids via symmetric fusion [19].

5. SOMATIC FUSION METHODS

The two primary somatic fusion methods are polyethylene glycol (PEG) induced fusion and electrofusion [33,34]. PEG induced fusion is advantageous in that it does not require special equipment, low cost, and high frequency of heterokaryon formation. Electrofusion relies on two different electrical pulses. Protoplasts are brought into intimate contact during the first pulse called di-electrophoresis; and the second pulse is a very short burst of intense direct current, which results in membrane fusion. Electrofusion has the advantages of convenience, no cell toxicity, and high frequency heterokaryon formation.

6. SELECTION SCHEMES FOR SOMATIC HYBRIDIZATION

For successful somatic hybrid regeneration, it is necessary to select the hybrid products from among the unfused and homo-fused protoplasts. An efficient selection system avoids the tedious identification of somatic hybrids among large numbers of regenerated calli or plants. Several schemes have been developed for somatic hybrid selection. These schemes include selective media; metabolic inhibitors [35], complementation systems such as chlorophyll deficiency complementation [36], auxotroph complementation, resistance markers and double mutants [37]; individual selection and culture, and application of the green fluorescent protein (GFP) marker gene.

The GFP gene has been a newly exploited marker to select somatic hybrids. It originates from the aquatic jellyfish *Aequorea victora* and emits stable and distinctive green fluorescence when expressed by living cells, without any cofactors or substrates but oxygen [38]. For this reason, transgenic plants expressing the GFP gene have been recently used as a parent in somatic hybridization. The potential of GFP as a somatic hybridization marker was first documented by using a transgenic citrange plant expressing GFP as a parent in a somatic fusion experiment [39]. GFP was shown to be useful for the continuous monitoring of the fusion process, identification of hybrid colonies, and selection of somatic hybrid embryos or plants. Guo & Grosser [40] further used the GFP marker in citrus somatic fusion and provided direct evidence of somatic hybrid vigor.

7. SEXUAL HYBRIDIZATION

Sexual hybridization is an important tool to plant breeders which enables the transfer of desirable traits from one species to another [41]. The steps of sexual hybridization involve a series of events which include germination of the pollen, pollinating the maternal taxa with pollen from the paternal taxa, growth of the pollen tube, fertilization, embryo and endosperm development and seed maturation [42].

8. TYPES OF SEXUAL HYBRIDIZATION

There are two main types of sexual hybridization which include intergeneric and interspecific hybridization. Interspecific hybridization involves the cross-fertilization between two species while intergeneric hybridization is the cross-fertilization between two genera that produces an offspring with phenotypic and genotypic traits of both parents promoting genetic diversity and evolution [43]. The major advantages of hybridization include the disease resistance, wider adaptation, increased fitness, higher yield and development of new improved crop varieties [44].

9. IMPACTS OF SEXUAL HYBRIDIZATION

9.1 Heterosis

Heterosis is a hybrid phenomenon which involves phenotypic superiority than their parents in terms of biotic and abiotic resistance, increased yield and growth rate [45]. Heterosis increases as the genetic variation of the crossing parental taxa increases [46]. In further hybridization generations, further disruptions of the parental linkages will result in decreased fitness or increased fitness than the parental taxa as extreme phenotypes such as superior fitness is selected [47]. Three models, dominance, overdominance and epistasis concepts have been proposed to demonstrate how heterosis occurs in hybrids [45]. Precisely, dominance concept involves the presence of recessive deleterious alleles in different loci of one parent masked by the beneficial alleles from the other crossing parental taxa in the F1 hybrid. Overdominance concept explains that at the loci controlling the heterosis, the presence of the heterozygote genotype that is superior to both the homozygous genotypes of the two crossing parents [9]. Epistasis involves the favorable interaction of gene combinations within the hybrids resulting in hybrid superiority [45]. Other studies explain that the exhibition of heterosis occurs as a result of multiple genetic occurrences due to simultaneous effects of dominance, overdominance, epigenetics and epistasis [9]. However, research has shown that heterosis in some cases can be as a result of a single over-dominant gene [45]. Additionally, small interfering RNA and micro-RNAs have been linked to heterosis by F1 hybrids showing an increased expression levels outside the parental taxa range [9]. For example, the intersubspecific hybridization between *Oryza sativa japonica* and *Oryza sativa indica* resulted in F1 hybrids exhibiting heterosis for spikelet fertility and harvest index [48]. Additionally, wheat and rye hybrids have showed heterotic effect on the yield due to increased spike density and biomass [49]. Additionally, *Zea mays* and *Tripsacum dactyloides* F1 hybrids exhibited increased salinity tolerance than both their parents [50,51].

9.2 Sterility and Inviability

Sterility and inviability are the main post-zygotic fertilization barriers to hybridization [52]. They limit gene flow resulting to fewer evolutionary consequences. However, when hybridization results to gene flow within different species, then evolutionary consequences manifest [44]. The main purpose of hybrid sterility is reproduction isolation to inhibit gene flow in order to maintain species identity [53]. Hybrid sterility is manifested by low grain yield, failure to form grain or pollen inviability [54]. Inviability is exhibited by formation of inviable seeds or weak and unfit germinated hybrids that are too frail to grow to maturity and survive [55].

Decreased fertility is as a result of reduced gamete formation and chromosomal rearrangements within the hybrids [56]. Hybrid sterility increases as the divergence between the crossing parental taxa increases [57]. Precisely, decreased fertility is more pronounced when divergence between crossing parental taxa is more than 4 million years [58]. This is because of the accumulation of inter-locus incompatibilities between the diverging populations [59].

Hybrid sterility and inviability is well explained by the Dobzhansky–Muller model which states that a genetic change due to divergence in loci in a population and a genetic change in the same loci in the second crossing population results to incompatibilities when the two genomes are hybridized resulting to post-zygotic incompatibilities and therefore, infertility and inviability is exhibited [60]. A cross between *Sorghum bicolor* and *Saccharum officinarum* resulted in a 53% fertility while previous crosses showed a fertility rate of 0.13% [61]. A cross between *Avena sativa* and *Zea mays* formed hybrids that exhibited partial fertility [62]. Inviability was evidently exhibited between *Zea mays* and *Trypsacum dactyloides* hybrids whereby 80% of the F1 hybrid seeds could not germinate. Furthermore, another study of the same cross showed the hybrids had pollen fertility ranging from 0% to 50% [63]. In certain

crosses, hybridization can result to absolute inviability. For example, *Triticum durum* and *Aegilops umbellulata* hybrid seeds were unable to germinate [64].

To overcome the phenomenon of sterility in hybrids, chromosome doubling can be employed by application of colchicine, Amiprophos-methyl or pronamid treatment [65]. Since the principle behind most infertility in plant hybrids is that chromosomes lack a pairing partner during meiosis, doubling of the parental sets of chromosomes ensures that pairing can take place within each set, allowing meiosis to proceed hence production of fertile gametes. The chromosome doubling technique results in amphidiploids as observed on *Syringa vulgaris* × *S. pinnatifolia* hybrids [66].

9.3 Hybrid Breakdown

Hybrid breakdown acts as a reproduction isolation at the second filial generation of the hybrids [67]. This phenomenon is manifested by the development of sterility and inviability in the F2 hybrids while their parental filial generation is fertile and viable [68]. This occurs due to the disrupted interaction of different loci during gene segregation creating incompatibility between the interacting genes after the first filial generation [69]. Previous studies in the F2 hybrids of *Indica sp.* and *Japonica sp.* cross revealed an occurrence of hybrid breakdown due to complimentary of recessive sterility genes between the two species genomes in the hybrid [70].

9.4 Arrested Pollen Tube Growth

Arrested pollen tube growth is a pre-zygotic reproduction isolation mechanism that restrict gene flow between different species by inhibiting the formation of zygote [71]. Pre-zygotic barriers are often very strong in plants and contributed more total reproductive isolation than post-pollination barriers [72].

Delayed and arrested growth of pollen tube within the stigma of the crossing maternal taxa inhibits successful fertilization of the ovules. This is evident in a cross between *Zea mays* and *Sorghum bicolor* whereby the sorghum pollen tube growth was arrested and could not grow past the micropyle to fertilize the ovule [73]. However, this barrier can be overcome by the supplementation of auxin hormone to the pollinated parental taxa. For example, successful hybridization between *Triticum estivum* and *Zea mays* was achieved by spraying of the pollinated silk with 2, 4-D that increased successful fertilization from 18.7% to 69.3% by increasing the number of pollen tubes growing down the pistil [74]. Additionally, crosses between *Triticum aestivum* and *Leymus arenarius* were supplemented with 2, 4-D to promote fertilization between the two taxa [75]. A commonly used technique to overcome this impediment is the somatic hybridization that involves fusion of protoplast. For example, pollen tube arrest in a cross between *Cucumis sativus* and *Cucumis melo* was overcome by protoplast fusion but successful hybridization is limited [76].

9.5 Embryo Abortion

In some crosses, a hybrid embryo can be formed but the maternal plants perceive it as foreign and aborts it in a degeneration process characterized by shrivelling of the embryo [77]. Embryo abortion occurs due to failed development during the early stages of cell differentiation of the hybrid zygote [78]. Furthermore, embryo abortion is positively related to the asymmetry of the pollen donor and recipient parents [79]. Nevertheless, formed hybrid embryo can be salvaged through a tissue culture technique called embryo rescue [80,81]. Embryo rescue overcomes this barrier by culturing the immature embryo prior to abortion by the maternal plant [82]. This technique was successfully implemented in an interspecific hybridization within the *Leucadendron* genus [10]. In another study, an interspecific cross between wild and cultivated *Vigna unguiculata* was achieved by embryo rescue to overcome embryo abortion [83]. Furthermore, embryo rescue is used to overcome reproduction barrier in intergeneric hybridization between *chrysanthemum* and *Ajania przewalskii* [84].

9.6 Selection Schemes for Sexual Hybrids

There are various techniques of determining hybridity which include use of morphological markers, molecular markers, cytogenetic analysis and fluorescent *in situ* hybridization. Determination of the hybridity is important because sometimes the hybrid embryo may lose chromosomes of one parent in early development [76]. Phenotype of the hybrids is determined by observing specific morphological markers such as grain quality, leaf size and shapes, plant height, yield and duration [85].

However, these markers are quiet limited for hybrid recognition [86].

Molecular markers involve amplification of specific amplified fragment length polymorphism (AFLP) [87], rapid amplification of polymorphic DNA (RAPD) [88] and single sequence repeat polymorphism (SSR) [89] markers related to fertility restoration and specific ribosomal DNA sequences. Molecular markers are the most reliable for identification of hybrids due to their unlimited number in the genome in comparison to chemical profiling which is time-consuming and limited in predicting hybrid ancestry [86].

In most studies, hybridity test involves the use of various tests to determine true hybrids. A study involving *Sorghum bicolor* and *Sorghum macrospermum* hybrids involved determination of true hybrids by evaluation of the pubescence of leaves of the hybrids, a characteristic of *Sorghum macrospermum*, determination of chromosomes number, fluorescent *in situ* hybridization targeting CEN38 marker present in *Sorghum bicolor* while absent in *Sorghum macrospermum*, and specific amplification of the AFLP markers specific to each parent [80].

Screening for secondary metabolites is a reliable technique for hybridity test as hybrids express secondary metabolites quantitatively and qualitatively different from their parents [86,90]. Precisely, hybrids may express novel secondary metabolites, some of the parental taxa secondary metabolites in different quantities and qualities than their parents' secondary metabolites or completely fail to express some of the parents' secondary metabolites [91]. Therefore, hybrid secondary metabolites normally have complex patterns of inheritance in hybrids. The commonly evaluated secondary metabolites are the phenolic, terpenoid, alkaloid, isothiozyanates and flavonoid compounds and the commonly studied secondary metabolite is the flavonoid compound due to its high variability and stability [86].

10. CONCLUSION

Over the years, wide hybridization has provided a platform for non-transgenic approaches in crop improvement programmes. Despite the great potential it provides, it is still limited by the various disadvantages of certain hybrid disgenesis like sterility, segregation and distortions in sex ratios, high frequency mutations, changes in the structure of chromosomes, non-disjunctions and rearrangements in chromosomes as well as variegations in leaves and stems. There is need for future improvements in the wide hybridization techniques as a potential alternative to transgenic crop improvement strategies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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