1 Plant Hybridization as an Alternative Technique in Plant Breeding Improvement

2 ABSTRACT

For ages, plant breeders have relied on the genetic variability that results from sexually crossing 3 plants within the same species. However, the variability that exists within species populations is 4 5 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; 6 sexual and somatic. Sexual hybridization, also referred to as wide or distant hybridization involves 7 combining two genomes from different parental taxa through pollination, either naturally or by 8 induction. Somatic hybridization involves the fusion of somatic cells instead of gametes, which 9 highly depends on the ability to obtain viable protoplasts and eventually differentiate them to 10 whole plants *in vitro*. The impacts of hybrids can either be positive or negative. Among the positive 11 attributes of hybrids that has been exploited is heterosis, which results either from dominance, 12 over-dominance or epistasis. Negative ones include sterility, arrested growth of the pollen tube 13 and embryo abortion. To overcome these problems, chromosome doubling, the use of hormones 14 such as 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and embryo rescue have been employed to 15 overcome sterility, arrested growth of pollen tubes and embryo abortion respectively. After the 16 development of hybrids, different hybrid identification techniques have been used to test them 17 such as the use of molecular and morphological markers, cytogenetic analysis and fluorescent *in* 18 *situ* hybridization. The use of hybridization techniques in plant improvement remains a vital tool 19 to cross species barriers and utilization of important attributes in unrelated crop plants which could 20 not have been achieved through conventional techniques of plant breeding. 21

22 Key words: Hybridization, reproductive barriers, molecular markers

23 INTRODUCTION

Genetic variability within the species has been efficiently utilized by breeders in their efforts to 24 25 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 26 pool of crops [2,3]. Introduction of new traits in plants largely relied on sexual crosses between 27 different genotypes within or between closely related species [1]. However, due to the presence of 28 29 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 30 agronomically-interesting traits may only be found in distantly related species or even in unrelated 31 plants [5]. Since they constitute a genetic resource potential, an array of techniques identifies and 32 isolates these genes and transfer them into crops [2,5]. Therefore, in cases in which genetic 33 variation is limited, then the most feasible approaches involve the application of transgenic and 34 hybridization approaches to exploit the desirable traits genes from different species [6,7]. 35 Hybridization between distant plant genera is a driver of genome evolution and new species 36 formation. Distant hybridization generates novel germplasm by causing genetic recombination [8]. 37 Where interesting genes have been identified and isolated, they have been transferred by 38 transformation, however in cases where genes coding for certain traits have not been identified, 39 wide hybridization has been the technique of preference. 40

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 44 problems such as sexual incompatibility, polyembryony, and male or female sterility encountered 45 in conventional sexual crossing [10]. In crop improvement, hybridization is done for one of the 46 47 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 48 desirable characteristics in certain crops into a single individual or thirdly, to exploit and utilize 49 hybrid varieties. Whatever the intension of the breeder, the overall aim of hybridization is always 50 51 to create genetic variation when two genetically different plants are brought together in the first 52 filial generation.

53 There are two main categories of hybridization techniques; sexual and somatic. Sexual hybridization, commonly known as wide or distant hybridization, hybrid combinations are 54 produced within specific taxonomic distances. Sexual hybridization techniques have been used 55 over time to produce better as well as new crops such as triticale, which is a crop species produced 56 from the sexual cross between wheat (Triticum vulgar) and rye (Secale cereale) in 1875 [11]. 57 However wide/distant hybridizations of individuals in different species and even genera have been 58 59 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 60 hybridization. These kinds of crossing are important because they break species barriers for 61 transfer of genes and therefore, make it possible to transfer genomes of one species to another 62 63 which results in phenotypic or genotypic changes in the progeny [12].

64 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 65 materials to be fused [6]. Precisely, somatic hybridization is done via protoplast fusion and it has 66 67 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 68 allotetraploid and autotetraploid genetic combinations [13]. After the successful establishment of 69 70 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, 71 the first ever somatic hybrid plantlet was a protoplast fusion of rice (Oryza sativa L.) and barnyard 72 grass (Echinochloa oryzicola), which was done in 1987 [15]. 73 74 This technique can facilitate conventional breeding, transfer of genes such as disease resistance

rans 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.

- 85
- 86 I. Somatic hybridization

Plant protoplasts can be prepared by treatment of plant cells with specific lytic enzymes which 87 remove the cell wall [16]. Protoplast fusion is a physical process during which two or more 88 protoplasts come into contact with each other in the presence of fusion-inducing agents like 89 90 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 91 protoplasts containing heteroplasmic cytoplasm and two fused parent nuclei [16]. Protoplasts of a 92 variety of plants can be fused using PEG, and the hybrid products will regenerate cell walls and 93 94 divide [16,18].

94 ulviue [10,16].

95 Classification of somatic hybrids

96 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 97 somatic hybridization refers to the combination of nuclear and cytoplasmic genetic information of 98 both parents [20]. Asymmetric somatic hybridization is incomplete, with the loss of some 99 cytoplasmic or nuclear DNA, and this type of hybridization has been used to introduce fragments 100 of the nuclear genome from one parent (donor) into the intact genome of another one (recipient) 101 102 [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 103 104 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 105 hybrids. The evolution of such techniques is continuing, as [24] recently obtained asymmetric 106 hybrids in sunflower via microprotoplast fusion with partial chromosome transfer from the 107 108 micronuclear parent.

109 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.

115 **1. Asymmetric fusion treatment**

Cybrids can be obtained by asymmetric fusion between irradiated donor protoplasts whose nuclei 116 have been destroyed, and recipient protoplasts whose organelle genomes usually have been 117 metabolically inhibited by iodoacetate (IOA). As a result, the heterokaryons combine vital 118 cytoplasm from the donor parent with the intact nucleus from the recipient parent, resulting in the 119 creation of asymmetric hybrids or cybrids [25]. In addition to donor-recipient asymmetric 120 hybridization, IOA treatment of one parent (or irradiation of one parent) and keeping the other 121 parent intact can also be applied to create cybrids. Some previous researchers [26] once obtained 122 cybrids via protoplast fusion between mesophyll protoplasts of a chlorophyll deficiency mutant 123 Lycopersicon peruvianum var. dentatum and gamma-irradiated mesophyll protoplasts of L. 124 125 esculentum.

126 **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.

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].

143 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.

151 Selection schemes for somatic hybridization

For successful somatic hybrid regeneration, it is necessary to select the hybrid products from 152 among the unfused and homo-fused protoplasts. An efficient selection system avoids the tedious 153 identification of somatic hybrids among large numbers of regenerated calli or plants. Several 154 schemes have been developed for somatic hybrid selection. These schemes include selective 155 media; metabolic inhibitors [35], complementation systems such as chlorophyll deficiency 156 complementation [36], auxotroph complementation, resistance markers and double mutants [37]; 157 individual selection and culture, and application of the green fluorescent protein (GFP) marker 158 159 gene.

160 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 161 expressed by living cells, without any cofactors or substrates but oxygen [38]. For this reason, 162 transgenic plants expressing the GFP gene have been recently used as a parent in somatic 163 hybridization. The potential of GFP as a somatic hybridization marker was first documented by 164 165 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 166 hybrid colonies, and selection of somatic hybrid embryos or plants. Guo & Grosser [40] further 167 168 used the GFP marker in citrus somatic fusion and provided direct evidence of somatic hybrid vigor.

169 II. 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 173 paternal taxa, growth of the pollen tube, fertilization, embryo and endosperm development and

seed maturation [42].

175 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].

182 Impacts of sexual hybridization

183 Heterosis

184 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 185 the genetic variation of the crossing parental taxa increases [46]. In further hybridization 186 generations, further disruptions of the parental linkages will result in decreased fitness or increased 187 fitness than the parental taxa as extreme phenotypes such as superior fitness is selected [47]. Three 188 models, dominance, overdominance and epistasis concepts have been proposed to demonstrate 189 190 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 191 the other crossing parental taxa in the F₁ hybrid. Overdominance concept explains that at the loci 192 controlling the heterosis, the presence of the heterozygote genotype that is superior to both the 193 homozygous genotypes of the two crossing parents [9]. Epistasis involves the favorable interaction 194 of gene combinations within the hybrids resulting in hybrid superiority [45]. Other studies explain 195 196 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, 197 research has shown that heterosis in some cases can be as a result of a single over-dominant gene 198 199 [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, 200 the intersubspecific hybridization between Oryza sativa japonica and Oryza sativa indica resulted 201 in F₁ hybrids exhibiting heterosis for spikelet fertility and harvest index [48]. Additionally, wheat 202 and rye hybrids have showed heterotic effect on the yield due to increased spike density and 203 204 biomass [49]. Additionally, Zea mays and Tripsacum dactyloides F_1 hybrids exhibited increased salinity tolerance than both their parents [50,51]. 205

206 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 217 crossing parental taxa is more than 4 million years [58]. This is because of the accumulation of 218 inter-locus incompatibilities between the diverging populations [59].

Hybrid sterility and inviability is well explained by the Dobzhansky–Muller model which states 219 that a genetic change due to divergence in loci in a population and a genetic change in the same 220 loci in the second crossing population results to incompatibilities when the two genomes are 221 hybridized resulting to post-zygotic incompatibilities and therefore, infertility and inviability is 222 exhibited [60]. A cross between Sorghum bicolor and Saccharum officinarum resulted in a 53 % 223 fertility while previous crosses showed a fertility rate of 0.13 % [61]. A cross between Avena sativa 224 and Zea mays formed hybrids that exhibited partial fertility [62]. Inviability was evidently 225 exhibited between Zea mays and Trypsacum dactyloides hybrids whereby 80 % of the F1 hybrid 226 seeds could not germinate. Furthermore, another study of the same cross showed the hybrids had 227 pollen fertility ranging from 0 % to 50 % [63]. In certain crosses, hybridization can result to 228 absolute inviability. For example, Triticum durum and Aegilops umbellulata hybrid seeds were 229 unable to germinate [64]. 230

- 231 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* \times *S. pinnatifolia* hybrids [66].

237 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 F_2 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 F_2 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].

245 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
- 249 barriers [72].
- Delayed and arrested growth of pollen tube within the stigma of the crossing maternal taxa inhibits 250 251 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 252 micropyle to fertilize the ovule [73]. However, this barrier can be overcome by the 253 supplementation of auxin hormone to the pollinated parental taxa. For example, successful 254 hybridization between Triticum estivum and Zea mays was achieved by spraying of the pollinated 255 silk with 2, 4-D that increased successful fertilization from 18.7 % to 69. 3% by increasing the 256 number of pollen tubes growing down the pistil [74]. Additionally, crosses between Triticum 257 aestivum and Leymus arenarius were supplemented with 2, 4-D to promote fertilization between 258 the two taxa [75]. A commonly used technique to overcome this impediment is the somatic 259

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].

263 Embryo abortion

In some crosses, a hybrid embryo can be formed but the maternal plants perceive it as foreign and 264 aborts it in a degeneration process characterized by shrivelling of the embryo [77]. Embryo 265 abortion occurs due to failed development during the early stages of cell differentiation of the 266 hybrid zygote [78]. Furthermore, embryo abortion is positively related to the asymmetry of the 267 pollen donor and recipient parents [79]. Nevertheless, formed hybrid embryo can be salvaged 268 through a tissue culture technique called embryo rescue [80,81]. Embryo rescue overcomes this 269 barrier by culturing the immature embryo prior to abortion by the maternal plant [82]. This 270 technique was successfully implemented in an interspecific hybridization 271 within the Leucadendron genus [10]. In another study, an interspecific cross between wild and cultivated 272 Vigna unguiculata was achieved by embryo rescue to overcome embryo abortion [83]. 273 Furthermore, embryo rescue is used to overcome reproduction barrier in intergeneric 274 275 hybridization between chrysanthemum and Ajania przewalskii [84].

276 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

- 302 flavonoid compounds and the commonly studied secondary metabolite is the flavonoid compound
- due to its high variability and stability [86].

304 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.

333 **REFERENCES**

- Govindaraj M, Vetriventhan M, Srinivasan M. Importance of genetic diversity assessment in crop plants and its recent advances: An overview of its analytical perspectives. Genetics Research International. 2015;1-14. doi:10.1155/2015/431487.
- 337
- Fu YB. Understanding crop genetic diversity under modern plant breeding. Theoretical and
 Applied Genetics. 2015;128(11):2131-2142.
- Migicovsky Z, Myles S. Exploiting Wild Relatives for Genomics-assisted Breeding of
 Perennial Crops. Front Plant Sci. 2017;8(460).
- Limera C, Sabbadini S, Sweet JB, Mezzetti B. New Biotechnological Tools for the Genetic
 Improvement of Major Woody Fruit Species. Front Plant Sci. 2017;(8):1418.
- Waara S, Glimelius K. The potential of somatic hybridization in crop breeding. Euphytica.
 1995;85(1-3):217-233.
- 6. Glimelius K. Sexual and somatic hybridization. Hereditas Suppl. 1985; (3):41-47.
- Hodnett GL, Hale AL, Packer DJ, Stelly DM, Silva J, Rooney WL. Elimination of a reproductive barrier facilitates intergeneric hybridization of *Sorghum bicolor* and *saccharum*. Crop Sci. 2010;50(4):1188–1195.
- Gaskin JF, Wheeler GS, Purcell MF, Taylor GS. Molecular evidence of hybridization in Florida's sheoak (*Casuarina spp.*) invasion. Mol Ecol. 2009;18(15):3216–3226.
- Goulet BE, Roda F, Hopkins R. Hybridization in Plants: Old Ideas, New Techniques. Plant
 Physiol. 2017;173(1):65–78. doi.org/10.1104/pp.16.01340
- Liu J, Xu X, Deng X. Intergeneric somatic hybridization and its application to crop genetic
 improvement. Plant Cell Tissue Organ Cult. 2005;82(1):19–44.
- MacIntyre R, Campbell M. Triticale. Proceedings of an international symposium [Internet].
 Mexico: International Development Research Centre; 1973 [cited 14 March 2019]. p. 1314. Available from: https://idl-bnc-
- idrc.dspacedirect.org/bitstream/handle/10625/18349/IDL-18349.pdf?sequence=1.
- Liu D, Zhang H, Zhang L, Yuan Z, Hao M, Zheng Y. Distant Hybridization: A Tool for
 Interspecific Manipulation of Chromosomes. In: Alien Gene Transfer in Crop Plants. New
 York, NY: Springer; 2014. p. 25–42.
- 363 13. Grosser JW, Gmitter FG. Protoplast fusion for production of tetraploids and triploids:
 364 Applications for scion and rootstock breeding in citrus. Plant Cell Tissue and Organ Cult.
 365 2011. 104(3):343-357. doi: 10.1007/s11240-010-9823-4.
- 14. Carlson PS, Smith HH, Dearing RD. Parasexual Interspecific Plant Hybridization. Proc Natl
 Acad Sci. 1972;69(8):2292–2294.
- Terada R, Kyozuka J, Nishibayashi S, Shimamoto K. Plantlet regeneration from somatic
 hybrids of rice (*Oryza sativa L.*) and barnyard grass (*Echinochloa oryzicola Vasing*). Mol
 Gen Genet. 1987;210(1):39–43.
- Abomohra AE-F, El-Sheekh M, Hanelt D. Protoplast fusion and genetic recombination
 between *Ochromonas danica* (Chrysophyta) and *Haematococcus pluvialis* (Chlorophyta).
 Phycologia. 2016; 55(1).
- 17. Durieu P, Ochatt SJ. Efficient intergeneric fusion of pea (*Pisum sativum L*.) and grass pea
 (*Lathyrus sativus L*.) protoplasts. J Exp Bot. 2000;51(348):1237–1242.
 doi:10.1093/jexbot/51.348.1237.
- Mishra D, Vishnupriya MR, Anil MG, Konda K, Sonti R V. Pathotype and Genetic
 Diversity amongst Indian Isolates of *Xanthomonas oryzae pv*. oryzae. PLoS One.

- 379 2013;8(11):1–11.
- Guo WW, Xiao SX, Deng XX. Somatic cybrid production via protoplast fusion for citrus
 improvement. Scientia Horticulturae. 2013;163:20-26. doi:10.1371/journal.pone.0081996.
- Polzerová H, Patzak J, Greplová M. Early characterization of somatic hybrids from
 symmetric protoplast electrofusion of *Solanum pinnatisectum Dun*. and *Solanum tuberosum*
- 284 *L*. Plant Cell Tissue Organ Cult. 2011; 104(2): 163-170. doi: 10.1007/s11240-010-9813-6.
- Tapingkae T, Zulkarnain Z, Kawaguchi M, Ikeda T, Taji A. Somatic (asexual) procedures
 (haploids, protoplasts, cell selection) and their applications. In: Plant Biotechnology and
 Agriculture. 2012;141-162.doi: 10.1016/B978-0-12-381466-1.00010-9.
- Fatta Del Bosco S, Napoli E, Mercati F, Abbate L, Carimi F, Ruberto G. Somatic cybridization for Citrus: polyphenols distribution in juices and peel essential oil composition of a diploid cybrid from Cleopatra mandarin (*Citrus reshni Hort. ex Tan.*) and sour orange (*Citrus aurantium L.*). Genet Resour Crop Evol. 2017;64(2):261-275. doi: 10.1007/s10722-015-0348-x.
- Zhang F, Wang P, Ji D, Kang G, Xiang F. Asymmetric somatic hybridization between
 Bupleurum scorzonerifolium Wild. and *Taxus chinensis var. mairei.* Plant Cell Rep.
 2011;30(10):1857-1864. doi: 10.1007/s00299-011-1093-z.
- Binsfeld PC, Wingender R, Schnabl H. Characterization and molecular analysis of
 transgenic plants obtained by microprotoplast fusion in sunflower. Theor Appl Genet.
 2000;101(8):1250-1258.
- Varotto S, Nenz E, Lucchin M, Parrini P. Production of asymmetric somatic hybrid plants
 between *Cichorium intybus L.* and *Helianthus annuus L.* Theor Appl Genet. 2001;
 102(6):950-956.
- 402
 403
 403
 404
 404
 405
 26. Kochevenko A, Ratushnyak Y, Kornyeyev D, Stasik O, Porublyova L, Kochubey S, Suprunova T, Gleba Y. Functional cybrid plants of *Lycopersicon peruvianum var 'dentatum'* with chloroplasts of *Lycopersicon esculentum*. Plant Cell Rep. 2000;19(6):588-597.
- 406 27. Maliga P, Lörz H, Lázár G, Nagy F. Cytoplast-protoplast fusion for interspecific chloroplast transfer in *Nicotiana*. Mol Gen Genet. 1982;185(2):211–215.
- 408 28. Bracha M, Sher N. Fusion of enucleated protoplasts with nucleated miniprotoplasts in onion
 409 (*Allium cepa L.*). Plant Sci Lett. 1981; 23(1):95-101.
- 410 29. Lörz H, Paszkowski J, Dierks-Ventling C, Potrykus I. Isolation and characterization of
 411 cytoplasts and miniprotoplasts derived from protoplasts of cultured cells. Physiol Plant.
 412 1981;53(3):385–391. doi.org/10.1016/0304-4211(81)90030-4.
- 30. Omar AA, Murata M, Yu Q, Gmitter FG, Chase CD, Graham JH, et al. Production of three new grapefruit cybrids with potential for improved citrus canker resistance. Vitr Cell Dev Biol Plant. 2017;53(3):256–269. doi: 10.1007/s11627-017-9816-7.
- 416 31. Sigareva MA, Earle ED. Direct transfer of a cold-tolerant Ogura male-sterile cytoplasm into cabbage (*Brassica oleracea ssp. capitata*) via protoplast fusion. Theor Appl Genet. 1997;
 418 94(2):213-220.
- 419 32. Fitter JT, Thomas MR, Niu C, Rose RJ. Investigation of *Nicotiana tabacum* (+) *N.*420 *suaveolens* cybrids with carpelloid stamens. J Plant Physiol. 2005;162(2):225–235. doi: org/10.1016/j.jplph.2004.02.006.
- 33. Belete T. A Review on Somatic Hybridization and Its Utilization in Crop Improvement. Int
 J African Asian Stud. 2018;43:24–34.

- 424 34. Grosser, J. W., and Gmitter, F. G. (1990). Protoplast fusion and citrus improvement. Plant
 425 Breed. Rev. 8, 339–374. doi: 10.1002/9781118061053.ch10.
- Shimonaka M, Hosoki T, Tomita M, Yasumuro Y. Production of Somatic Hybrid Plants
 between Japanese Bunching Onion (*Allium fistulosum L.*). and Bulb Onion (*A. cepa L.*)
 via Electrofusion. Japan Soc Hort Sci. 2002; 71(5):623-631.doi: 10.2503/jjshs.71.623.
- 429 36. Dragoeva A, Atanassov I, Atanassov A. CMS due to tapetal failure in cybrids between
 430 *Nicotiana tabacum* and *Petunia hybrida*. Plant Cell Tissue Organ Cult. 1998; 55(1):67-70.
- 431 37. Conner AJ, Tynan JL. A maternally inherited, chlorophyll-deficient mutant of a transgenic
 432 kanamycin-resistant *Nicotiana plumbaginifolia* plant. Int J Plant Sci. 1995; 156(3):320-325.
- 433 38. Tsien RY. The green fluorescent protein. Annu Rev Biochem. 1998; 67:509-544. doi:
 434 10.1086/297253.
- 435 39. Olivares-fuster O, Pena L, Duran-Vila N, Navarro L. Green Fluorescent Protein as a Visual
 436 Marker in Somatic Hybridization. Ann Bot. 2002;89:491–497. doi: 10.1093/aob/mcf054.
- 437 40. Guo W, Grosser JW. Somatic hybrid vigor in Citrus : Direct evidence from protoplast fusion
 438 of an embryogenic callus line with a transgenic mesophyll parent expressing the GFP
 439 gene.Plant science. 2005;168:1541–1545.doi:10.1016/j.plantsci.2005.02.029.
- 41. Zeliang PK, Pattanayak A. Wide Hybridization in the Genus *Oryza*: Aspects and Prospects.
 441 Indian J Hill Farming. 2013;26(2):71–77.
- 42. Debbarama C. Wide Hybridization and Embryo-Rescue for Crop Improvement in Capsicum. Agrotechnology. 2013;01(S11). doi: 10.4172/2168-9881.S11-003.
- 444 43. Chen J, Luo M, Li S, Tao M, Ye X, Duan W, Zhang C, Qin Q, Xiao J, Liu S. A comparative study of distant hybridization in plants and animals. Science China Life Sciences. 2018.
 446 61(3):285-309. doi: 10.1007/s11427-017-9094-2
- 447 44. Zelaya I, Micheal D. K. Owen MJV. Transfer of glyphosate resistance : evidence of. hybridization in Conyza (Asteraceae). Am J Bot.2007;94(4):660–673. doi:10.3732/ajb.94.4.660.
- 45. Yang L, Li B, Zheng XY, Li J, Yang M, Dong X, He G, An C, Deng XW. Salicylic acid
 451 biosynthesis is enhanced and contributes to increased biotrophic pathogen resistance in
 452 Arabidopsis hybrids. Nat Commun. 2015;6:1–11. doi: 10.1038/ncomms8309.
- 46. Birchler JA. In Search of the Molecular Basis of Heterosis. Plant Cell Online [Internet].
 2003;15(10):2236–2239. doi: 10.1105/tpc.151030.
- 47. Edmands S, Feaman H V., Harrison JS, Timmerman CC. Genetic consequences of many generations of hybridization between divergent copepod populations. J Hered.
 457 2005;96(2):114–123. doi: 10.1093/jhered/esi014.
- 48. Murayama S, Abu M, Sarker Z. Agronomic Performance of F 1 Hybrids of Rice (*Oryza sativa L*.) in *Japonica-Indica* Crosses. Plant Prod Sci. 2015;1008. doi:10.1626/pps.5.203.
- 460 49. Owuoche JO, Sears RG, Brown-Guedira GL, Gill BS, Fritz AK. Heterotic effects of wheat461 rye chromosomal translocations on agronomic traits of hybrid wheat (*Triticum aestivum L.*)
 462 under an adequate moisture regime. Euphytica. 2003;132(1):67–77.
- 463 50. Pesqueira J, García MD, Molina MC. NaCl tolerance in maize (*Zea mays ssp. mays*) x
 464 *Tripsacum dactyloides L*. hybrid calli and regenerated plants. Spanish J Agric Res.
 465 2003;1(2):59–63.

- Shavrukov Y, Sokolov V. Maize-gamagrass interspecific hybrid, *Zea mays x Tripsacum* dactyloides, shows better salinity tolerance and higher NA + exclusion than maize and sorghum. Electronic journal of Biotech. 2015;4 (1):128-133. doi: 10.2225/vol9-issue3-fulltext-25.
- 470 52. Bushell C, Spielman M, Scott RJ. The basis of natural and artificial postzygotic
 471 hybridization barriers in Arabidopsis species. Plant Cell. 2003;15:1430–1442. doi:
 472 10.1105/tpc.010496.
- Koide Y, Ogino A, Yoshikawa T, Kitashima Y, Saito N, Kanaoka Y, Onishi K, Yoshitake
 Y, Tsukiyama T, Saito H, Teraishi M, Yamagata Y, Uemura A, Takagi H, Hayashi Y, Abe
 T, Fukuta Y, Okumoto Y, Kanazawa A. Lineage-specific gene acquisition or loss is
 involved in interspecific hybrid sterility in rice. Proc Natl Acad Sci. 2018; 115(9):19551962. doi: 10.1073/pnas.1711656115.
- 478 54. Kubo T. Genetic mechanisms of postzygotic reproductive isolation: An epistatic network
 479 in rice. Breed Sci. 2013; 63(4): 359–366. doi: 10.1270/jsbbs.63.359.
- 480 55. Scopece G, Widmer A, Cozzolino S. Evolution of Postzygotic Reproductive Isolation in a
 481 Guild of Deceptive Orchids. Am Nat. 2008; 171(3):315-326.
- 482 56. Johnson N. Hybrid Incompatibility and Speciation. Nature Education. 2008; 1(1):20.
- 483 57. Edmands S. Does parental divergence predict reproductive compatibility? Trends in
 484 Ecology and Evolution. 2002; 17(11):520-527. doi: 10.1086/527501.
- 485 58. Levin DA. The long wait for hybrid sterility in flowering plants. New Phytol.
 486 2012;196(3):666–670. doi: 10.1111/j.1469-8137.2012.04309.x.
- 59. Sweigart AL, Fishman L, Willis JH. A simple genetic incompatibility causes hybrid male sterility in mimulus. Genetics. 2006;172(4):2465–2479. doi: 10.1534/genetics.105.053686.
- Wang H, Bennetzen JL. Centromere retention and loss during the descent of maize from a tetraploid ancestor. Proc Natl Acad Sci. 2012;109(51):21004–21009. doi: 10.1073/pnas.1218668109.
- 492 61. Nair N V. Production and cyto-morphological analysis of intergeneric hybrids of *Sorghum* x *Saccharum*. Euphytica. 1999; 108(3):187-191. doi: 10.1023/A:1003633015836.
- 494 62. Riera Lizarazu O, Rines HW, Phillips RL. Cytological and molecular characterization of
 495 oatXmaize partial hybrids. Theor appl Genet.1996;93(1/2):123–135. doi:
 496 10.1007/BF00225737.
- 497 63. Molina MDC, García MD, Chorzempa SE. Meiotic study of *Zea mays ssp. mays* (2n = 40)498 x *Tripsacum dactyloides* (2n = 72) hybrid and its progeny. Electron J Biotechnol. 499 2006;9(3):276–280.
- Hadzhiivanova B, Bozhanova V, Dechev D. Interspecific hybridization between durum
 wheat and *Aegilops umbellulata (Zhuk.)*. Bulg J Agric Sci. 2012;18(5):713–921.
- Melchinger AE, Molenaar WS, Mirdita V, Schipprack W. Colchicine alternatives for chromosome doubling in maize haploids for doubled- haploid production. Crop Sci. 2016;56 (2):1-41. doi: 10.2135/cropsci2015.06.0383.
- 505 66. Rose JB, Kubba J, Tobutt KR. Chromosome doubling in sterile *Syringa vulgaris* \times *S.* 506 *pinnatifolia* hybrids by *in vitro* culture of nodal explants. Plant Cell Tissue Organ Cult.

- 507 2000; 6(2):127–132.
- 508 67. Stelkens RB, Schmid C, Seehausen O. Hybrid breakdown in cichlid fish. PLoS One. 2015;
 509 10(5): e0127207. doi: 10.1371/journal.pone.0127207.
- 510 68. Kubo T, Yoshimura A. Genetic basis of hybrid breakdown in a *Japonica/Indica* cross of
 511 rice, *Oryza sativa L*. Theor Appl Genet. 2002;105(6–7):906–911. doi:
 512 10.1371/journal.pone.0127207.
- 69. Oka A, Mita A, Sakurai-Yamatani N, Yamamoto H, Takagi N, Takano-Shimizu T,
 Toshimori K, Moriwaki K, Shiroishi T. Hybrid Breakdown Caused by Substitution of the
 X Chromosome between Two Mouse Subspecies. Genetics. 2004; 166(2):913-924.
- 516 70. Li Z, Pinson SRM, Paterson AH, Park WD, Stansel JW. Genetics of hybrid sterility and hybrid breakdown in an intersubspecific rice (*Oryza sativa L.*) population. Genetics.
 518 1997;145(4):1139–1148.
- 519 71. Dickinson GR, Lee DJ, Wallace HM. The influence of pre- and post-zygotic barriers on interspecific *Corymbia* hybridization. Ann Bot. 2012; 109(7): 1215–1226. doi: 10.1093/aob/mcs050.
- 522 72. Baack E, Melo MC, Rieseberg LH, Ortiz-Barrientos D. The origins of reproductive isolation
 523 in plants. New Phytol. 2015;207(4):968–984. doi: 10.1111/nph.13424.
- Find the state of the
- 527 74. Wędzony M, Lammeren A Van. Pollen tube growth and early embryogenesis in wheat×
 528 maize crosses influenced by 2, 4-D. Ann Bot. 1996;(1991):639–647. doi:
 529 10.1093/aob/77.6.639.
- Anamthawat-Jonsson K. Wide-hybrids between wheat and lymegrass: breeding and agricultural potential. Buvusindi. 1996;10:101–113.
- JinFeng C, Adelberg J. Interspecific hybridization in Cucumis progress, problems, and
 perspectives. HortScience. 2000;35(1):11–5.
- 534 77. Liu H, Yan G, Sedgley R. Interspecific hybridization in the genus *Leucadendron* through
 535 embryo rescue. South African J Bot. 2006;72(3):416–20. doi: 10.1016/j.sajb.2005.11.003.
- 78. Pannanee Rodrangboon, Pradit Pongtongkam SS and TA. Abnormal Embryo Development
 and Efficient Embryo Rescue in Interspecific Hybrids, *Oryza sativa × O. minuta* and *O. sativa × O. officinalis*. Breed Sci. 2002;52:123–9.
- 539 79. Moller AP. Developmental stability of flowers, embryo abortion, and developmental selection in plants. Proc R Soc B Biol Sci. 1996;263:53-56. doi: 10.1098/rspb.1996.0009.
- 541 80. Kuhlman LC, Burson BL, Klein PE, Klein RR, Stelly DM, Price HJ, Rooney WL. Genetic
 542 recombination in *Sorghum bicolor* x *S. macrospermum* interspecific hybrids. Genome.
 543 2008;51(9):749–56. doi: 10.1139/G08-061.
- 81. Hegde SG, Waines JG. Hybridization and introgression between bread wheat and wild and
 weedy relatives in North America. Crop Sci. 2004;44:1145–55. doi:
 10.2135/cropsci2004.1145.
- 82. Reed SM,Jones DK, Rinehart TA. Production and characterization of intergeneric hybrids
 between *Dichroa febrifuga* and *Hydrangea macrophylla*. J Am Soc Hortic Sci. 2008; 133(1)
 : 84-91. doi: 10.21273/JASHS.133.1.84.
- 550 83. Fatokun CA. Wide hybridization in cowpea: problems and prospects. Euphytica.

- 551 1991;54(2):137–40.
- 552 84. Deng Y, Chen S, Chen F, Cheng X, Zhang F. The embryo rescue derived intergeneric hybrid
 553 between *chrysanthemum* and *Ajania przewalskii* shows enhanced cold tolerance. Plant Cell
 554 Rep. 2011. 30 (12): 2177-2186. doi: 10.1007/s00299-011-1123-x.
- 85. Roy PS, Dash AK, Subudhi HN, Rao RN, Rao JN. Molecular and morphological characterization of Indian rice hybrids. Aust J Crop Sci. 2014;8(12):1607–1614.
- 557 86. Lopez-Caamal A, Tovar-Sánchez E. Genetic, morphological, and chemical patterns of plant
 558 hybridization. Rev Chil Hist Nat. 2014;87(1):1–15.
- 87. Pooler MR, Riedel LGH, Bentz SE, Townsend AM. Molecular Markers Used to Verify
 Interspecific Hybridization between Hemlock (*Tsuga*) Species. J Am Soc Hortic Sci.
 2002;127(4):623–7.
- 88. Ballester J, De Vicente MC. Determination of F1 hybrid seed purity in pepper using PCRbased markers. Euphytica. 1998; 103(2): 223–226.
- 564 89. Turchetto C, Segatto ALA, Beduschi J, Bonatto SL, Freitas LB. Genetic differentiation and
 565 hybrid identification using microsatellite markers in closely related wild species. AoB
 566 Plants. 2015;7: plv084.
- 567 90. Kirk H, Young HC, Hye KK, Verpoorte R, Van Der Meijden E. Comparing metabolomes:
 568 The chemical consequences of hybridization in plants. New Phytol. 2005;167(2):613–22.
 569 doi: 10.1111/j.1469-8137.2005.01448.x.
- Orians CM. The effects of hybridization in plants on secondary chemistry: Implications for
 the ecology and evolution of plant Herbivore interactions. Am J Bot. 2000;87(12):1749–
 doi: 10.2307/2656824.
- Negrutiu I, Hinnisdaels S, Mouras A, Gill BS, Gharti-Chhetri GB, Davey MR, Gleba YY,
 Sidorov V, Jacobs M. Somatic versus sexual hybridization: features, facts and future. Acta
 Bot Neerl. 1989;38(3):253–72. doi: 10.1111/j.1438-8677.1989.tb01350.x.
- 576
- 577