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Evaluation of Interspecific-Interploid Hybrids (F_1) and Back Crosses (BC_1) in *Hylocereus* Species (Cactaceae)

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1. Introduction

1.1 Background - *Hylocereus* species

Vine cacti are night-blooming epiphytes plants, endemic to the Americas, and belong to Cactaceae, subfamily Cactoideae, tribe Hylocereeae (Br. and R.) Buxbaum (Barthlott & Hunt, 1993). According to the New Cactus Lexicon (Hunt, 2006), the genera *Hylocereus* (Berger) Br. and R., comprises 14 species, and they are widely distributed in tropical and subtropical regions of the Americas from Mexico to North Argentina (Mizrahi & Nerd, 1999; Merten, 2003). They inhabit a wide range of ecosystems, including coastal areas, high mountains and tropical rainforests (Ortiz, 1999). These species, known as pitahaya or Dragon fruit, are currently being marketed worldwide and have a high economic potential as exotic fruit crops in arid regions where water is scarce, since they use a Crassulacean acid metabolism (CAM) pathway and are exceptionally drought-tolerant (Raveh et al., 1998; Mizrahi & Nerd, 1999; Nobel & de la Barrera, 2004).

Hylocereus species are characterized by triangular stems, bearing large edible fruits (200-600 g) with broad scales and various peels and flesh colours (Lichtenzveig et al., 2000). These species have big flowers (25-30 cm) with large male and female reproductive organs, facilitating manual pollination and manipulation for breeding studies (e.g. the anthers can be easily removed to avoid contamination). The number of ovules per flower is very high, with ~7,200, 5,300 and 2,000 ovules per locule in *Hylocereus undatus* (Haw.) Britton et Rose, *H. monacanthus* (Lem.) Britton et Rose, and *H. megalanthus* (K. Schumann ex Vaupel) Bauer, respectively (Nerd & Mizrahi, 1997; Bauer, 2003; Tel-Zur et al., 2005). *H. megalanthus*, resembles *Hylocereus* in its vegetative appearance, but bears medium-sized (80-200 g) yellow fruit with a spiny peel and a white tasty pulp (Weiss et al., 1994). Seed viability varies among different *Hylocereus* species, as well as within the same species. The pollen donor has an effect on fruit weight, and a positive correlation was found between fruit weight and total seed number when self-pollinated fruit was compared with cross-pollinated fruit (Weiss et al., 1994; Lichtenzveig et al., 2000). *H. monacanthus* was reported to be self-incompatible (Weiss et al., 1994; Lichtenzveig et al., 2000), while *H. undatus,* under growing

conditions in Israel, is self-compatible at the end of the season, yet its fruits are smaller than those obtained following cross-pollination. Because of the low yields obtained after self-pollination, hand-cross pollination is routinely carried out for both *H. monacanthus* and *H. undatus* (Mizrahi et al., 2004).

Over the past 20 years, in order to develop this crop to its full economic potential, a traditional breeding program has been developed at Ben-Gurion University of the Negev, focused on the production of superior hybrids in terms of fruit quality, though few of them currently grow at a commercial scale (Tel-Zur et al., 2004, 2005).

2. Polyploidy

Polyploidy is the state of having more than two full sets of homologous chromosomes. Polyploidization may have played a major role in the diversification and speciation of the plant kingdom, generating the genetic and epigenetic novelty that has resulted in the significant diversity observed today (Stebbins, 1971; Soltis & Soltis, 1993). Two forms of polyploidization are autopolyploidization and allopolyploidization. Allopolyploids result from the combination of two genetically and evolutionarily different (or homeologous) genomes. Allopolyploids originated from wide hybridization, as in the case of *Triticum turgidum* L. (Zhang et al., 2010). Enzymes encoded by both parents' alleles and novel enzymes that are encoded by new allelic combinations may be produced by allopolyploids, possibly contributing to their evolutionary success (Soltis & Soltis, 1993). Autopolyploids result from the combination of genomes from two individuals of the same species, producing multiple chromosome sets with similar (homologous) genomes. Autopolyploids can arise from a spontaneous, naturally occurring genome doubling, such as *Galax urceolata* (Soltis et al., 2007).

3. Meiosis and fertility of polyploids

The ploidy level refers to the number of sets of chromosomes (basic number) and is notated by an "*x*". The basic number is the haploid number of the diploid species, being the chromosome number in a polyploid series, divisible by its basic number (Ranney, 2000).

Both diploid and polyploid organisms can produce viable germ cells. Those of tetraploid organisms are diploid. Since four chromosomes are homologous, quadrivalents are frequently formed during meiosis. Their stability is much less than that of bivalents, leading to an increased ratio of mistakes and, therefore, to reduced fertility and, in extreme cases, even to gamete sterility. Some species have an undisturbed quadrivalent development, while, in others, it does not take place at all (Elliot, 1958).

Polyploid crop species include potatos, coffee, bananas, peanuts, tobacco, wheat, oats, sugarcane, plums, loganberries and strawberries (Stebbins, 1950). Frequently, polyploidization results in bigger organs and improved traits; thus, plant breeders have become interested in the artificial induction of polyploids. It must be pointed out that, in many seed crops, polyploidy lines have shown lower fertility rates than their diploid prototypes; also, in general, there is an optimum range of polyploidy, beyond which growth may be depressed along with increasing chromosome numbers (Elliot, 1958; Wolfe, 2001).

3.1 Meiosis of triploids

Triploids have generally been considered an evolutionary dead end because they have very low fertility and tend to produce aneuploid or unbalanced gametes (Ollitrault et al., 2008). Triploid formation is commonly caused by the fertilization of a haploid egg with diploid pollen or vice versa (Figure 1). During the meiosis of triploids, trivalents are frequently formed. During Anaphase I, the chromosomes are distributed onto both daughter cells. Only in rare cases does one of them receive exactly double the amount (2*n*) of the simple set (1*n*). Generally, both of them are equipped with incomplete sets (aneuploidy; Figure 1). This nearly always results in an imbalance of the chromosome composition, leading to lethality. Therefore, triploidy causes , with a few rare exceptions, sterility of the pollen (or strongly reduced fertility). However, triploids can produce haploid, diploid, or triploid gametes at low rates, which can lead to diploid, triploid, and tetraploid progenies (Otto & Whitton, 2000).

There have been numerous reports in the literature of meiotic behaviour in triploid plants, resulting in valuable cytogenetic information regarding the species investigated. Studies of *Solanum tuberosum* triploids have also provided evidence that the amount of lagging chromosomes is, more or less, proportional to the average number of univalents (Lange & Wagenvoort, 1973). In the case of the wild citrus 'Hong Kong' Kumquat (*Fortunella hindsii* Swing), early cytogenetic studies have described triploid meiosis, showing either trivalent pairing and some univalents (Longley, 1926) or the predominance of trivalents but, also, the presence of numerous bivalents and univalents, as well as great variation in the number of some genotypes (Frost & Soost, 1968).

In general, the triploid plants have failed to set seed with its own pollen; therefore, only in rare cases in citrus and only under controlled conditions was possible to obtain seeds (Ollitrault et al., 2008). In the case of citrus, the seeds obtained from the triploid plants were produced with pollen from diploid species (Ollitrault et al., 2008). Wakana et al. (1981) have shown that the formation of triploid embryos are associated with a pentaploid endosperm, which is a strong indication that triploid hybrids result from the fertilization of unreduced (2*n*) ovules by normal haploid (1*n*) pollen (Esen et al., 1979).

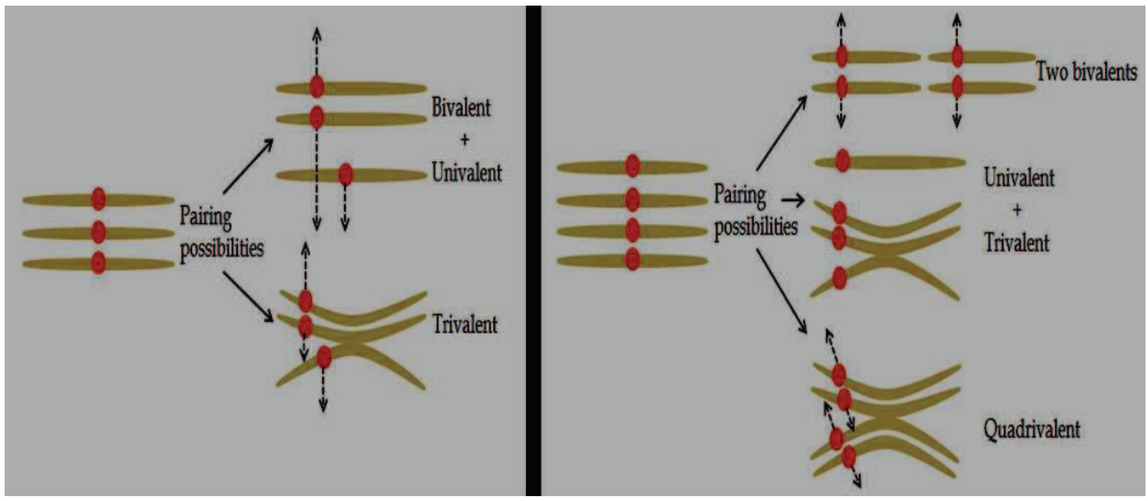
3.2 Meiosis of tetraploids

Polyploidy plays an important role in plant evolution, and it is known that the genomes of flowering plants, including many crop plants of worldwide importance, are polyploidy (Doyle et al., 2008; Leitch & Leitch, 2008).

The meiotic behaviour of plant chromosomes is affected by both genetic and environmental factors (Rezaei et al., 2010). Gametic viability is generally lower in autotetraploid genotypes that have multivalent chromosome association during meiosis than in allotetraploids that form bivalents, leading to equilibrated disomic segregation (Ollitrault et al., 2008). In citrus, it has been shown that the degeneration of pollen mother cells is more frequent in autotetraploids than in their diploid parental genotypes (Frost & Soost, 1968). These authors also observed a great variability in chromosome conjugation (quadrivalents, trivalents, bivalents, and univalents) during Metaphase I and showed that one third to one half of sporads have more than the normal number of four microspores (generally six or seven).

Meiotic restitution (working in functional unreduced female and male gametes) plays a predominant role in producing allopolyploids in flowering plants. This phenomenon, including first division restitution (FDR) and second division restitution (SDR), has been documented in cereal crops (Jauhar, 2003, 2007; Matsuoka & Nasuda, 2004; Zhang et al., 2007, 2010; Wang et al., 2010). It has been suggested that the unreduced gametes produced through meiotic restitution may have been a major mechanism for the widespread occurrence of polyploidy in nature (Jauhar, 2003, 2007; Ramanna & Jacobsen, 2003).

During the meiosis of tetraploids, trivalents and quadrivalents are formed. In Figure 1, the possible combination of balanced and unbalanced gametes in tetraploids during meiosis and in the following Anaphase I and the chromosome distribution onto both daughter cells are shown. Contrary to triploids, tetraploids frequently result with exactly double the amount (2*n*) of the simple set (1*n*).



(a) (b)

Fig. 1. Production of balanced and unbalanced gametes in (a) triploids and (b) tetraploids during meiosis that may to lead balanced and unbalanced segregation and partitioning of chromosome sets.

4. Interspecific-interploid crosses

The following are crucial questions in allopolyploid research: what are the genetic and functional consequences of combining two genetic systems into a common nucleus (Huxley, 1942), and what are their ultimate effects on plant development (Steimer et al., 2004), flowering time (Simpson, 2004) and plant evolution (Kalisz & Purugganan, 2004). The critical period of allopolyploid formation seems to be immediately after fertilization, which probably involves intensive genetic, genomic and physiological changes (McClintock, 1984). In maize, for example, reciprocal interploidy crosses resulted in very small and largely infertile seeds with defective endosperms (Pennington et al., 2008). In *Arabidopsis thaliana*, reciprocal crosses between diploid and tetraploid plants resulted in viable triploid seeds; however, the seed development pattern and, in particular, the endosperm were abnormal (Scott et al., 1998). Additionally, Bushell et al. (2003), working with interspecific-interploid crosses between the diploid *A. thaliana* (2n = 2x = 10) as the seed parent and the tetraploid *A.*

arenosa (2n = 4x = 32) as the pollen donor, produced fruits with aborted seeds, while the reciprocal cross failed to produce fruits, and the flowers collapsed one day after pollination. Several theories have been developed to explain the success or failure of such crosses. Müntzing (1930) postulated that any deviation from the 2:3:2 maternal:endosperm:embryo genome ratios would result in seed failure, but his theory was widely disproved. Von Wangenheim (1957) proposed that seed failure in interploid crosses is caused by shifts in the initial quantity of cytoplasm per nuclear chromosome set, a theory later refuted by Lin (1982). Johnston et al. (1980) put forward the endosperm balance number (EBN) theory, which stated that hybrid success is based on an effective maternal:paternal genome ratio that must be 2:1 for normal endosperm development. This simplified theory was useful for plant breeders for predicting the success of a cross, especially among *Solanum* species (Carputo et al., 1997), but it failed to predict crossing success in other plant species. Haig & Westoby (1989) pointed out that the effect of "parental conflict of interests" on the growth of offspring suggested that maternal and paternal growth-promoting genes would be expressed differentially. Nowack et al. (2006) found that endosperm development is initiated by fertilizing the egg cell only and not the central cell with a mutant of the *A. thaliana* Cdc2 homolog CDC2A (cdc2a mutant pollen produces one sperm cell instead of two), suggesting that a positive signal for proliferation of the central cell triggered endosperm formation following egg fertilization. Nowack et al. (2007) showed that endosperm, exclusively derived from maternal origin, is able to sustain complete embryo development. These findings support Strasburger's (1900) hypothesis that, during evolution, the female gametophyte was reduced to the central cell of modern angiosperms and that fusion of the second sperm is used as a trigger to start endosperm development.

The fertilization process includes the fusion of the gametic membranes and the uptake of sperm cytoplasm by the female gametes, after which the fertilized egg is covered by a cell wall; thus, the endosperm and embryo develop in parallel to form a mature seed.

Crosses between the tetraploid *Hylocereus megalanthus* as the female parent and the diploid *H. undatus* or *H. monacanthus* as the male parent yielded pentaploid, hexaploid and 6x-aneuploid hybrids, while the reciprocal cross, using *H. monacanthus* as the female parent, yielded triploid and 3x-aneuploid hybrids (Tel-Zur et al., 2003). In *H. monacanthus* × *H. megalanthus* crosses, a higher number of truth hybrids were found about 92 %, of them were 3x while the reciprocal cross resulted in 5 % of truth hybrids with a ploidy level higher than expected (Tel-Zur et al., 2003, 2004). Endosperm breakdown is widely believed to be the cause of seed failure; however, seed abortion in vine cactus was probably due to a genomic imbalance between the seed parent and the embryo, rather than the maternal:paternal genome ratio in the endosperm (Tel-Zur et al., 2005) (Table 1). Endosperm dysfunction was the primary reason for hybrid abortion in a range of Angiosperm families (Brink & Cooper, 1947). Nowack et al. (2010) showed that the fertilized egg transmits a signal for development, but it cannot continue without the fertilization of the central cell. On the basis of this hypothesis, Cisneros et al. (2011) postulate, for vine cacti, that: 1) double fertilization happened but the zygote aborted, resulting in empty seeds due to post-zygotic barriers; 2) endosperm formation is necessary (and double fertilization is required) for normal seed coat development, but the presence of an embryo is not essential for the development of a normal black seed coat; and 3) increased genome dosage in the polyploid results in reduced seed viability, which may be attributable to a maternal/paternal imbalance or a lack of double fertilization.

Hybridization is an important source of improved genotypes for cultivation. Hybrids have been produced recently in our laboratory, following interspecific-interploidy crosses among *Hylocereus* species, setting fruits with a range of 5 to 15 % of unviable seed/fruit. The number of the unviable seeds was higher than that observed in the diploid *Hylocereus* species and lower than that observed in the female parent (S–75 or 12-31), suggesting that the triploid and tetraploid level of the hybrids result in a better seed viability. Few of the interspecific-interploidy hybrids set small fruits with a low seed number.

	Seed parent ploidy	Pollen parent ploidy	Predicted seedling ploidy	Predicted endosperm ploidy (m:p)	Seedling ploidy (m:p)	Average seed weight (mg ± SE)
Homoploid crosses	2x	2x	2x	3x (2m:1p)	2x (1m:1p)	2.44 ± 0.05
	4x	4x	4x	6x (4m:2p)	4x (2m:2p)	7.73 ± 0.39
Paternal excess	2x	4x	3x	4x (2m:2p)	3x (1m:2p)	4.37 ± 0.19
Maternal excess	4x	2x	3x	Unreduced female gamete[1]		
				9x (8m:1p) and not 5x (4m:1p)	5x (4m:1p)	3.1 to 5.9
				Chromosome doubling[2]		
				5x (4m:1p)	6x (4m:2p)	3.1 to 5.9
				Aborted seeds[3]		
				5x (4m:1p)	3x (2m:1p)	<3.0

[1]Assuming that all the embryo sac cells (including the egg cell) are a result of unreduced meiosis, since megasporogenesis occurs before megagametogenesis.
[2]Ploidy in the embryo sac cells was assumed as remaining normal, while the hexaploid hybrids are probably a result of chromosome doubling (*see* Tel-Zur et al., 2003).
[3]Triploid hybrids were obtained in this cross direction using embryo rescue (*see* Cisneros, 2009).
(m:p): maternal to paternal genome ratios.
2x: *Hylocereus* species, 4x: *H. megalanthus*.

**The results presented in this table are a composite of unpublished and previously published data in Tel-Zur et al. (2005).*

Table 1. Outcome of homoploid and interspecific-interploid crosses in species of vine cacti*

5. Phenotypic and genomic evaluation

At the beginning of the 90s, there was very little scientific information available on the cultivation and the biological background of vine cacti. As of now in contrast to the early 90s, investigations in both horticultural and physiological aspects of climbing cacti have been covered and published in the professional literature; beginning with its reproductive biology (Weiss et al., 1994; Nerd & Mizrahi, 1997), shading requirements (Raveh et al., 1998), and fruit development, ripening, and post-harvest handling (Nerd & Mizrahi, 1998; Nerd et al., 1999; Ortiz, 1999).

Phenotypic and genomic characterization of the vine cactus core collection was reported by Tel-Zur et al. (2011), showing a high level of variability for most of the traits studied.

Although the heritability of these traits has yet to be studied, and some are likely to have a substantial environmental component, the levels of variation reported strongly suggested to us that vine cacti have high potential for breeding programs as exotic fruit crops, intrinsically adapted to dry areas (Tel-Zur et al., 2011).

Morphological traits, such as fruit shape, peel and flesh colour, stem size or height, often have one to one correspondence with the genes controlling the traits. In such cases, the morphological characters (the phenotypes) can be used as reliable indicators for specific genes that can be linked with quantitative trait loci (QTL), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), among others and must be used to build the genetic linkage map of the vine cacti.

5.1 Phenological characterization

Morphological characteristics have not only been used for formal taxonomical overviews of large germplasm collections, but also for studying geographical patterns, as was done for durum wheat (Yang et al., 1991), bread wheat (Börner et al., 2005), barley (Tolbert et al., 1979) and triticale (Furman et al., 1997). Large variations in morphological characteristics were reported in garlic (Baghalian et al., 2006), different species of onions (Ozodbek et al., 2008; Zammouri et al., 2009), bananas (Uma et al., 2004) and pomegranates (Zamani et al., 2007).

Morphological traits are considered easy to observe, and it is possible to screen and categorize large amounts of genotypes at a low cost, which is a great advantage when managing large germplasm collections (Diederichsen, 2008). However, the optimal utilization of morphological descriptors involves the evaluation of agronomic performance in the farm (Zammouri et al., 2009). The exploitation of such traits increases our knowledge of the genetic variability available and strongly facilitates breeding for wider geographic adaptability, with respect to biotic and abiotic stresses (Diederichsen, 2004).

The vine cacti plants grow up tree trunks and are anchored by aerial roots. The fruits have red, purple or yellow peels, while the colour of the flesh can range from white to red or magenta. The skin is covered with bracts or "scales", hence the name dragon fruit. The seeds are small and are consumed with the fruit. The fruit can weigh up to 900 grams, but the average weight is between 350 and 450 grams (Mizrahi & Nerd, 1999; Merten, 2003). The weight depends on pollination management as well as the genotype (Weiss et al., 1994; Nerd & Mizrahi, 1997). The fruits are most often consumed fresh. In some parts of South America, the pulp is used to prepare drinks (Ortiz, 2001).

One of the major problems in cultivating vine cacti under desert conditions is their sensitivity to both low and high temperatures (Mizrahi & Nerd, 1999). Since variability in these characteristics exist among genotypes, and since there is no genetic barrier among species and even genera, breeding may solve these problems (Lichtenzveig et al., 2000; Tel-Zur, 2001). Another important problem with these fruits that might be solved with breeding is the poor taste of the red pitahayas, *H. monacanthus* and *H. undatus* (Mizrahi et al., 2002). The delicious yellow pitahaya, *H. megalanthus*, can tolerate high temperatures better than the other species, but the fruits are smaller (Mizrahi et al., 2002; Dag & Mizrahi, 2005).

The initial interploid crosses between the diploid *H. monacanthus* as a female parent and the tetraploid *H. megalanthus* as a male parent resulted in the release of two triploid hybrids

(named S–75 and 12–31) to growers (Tel-Zur et al., 2004). These hybrids offered better taste than their diploid female parent and larger fruits than their tetraploid male parents, but, still, their spiny peel (a dominant trait), which is characteristic of *H. megalanthus*, was a limitation to further cultivation.

Recently, we performed an evaluation of 109 hybrids for three consecutive years; of this number, 40 were interspecific interploid F_1 (IH) hybrids (among the triploids S–75 or 12–31 and the diploid species *H undatus*), and 69 were first-back crosses (BC_1) between the triploids (S–75 and 12–31) and their parental lines. The morphological and agronomical fruit traits from the interspecific interploid F_1 hybrids, as well as the BC_1, under study are presented in Table 2, while the characteristics of the parental line were summarized in Tel-Zur et al. (2004, 2005). Variations in fruit form from round to elongated ellipse were observed in the basis of the dissimilarities of the fruit shape index (lower values were shown in hybrids not listed in the Table 2).

The number of flowers per hybrid per year was evaluated in 78 hybrids that set fruits. Among them, a wide range was observed, from one for BC–022 (not listed in Table 2) to 21 for IH–052 (Table 2). The weight of the fruits ranged from 119 to 273 grams and was intermediate between the fruit weights of the parental lines. Comparing these results with the parental genotypes, the majority of the hybrids were intermediate or lower than those of the parents, indicating partial dominance or co-dominance, similar to that described by Tel-Zur et al. (2004).

The potential yield per plant, a very important agricultural trait and a target trait in breeding programs, was calculated on the basis of the number of flowers per year and mean fruit weight (Tel-Zur et al., 2011). Two accessions demonstrated high potential yield, IH–003 and IH–052 with 3.25 and 3.72 kg/plant/year, respectively (Table 2). This trait was probably underestimated and will increase when the plants are older. Tel-Zur et al. (2005) reported the fruit weight in the female parent (hybrids S–75 and 12–31) to be between 192 to 267 grams, depending on the pollen donor used. This trait was intermediate between the parents, indicating partial dominance or co-dominance, as was previously described for others hybrids (Tel-Zur et al., 2004, 2011).

The number of viable seeds/fruit was quantified in several hybrids studied, showing a very high percentage of viable seeds (more than 85%), and their total number did not affect fruit size. In general, the phenotype of the seeds was similar to that described in tetraploids vine cacti (Cisneros et al., 2011); thus, we assume that this is a characteristic inherited from *H. megalanthus*.

The flowering season of the F_1 hybrids and BC_1 was from August to November. The time to full ripeness was extremely variable among the studied plants. The results showed variation within hybrids belonging to the same cross and ranged from 52 days in IH–011 (12–31 × *H. undatus*) to 156 days in IH–057 (12–31 × *H. undatus*), while in BC_1 (S–75 × *H. monacanthus* or *H. megalanthus*), the variation was lower but more similar to that reported in *H megalanthus*. The ripening time was found to be a genotype-specific trait with considerable variability among *H. megalanthus* accessions (Dag & Mizrahi, 2005). Mizrahi & Nerd (1999); later, Tel-Zur et al. (2004, 2011) reported that flowers of *H. megalanthus* that bloomed in early autumn matured in 90 days while those that bloomed later (November and December) matured in 160 days. Thus, this characteristic had an intermediate behaviour, implying that its inheritance was co-dominant and that the influence of low temperature was minimal.

Plant code[1]	Flowers/ plant/year	Fruit shape index (FL/FW) ± SE	Fruit weight (g) ± SE	Flesh/Peel ratio ± SE	Potential yield/plant (kg)*	Ave. days to ripening
IH-001	8	1.9 ± 0.04	131 ± 18	0.88 ± 0.11	1.05	58
IH-002	5	1.9 ± 0.08	273 ± 33	2.36 ± 0.36	1.36	92
IH-003	16	1.8 ± 0.03	203 ± 15	1.39 ± 0.14	3.25	75
IH-004	12	1.8 ± 0.04	119 ± 10	1.74 ± 0.22	1.43	67
IH-005	6	1.8 ± 0.06	199 ± 27	1.23 ± 0.19	1.19	83
IH-006	12	1.8 ± 0.05	169 ± 14	2.12 ± 0.29	2.03	85
IH-007	8	1.8 ± 0.05	231 ± 25	1.68 ± 0.24	1.85	88
IH-008	9	1.9 ± 0.04	203 ± 26	1.25 ± 0.23	1.83	86
IH-009	13	2.0 ± 0.07	197 ± 17	1.39 ± 0.16	2.56	82
IH-011	11	1.6 ± 0.06	203 ± 10	2.22 ± 0.19	2.23	52
BC-026	10	1.7 ± 0.08	170 ± 25	1.82 ± 0.21	1.70	81
BC-027	7	1.7 ± 0.13	204 ± 26	2.61 ± 0.32	1.43	101
BC-028	12	1.9 ± 0.05	220 ± 17	1.85 ± 0.17	2.64	90
BC-029	10	1.8 ± 0.03	150 ± 14	1.87 ± 0.23	1.50	87
BC-036	8	1.9 ± 0.04	180 ± 16	1.47 ± 0.06	1.44	85
BC-045	11	1.8 ± 0.04	171 ± 12	2.04 ± 0.20	1.88	81
BC-047	10	2.0 ± 0.06	158 ± 24	1.39 ± 0.17	1.58	78
BC-049	8	1.9 ± 0.05	200 ± 21	1.20 ± 0.16	1.60	88
IH-050	16	1.8 ± 0.03	181 ± 24	1.81 ± 0.16	2.90	94
IH-051	12	1.9 ± 0.04	176 ± 9	1.86 ± 0.13	2.12	88
IH-052	21	1.9 ± 0.03	177 ± 13	1.43 ± 0.10	3.72	63
IH-057	8	1.6 ± 0.04	153 ± 32	1.11 ± 0.15	1.23	156
BC-066	7	1.9 ± 0.09	149 ± 29	1.59 ± 0.30	1.04	103
IH-070	10	1.7 ± 0.09	134 ± 19	2.03 ± 0.25	1.34	94
IH-074	6	1.8 ± 0.06	177 ± 24	2.09 ± 0.31	1.06	82
BC-075	9	1.9 ± 0.09	169 ± 23	1.78 ± 0.21	1.52	80
BC-077	6	1.9 ± 0.05	228 ± 28	1.69 ± 0.24	1.37	91
BC-098	8	1.8 ± 0.07	152 ± 17	1.90 ± 0.18	1.22	61
IH-106	6	1.8 ± 0.07	177 ± 19	1.64 ± 0.46	1.06	72
IH-107	7	1.9 ± 0.08	191 ± 19	1.70 ± 0.28	1.33	78

[1] Plant codes refer to IH: Interspecific-interploid cross and BC: Back crosses

* Potential yield per plant was calculated as a number of flowers/year × mean fruit weight

Table 2. F_1 and BC_1 characterization: flowers per plant, fruit shape index (fruit length/fruit width), fresh/peel ratio, fruit weight, potential yield and average number of days until ripening

5.2 Genome size analyses and ploidy estimation

Estimating genome size and ploidy level in putative hybrids is the first step in evaluating the success of the interploid cross. Two methods are generally used: chromosome count and flow cytometry. Flow cytometry is a technique of genome quantification, initially developed for biomedical research and adapted for genetic plant analysis (Segura et al., 2007), that provides an accurate method to estimate ploidy level by measuring the proportions of cells

in the G1, S and G2/M stages of the cell cycle (Doležel et al., 1989). The nuclear phase status is, by convention, indicated using the letter "n". The designations (meiotically) reduced and non-reduced, or haplophasic and diplophasic, are preferable to haploid and diploid, respectively, because their meaning is unambiguous. "n" indicates the meiotically reduced chromosome number, 2n the non-reduced number (Greilhuber et al., 2005; Murray, 2005).

Determining the intra- and inter-specific variation of DNA content is important to prove the success of the hybridization event, as well as for breeding programs and for genetic manipulation (Doležel et al., 1994; Doležel & Bartoš, 2005). These data can also be used to calculate cell cycle times, which are needed in genetic studies, and are useful for analysis of plant growth and development (Loureiro et al., 2007).

There are many examples of correlations between C-value variation between species and cellular parameters, such as the duration of the mitotic and meiotic cell cycle and the sizes of cells. From a taxonomic standpoint, intraspecific C-value variation is probably the most significant indicator that proves the presence of more than one genome combined within a species (Bennett, 1972; Murray, 2005; Záveský et al., 2005). The nuclear replication status (G_1 for non-replicated, S for replicating, G_2 for replicated) leads to DNA content changes expressed in terms of 'C'. For instance, 1C can be the DNA content of a young pollen cell nucleus just after meiosis, and 2C the content of a Telophase root tip nucleus (Moscone et al., 2003; Greilhuber et al., 2005).

Bennett & Leitch (2005) developed a Plant DNA C-values database that currently contains data for 7,058 plant species. It combines the DNA C-values database from the Angiosperm, Gymnosperm, the Pteridophyte, and the Bryophyte, together with the addition of the Algae DNA C-values database. Also included in the database are two Cactaceae species, *Opuntia microdasys* (1C = 2.24 pg) and *Rebutia albiflora* (1C = 1.91 pg), and a number of succulent species from the families Asparagaceae, Bromeliaceae, Crassulaceae, Apocynaceae, Xanthorrhoeaceae and a small number of genera in Asteraceae, in which the 2C-DNA values ranged from 1.11 to 16.85 pg. Segura et al. (2007) reported four different ploidy levels of 23 *Opuntia* species determined by flow cytometry, and the amounts of 2C-DNA ranged from 4.17 pg in *Opuntia incarnadilla* Griffiths to 6.53 pg in *Opuntia heliabravoana* Scheinvar.

A flow cytometric analysis was used to determine chromosome numbers and ploidy in *Consolea* species (Cactaceae). Compared to the base number, the mitotic and meiotic counts indicated hexaploid (2n = 66) and octoploid (2n = 88) species and no diploids, with the 2C-DNA values ranging from 4.88 to 9.50 pg (Negron-Ortiz, 2007). The 2C-DNA content was studied for species of *Hylocereus*, *Selenicereus* and *Epiphyllum,* showing a diploid level for all the species studied, except for two cases of a tetraploid level in *H. megalanthus* and *S. vagans* (Bgek.) Britton et Rose (Tel-Zur et al., 2011). The range of the 2C-DNA content varied from 3.21 pg for *S. grandiflorus* spp. *grandiflorus* (L.) Britton et Rose to 8.77 pg for *H. megalanthus* (Tel-Zur et al., 2011).

2C-DNA content and ploidy estimation was studied in the 109 F_1 and BC_1 of vine cacti using flow cytometry (Table 3). Table 3 shows a random sample chosen from all of them. The 2C-DNA amount in these hybrids ranged from 3.30 pg for IH–051 to 11.67 pg for BC–031 (Table 3), comparable with that reported in different *Hylocereus* and *Selenicereus* species (Tel-Zur et al., 2011) and in other cacti species (Negron-Ortiz, 2007; Segura et al., 2007). These results showed that the ploidy level of the hybrids were diploid, triploid, tetraploid and

Plant code[1]	Nuclear DNA content pg/ 2C ± SD	Genome size 1C (Mbp)	Ploidy analyzed*	Ploidy estimated
IH-001	8.52	4166	N.D	4x
IH-002	6.67	3300	N.D	3x
IH-003	8.42	4044	N.D	4x
IH-004	8.43	4122	N.D	4x
IH-005	6.62	3237	N.D	3x
IH-006	8.22	4068	N.D	4x
IH-011	4.38	2141	N.D	2x
BC-016	8.52	3921	N.D	4x
BC-017	8.66	4234	N.D	4x
BC-018	4.04	1975	N.D	2x
BC-019	6.80	3325	N.D	3x
BC-020	8.08	4469	N.D	4x
BC-021	6.55	3281	N.D	3x
BC-023	11.44	5594	N.D	6x
BC-025	7.67	4528	44	4x
BC-026	6.03	3041	N.D	3x
BC-027	6.20	3022	N.D	3x
BC-028	4.22	2063	N.D	2x
BC-029	3.80	1858	N.D	2x
BC-031	11.67	5706	66	6x
BC-032	8.51	4811	N.D	4x
BC-033	5.72	2934	N.D	3x
BC-034	8.00	3735	N.D	4x
BC-035	6.22	2997	33	3x
BC-036	7.25	3545	N.D	4x
BC-037	6.98	3413	N.D	3x
BC-045	6.87	3359	29 – 33	3x or mix
IH-050	3.40	1662	N.D	2x
IH-051	3.30	1613	N.D	2x
IH-052	4.53	2215	N.D	2x
IH-057	9.00	4401	N.D	4x
IH-062	4.33	2117	N.D	2x
IH-106	4.57	2234	N.D	2x

[1] Plant codes refer to IC: Interspecific-interploid cross and BC: Back crosses

***** Ploidy analyzed was based on the number of chromosomes counted using acetocarmine stain

Table 3. Genome size and ploidy estimation in F_1 and BC_1.

hexaploid, with only one exception, BC–045, that was triploid or aneuploid (Table 3). In different species of *Hylocereus* and *Selenicereus*, diploid and tetraploid were reported (Tel-Zur et al., 2011). In the BC_1, we expected to find ploidy levels ranging from 2x to 4x, according to the ploidy of the parents, [for example, the cross between the female triploid S–75 or 12–31 (2n=3x=33) and the diploid male parent *H. monacanthus* (2n=2x=22) or the tetraploid *H. megalanthus* (2n=4x=44)], but never 6x, as was obtained for the BC–023 (12–31 × *H. monacanthus*) and BC–031 (S–75 × *H. monacanthus*). In these cases, we assumed that the

genome was duplicated following a fertilization event with an unreduced (2*n*) female gamete from the triploid female parent (S–75 or 12–31) and a normal reduced (*n*) male gamete from the diploid *H. monacanthus*. Cisneros (2009) reported analogous unexpectedly high ploidy levels (6x) obtained in similar interspecific-interploid crosses between the triploid S–75 and the tetraploid *H. megalanthus* accession 96-667. Ploidy levels higher than 3x, observed in the hybrids under study, were probably due to unreduced gametes produced by the mother plant or by the pollen donor.

6. Cytology of chromosome non-disjunction

One of the major routes for polyploidization involves gametic "non-reduction" or "meiotic nuclear restitution" during microsporogenesis and megasporogenesis, resulting in unreduced 2*n* gametes. Non-reduction could be due to meiotic non-disjunction, failure of cell wall formation or formation of gametes by mitosis instead of meiosis (Elliot, 1958; Grant, 1981). Unreduced gametes (2*n*) are recognized as a common mechanism of origin of most polyploids in plants (Sang et al., 2004; Otto, 2007; Matthew et al., 2009). Generally, 2*n* gametes originate due to deviating meiosis in plants. Deviations can occur in plants with normal chromosome pairing, as well as in those with disturbed chromosome pairing as, for example, in distant interspecific hybrids or synaptic mutants. The process that leads to the formation of *2n* gametes is called meiotic nuclear restitution and occurs either during micro- or megasporogenesis (Ramanna & Jacobsen, 2003).

Cytological disturbances may lead to sterility or reduced seed viability. Cytological disturbances and anomalous behaviours, such as heavy-walled coenocytes, uncoiled chromosomes, supernumerary chromosomes, production of cross-bridges at second division and the occurrence of globular structures in the microsporocytes, were reported for several species and are strongly associated with a high level of sterility in polyploids, e.g., in the triploid hybrid of *Gossypium hirsutum-herbaceum* (Beasley, 1940), in the allohexaploid of *Phleum pratense* (Nath & Nielsen, 1961) and in the tetraploids of *Brachiaria decumbens* (Simioni & Borges do Valle, 2011).

Winge (1917) proposed a theory of "hybridization followed by chromosome doubling" as a mechanism enabling the survival and development of the hybrid zygote by providing each chromosome with a homologue with which to pair. Despite the lack of well-documented evidence, generations of biologists believed that polyploids were generated by somatic doubling (zygotic or meristematic). Conversely, Harlan & de Wet (1975) listed 85 plant genera known to produce 2*n* or "unreduced" gametes (pollen or egg cells carrying the somatic chromosomal numbers), which reinforced the theory of Karpechenko (1927) and of Darlington (1956) that sexual polyploidization, resulting from 2*n* gamete fusion, is the driving force behind the origin of polyploid species. Currently, most of the evidence attributes polyploid formation to: (1) sexual polyploidization through fusion of 2*n* gametes; (2) somatic mutations in meristematic cells, namely, chimera (Morgan et al., 2001; Karle et al., 2002); (3) somatic polyploidization by nuclear fusion (Baroux et al., 2004); or (4) polyspermy, the fertilization of an egg by more than one sperm (Virfusson, 1970).

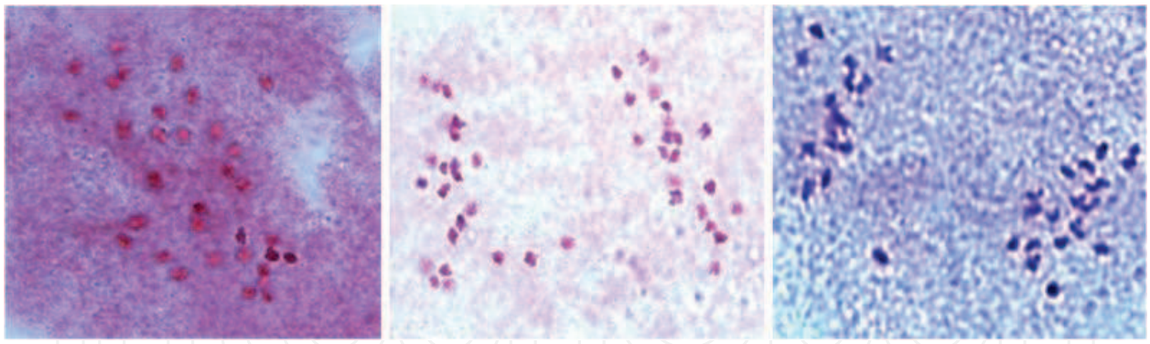
There is, however, some scientific evidence supporting the occurrence of "hybridization followed by chromosome doubling", the best known example being that of *Primula* "*kewensis*", a first-generation hybrid between *P. floribunda* and *P. verticillata* (Newton &

Pellew, 1929). For some unknown reason, the chromosomes in one branch of the plant had spontaneously doubled (somatic doubling), a process that apparently restored fertility by providing each chromosome with an identical partner with which to pair. Other examples are the spontaneous appearance of the tetraploid *Oenothera lamarckiana* Ser. (Gates, 1924) and the amphidiploids *Nicotiana glutinosa* L. and *N. tabacum* L. (Clausen & Goodspeed, 1925), which provide empirical evidence for polyploidization, presumably, by chromosome doubling. Furthermore, corn plants exposed to heat shock after pollination produced diploid, tetraploid and octaploid seedlings. The latter two states of polyploidy seem to be the result of somatic doubling in the zygote or young embryo (Müntzing, 1933). Nath & Nielsen (1961) reported that the origin of the hexaploid level in *Phleum pratense* was due to the trebling of the diploid genome complement from the *P. nodosum* species.

Lately, evidence supporting Winge's theory has been reported by Tel-Zur et al. (2003) in vine cacti crosses between the tetraploid *Hylocereus megalanthus* as the female parent and the diploid *H. undatus* or *H. monacanthus* as the male parent, yielding several hybrids with an unexpectedly high ploidy level, always higher than that of the female parent. Since unreduced gametes were not observed in the diploid *Hylocereus* species (Tel-Zur et al., 2003), the origin of *6*x is still not clear, and could be the result of chromosome doubling following interspecific-interploid crosses. Cisneros (2009) found similar results following interspecific-interploid crosses by using the embryo rescue technique, in which only one hybrid out of 22 tested showed the expected (3x) ploidy level that was lower than the ploidy of the female parent.

Univalents and multivalents were observed in the pollen mother cells (PMCs) of the tetraploid *H. megalanthus* at Metaphase I, even though chromosome disjunction at Anaphase I was very balanced (Lichtenzveig et al., 2000). The large pollen grains observed in this species, about 12% of the sample, were probably unreduced gametes formed due to meiotic irregularities during Anaphase II (Tel-Zur et al., 2003). Therefore, the pentaploid hybrids reported were probably the result of a fertilization event between an unreduced egg cell (from the tetraploid *H. megalanthus*) and a reduced pollen grain (from the diploid *H. undatus* or *H. monacanthus*). However, the hexaploid and 6x-aneuploid hybrids are an exceptional case, since the diploid *H. undatus* and *H. monacanthus* showed a regular chromosome disjunction at Anaphase I and a uniform pollen diameter, and all the interspecific-homoploid (2x) *Hylocereus* × *Hylocereus* hybrids were diploids, which strongly indicated insignificant or null production of unreduced gametes (Lichtenzveig et al., 2000; Tel-Zur et al., 2003, 2004).

Consequently, the hexaploid hybrids obtained as a result of the interspecific-interploid cross occurred at a frequency much higher than that expected for a possible fusion of 2*n* gametes from both egg and pollen donor parents. In interspecific-interploidy triploid and 3x-aneuploid hybrids, rod and ring bivalents were observed in the PMCs at Metaphase I. The frequency of the ring bivalents was much lower than that of the rod bivalents, followed by a balanced segregation in Anaphase I (Tel-Zur et al., 2005). Abnormal spindle geometry during Metaphase I (parallel and tripolar) and lagging chromosomes were also observed (Figure 2). Therefore, the relatively high percentages of functional female and male gametes (9.8 - 18.6% of viable pollen and 6.0 - 35.5% of viable seeds) produced by 3x hybrids are most likely the result of balanced chromosome segregation during meiosis (Tel-Zur et al., 2005). Moreover, all the hybrids were fertile or partially fertile, indicating that pre- and post-zygotic barriers are not a major factor blocking the development and viability of hybrids among vine cacti species (Tel-Zur et al., 2003, 2004, 2005).



(a) (b) (c)

Fig. 2. Meiotic abnormalities observed in interspecific-interploid hybrids. (a) Hybrid BC–045 (3x aneuploid): Prophase I showing 32 chromosomes. (b) Hybrid BC–025 (4x): later Anaphase I showing balanced chromosome disjunction and lagging chromosomes. (c) Hybrid BC–035 (3x): later Anaphase I showing unbalanced chromosome disjunction and a lagging chromosome.

7. Cytomixis

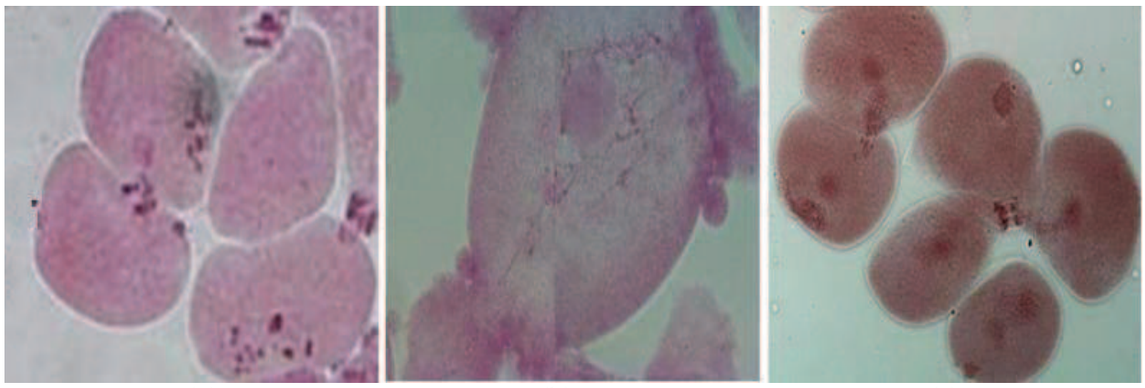
Cytomixis was defined as the migration of chromatin between adjacent cells through a cytoplasmic connection channel, i.e., chromatin migration between meiocytes, and was reported for the first time in the PMCs of *Crocus sativus* by Körnicke in 1901 (as cited in Bellucci et al., 2003). Cytomixis has been extensively studied in a great number of species; however, its origin and significance are still unclear, and its role in evolution, as well as its genetic control, remains speculative and controversial. Cytomixis is now considered to be a cytological phenomenon, though infrequent, and not an artefact that occurred during slide preparations (Bellucci et al., 2003). This phenomenon was observed more frequently during microsporogenesis, mostly during Prophase of the meiotic division, but can occur in all stages of the meiosis, especially in genetically unbalanced genotypes such as haploids, triploids, aneuploids, mutants and hybrids (de Nettancourt & Grant, 1964; Gottschalk, 1970; Salesses, 1970; Mantu & Sharma, 1983). Usually, a few cells (two to four) participated in the process, while a large numbers of PMCs were involved (Malallah & Attia, 2003).

The process of chromatin transference occurs mainly from the donor to the recipient cell and could include a small part of the chromatin material or the whole genome of the donor cell. Negron-Ortiz (2007) reported, in *Consolea* species, the occurrence of cytomixis, and the number of cells involved varied from two to nine, depending on the species, resulting, occasionally, in the establishment of empty microsporocytes after a total chromatin migration. The meiocytes with no chromatin were lost during the meiotic division, and those with abnormal genome size formed unbalanced gametes.

Previous reports indicated a genetic control of the cytomixis process (Omara, 1976; Morikawa & Legget, 1996), which can be affected by extremely high temperatures (Mantu & Sharma, 1983), herbicides (Bobak & Herich, 1978), or by pathological agents (Bell, 1964).

During observations of immature anthers of the F_1 and BC_1 vine cacti, multiple chromatin bridges between microsporocytes were observed at Prophase I; such bridges allow chromatin transfer between cells (Figure 3). These observations of PMCs showed that the

cytomixis process detected during the Prophase of the meiosis was more frequent in some of the interspecific interploid hybrids between the allotriploid S–75 as the mother parent, and the diploid *H. undatus*, as the pollen donor. Most likely, this phenomenon took place in these hybrids because of the convergence of three different genomes (*H. monacanthus*, *H. megalanthus* and *H. undatus*), and may thus imply selective DNA elimination as a response to the allopolyploidization process. The number of cells involved in the phenomenon varied from two to four. Completely empty microsporocytes were not observed. In some PMCs' donor, the remark of some chromosomes indicated that the migration to its attached recipient cell was not complete (Figure 3). Another interesting phenomenon observed was the formation of vesicle-like objects in the walls of the PMCs (Figure 3). Chromosome segments were observed in some of those vesicles and around them, which seems to be a way to remove DNA from the PMCs before the meiotic division. To the best of our knowledge, no previous reports were found in the literature addressing similar vesicle-like formation.



(a) (b) (c)

Fig. 3. Cytomixis process in PMCs in vine cacti. (a) Cytoplasmic connections in IH–006. (b) Vesicle-like formation with DNA material during Prophase in BC–023. (c) Prophase showing chromosomes that were not involved in the migration process in BC–032.

The ability of cytoplasmic channels to penetrate the callus walls, which are usually formed around microsporocytes at the end of Prophase, determines the beginning of the cytomixis process. Still unknown are the trigger that induces the PMCs to develop a connection channel with its neighbour; what points are involved in the process of recognizing the potential receptor cell; and if this process is a result of the genetic instability and/or incompatibility due to the combination of different genomes in a single cell.

8. Conclusions

Classical breeding methods can be defined as a set of tools that allows researchers to improve and characterize plants or living organisms. Several technologies, such as fluorescent activated cell sorting (FACS), molecular markers [e.g., random amplified polymorphism (RAP), amplified fragment length polymorphism (AFLP) and fluorescent *in situ* hybridization (FISH)], marker assistant selection (MAS), and quantitative trait loci (QTL) are currently extensively used for the purpose of plant evaluation and characterization. Combining these technologies with phenotypic, genomic and cytological

evaluations has given us a model system by which to associate the agronomical trait with the gene controlling its expression and making it possible to infer the inheritance. Additionally, this model system provides an excellent research tool to elucidate the pathways of polyploid formation and seed development following interspecific-interploid hybridization and to solve critical hypotheses related to the origin of vine cacti species, contributing to the new discoveries of improved nutritional value, potential yield enhancement, breaking the self-incompatibility of the diploid lines and improving resistance to abiotic factors.

Two major basic mechanisms are most likely involved in polyploidization in vine cacti: (1) unreduced (2*n*) gametes, mostly arising from the nuclear division restitution during the meiosis of the micro- or megasporegenesis that can produce hybrids with a ploidy level higher than that of the parents in triploid × diploid and triploid × tetraploid hybridizations; and (2) duplication of the chromosomes after hybridization that gives rise to polyploidy.

In general, the meiocytes resulting from the meiosis in triploids and tetraploids can display more than one possible pairing association (*see* Figure 1); thus, the probability to produce unreduced (2*n*, 3*n* or 4*n*) and unbalanced gametes is high.

The results summarized here provide experimental support to the hypothesis of polyploidization through somatic chromosome doubling or due to meiotic non-disjunction, although both the timing of the polyploidization event and the nature of the trigger remain unclear, as does the entire process leading to genome doubling. Further work on this topic will be directed to elucidate this phenomenon.

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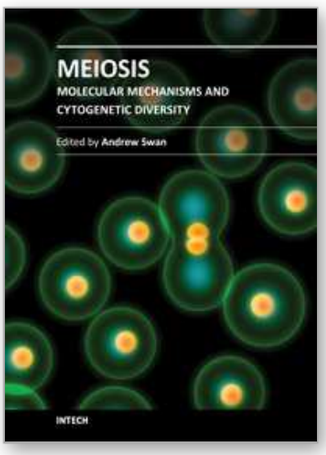

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Meiosis, the process of forming gametes in preparation for sexual reproduction, has long been a focus of intense study. Meiosis has been studied at the cytological, genetic, molecular and cellular levels. Studies in model systems have revealed common underlying mechanisms while in parallel, studies in diverse organisms have revealed the incredible variation in meiotic mechanisms. This book brings together many of the diverse strands of investigation into this fascinating and challenging field of biology.

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