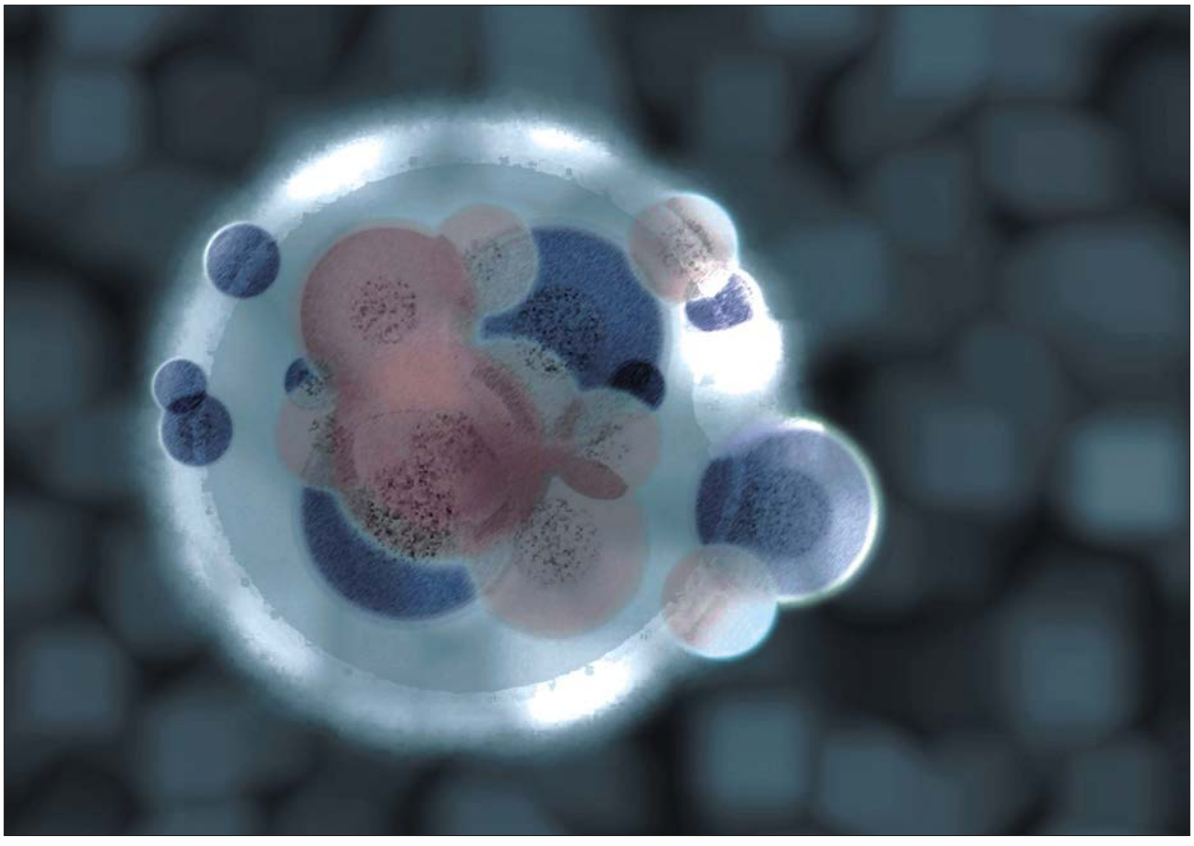

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A Derived Pentaploid Hybrid from *Solanum macrocarpon* L. (Solanaceae) and its Induced Multiploid Mutant

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Abstract: Incorporation of novel genes into crop plants could be enhanced by a combination of mutation breeding and hybridization. The backcross between *Solanum macrocarpon* L. and its induced multiploid mutant yielded a vigorous hybrid. The hybrid was erect (105 cm high) with larger expanded leaves (35×21 cm) than either the natural (75 cm high, 27×15 cm leaf size) or the mutant (21 cm, 14×11 cm) parent. Inflorescence was umbellate as in both parents but eight flowered in hybrid and 1-4 in the parents. Pollen sizes nearly doubled (52.5 µm) in the hybrid with 85.7% viability and respectively 97.3 and 58.2% in the maternal-natural and paternal-mutant parent. Self-pollination failed in the hybrid but an F_2 fruit was obtained through repeated hand pollination. Fruit was light brown and contained 78 seeds but yellow in both parents with 107 and 67 seeds. Whereas meiotic chromosomes segregated normally into n = 12 gametes, the mutant produced varied, n = 11-18, gametes. Exceptionally, the hybrid polyploidized into 2n = 60. Possible mechanism through endoduplication and the conferment of vigour and gigas morphological features on the hybrid were expatiated.

Key words: Backcross, endoduplication, hybrid vigour, mutation, polyploidization, *Solanum*

INTRODUCTION

Genetic changes through allo- and/or autopolyploidization have been a major research focus (Oyelana and Ogunwenmo, 2005; Albertin *et al*., 2005) aimed at expanding species genetic base to enhance value and species status (Stupar *et al*., 2007; Auger *et al*., 2005). Combination of mutation breeding with hybridization (Perhald *et al*., 2006; Jain, 2005; Kinoshita *et al*., 2004) offers a blend of techniques in genome improvement practices with outstanding results. Consequently, rare or novel genes have been incorporated into genomes of crop plants (Lashermes *et al*., 2000; Ugborogho and Oyelana, 1999) by hybridization and mutagenesis accompanied by polyploidization (Oyelana and Ogunwenmo, 2005; Conklin *et al*., 1999). The use of foreign chromosome constitution as background or platform for creating novel phenotypes (Ji and Chetelat, 2003) is fairly common and cultivars developed through autopolyploidization abound (Johnson *et al*., 2001; Paz and Veilleux, 1999; Liu *et al*., 1998). A number of genomic variants and clones including aneuploids, mixoploids and monoploids (Gavrilenko *et al*., 1999; Stupar *et al*., 2007) might have emerged through irregularities in meiosis in few of the synthetic polyploids. General low fertility and other forms of negative gene interactions in clones have equally been reported by Vuylsteke *et al*. (2005).

Polyploids originate from either sexual reproduction via 2n gametes or chromosome doubling. The latter has been achieved in a number of *Solanum* species including *S. melongena*×*S. scabrum* (Oyelana *et al*., 2009), *S. macrocarpon* L. (Oyelana and Ogunwenmo, 2005) and *S. tuberosum* L. (Ercolano *et al*., 2004). However, the hybrid vigour that often characterize polyploid phenotypes were suppressed in these and other variants. Similar observations also revealed qualitative gene expression changes, ranging from complete gene silencing and depression to breakdown and loss of viability in some mutants (Song *et al*., 1995; Wang *et al*., 2004; Udali and Wendel, 2006; Albertin *et al*., 2006). The stability of mutants has always been a major constraint in mutagenic manipulations (Herrera *et al*., 2002; Oyelana and Ugborogho, 2008). However, vigour has been restored through repeated backcrosses (Ugborogho and Oyelana, 1999; Rieseberg *et al*., 2000) made possible by genomic reconstruction through re-alignment of alleles and exploitation of heterosis (Frascaroli *et al*., 2007; Springer and Stupar, 2007; Auger *et al*., 2005).

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A mutant (M3) from a set of three aneuploid series (M1, M2, M3) was backcrossed to the natural parent mainly to restore meiosis, stabilize the genome and re-establish homology. This was aimed at developing phenotypes with higher fertility and fecundity, improved vigour and better market values.

MATERIALS AND METHODS

Colchicine treatment: Colchicine treatment followed Oyelana and Ogunwenmo (2005). Seeds were presoaked in deionised water for 24 h to activate the embryos. They were soaked in 0.1-1.0% Colchicine for 24 and 36 h. One (M3) of the four (M1-M4) seedlings from 0.6% 36 h treatment, which consistently showed distinct morphological and cytological variations from the natural species, was used for the hybridization experiment.

Hybridization: Twenty-four reciprocal crosses were effected between the natural species and its mutant (M3) (Fig. 1a, b). The only successful cross was obtained when the mutant cultivar was the pollen donor:

Solanum macrocarpon ♀ natural species × ♂ mutant cultivar (M3)

Emasculation and pollination of flowers: Twenty unopened flower buds were bagged 48 h prior to pollination. Emasculation was effected 18 h prior to anthesis while flowers remained in bags. Pollen from freshly dehisced anthers from the mutant cultivar were rubbed on the stigmatic surfaces with the help of a hand brush. The process was repeated at intervals of 2 h and discontinued 1 h before the flowers closed. The pollinated flowers were left in bags till the corolla withered.

Cultivation: The seedlings from the F_1 seeds were allowed to sufficiently root before they were transferred into polythene bags and raised in the nursery. The only surviving F_1 hybrid plant was nursed under shade for upward of 2 weeks before exposure to the field environment and cultivated alongside the parent species.

Morphometric analysis: Detailed observation of the morphological and floral features was carried out with the help of a stereomicroscope, while the measurements of the different parts were effected with a meter rule.

Cytological analysis

Stomata: The epidermal strips from leaves were fixed in 50% glycerol for 24 h under room temperature and later observed under a Wild compound microscope for stomata types and distribution. The dimension of stomata was taken with the help of an eyepiece graticule at x400 (Ogunwenmo, 1999).

Pollen: Pollen from matured anthers were teased in 1:1 glyceroacetocarmine on a glass slide and placed in an oven at 60°C for 48 h (Oyelana and Ugborogho, 1997). The slides were observed under a Wild compound microscope for detail pollen morphology and stainability. The diameters of pollen were measured with the help of an eyepiece graticule at x400.

Meiotic chromosomes: Young flower buds were fixed in 1:3 acetic alcohol for 24 h. The Pollen Mother Cells (PMC) were teased out from the young anthers on a glass slide and stained with 2% acetocarmine. The meiotic stages were observed and photographed under a Wild compound microscope at x400 (Ugborogho and Oyelana, 1992).

Mitotic chromosomes: Excised tips from young radicles were hydrolyzed in 1 N HCl at 60°C. They were rinsed in water and later stained in 2% acetocarmine. A gentle tap on the cover slips ensured even spread of the cells. The slides were viewed under a Wild compound microscope and the lengths of the different chromosomes measured with the help of an eyepiece graticule at x400 (Ogunwenmo, 2005).

Statistical analysis: Statistical analyses were carried out on the morphological and floral characteristics of the hybrid vis-à-vis its maternal-natural and paternal-mutant parents using SPSS for Windows, version 14.0. (SPSS Inc. Chicago, IL. USA). Statistical significance was established using One-Way Analysis of variance (ANOVA) and data were reported as Mean±SE. The one way ANOVA was further subjected to Post Hoc Test (LSD).

RESULTS

Description of species

***Solanum macrocarpon* L.-natural species:** The species was an annual shrub with erect and woody stem (75 cm). Internodal length was 7-8.5 cm. Leaves were simple, glabrous on both surfaces, pinnately veined, deeply lobed and attenuate at base. Leaf length was about twice the breadth, 27.2×15.3 cm (Fig. 1b). Stipules were profuse and two-leaflets, which may equal the size of normal leaves. Stomata were anomocytic and evenly distributed on both surfaces of leaves. Inflorescence was simple umbellate to subumbellate, often leaf opposed, subtending 2-4 flowers.

Table 1: Morphological and floral characteristics of *Solanum macrocarpon*, its mutant and the pentaploid hybrid

Characters	*S. macrocarpon* (natural species)[a]	F_1 hybrid[b]	*S. macrocarpon* (mutant)[c]
Habit	Erect	Erect	Shrubby
Height (cm)	75.0	105.0	21.0
Internode* (cm)	7.0-8.5	14.0-19.0	2.5-3.0
Leaf length* (cm)	26.9-30.0	33.6-37.8	13.8-17.0
	27.2±1.4	35.3±0.6	14.1±1.7
Leaf breadth* (cm)	14.3-18.3	20.0-25.5	8.8-12.65
	15.3±2.1	21.1±1.3	10.7±1.5
Petiole length* (cm)	4.7-7.0[†ab]	5.8-7.6	4.2-5.1
	5.5±1.1	6.4±1.32	4.5±0.9
Inflorescence/No. of flowers	Umbellate/2-4	Umbellate/8	Umbellate/1-3
Petal colour	Purple	Purple	Purple
Petal length* (mm)	16.0-19.5[†ab]	17.5-20.1	9.7-12.5
	18±0.3	18.0±0.6	10.5±1.5
Petal breadth* (mm)	7.5-10.1[†ab]	6.7-9.8	5.6-8.5
	8.0±0.9	7.0±0.8	7.0±0.7
Pedicel length* (mm)	12.0-16.0	6-10	5.0-7.0
	14.0±1.4	8.0±0.9	6.0±0.7
Fruit colour	Yellow	Light brown	Yellow
Fruit diameter* (mm)	39.0-48.0	48.2	12.0-15.0
	46.0±0.95	-	13.3±1.2
No. of seeds per fruit*	88-125	78[†bc]	58-78
	107±2.8	-	67±2.57
Pollen viability (%)	97.3	85.7	58.2
Pollen diameter* (μm)	33.6-43.4	46.5-56.8	29.7-40.4[†ac]
	35.4±1.7	52.5±1.6	36.1±1.9
Adaxial stomatal	39.0-44.7	29.7-33.5	36.5-40.0
length* (μm)	41.0±2.5	31.6±1.7	38.2±1.6
Adaxial stomatal	27.6-30.0	23.8-26.9	33.4-36.9
breadth* (μm)	28.1±1.9	24.2±1.9	34.3±1.8
Abaxial stomata	36.8-39.1	29.6-32.6	36.8-38.6[†ac]
length* (μm)	38.9±1.4	31.3±1.8	37.8±1.6
Abaxial stomata	25.7-28.8	23.6-27.0	28.5-31.2
breadth* (μm)	26.6±1.7	24.0±1.9	29.5±1.7

*Significant at $p<0.05$ †Not significant, Post Hoc (LSD) Test: ab: Maternal-natural parent vs F_1 hybrid, bc: F_1 hybrid vs paternal-mutant parent, ac: Maternal-natural parent vs paternal-mutant parent. All significant at $p<0.05$ except where indicated †

Flowers were pentamerous, actinomorphic and bisexual. Corolla was purple and lobed. Calyces were narrowly lobed and may accrescent on fruits. Pollen were regular and highly viable (97.3%). Fruits were round, yellow to golden colour and 46 mm in diameter (Table 1).

***S. macrocarpon*-mutant cultivar (M3):** It was a shrub-like plant with short erect and woody stem (21 cm). Internodal length was short, 2.5-3 cm. Leaves were simple and few, glabrous on both surfaces, pinnately veined, obovate to oblanceolate and attenuate at base, 14.1×10.7 cm (Fig. 1a). Stipule was sessile. Stomata were anomocytic and evenly distributed on leaf surfaces. Inflorescence was simple umbellate, subtending 1-3 flowers. Flowers were pentamerous, actinomorphic and bisexual. Corolla was purple and lobed. Calyces were narrowly lobed and may accrescent on fruits. Pollen were regular and 58.2% viable. Fruits were round, yellow and 13 mm diameter (Table 1).

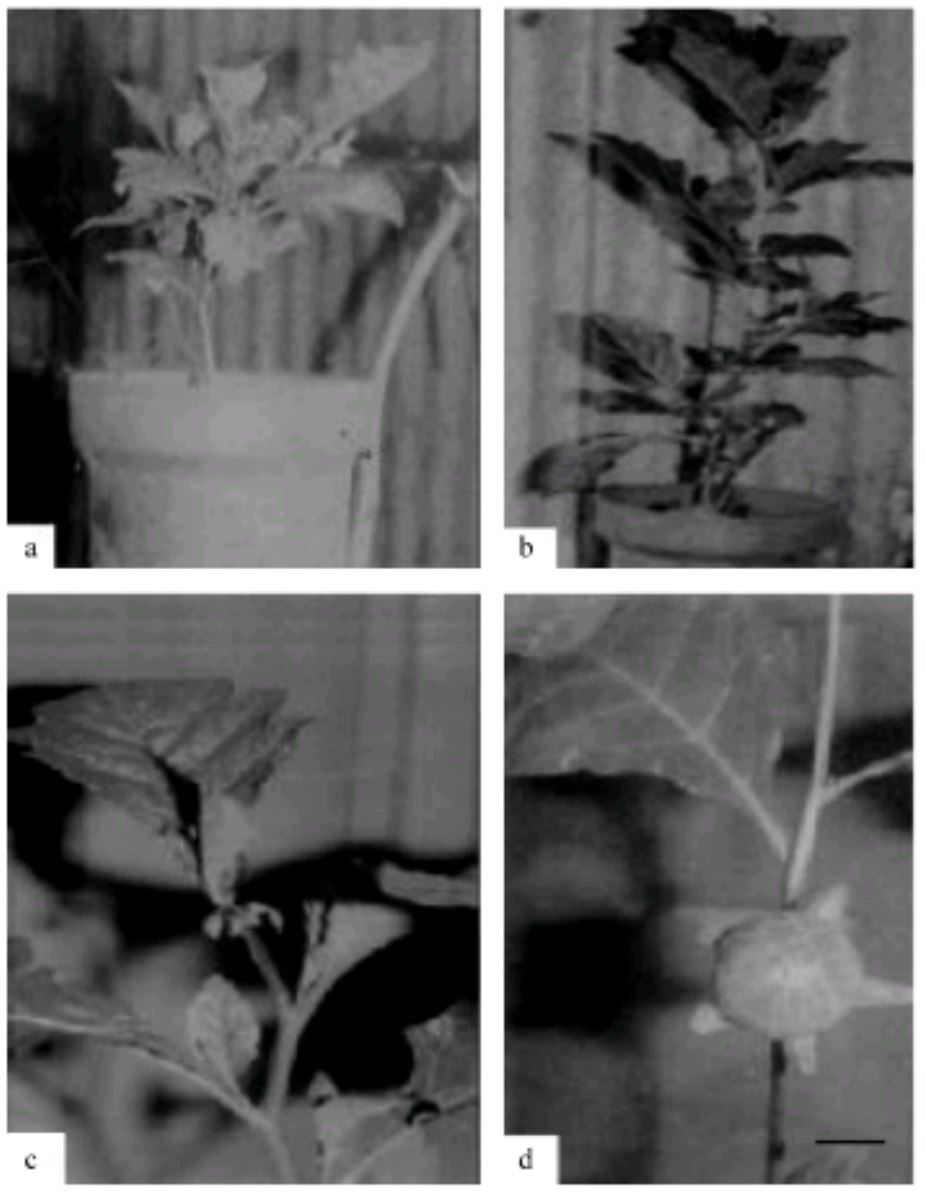

Fig. 1: *Solanum macrocarpon*, Parent and hybrid: (a) Multiploid mutant parent, (b) Natural species, (c) F_1 hybrid showing eight-flowered inflorescence and (d) Hybrid with F_2 fruit-Scale bar: 2 cm

Hybrid: The hybrid significantly differed from its either parent ($p<0.05$) in almost all its morphological and floral characteristics (Table 1).

Germination: Four seeds germinated from the 20 seeds planted and one seedling eventually flowered and produced the only F_2 fruit.

Growth and morphological characters: The F_1 plant was erect, stout and vigorous, 105 cm (Fig. 1c, d). Internodes were elongated (14-19 cm). The leaves were well expanded, significantly larger (35.3×21.1 cm) than parents ($p<0.05$) and glabrous on both surfaces. Stipules were rare. Stomata were anomocytic and evenly distributed on both leaf surfaces (Table 1).

Inflorescence and floral characters: Inflorescence was umbellate and consisted of eight flowers (Fig. 1c). Some flowers possessed short styles making pollination difficult. The stamens were arranged loosely around the style while the anther heads projected above the stigmatic surface. The flowers that were characterized by long and exerted styles had long and stout calyces which

accrescent on fruit. Pollen were regular and viability was restored to 85.7%. Pollen sizes nearly doubled in the hybrid (52.5 μm). The mutant parent had the smallest pollen while the hybrid had the largest. The natural parent was intermediate in values between the mutant and the hybrid ($p<0.05$) (Fig. 2).

Fruit: The F_1 hybrid was unable to set fruit through natural shedding of pollen. The only F_2 fruit obtained was through repeated hand pollination (Fig. 1d). The F_2 fruit was light brown, 48.2 mm and contained 78 seeds (Table 1).

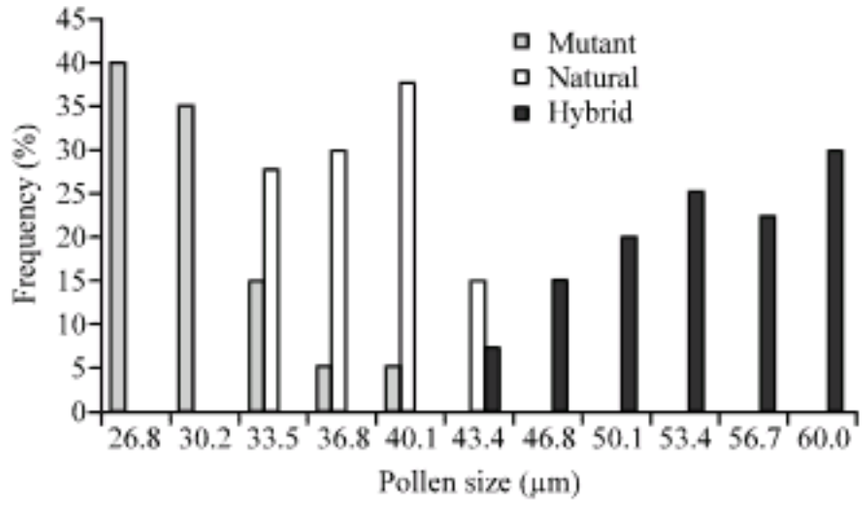

Fig. 2: Pollen size distribution of hybrid and parent-*Solanum macrocarpon*

Meiotic chromosomes: Chromosomes segregated into n = 12 (Fig. 3a) in the natural species. Meiosis was normal and bivalents were regularly paired. Mutant cultivar was a multiploid with n = 11, 12, 13, 14, 15, 16 and 18 (Fig. 3b-f). Meiosis was generally irregularly. Bivalent pairing was fairly regular, though few multivalents and clumps characterized some cells (Fig. 3f, g). Segregation of bivalents at anaphase I and II was normal. No diads were observed.

Mitotic chromosomes: The diploid number of 2n = 60 (Fig. 3h) was obtained for the hybrid.

DISCUSSION

The application of mutation breeding technique has greatly enhanced the development of new crop varieties (Shu and Lagoda, 2007) and subsequent incorporation of induced genotypes into functional genomes through backcrosses (Herrera *et al*., 2002) to related species. This combination of techniques explores the possibilities of increasing the efficiency and efficacy of mutation as a breeding strategy in crop improvement practices.

A number of mutant genes have been so integrated (Osborn *et al*., 2003; Rutger, 2006) to produce modern crop varieties. The common barley varieties in Europe and

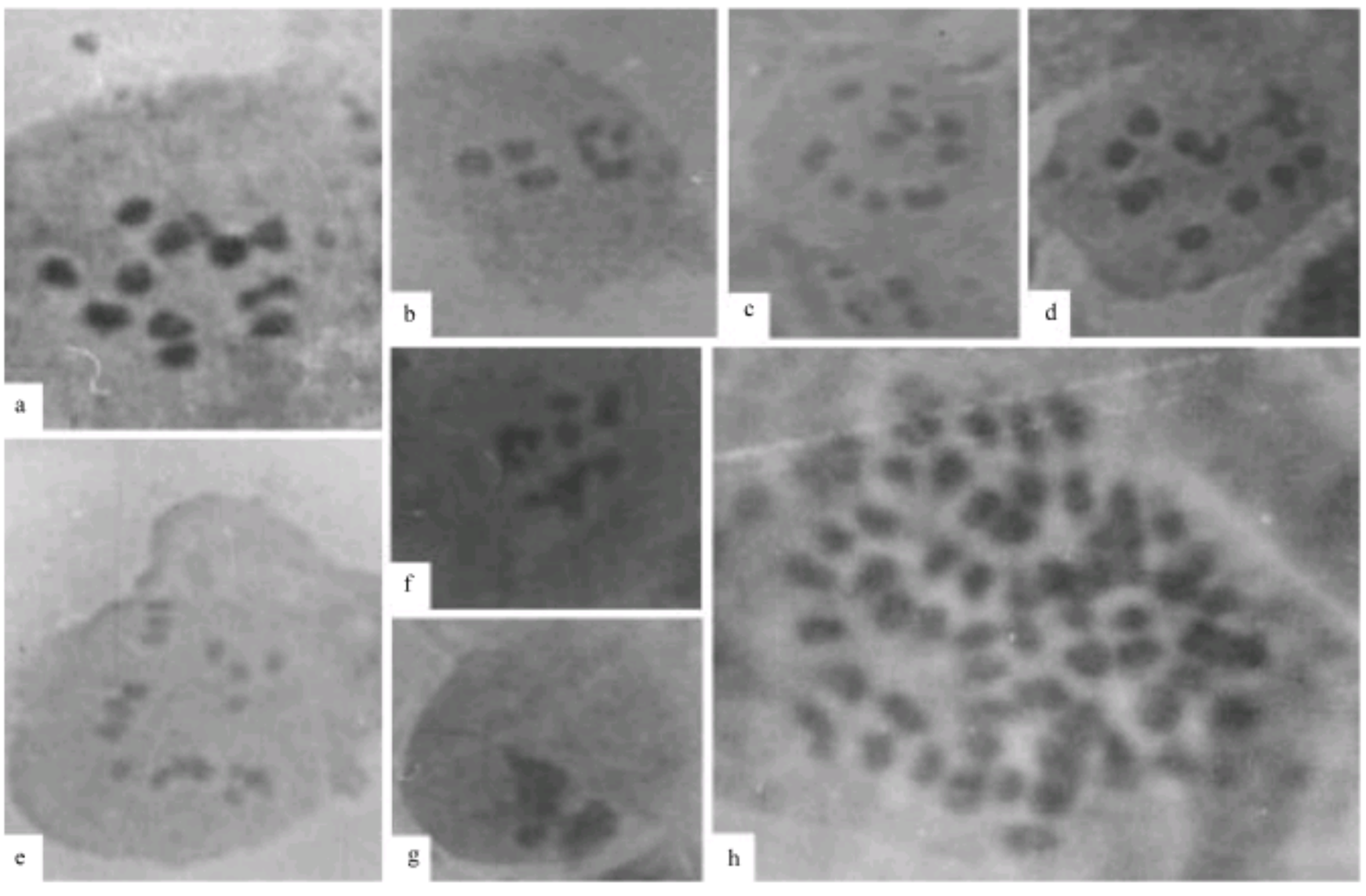

Fig. 3: Meiotic and mitotic chromosomes of *Solanum macrocarpon* and hybrid, (a) Natural parent species, n = 12, (b-g) Multiploid mutant parent, (b) n = 11, (c) n = 12, (d) n = 13, (e) n = 18, (f) Multivalents, 3 II, 1 IV, 1 VI, 1 VIII, (g) Clumps and (h) F_1 hybrid, 2n = 60

Australia contain various mutant alleles for disease resistance (Stupar *et al*., 2007). The technique has also been employed to improve tolerance to abiotic (salinity, cold and acidity) stress, nutrition and food quality and market preference (Guo *et al*., 1996) of some important grains. The rapid advances in molecular biology and DNA technology (Shu and Lagoda, 2007) create platform for increasing the efficiency of hybrid mutants and provide the needed tools for genome manipulation in crop research.

The crossing of the multiploid mutant with its pure species further affirmed the importance of homogeneity in gene expression. The phenotype of the pentaploid hybrid was exceptional and offered a number of advantages over the parents. The mitotic chromosomes revealed configurations of a typical autoploid (Auger *et al*., 2005) and suggest the likely origin of the tetraploid group of species including *Solanum scabrum* Scabrum (2n = 48). Oyelana (2005) classified *S. scabrum* Scabrum (*S. nigrum*), an autotetraploid, which probably arose from chromosome doubling in related diploid species. The success of polyploids, either from 2n gametes (Shu and Lagoda, 2007; Auger *et al*., 2005) or chromosome doubling (Oyelana and Ogunwenmo, 2005; Oyelana *et al*., 2009) depends on the degree of similarities between the crossing genotypes.

Cell size and the dimension of morphological characters (Stupar *et al*., 2007; Lavania, 2005) may correlate with level of ploidy. Plants with increased ploidy (> 3x) are usually vigorous in growth and in sizes of most morphological features (Adams *et al*., 2003; Carputo *et al*., 2003; Comai, 2005; Udalli and Wendel, 2006). The high number of flowers (8) on the inflorescence of the pentaploid hybrid compared to the parents (1-3 and 2-4) is a polyploid feature. Tazhin (1980) observed a combination of floral structure, blossoming inflorescence and multifarious florets in F_1 hybrid from a cross between a mutant and its normal barley species. Some pentaploid hybrids were associated with plumb seeds which were developed from a cross involving rye and its multiploid mutant (Klindworth and Williams, 2003). A guard cell size in an autopolyploid potato was found to increase with ploidy (Stupar *et al*., 2007).

The pentaploid hybrid was vigorous in growth, with significantly large expanded leaves twice as those of a parent ($p<0.05$). High ploidies with similar phenotypes have been observed in potato clones (Uijtewaal *et al*., 1987). Organ thickness, especially in leaves (Stupar *et al*., 2007) increased with ploidy. A maize cultivar characterized by bigger combs, increased height and expanded leaves was obtained from a cross involving a diploid and its induced tetraploid (Riddle *et al*., 2006).

Pollen viability hitherto depressed (58.2%) in the mutant parent was restored to 85.7% in the hybrid. Pollen characteristic features and the degree of pollen viability had been employed by Adams *et al*. (2004) and Wang *et al*. (2004, 2006) in investigating extent of chromosome similarity, level of ploidy, gene flow, phylogeny and origin of species (Herrera *et al*., 2002; Carvalho, 1988) as well as extent of genetic changes (Peloquin *et al*., 1999) occurring in groups of related species. Natural species are often highly fertile (Ugborogho and Oyelana, 1999; Oyelana *et al*., 2009) and characterized by regular meiosis. Meiotic irregularities had been observed in the genome of the mutant parent (Oyelana and Ogunwenmo, 2005) implying negative colchicine effects. Invariably, some genes might have been heterochromatised with the subsequent depression of their allelic effects, which consequently resulted in low pollen fertility.

The inability of the pentaploid to set fruit from the natural shedding of pollen may be due to loss of allelic effects in a group of genes of paternal parent origin. However, the restoration of pollen fertility in the hybrid and the expression of gigas features (large expanded leaves, increased height and high flower number/inflorescence) may be ascribed to positive genetic changes. Regular meiosis in the hybrid adequately reflected this positive development, as homology was re-established. The fitness cost expected of a higher ploidy due to elevated DNA content with accompanying meiotic and mitotic irregularities were equally eliminated or suppressed, suggesting restoration of normalcy (Comai, 2005). The seemingly related genomes of both parents might have encouraged regular bivalent pairing in the pentaploid.

The composite genes might have been unregulated, due to autopolyploidization of the mutant genome (paternal parent), which resulted in larger cell size (Oyelana and Ogunwenmo, 2005), translating into development of gigas features in the pentaploid. The size of pollen was largest in the hybrid and the cellular differentiation might have provided the platform for positive nucleo-cytoplasmic interactions that enhanced a higher level of DNA transcription and consequently, the synthesis of high doses of growth hormones in the pentaploid. Guo *et al*. (1996) observed that expression of most genes increases with ploidy.

The exceptional large leaves would improve the table value and market acceptance of the pentaploid hybrid. It appears that the expression profile of the composite genes in the pentaploid was optimized for leave production, making this phenotypic superiority an economic advantage over its diploid relatives, including the parents.

The seemingly reproductive difficulty and poor fruit set in the pentaploid hybrid may improve over time. The development of an innate mechanism for right allelic pairing may be achieved through segregation and re-alignment of chromosomes in subsequent generations. Gene introgression has been employed to restore meiosis and re-establish homology for enhanced agronomic values in variety of domesticated crops (Herrera *et al*., 2002; Van der Vossen, 2001). The genus *Solanum* consists of a number of intermediate populations of synthesized species along different ploidy lines (Stupar *et al*., 2007; Peloquin *et al*., 1999; Hawkes, 1990) and effective introgression through backcrosses would help stabilize these fragmented genomes.

The hybrid possibly evolved by endoduplication of the 2n = 30 zygote from the n = 12 (natural species) and n = 18 (multiploid mutant) gametes producing an autopentaploid, 2n = 60. This phenomenon has been observed in a novel hexaploid (2n = 72) hybrid from interspecific cross between *S. melongena* (n = 12) × *S. scabrum* (n = 24) (Oyelana *et al*., 2009). The hybrid was unique and morphologically and genetically different from either parent. Thus, apart from mutation and other forms of polyploidization, endoduplication had contributed to the evolutionary mechanisms in *Solanum.*

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