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Novel inter-series hybrids in *Solanum*, section *Petota*

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Abstract Sexual hybrids between distantly related *Solanum* species can undergo endosperm failure, a post-zygotic barrier in inter-species hybridizations. This barrier is explained by the endosperm balance number (EBN) hypothesis, which states that parents must have corresponding EBNs for viable seed formation. Tests for inter-crossability were made involving the Mexican species *Solanum pinnatisectum* Dunal. (series *Pinnatisecta*, $A^{pi}A^{pi}$, 1EBN), autotetraploids of this species, *Solanum verrucosum* Schlechtd. (series *Tuberosa*, AA, 2EBN), haploids (2*x*, 2EBN) of the South American *S. tuberosum* L. (series *Tuberosa*, $A_1A_1A_2A_2$, 4EBN), and F_2 haploid-species hybrids with South American species (AA, 2EBN) *S. berthaultii* Hawkes, *S. sparsipilum* (Bitter.) Juz. and Bukasov and *S. chacoense* Bitter. The development of hybrid endosperms was investigated for these combinations by confocal microscopy with regard to cell-division timing and tissue collapse. Novel sexual diploid (AA^{pi}) and triploid ($AA^{pi}A^{pi}$) inter-series hybrids were generated from *S. verrucosum* × *S. pinnatisectum* crosses by using post-pollination applications of auxin. F_1 embryos were rescued in vitro. The hybrid status of recovered plants was verified by microsatellite marker analysis, and the ploidy was determined by chromosome counting. The application of phytohormones in inter-ploidy *S. pinnatisectum* × *S. tuberosum* crosses, however, did not delay endosperm collapse, and embryos were not formed. Other diploid, 1EBN species tested in remote hybridizations with Group *Tuberosum* were *S. cardiophyllum* Lindl., *S. trifidum* Correll, and *S. tarnii* Hawkes and Hjert., series *Pinnatisecta*, and *S. bulbocastanum* Dunal., series *Bulbocastana*. Based on the analysis of post-zygotic reproductive barriers among isolated species of section *Petota*, we propose strategies to overcome such incompatibilities.

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Introduction

Inter-species hybridizations are efficient means for broadening the genetic variation of cultivated potato, *Solanum tuberosum* L. (Watanabe et al. 1995). The barriers that limit sexual methods of reproduction have to be identified and circumvented to promote exchange of genetic variation and, eventually, germplasm improvement. These barriers in *Solanaceae* include nuclear-cytoplasmic male sterility (Shifriss 1997), polyploidy (Thompson and Lumaret 1992), endosperm failure (Friedman 1998), and stylar interactions (de Nettancourt 1997) and likely result in the reproductive isolation of *Solanum* species (Hanneman 1999).

Endosperm failure has been reported to prevent hybridization among the reproductively isolated *Solanum* species of section *Petota* (Johnston and Hanneman 1982), since in incongruous inter-species crosses, the seed fails to develop after fertilization. In compatible crosses, the two polar nuclei of the central cell normally fuse with the generative male nucleus to form the endosperm, whose nuclear constitution involves an extra set of maternal chromosomes, compared to the embryo. The endosperm is a unique specialized tissue, being a product of fertilization that does not form

germ cells yet is essential for seed development (Brink and Cooper 1947). In incompatible crosses, the endosperm fails to differentiate into the nutrition source of the seed. With this balance disturbed, an overgrowth of surrounding maternal tissue occurs, and the endosperm collapses, causing degeneration of the embryo.

The ability to generate sexual inter-series hybrids of the Group *Tuberosum* with wild species of the series *Pinnatisecta* and *Bulbocastana* is commonly limited by post-zygotic incompatibility in the endosperm, which is explained by the endosperm balance number (EBN) hypothesis (Johnston et al. 1980). Gametes of different potato species with equal EBNs can successfully interfertilize because of the compatibility of the maternal and paternal EBN in a 1:1 proportion in the F_1 embryo and in a 2:1 ratio in the F_1 endosperm and also because of the interactions between the embryo and/or endosperm with somatic tissue(s) of the maternal sporophyte. Potato species with different EBNs, and therefore, deviant EBN ratios, do not hybridize and cause endosperm failure. Hawkes and Jackson (1992) concluded on the taxonomic implications of the EBN hypothesis that the 1EBN condition represented the original state of wild diploid species in Mexico, and that the 2EBN condition arose in South America as an isolating mechanism against hybridization in the evolution of 2EBN species from 1EBN species. This effective incompatibility barrier maintains genome integrity among the sympatric species.

Diploid *S. verrucosum* is an ancestral parent of the polyploid Mexican series *Demissa* and a link towards the South American series *Tuberosa* of cultivated potatoes (Hawkes 1990). *S. verrucosum* (2EBN) is crossable as a female partner with 2EBN South American wild and cultivated *Solanum* species of various series. If *S. verrucosum* is crossed as a male with either diploid incompatible wild species or *S. tuberosum* haploids, the pollen is rejected through unilateral incompatibility (Hermsen and Ramanna 1976). The potential of using *S. verrucosum* as a bridging species for the introgression of 1EBN *Solanum* species has been emphasized by Abdalla and Hermsen (1972a). According to Abdalla and Hermsen (1972b), male sterility in F_1 due to interactions between *S. verrucosum* cytoplasm and plasmon-sensitive genes in the 1EBN Mexican species can hinder gene exchange between these species.

Crossability with either cultivated potato or bridging *Solanum* species is a prerequisite to attain remote sexual hybridizations and introgression of germplasm from the Mexican 1EBN distinct group of species that includes diploid *S. pinnatisectum* (Hermsen 1994). *S. pinnatisectum* is particularly desired for potato enhancement and has been proposed as a partner for concurrent introgression of multiple resistances (Hayes and Thill 2002). This species has strong barriers to hybridization with both 2EBN and 1EBN *Solanum* species (Novy and Hanneman 1991; Kuhl et al. 2002; Ramon and Hanneman 2002). Nevertheless, *S. pinnatisectum*, like *S. verrucosum*, carries high levels of resistance to pests, in addition to improved processing quality (Kuhl et al. 2001; Zlesak and Thill 2002; Hayes and Thill 2002).

Despite embryo–endosperm incompatibilities, reproductively separated taxa within *Solanum* section *Petota* of tuber-bearing potato species with distinct genomes (Matsubayashi 1991) can be crossed artificially to produce enhancement hybrids. Sexual gene transfer can be accomplished between species with different ploidy and EBN levels by means of chromosome manipulations involving haploids, 2*n* gametes, EBN manipulation, bridging species, double pollinations, and embryo rescue (Ehlenfeldt and Hanneman 1984; Peloquin et al. 1989; Singsit and Hanneman 1991). In this way, breeding schemes have been developed by Hermsen (1994) for introgression of *S. pinnatisectum* and *S. bulbocastanum* wild species, using *S. verrucosum* bridge and tetraploid *S. tuberosum*.

Attempts to directly cross the Mexican 1EBN diploid species with cultivated potato (4EBN) have been previously made by Jackson and Hanneman (1999), testing the crossability between these species as fruit and seed set and by stylar barriers. Although no stylar barriers were found, the Mexican primitive species of superseries *Stellata*, including series *Pinnatisecta* and *Bulbocastana*, did not set any fruit and seed in these crosses. Likewise, Novy and Hanneman (1991) tried without success to cross this group of Mexican isolated species, including 2*n* pollen-producing and chromosome-doubled 1EBN clones, with Group *Tuberosum* haploids, obtaining no fruit or parthenocarpic fruit in some combinations. Inter-species incompatibility barriers were established in these crosses, even with the 1EBN species used as a male parent.

Few reports exist in the literature on inter-EBN hybrids with Mexican diploids. One hypoploid ($2n = 22$) hybrid missing a chromosome pair, with low female and male fertility, was obtained by Ramon and Hanneman (2002) in direct crosses between diploid *S. tuberosum* and diploid *S. pinnatisectum* through double pollination and embryo culture. Diploid inter-EBN hybrids with bridging species have been obtained by Abdalla and Hermsen (1972a) between diploid *S. verrucosum* and diploid *S. bulbocastanum*. In the first introgression step, hybrids that are triploid can be produced by sexual hybridization (Masuelli and Camadro 1992). Louwes et al. (1992) reported on production of both diploid and triploid hybrids between South American diploid 1EBN *S. circaeifolium* and diploid *S. tuberosum* through the function of reduced and unreduced eggs. Based on EBN manipulation, Carputo et al. (1997) introgressed chromosomes from the South American 1EBN species *S. commersonii* chromosomally doubled genotypes as females in direct crosses with species of Group *Tuberosum*, obtaining triploid *S. commersonii–S. phureja–S. tuberosum* F_1 hybrids.

Auxins are involved in many aspects of plant development by stimulating cell division, elongation, growth, and differentiation, and have been used in particular to promote flowering, induce and maintain fruit, embryo,

endosperm, and seed maturation (reviewed by Davies 1995). Auxins, together with cytokinins, are also known to maintain undifferentiated cells in proliferation and division during in vitro culture (Eckardt 2001).

Ploidy and EBN manipulation, phytohormones, bridging species, and embryo rescue were all combined in our research to bypass cross-hybridization obstacles. Here we report on novel diploid and triploid hybrids created sexually between isolated *Solanum* species within section *Petota* and document endosperm and embryo development through confocal microscopy, fruit formation, seed production and viability, and plant regeneration in reciprocal inter- and intra-EBN crosses with and without application of auxin phytohormones and embryo rescue.

Materials and methods

Plant material

Plants analyzed and included in crosses and species abbreviations are listed in Table 1. The *Solanum* species germplasm was received as true seed from the USDA/ARS Inter-Regional Potato Introduction Project (NRSP-6), Sturgeon Bay, Wisconsin, and planted in the greenhouse for tuber increase. The parents were represented by six plants each of 12 genotypes of 2*x*-Pnt (*Solanum pinnatisectum* Dunal.) and six genotypes of 4*x*-Pnt, four genotypes of 2*x*-Cph (*S. cardiophyllum* Lindl.) and one genotype of 4*x*-Cph, one genotype of 2*x*-Blb (*S. bulbocastanum* Dunal.), two genotypes of 2*x*-Trf (*S. trifidum* Correll), one genotype of 2*x*-Trn (*S. tarnii* Hawkes and Hjert.) [all species of 1EBN Mexican (Mex) origin], 20 genotypes of 2*x*-Ver (*S. verrucosum* Schlechtd.), and 12 genotypes of 2*x*-Tbr (*Tuberosa* series) and F_2 2*x*-H-S (F_2 haploid-species hybrids). The accessions of the wild species 2*x*-Pnt, 2*x*-Cph, and 2*x*-Trf were selected by Zlesak and Thill (2002) for late blight resistance. The 4*x*-Pnt and 4*x*-Cph genotypes were derived from in vitro adventitious shoots on cultured leaf explants of the same diploid species selections. They were previously confirmed cytologically in mitosis as polyploids. Adapted parents included 2*x*-Tbr haploids US-W 463 and US-W 3611 of cultivar Katahdin and F_2 2*x*-H-S clones C159, C182, C189, C192, C213, C231, C305, C307, C380 with 2*x*-Ber (*S. berthaultii* Hawkes), 2*x*-Spl [*S. sparsipilum* (Bitter.) Juz. and Bukasov], 2*x*-Chc (*S. chacoense* Bitter.), and 2*x*-Tbr species ancestors (Table 1; Thill and Peloquin 1994).

Crossing experiments

Plants were grown in the greenhouse from tubers in 25-cm diameter pots under artificial long-day photoperiod. Emasculated flowers were pollinated in the morning with fresh or −20°C stored pollen 1–2 days before anthesis. Randomly selected inflorescences were sprayed with 10 ppm synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) solution 1 day after pollination (DAP) according to previous protocols in cereals (Rines et al. 1997). Three of the plants of each genotype were auxin-treated and three others remained untreated. Fruit were collected at ≥30 DAP for seed culture or at 14–21 DAP for embryo rescue.

The crosses evaluated were (1) 2*x*-Ver × 2*x*-Mex and 2*x*-Mex × 2*x*-Ver; (2) 2*x*-Ver × 4*x*-Mex and 4*x*-Mex × 2*x*-Ver; (3) 2*x*-Tbr × 4*x*-Mex and 4*x*-Mex × 2*x*-Tbr; (4) 2*x*-Tbr × 2*x*-Mex and 2*x*-Mex × 2*x*-Tbr; and (5) Control: 2*x*-Ver × 2*x*-Tbr, 2*x*-Ver × 2*x*-Ver, 2*x*-Tbr × 2*x*-Tbr and 4*x*-Mex × 4*x*-Mex. The percentage of fruit, seed, embryo formation, and seedling regeneration on culture media were analyzed.

Statistical procedures

To test for fruit set differences, 95% confidence intervals for percent fruit per pollination were estimated according to the Wilson (1927) formula without continuity correction. Significant differences in the frequency of fruit per pollination and regenerated plantlets per cultured seeds were identified by use of a chi-square (χ^2) test (Fisher 1922). Mean values and variances of seeds per fruit were calculated for 4*x*-Mex × 2*x*-Tbr, 2*x*-Ver × 4*x*-Mex, and/or 2*x*-Ver × 2*x*-Mex combinations. Means were compared using *t*-tests for unequal variances (Student 1908; Welch 1938).

Microscopy procedures

To document embryo and endosperm development using confocal microscopy, an average of 100 ovules were collected for each combination: 4*x*-Pnt × 2*x*-Tbr; 4*x*-Pnt × 2*x*-H-S; 2*x*-Ver × 2*x*-Pnt; 2*x*-Ver × 4*x*-Pnt; 2*x*-Ver × 2*x*-Tbr; and selfed 4*x*-Pnt at 2, 4, 7, 14, 17, and 20 DAP (both early and late in development). The specimens were dehydrated through a series of 70, 95, and 100% ethanol treatments for 15 min each and cleared in an ethanol/methyl salicylate (1:1) mixture for 2 h, followed by methyl salicylate for 2 h or overnight, then stained with a 1-μM solution of sytox green nucleic acid stain in dimethylsulfoxide for 30 min. Preparations were examined using a Bio-Rad MRC-1024 confocal microscope attached to a Nikon Diaphot inverted microscope (Bio-Rad Laboratories, Hercules, Calif., USA) equipped with a 15-mW Krypton/Argon laser with excitation filters of 488-, 568-, and 647-nm laser lines. Digital images were collected using LaserSharp, version 3.2 software (BioRad Laboratories) and processed using Image Pro Plus, version 4.5 software (Media Cybernetics, Silver Springs, Md., USA).

Pollen viability and the presence of 2*n* pollen were tested from the flowers of individual parent plants.

Table 1 Plant species and taxonomic relationships

Section	Origin	Series	Group	Species	Abbreviation	Ploidy	EBN	Accession
Petota	Mexican (Mex)[a]	*Pinnatisecta*		*Solanum pinnatisectum* Dunal.	Pnt	2*x*	1	PI 184764
						2*x*	1	PI 230489
						2*x*	1	PI 275232
						2*x*	1	PI 275233
						2*x*	1	PI 275236
						2*x*	1	PI 347766
						4*x* (autotetraploid)	2	PI 230489
						4*x* (autotetraploid)	2	PI 275233
				S. tarnii Hawkes and Hjert.	Trn	2*x*	1	PI 545742
				S. trifidum Correll	Trf	2*x*	1	PI 255541
						2*x*	1	PI 283065
		Bulbocastana		*S. cardiophyllum* Lindl.	Cph	2*x*	1	PI 283062
						2*x*	1	PI 283063
						4*x* (autotetraploid)	2	PI 283062
				S. bulbocastanum Dunal.	Blb	2*x*	1	PI 310963
		Tuberosa		*S. verrucosum* Schlechtd.	Ver	2*x*	2	PI 161173
						2*x*	2	PI 195171
	South American		*Tuberosum*	*S. tuberosum* L.	Tbr	4*x*	4	NA[b]
						2*x* (haploid)	2	NA
				S. berthaultii Hawkes	Ber	2*x*	2	Unknown
				S. sparsipilum (Bitter.) Juz. and Bukasov	Spl	2*x*	2	Unknown
		Yungasensa		*S. chacoense* Bitter.	Chc	2*x*	2	Unknown
				F_2 haploid-species hybrids	F_2 H-S	2*x*	2	NA

[a] *Mex* symbolizes a group of five 1 endosperm balance number (EBN) species from series *Pinnatisecta* and *Bulbocastana* included in crosses as described in "Materials and methods." *Ver* is a 2EBN Mexican species from series *Tuberosa*, treated separately for this purpose
[b] *NA* Not available

Pollen stainability was determined after staining in a drop of 2% (w/v) acetocarmine–glycerol mix for 5–10 min. The diameter of the stained pollen was measured by an eyepiece micrometer, and at least 300 pollen grains were randomly screened. Pollen was considered 2*n* if it was 1.25–1.4 times the diameter of the *n* pollen. Genotypes with ≥6% stainable pollen were classified as male fertile, and genotypes with ≥1% 2*n* pollen were classified as 2*n* pollen producers according to Hermundstad and Peloquin (1985).

Chromosome numbers of the selected hybrids were counted on Feulgen-stained mitotic metaphase preparations from squashed root tips. A minimum of ten metaphase cells were used from eight separate roots of four different plants. The root tips were pretreated in 2-mM 8-hydroxyquinoline for 4 h and then fixed in 3:1 (ethanol:acetic acid) Farmer's solution for 48 h. Following hydrolysis for 12 min in 1 N HCl at 60°C, the root tips were stained for 2 h with Schiff's reagent and squashed in 2% (w/v) aceto-orcein. Microscopic images were collected with a Magnafire CCD camera and processed using Adobe Photoshop, version 5.5 (Adobe Systems, San Jose, Calif., USA).

Embryo rescue and seed culture

Inter-series F_1 immature seeds were isolated from the fruit 14–21 DAP for embryo rescue and plant in vitro culture. After surface sterilization of the fruit with 70% ethanol for 1 min, the embryos were removed from plump, immature seeds and cultured on HLH medium (Neal and Topoleski 1983). In the case of flat, immature seeds, the micropylar end of the seed coat was opened with a dissection needle, and whole seeds were placed on the culture medium. In control experiments without embryo culture, fruit were harvested at 30 DAP, and mature seeds were cultivated in vitro on MS medium supplemented with 4% sucrose and 7% agar (Murashige and Skoog 1962). The embryo and seed cultures were

placed under low light intensity of cool-white fluorescent light at 24°C (16 h light, 8 h dark).

SSR analysis

Progeny families were screened with microsatellite markers identified from the EMBL (WEBIN at: http://www.ebi.ac.uk/embl/Submission/webin.html) and GenBank (BankIt at:http://www.ncbi.nlm.nih.gov/BankIt/) databases. DNA extraction and PCR were accomplished by using the REDExtract-N-AmpTM Plant PCR Kit (Sigma) according to the vendor's recommendations. Designed PCR primers marking the polymorphic CTT SSRs within the *waxy* gene of Ver, and the polymorphic AATT SSRs within the *patatin* pseudogene of Pnt were derived from Tbr (Veilleux et al. 1995) and used for species-specific amplification to discriminate the parental germplasm and identify the F_1 hybrids. The independently amplified polymorphic PCR products were mixed and separated in 3.5% agarose gel in 1× TAE pH 8.0 buffer for 3 h at 125 V. A true hybrid was considered to be identified when specific marker bands could be detected from both parents in the resulted progeny.

Results

Endosperm development and embryogenesis in inter-series *Solanum* hybrids

Confocal scanning microscopy revealed that early endosperm collapse was prevalent in these wide crosses (Fig. 1a–d). The collapse was associated with hyperplasia of the endothelium (Dinu and Thill 2004). Consistently observed in crosses of remotely related species, the ovules aborted at different stages of development, following endosperm and embryo collapse. With 4*x*-Pnt females, the endosperm collapsed and a proembryo was not formed (Fig. 1a, b). In contrast, embryos were formed and aborted later during endosperm collapse with 2*x*-Ver bridging species as females (Fig. 1c, d).

Throughout two crossing seasons among 1,975 intra-EBN reciprocal pollinations between 4*x*-Mex × 2*x*-Tbr and 2*x*-Tbr × 4*x*-Mex, only 15 parthenocarpic fruit were produced. Confocal microscopy evidenced seed failure advancing within 3–4 days after pollination due to endosperm cell breakdown in 4*x*-Pnt × 2*x*-Tbr and 4*x*-Pnt × 2*x*-H-S crosses, despite matching EBN ratios (Fig. 1a, b). Because of the premature endosperm and seed collapse, it was difficult to discriminate between the

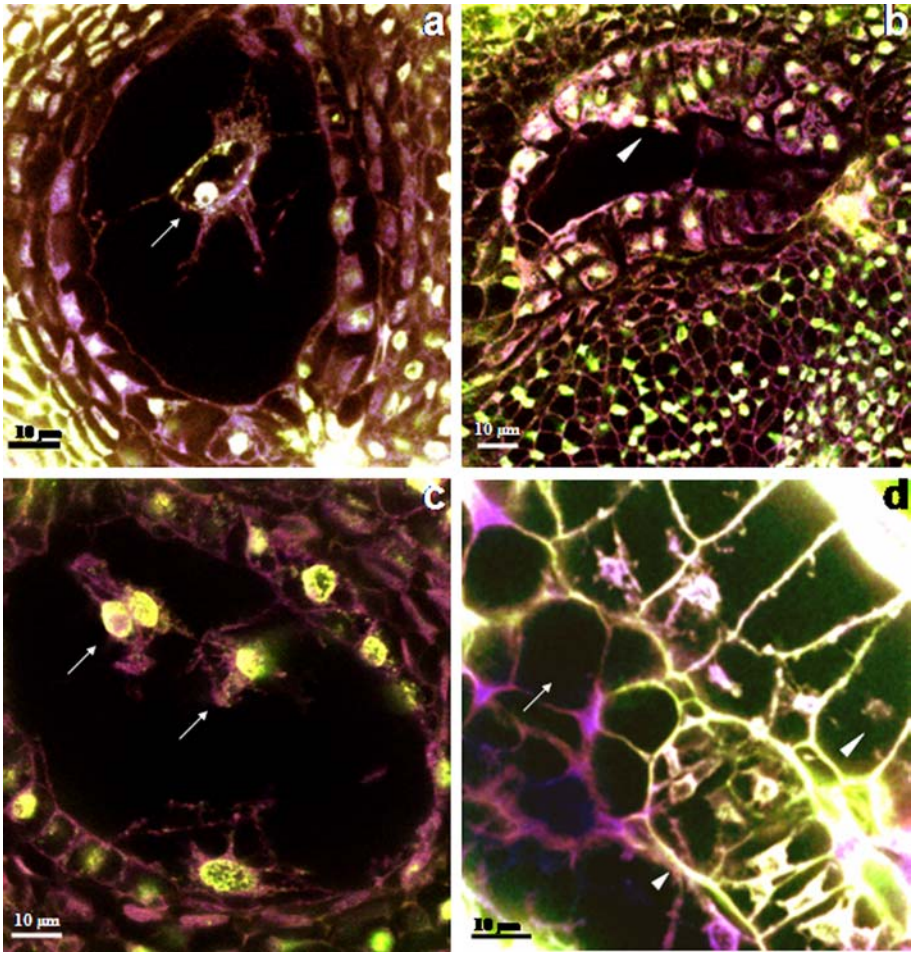

Fig. 1 Early versus late endosperm and embryo collapse during ovule abortion in incongruous inter-series intra-endosperm balance number (EBN) direct 4*x*-Pnt (*Solanum pinnatisectum* Dunal.) × 2*x*-Tbr (*S. tuberosum* L.) (**a–b**) and inter-EBN bridging crosses 2*x*-Ver (*S. verrucosum* Schlechtd.) × 2*x*-Pnt (**c–d**) with no 2,4-D auxin treatment. **a** Developing endosperm (*arrow*) in the embryo sac of the young ovule at 2 days after pollination (DAP), with 60× magnification. **b** Invasive endothelial cells (*large arrow head*) in the aborting ovule at 7 DAP, 60×. **c** Endosperm division (*arrow*) in the 4 DAP ovule, 60× magnification. **d** Globular-stage zygotic embryo (*small arrow head*), degenerating endosperm (*arrow*), and enlarged endothelial cells (*large arrow head*) in the immature seed at 14 DAP, 60×

unfertilized central cell and the primary cell of the endosperm. A multicellular endosperm was never detected, and potentially, no endosperm development occurred (Fig. 1a). Because of very early endosperm collapse, zygote division was not observed and true embryos were not found. An undifferentiated mass of cells proliferated instead of forming an embryo (data not shown). The inner layer of the integument, the endothelium tissue was actively dividing in multiple cell layers. Dense cytoplasmatic endothelial cells migrated to the central cavity of the ovules, already at 7 DAP (Fig. 1b). Among 208 (during 2002) and 32 crosses (during 2003) of 4*x*-Mex × 2*x*-Tbr and 2*x*-Tbr × 4*x*-Mex with auxin treatment applied to ovaries post-fertilization, 76 and 19 fruit, respectively (inclusive parthenocarpic), yielded 119 and 123 flat immature seeds cultured in vitro at 14–21 DAP. Even with phytohormone treatment in these crosses, in parallel with early endosperm and seed failure, embryos were not formed and could not be rescued.

In 2*x*-Ver × 2*x*-Pnt and 2*x*-Ver × 4*x*-Pnt crosses, irrespective of differences between EBN ratios, incipient normal endosperm and zygote division, and occasional embryo growth were observed with no treatment, followed by aborting mature seeds (Fig. 1c, d). The large, vacuolated cells of the endosperm with enlarged, well-stained nuclei were developed at 4 DAP, in close contact with the chalazal and micropylar ends. The endosperm cells were still present at 14 DAP (Fig. 1c). We noticed the lack of further differentiation of these cells, which failed to enlarge further, becoming smaller and lacking stainable nuclei. The embryo began development after zygote division, in spite of the failing endosperm, such as at 14 DAP a globular embryo was detected in the inter-EBN 2*x*-Ver × 2*x*-Pnt crosses. The endothelial cells underwent radial elongation with occasional transverse divisions (Fig. 1d). Following endosperm collapse, the embryo ceased further development and degenerated, and no viable embryos could be rescued from Ver combinations with no auxin treatment (Table 2D). With

Table 2 The effect of 2,4-dichlorophenoxyacetic acid (2,4-D), embryo rescue, ploidy manipulation and maternal contribution on fruit set, seed set, and quality (viability, maturity, regeneration efficiency) in crosses of South American 2*x* (2EBN) *S. tuberosum* (*Tbr*) and Mexican (*Mex*) 2*x* (2EBN) *S. verrucosum* (*Ver*) series *Tuberosa* species with 2*x* 1EBN and chromosomally doubled 4*x* 2EBN Mex wild species with and without embryo rescue. *Pol* Pollinations

Combination	Number of		Fruit per pollination		Number of seeds cultured			Regeneration	
	Pol	Fruit[a,b]	Percentage	95% CI	Flat immature[c]	Plump immature[d]	Mature[e]	Number	Percentage
A. 2,4-D treatment, embryo rescue[f] treatment									
2*x*-Ver × 2*x*-Mex	21	17[b]	81	60–92	5	39	0	12	27
2*x*-Ver × 4*x*-Mex	86	67[b]	78	68–85	3	67	0	12	17
4*x*-Mex × 2*x*-Tbr	32	19[b]	59	42–74	123	0	0	0	0
Control 2*x*-Ver × 2*x*-Tbr	1	1	100	21–100	0	0	30	23	77
Control 4*x*-Mex × self	5	2	40	12–77	0	0	17 (68)[g]	16	94
Total									22
X^2, 4*df*									139*
B. 2,4-D treatment, no embryo rescue[h] treatment									
2*x*-Ver × 2*x*-Mex	302	26[a]	9	6–12	0	0	1	0	0
2*x*-Ver × 4*x*-Mex	2	2	100	34–100	0	0	2	0	0
4*x*-Mex × 2*x*-Tbr	102	16[b]	16	10–24	0	0	40[I]	0	0
C. No 2,4-D treatment, embryo rescue[f] treatment									
2*x*-Ver × 2*x*-Mex	39	15	38	25–54	0	0	0	0	0
4*x*-Mex × 2*x*-Tbr	26	4	15	6–34	0	0	0	0	0
D. No 2,4-D treatment, no embryo rescue[h] treatment									
2*x*-Ver × 2*x*-Mex	1321	233[a]	18	16–20	0	0	1	0	0
2*x*-Ver × 4*x*-Mex	456	67[a]	15	12–18	0	0	0	0	0
2*x*-Mex × 2*x*-Ver	113	0[a]	0	0–3	0	0	0	0	0
2*x*-Mex × 2*x*-Tbr	18	0[a]	0	0–18	0	0	0	0	0
4*x*-Mex × 2*x*-Ver	240	1[a]	1	0–2	0	0	0	0	0
4*x*-Mex × 2*x*-Tbr	258	15[a]	6	4–9	0	0	8[I]	0	0
2*x*-Tbr × 2*x*-Mex	18	0[a]	0	0–18	0	0	0	0	0
2*x*-Tbr × 4*x*-Mex	59	0[a]	0	0–6	0	0	0	0	0
Control 2*x*-Tbr/2*x*-Ver × 2*x*-Tbr/2*x*-Ver	123	36	29	22–38	0	0	30 (3,845)[g]	25	83.4
Control 4*x*-Mex × self	67	12	18	10–29	0	0	30 (114)[g]	18	60

Significance level: $*P < 0.01$

[a]Fruit set followed by this letter are significantly lower than control pollinations ($P < 0.01$)

[b]Fruit set followed by this letter are significantly higher than no treatment ($P < 0.01$)

[c]14–21 Days after pollination (DAP) seeds with collapsed endosperm

[d]14–21 DAP seeds with endosperm present

[e] > 21 DAP seeds

[f]14–21 DAP

[g]Total number of seeds generated

[h] > 30 DAP

[I]Seeds collapsed

2,4-D application, endosperm collapse was delayed, and embryo development continued to mature stages, allowing successful embryo rescue for these combinations (Table 2A). Control crosses of 2*x*-Ver × 2*x*-Tbr and selfed 4*x*-Pnt developed normally.

Genome dosage and EBN dependence in *Solanum* hybridizations

Ploidy and EBN manipulations via chromosome doubling were not effective to increase fruit and seed set (Tables 2, 3). Ploidy manipulation for direct inter-ploidy 4*x*-Mex × 2*x*-Tbr and 2*x*-Tbr × 4*x*-Mex crosses was unsuccessful, resulting in eight collapsed and zero seeds (Table 2D) and producing significantly fewer fruit (6 or 0%), in comparison to 2*x*-2EBN × 2*x*-2EBN control crosses—χ^2 test: $1df = 52.1$, $P < 0.01$; and 4*x*-2EBN × 4*x*-2EBN crosses— χ^2 test: $1df = 14.7$, $P < 0.01$ (Table 2D). Fruit set and size in 4*x*-Mex × 2*x*-Tbr and 2*x*-Tbr × 4*x*-Mex crosses (26%) were significantly increased (χ^2 test: $1df = 43.7$, $P < 0.01$) with 2,4-D application, whereas viable seed production and seed quality was not improved (Table 2A and B). Furthermore, no viable seeds were recovered by combining 2,4-D and embryo rescue in 4*x*-Mex × 2*x*-Tbr and 2*x*-Tbr × 4*x*-Mex crosses (Table 2A).

Crosses with 2*x*-Ver as female with 2*x* 1EBN or 4*x* 2EBN males with no 2,4-D (Table 2D) produced fruit in 18 and 15% of pollinations (not significantly different—χ^2 test: $1df = 1.8$, $P > 0.01$), respectively. The fruit was small in size and the frequency of fruit production for all crosses using 2*x*-Ver as a female was significantly lower than in 2*x* 2EBN × 2*x* 2EBN control crosses— χ^2 test: $2df = 14.4$, $P < 0.01$ (Table 2D). No stylar barriers were found in 2*x*-Ver × 2*x*-Pnt and 2*x*-Ver × 4*x*-Pnt crosses (data not shown). One non-viable mature seed from 2*x*-Ver PI 195171 × 2*x*-Trn was recovered from 1,321 pollinations (Table 2D). Ploidy manipulation of the Mexican species was not effective at increasing seed set with, without, or by combining 2,4-D treatment and embryo rescue in crosses with 2*x*-Ver females, and in inter-ploidy crosses with either 2*x*-Ver or 2*x*-Tbr and 4*x*-Mex (Table 3).

Irrespective of embryo rescue, application of 2,4-D to 2*x*-Ver × 2*x*-Mex and 2*x*-Ver × 4*x*-Mex crosses overall increased fruit size and significantly increased fruit set by 28% (χ^2 test: $1df = 23.5$, $P < 0.01$; Table 2A and B), of which fruit set increased by 9% (χ^2 test: $1df = 14.9$, $P < 0.01$) in 2*x*-Ver × 2*x*-Mex crosses (Table 2B and D). Without embryo rescue, three non-viable mature seeds were recovered from 28 fruit out of 304 pollinations, two seeds from 2*x*-Ver PI 195171 × 4*x*-Pnt PI 275233 and one seed from 2*x*-Ver PI 195171 × 2*x*-Pnt PI 275236 crosses (Table 2B). No significant difference (χ^2 test: $1df = 0.1$, $P > 0.01$) was detected with ploidy manipulation in the fruit set between 2*x*-Ver × 2*x*-Mex and 2*x*-Ver × 4*x*-Mex crosses (Table 2A).

Overall, 106 plump immature seeds and embryos were cultured from 84 fruit of 2*x*-Ver × 2*x*-Mex or 2*x*-Ver × 4*x*-Mex by combining 2,4-D treatment and embryo rescue (Table 2A). Seed quality in terms of viability, maturity, size, and regeneration efficiency was significantly increased for combinations that involved 2*x*-Ver when both treatments were applied (Table 2A). In contrast, embryo rescue alone (Table 2C), 2,4-D application without embryo rescue (Table 2B), or no treatment (Table 2D) yielded no viable seeds in these crosses. 2,4-D application in combination with embryo rescue at 14–21 DAP increased significantly the seed set in 2*x*-Ver × 4*x*-Mex and 2*x*-Ver × 2*x*-Mex (mean 2.3) and in 4*x*-Mex × 2*x*-Tbr (mean 8.5) crosses (Table 4). At more than 30 DAP, in the absence of embryo rescue, seed set was significantly reduced (mean 0.1) in 2*x*-Ver × 2*x*-Mex and 2*x*-Ver × 4*x*-Mex crosses when using 2,4-D, and no significant difference was detected in the same treatments of the 4*x*-Mex × 2*x*-Tbr crosses (Table 4). Combining crossing with Ver as a female, 2,4-D and embryo rescue resulted in higher frequencies of offspring with diploid and autotetraploid Pnt species (Table 5).

Unilateral incompatibility was evident in many combinations. The diploid crosses with 2*x*-Ver males were unsuccessful (Table 2D), probably due to stylar barriers of unilateral incompatibility (Hermsen and Ramanna 1976). Inter-species incompatibility was maintained by crossing 4*x*-Mex self-compatible females with 2*x*-Ver self-compatible males, with one parthenocarpic fruit resulting from 240 pollinations (Table 2D). Likewise, no fruit was obtained in the crosses between self-incompatible 2*x*-Tbr × self-com-

Table 3 The effect of ploidy manipulation on seed set as determined by seeds per fruit means and variances in crosses of 2*x*-Ver × 2*x*- or 4*x*- (chromosomally doubled) Mex species, and 4*x*-Mex × 2*x*-Tbr with and without 2,4-D and embryo rescue

	Combination		
	2*x*-Ver × 2*x*-Mex (Inter-EBN)	2*x*-Ver × 4*x*-Mex (Intra-EBN)	4*x*-Mex × 2*x*-Tbr (Intra-EBN)
2,4-D and no embryo rescue[a]			
Number of fruit	11	2	9
Mature seeds/fruit			
Mean	0.02	0.8	3.0
Variance	0.01	0.1	33.2
2,4-D and embryo rescue[b]			
Number of fruit	35	9	12
Immature seeds/fruit			
Mean	1.4	6.0	8.5
Variance	5.5	35.3	33.2
No treatments[a]			
Number of fruit	112	26	6
Mature seeds/fruit			
Mean	0.1	0	1.3
Variance	0.9	0	11.0

[a] > 30 DAP
[b] 14–21 DAP

Table 4 The effect of 2,4-D on seed set as determined by the seeds per fruit means and variances in crosses of 2*x*-Ver × 2*x*- and 4*x*-Mex wild species, and 4*x*-Mex × 2*x*-Tbr with and without embryo rescue

	Combination			
	2*x*-Ver × 4*x*-Mex/2*x*-Mex		4*x*-Mex × 2*x*-Tbr	
	2,4-D treatment	No 2,4-D treatment	2,4-D treatment	No 2,4-D treatment
Embryo rescue treatment[a]				
No. of fruit	44	8	12	4
Immature seeds/fruit				
Mean	2.3*	0	8.5*	0
Variance	14.3	0	33.2	0
No embryo rescue treatment[b]				
No. of fruit	13	138	9	6
Mature seeds/fruit				
Mean	0.1*	0.7	3.0	1.3
Variance	0.2	0.7	33.2	10.6

Significance level with use of 2,4-D compared to no 2,4-D application: $*P < 0.01$
[a] 14–21 DAP
[b] > 30 DAP

Table 5 Combination type, pollen viability and 2*n* pollen of the male parent, and ploidy level of the parents and the progeny combinations derived from crossing the Mexican 2*x* 2EBN Ver species series *Tuberosa* with diploid 1EBN and chromosomally doubled tetraploid 2EBN Pnt Mexican species with 2,4-D treatment and embryo rescue

Female	Male			Number of pollinations	Number of fruit	Number of embryos rescued	Combination	
Ploidy-Genotype	Ploidy-Genotype	Pollen stainability[a] (%)	2*n* pollen[b] (%)				Progeny (ploidy)	Number of descendents
2*x*-Ver 195171-9	4*x*-Pnt 275233-4	67.8	3	1	1	16	MNDI05 (3*x*)	2
2*x*-Ver 195171-2	2*x*-Pnt 275232-1	98	8	2	2	5	MNDI17 (3*x*)	4
2*x*-Ver 195171-2	2*x*-Pnt 275232-1	98	8	2	2	6	MNDI19 (3*x*)	2
2*x*-Ver 195171-2	2*x*-Pnt 275232-1	98	8	2	2	8	MNDI22 (2*x*)	2
2*x*-Ver 161173-2	2*x*-Pnt 275236-1	100	0	1	1	6	MNDI24 (2*x*)	4
2*x*-Ver 161173-2	4*x*-Pnt 275233-4	67.8	3	1	1	15	MNDI25 (3*x*)	10
Total								24

[a] Genotypes with ≥6% stainable pollen are considered male fertile (Hermundstad and Peloquin 1985)
[b] Genotypes with ≥1% 2*n* pollen are considered 2*n* pollen producing (Hermundstad and Peloquin 1985)

patible 4*x*-Mex (Table 2D), implicating unilateral incompatibility as a potential barrier to hybridization (data not shown).

Control (no treatment) 2*x*-2EBN × 2*x*-2EBN and 4*x*-2EBN × 4*x*-2EBN crosses were successful, producing fruit 29% and 18% of the time and containing 3,845 and 114 seeds, respectively (Table 2D). The 4*x*-Pnt genotypes were self-compatible, producing nine fruit and 245 seeds from 58 self-pollinations (data inclusive in control crosses, Table 2D). 2*x*-Ver genotypes were also self-compatible in controlled self-pollinations. Out of 61 2*x*-Ver self-pollinations, 12 fruit were formed and 1,355 seeds recovered (data inclusive in control crosses, Table 2D).

Novel hybrids in section *Petota*

A total of 106 embryos from immature seeds were rescued in vitro (Table 2A) from the crosses 2*x*-Ver × 2*x*-Pnt and 2*x*-Ver × 4*x*-Pnt with 2,4-D treatment. About 23% (24/106) of these F_1 embryos germinated and developed into vigorous plantlets. These 24 regenerated seedlings from four families and six combinations (Table 5) were analyzed by molecular means. The Ver- and Pnt-specific SSR markers used verified that all the F_1 genotypes were true hybrids (Fig. 2). The ploidy levels of the hybrid plants and their parents were determined cytologically (Fig. 3; Table 5). The resulting diploid (inter-EBN, AA^{pi}) and triploid

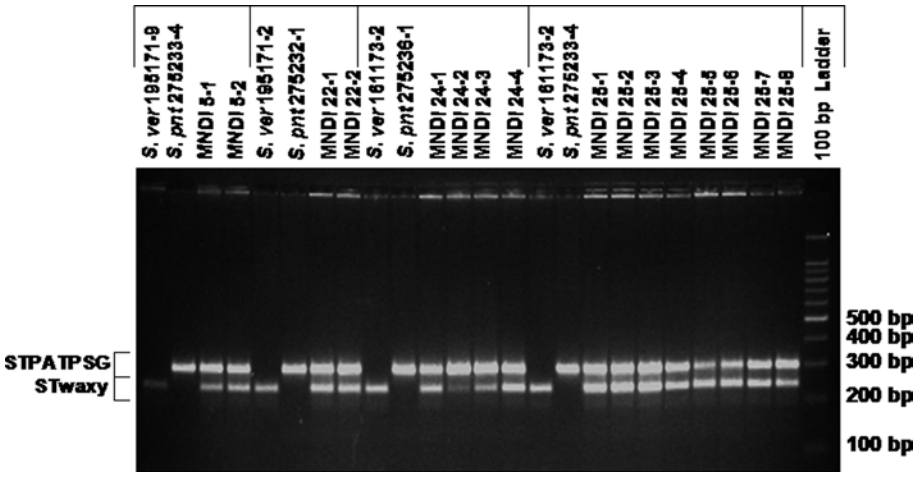

Fig. 2 Microsatellite marker polymorphism using primers flanking the CTT repeats within the *waxy* gene locus of Ver and the AATT repeats within the *patatin* pseudogene of Pnt in the parents. *Lanes 1*, *5*, *9*, and *15* Ver; *lanes 2*, *6*, *10*, and *16* Pnt; *lanes 7–8* MND122 (diploid); *lanes 11–14* MND124 (diploid); *lanes 3–4* MND105 (triploid hybrid progeny combination); *lanes 17–24* MND125 (triploid hybrid progeny combination); *lane 25* 100-bp ladder

(intra-EBN, $AA^{pi}A^{pi}$) F_1 hybrids have complete sets of chromosomes derived from both parental species (Fig. 3; Table 5).

The combinations MNDI17, MNDI19 and MNDI22 from three independent crosses represent one family (Table 5) in which it was possible to obtain both diploid and triploid progeny, owing to 8% functional 2*n* pollen. The genotypes of the combination MNDI24 were diploid and of combinations MNDI05 and MNDI25 were triploid (Table 5).

The sexual hybrids showed intermediate leaf shape and corolla color between the parents. Morphological characters were polymorphic between the F_1 full-sib genotypes. More vigor in leaf size, leaf branches, interstitial leaflets, number of flowers per inflorescence, radius of corolla, fruit length, plant height, and general habit of the plants was seen in hybrids versus parents and in triploids versus diploids, likely due to parental genome dosage (data not shown).

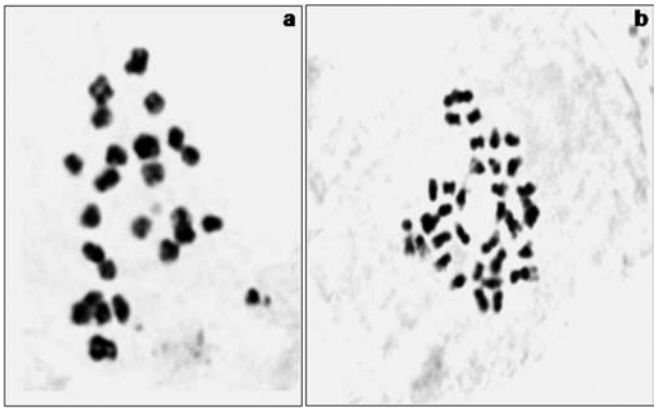

Fig. 3 Mitotic analysis of somatic metaphase cells. **a** Diploid hybrid MNDI24-2 with chromosome number $2n = 2x = 24$. **b** Triploid sexual hybrid MNDI05-1 with chromosome number $2n = 3x = 36$

Discussion

The dynamic taxonomy recognizes one cultivated and 196 wild tuber-bearing potato species in the genus *Solanum* section *Petota*, divided in four clades (Spooner and Hijmans 2001). The ancestral group of 1EBN species is paraphyletic in Mexico and Central America and includes the members of series *Pinnatisecta* Hawkes ($A^{pi}A^{pi}$). The species Ver of the series *Tuberosa* Hawkes (AA) is the only Mexican 2EBN diploid species that crosses with some of the Mexican diploid and polyploid species and with South American species and is considered to be evolved from the primitive Mexican species genome (Spooner et al. 2003). Incongruity in the genus *Solanum* between these 1EBN and 2EBN species arises from pre- and post-zygotic reproductive barriers to gene exchange, resulting in the isolation of the taxa. The failure of effective partner relationships is caused by a mismatch of genetic and biochemical factors, present in the isolated species (de Nettancourt 1997). This present research focuses on the interspecies incongruity effects on endosperm and seed development.

In the absence of 2,4-D treatment or embryo rescue, all tested inter-series pollinations were unsuccessful. Inter-species incongruity was maintained in the intra-EBN crosses with the cytoplasm of wild chromosomally doubled 1EBN species. Endosperm structure and growth abnormalities have been proposed as the explanations for incongruity in these crosses. The collapse of the endosperm cells occurred at different time intervals and rates after pollination, causing seed failure, which was earlier with 2*x*-Tbr combinations and later with 2*x*-Ver crosses. The loss of control during the hybrid endosperm development was associated with the endosperm cells failing early to divide normally and therefore, the zygote did not differentiate for embryo development; however, we observed division of the hyperplastic endothelial cells in the embryo sac. In these crosses, seeds collapsed early, and the genetic imbalance

was maintained in the hybrid endosperm despite the applied auxin and seed culture treatments.

In our crosses, the mechanisms of genetic isolation appear to operate directly at the endosperm level in incongruous 2*x*-Ver female crosses with 2*x*-Pnt and 4*x*-Pnt. The endosperm cells started to divide with no treatment, and the failure of the endosperm cells occurred later during endosperm development. The endosperm cells failed to enlarge and differentiate in a normal manner, likely resulting from a failure of mitotic activity and cell division. This barrier of the cell cycle had direct implications on the progression of development of the cellular type of endosperm. The embryo growth was occasionally promoted to the globular stage within further stages of endosperm and seed collapse. The use of Ver species as a female in the bridging crosses with the diploid (1EBN) and autotetraploid (2EBN) Mex species was a prerequisite for viable seed set. The fruit set, seed set, and regeneration efficiency were improved in these crosses only with combined treatments of auxin application and embryo rescue. As female parents in our crosses, 2*x*-Ver showed no discrimination against the fertilization with pollen of either 2*x*-Pnt or 4*x*-Pnt species, with or without auxin application. Our data support the idea that endosperm barriers can act in succession, if early developmental requirements are first met. In these particular cases, an initial compatibility of genetic factors in the endosperm mitotic cycle must have been present. In a contrary situation, it would not be possible to induce hybrid formation, with combined phytohormone and embryo rescue treatments.

Breakdown of the endosperm and hyperplasia of the endothelium were previously described in inter-species *Solanum* matings by other authors (Lester and Kang 1998). They also presented cells without nuclei in the endosperm and adjacent embryo parts or found absence of the embryo in inter-species *Solanum* hybrids. These authors suggested that the endosperm controls the morphogenesis of the embryo and digesting enzymes are produced when the control of biosynthesis is not coordinated in hybrid nuclei with genetic imbalances. In inter-ploidy and inter-EBN incongruous *Solanum* combinations, similar observations were made by Masuelli and Camadro (1997), who found embryoless embryo sacs and proposed that lethal genes act at the embryo level, independently of the paternal contribution and gene dosage. They explained triploid hybrid production in these $4x \times 2x$ crosses by the segregation of the EBN loci, suggesting that the genetic differences of endosperm failure in inter-species crosses may be more quantitative than qualitative (Hermsen 1987).

Ploidy of the endosperm in inter-ploidy crosses is considered an important factor for successful seed development. Our studies show that there was no effect of ploidy and consequently, of the EBN manipulation with the ploidy on fruit or seed set in crosses of 2*x*-Ver or 2*x*-Tbr with Mexican 1EBN diploids. Based on the hybrid seed development and viability, the polar-nuclei activation hypothesis of Nishiyama and Yabuno (1978) proposed that the development of hybrid endosperm depends on the 2:1 ratio of the relative strength of specific female and male genes expressed in this tissue. With a similar concept, the EBN hypothesis reflects on exceptions to the two maternal:one paternal EBN ratio in the endosperm, where viable seed is produced while the ratio is not maintained, but not produced while the ratio is met (Johnston et al. 1980; Katsiotis et al. 1995). Therefore, the endosperm ratio demonstrates that seed viability is not always dependent on the actual ploidy composition, but is determined by ratios of genetic factors irrespective of the ploidy, i.e., EBN. In potato, at least two or three genes are believed to be responsible for the EBN system (Ehlenfeldt and Hanneman 1988; Camadro and Masuelli 1995; Johnston and Hanneman 1996). Furthermore, speciation via EBN might involve a duplication of the EBN genes, without consequent doubling of the genomes, or a change in the level of regulation of these genes.

The effect of Mexican Pnt or Ver species genomes, and potentially EBNs, on seed viability can also be interpreted in the context of differential gametic imprinting. The imprinting hypothesis states that a two maternal:one paternal genome ratio is required in the endosperm for the normal seed development (Lin 1984; Haig and Westoby 1991). In maize, Charlton et al. (1995) crossed a diploid female plant with diploid and autotetraploid males, producing viable triploid endosperm, and degenerated tetraploid endosperm, respectively. They proposed that gametically imprinted genes act at an early developmental stage and involve a regulatory protein composed of two polypeptides in a proportion determined by the ratio of maternal and paternal genomes. In *Arabidopsis*, Scott et al. (1998) found dosage effects of the parental genomes and concluded on the parent-of-origin effect of imprinted loci affecting endosperm and seed development. Masuelli (2001) established a correspondence between the dosage effect and transgene silencing, suggesting that multiple copies of the genes for endosperm development are inducing a mechanism of gene silencing that acts as a genome defense system, inactivating and methylating the extra maternal or paternal genes.

The effects of the combined factors in the parental genomes on endosperm development are evident. As previously discussed, one aspect is the effect of genome dosage on central cell and endosperm development. As demonstrated by other researchers, the endosperm differences are not entirely due to species differences or genome incompatibility. These barriers can be overcome with unreduced gametes or somatic chromosome doubling in one of the species, for example, through tetraploidization of the diploid 1EBN species (Bamberg et al. 1994; Carputo et al. 1997, 2000). As our results also show, these were prerequisites for balancing the ploidy and EBN levels in the gametes of the inter-series, inter-ploidy triploid hybrids between 2*x*-Ver species females and diploid genotypes producing 2*n* pollen or *n* pollen in autotetraploids of Pnt 1EBN species males as parents.

The non-reduction in diploid gametes has shown to facilitate polyploid formation through the triploid bridge in inter- and intra-species hybridizations (Ehlenfeldt and Ortiz 1995; Ramsey and Schemske 1998). Johnston and Hanneman (1995) hypothesized an incomplete penetrance of the EBN ratio, environmental effects, or two pollen tubes fertilizing the same ovule, allowing an occasional endosperm to develop normally. Carputo et al. (1999) also determined that endomitosis in the polar nuclei represented the cause of the chromosome duplication without cell division, explaining for the exceptions of the inter-EBN crosses and triploid formation.

In addition, we obtained inter-series, intra-ploidy diploid hybrids between the diploid species genotypes, without 2*n* gametes or somatic doubling. These genotypes resulted from the fusion of reduced gametes, when no functional unreduced pollen was produced by the genotype of the Pnt species, representing an inter-EBN cross, and thus, an exception from the EBN hypothesis. Pnt was the only successful species in these cross-hybridizations. Therefore, we assume that this effect is genotype-specific, since particular genotype combinations were preferentially represented in the resulted hybrid families in our studies. Our findings confirm that mechanisms of endosperm development other than genome and ploidy dosage for EBN are involved during early endosperm formation and/or embryogenesis in viable seed formation in crosses between these remote *Solanum* species.

The collapse of the endosperm in inter-EBN *Solanum* crosses can be explained by genotype-specific control of mitotic-cycle time of the parental genomes. Based on differences in mean cell-doubling time between the parents, Masuelli (2001) proposed a model for the formation of the hybrid endosperm. This model implies that cyclin-dependent kinase complexes control the induction of DNA synthesis and mitosis in hybrid endosperms in *Solanum*. Enlarged endosperm nuclei are undergoing endoreduplication in the incompatible endosperm genome, increasing DNA synthesis (Phillips et al. 1985; Schweizer et al. 1995). The nuclear size of hybrid endosperm was therefore correlated with endosperm development factors and EBN differences of parental gametes (Masuelli 2001).

Our results confirm that post-pollination barriers can be circumvented, and viable hybrids can be produced by in vitro embryo rescue, following 2,4-D application. Post-zygotic inter-species incongruity can be overcome in inter-series crosses irrespective of EBN ratios (Thill et al. 2003). With the combined treatments, inter-EBN as well as intra-EBN hybrids could be obtained between isolated *Solanum* species. This occurred by inducing hybrid endosperm development in a case that would normally represent a rare event. The culture of excised embryos on nutrient medium has been used in many crop species including potatoes, as a means to obtain inter-species hybrids in difficult crosses (Singsit and Hanneman 1991). In wide crosses, it has been reported that post-pollination application of auxin phytohormones such as the synthetic 2,4-D maintains the developing seed and facilitates normal division of the hybrid endosperm and zygote (Mujeeb-Kazi et al. 1987).

The hybrids reported here between Ver and Pnt and the strategy used to obtain them represent a substantial advancement in potato enhancement. Genotypes of Ver and Pnt can be identified by combining multiple resistances and quality traits, thereby allowing concurrent introgression, an efficient breeding method (Hayes and Thill 2002). According to the EBN rule, our triploid hybrids created in intra-EBN crosses involving somatic doubling or unreduced pollen have one genome of 2*x*-Ver (1EBN) and two genomes of 2*x*-Pnt (0.5EBN + 0.5EBN), equaling an EBN of 2. These genotypes should be crossable with 2*x*-Ver or 2*x*-Tbr through fertile *n* gametes and with 4*x*-Tbr through functional 2*n* gametes. The 3*x* 2EBN hybrids could be used in crosses with 2*x* 2EBN genotypes to generate trisomics and aneuploids with variable numbers of extra chromosomes, as previously shown by Carputo (1999). Gametes with a higher chromosome number have a higher probability of matching EBNs with the gametes of the 2*x* parent (Ehlenfeldt and Hanneman 1988). In addition, through the function of 2*n* gametes in the triploids balancing the parental EBNs, traits of interest could be transferred to the pentaploid level with 4*x*-Tbr. In natural triploid hybrids (3EBN) of *Solanum*, selection due to EBN favors gametes with lower numbers of extra chromosomes in backcrosses with diploid (2EBN) males, increasing the probability of the same EBN value in the gametes (Masuelli and Camadro 1992). In inter-species triploid (2EBN) *Solanum* hybrids, EBN is exercising selection for gametes with high chromosome numbers and EBN values (Carputo 1999; Carputo et al. 1999). Our diploid inter-EBN hybrids have one genome of 2*x*-Ver (1EBN) and one genome of Pnt (0.5EBN), resulting in an EBN of 1.5. The resulting EBN in the gametes could vary between 0.5EBN, 1EBN or 1.5EBN. 0.5EBN gametes with higher chromosome number, 1EBN gametes, and 1.5EBN gametes with lower chromosome number would be normally functional in a cross with a diploid 2EBN parent. These backcrosses present possibilities for the introgression via inter-series hybrids.

This is the first documented research on the utility of combining auxin treatment and embryo rescue, together with exploring endosperm and embryo development through confocal microscopy in crosses within the genus *Solanum*.

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