Chapter 3
Experimental Interspecific Hybrids in *Nicotiana* and Barriers to Hybridization

3.1 Overview of History and Extent of Interspecific Hybridization in *Nicotiana*

Interspecific hybrids may arise spontaneously in laboratories that maintain their in-house collections of different *Nicotiana* species. Unlike these rare events, such as the hybrid 2x (*N. setchellii* × *N. otophora*) reported by Bawolska et al. (1978), the vast majority of the known hybrids within the genus were the result of purposeful manipulation. The first experimental crossing of two different species of *Nicotiana* is generally credited to the German scholar J. G. Koelreuter, who produced a hybrid between *N. paniculata* and *N. rustica* while working in St. Petersburg, Russia in 1760 (Kostoff, 1943; Ternovsky, 1962; Mayr, 1986). Koelreuter's classical work confirmed the existence of sexuality in plants and demonstrated the equivalency of maternal and paternal parents in contributing to their offspring. The first interspecific hybrid that involved *N. tabacum* was probably also created by Koelreuter, who backcrossed the hybrid *N. rustica* × *N. paniculata* to *N. paniculata* and mated the offspring with *N. tabacum* (Kostoff, 1943). One may speculate that the trispecific combination thus produced was probably composed of aneuploid plants possessing full haploid genomes of *N. paniculata* and *N. tabacum* plus varying numbers of chromosomes contributed by *N. rustica*. Koelreuter also produced the hybrid 2x *(N. glutinosa* x *N. rustica*) and created the first true amphihaploid hybrids between *N. tabacum* and other *Nicotiana* species: 2x (*N. glutinosa* × *N. tabacum*), 2x (*N. paniculata* x *N. tabacum*) and 2x (*N. rustica* x *N. tabacum*) (Kostoff, 1943; Mayr, 1986).

In the nineteenth century, several other interspecific hybrid combinations with *N. tabacum* were synthesized. According to the accounts by East (1928) and Kostoff (1943), Sageret crossed *N. suaveolens* with *N. tabacum* in the 1820s, Gaertner produced N. *rustica* × *N. tabacum* and *N. quadrivalvis* × *N. tabacum* in the 1840s, and in the 1850s, Naudin was the first to produce the hybrid N. *tabacum* × *N. alata*.

A. Berbeć, *A Century of Interspecific Hybridization and Introgression in Tobacco*,
https://doi.org/10.1007/978-3-031-54964-9_3

Brongniart and Gris (1861) were the first to report the hybrid N. *glauca* × *N. tabacum*.

East (1928), one of the early cytogeneticists of *Nicotiana*, divided artificially made interspecific hybrids of *Nicotiana* into 'pre-Mendelian' and 'post-Mendelian'. The former were produced by Koelreuter and by nineteenth century investigators such as Gartner, Focke, Gudron, Naudin, and Sageret. The post-Mendelian hybrids were those created by East himself and by his contemporaries. East's list of viable interspecific hybrids includes 22 'pre-Mendelian' hybrid combinations and 43 hybrids produced later, 65 interspecific hybrids altogether, not including reciprocals. Kostoff (1943) described 181 interspecific hybrids, and Goodspeed (1945) and Goodspeed and Thompson (1959) elevated their number to 243. Citing those authors, Smith (1968) estimated that more than 300 interspecific hybrids had been reported in the genus. Apparao and Ramavarma (1974) raised the estimated number to 340. The author of this volume found references to 455 interspecific hybrid combinations within the genus *Nicotiana* that have been produced by 2023, including several that were reported as nonsurviving seedlings or as mere viable seeds (see Chap. 7). The former, mostly included in the type II seedling death category, have been included on the grounds that hybrid seedling lethality, especially type II, was recently demonstrated as a remediable condition controlled by a simple genetic system. The hybrids, together with literature references limited mostly to the earliest ones, were compiled in Chap. 7 of this review. The supplement chapter lists hybrid combinations and the parentage of each including the reciprocal hybrid, if reported. Multispecies crosses have not been included (those that involve *N. tabacum* as one of the parents are treated separately in Chap. 4 and listed in Tables 4.5 and 4.6).

Table 3.1 lists *Nicotiana* species and the numbers of hybrid combinations with other *Nicotiana*e reported for each of them. It also includes information on whether a particular species was successfully crossed with the cultivated species *N. tabacum*. Included in Table 3.1 is also information about the degree of chromosome homology between *N. tabacum* and the other *Nicotiana* species. Structural similarity between the chromosomes of *N. tabacum* and those of its wild relatives can be treated as a preliminary element of the assessment of how much restriction to the genetic flow exists between the species of interest and cultivated tobacco. As can be readily seen from Table 3.1, this piece of information, essential from the standpoint of a potential interspecific breeder, is either lacking or difficult to locate for nearly half of the species that have been successfully hybridized with *N. tabacum*.

The majority of *Nicotiana* species (fifty-nine) listed in Table 3.1 have a record of having been hybridized with both *Nicotiana tabacum* and at least one other sister *Nicotiana*. Some (*N. mutabilis*, *N. petunioides*, *N. attenuata*, *N. corymbosa*, *N. linearis*, *N. burbidgeae*, *N. thyrsiflora* and *N. wigandioides*) were hybridized with at least one other *Nicotiana* but not with *N. tabacum*. Twenty-eight species listed in Table 3.1, the majority of which were discovered only recently (*N. gandarela*, *N. azambujae*, *N. acaulis*, *N. ameghinoi*, *N. cutleri*, *N. paa*, *N. longibracteata*, *N. spegazzini*, *N. bilybara*, *N. candelabra*, *N. fatuhivensis, N. faucicola*, *N. gascoynica, N. heterantha*, *N. hoskingii*, *N. insecticida*, *N. karijini, N. monoschizocarpa*, *N. murchinsonica*, *N. nota*, *N. paulineana,*

Table 3.1 Potential of *Nicotiana* species to hybridize with *Nicotiana tabacum* and with other sister *Nicotianae*

Species	Hybrid with *N. tabacum* Y = reported N = not reported	Chromosome pairing in F_1 hybrids with *N. tabacum*		Hybrids with species other than *N. tabacum* (number of species)[3]
		No. of bivalents: range and modal number (in parentheses)	Reported by	
Alatae				
N. alata	Y	5–8 (?) 1–6 (3–4) 0–10 (6)	Kostoff (1943), Patel, 1960 Takenaka (1954, 1956c) Berbeć (1987b)	24
N. azambujae	N			N
N. bonariensis	Y			12
N. forgetiana	Y	1–8 (3)	Takenaka (1963)	10
N. gandarela	N			
N. langsdorffii	Y	5–12 (11)[1] 0–5 (0)	Takenaka et al. (1955), Takenaka (1958), Hu (1956) Burk (1972)	21
N. longiflora	Y	5–7 (4–9) 0–4 (0–1)	Kostoff (1943) Takenaka (1957), Takenaka (1962a)	21
N. mutabilis	N			5
N. plumbaginifolia	Y	10–12 1–6 (3)	Patel (1960) Goodspeed (1954)	22
N. sp. ‘Rastroensis	N			6
N. x *sanderae*	Y	5–8 (6)	Kostoff (1943) Malecka (1977)	20
Nicotiana				
N.tabacum	N	x	x	x
Noctiflorae				
N. acaulis	N			N
N. ameghinoi	N			N

(continued)

Table 3.1 (continued)

Species	Hybrid with *N. tabacum* Y = reported N = not reported	Chromosome pairing in F_1 hybrids with *N. tabacum*		Hybrids with species other than *N. tabacum* (number of species)[3]
		No. of bivalents: range and modal number (in parentheses)	Reported by	
N. glauca	Y	9–12 0–7 (4) 9–10 7–12 0–8 (2–3) [2] 0–7 (0) 0–5 (0)	Kostoff (1943) Goodspeed (1954) Kehr and Smith (1952) Patel (1960) Berbeć (1971) Trojak-Goluch and Berbeć (2002)	34
N. noctiflora	Y			7
N. paa	N			N
N. petunioides	Y			3
Paniculatae				
N. benavidesii	Y	0–6 (3) 0–5 0–6 (0)	Goodspeed (1954) Takenaka (1958) Berbeć (1987a)	7
N. cordifolia	Y			4
N. cutleri	N			N
N. knightiana	Y	0–5 (0) 2–12 0–1, 0–3 (0)	Takenaka (1958, 1962c) Patel (1960) Berbeć et al. (1979), Berbeć (1987a)	8
N. paniculata	Y	0–4 (0) 0–4 (1) 2–5	Goodspeed (1954), Berbeć et al. (1979) Takenaka et al. (1956), Takenaka (1962a) Kostoff (1943)	24

N. raimondii	Y	3–5, 0–6 0–7 (1) 0–3 (0)	Kostoff (1939a, 1939b) Goodspeed (1954) Berbeć (1987a)	7
N. solanifolia	Y	0–7 (2) 1–5 (1)	Goodspeed (1954) Takenaka et al. (1955); Takenaka (1956b)	11
Polydicliae				
N. clevelandii	Y			9
N. quadrivalvis	Y	0–6 0–10 (4)	Patel (1960) Goodspeed (1954)	27
Petunioides				
N. acuminata	Y			3
N. attenuata	N			8
N. corymbosa	N			3
N. longibracteata	N			N
N. linearis	N			1
N. miersii	N			11
N. pauciflora	Y	0 0–4	Gentscheff (1931) Patel (1960)	3
Repandae				
N. nesophila	Y			4
N. nudicaulis	Y	0 1–5 (?) Max. 4	Gentscheff (1931) Kostoff (1943), Burk and Neas (1964)	15
N. repanda	Y			15
N. stocktonii	Y	0–4 (?)	Wong (1975)	4

(continued)

Table 3.1 (continued)

Species	Hybrid with *N. tabacum* Y = reported N = not reported	Chromosome pairing in F_1 hybrids with *N. tabacum*		Hybrids with species other than *N. tabacum* (number of species)[3]
		No. of bivalents: range and modal number (in parentheses)	Reported by	
Rusticae				
N. rustica	Y	0–7 (3) 1–10 (?) 0–6 (2–3)	Goodspeed (1954) Takenaka (1953) Moav and Cameron (1961)	30
Suaveolentes				
N. africana	Y	0–4 (?) 0–5 (0–1)	Gerstel et al. (1979) Doroszewska and Berbeć (1996)	4
N. amplexicaulis	Y	0–4 (0)	Berbeć et al. (1979)	12
N. benthamiana	Y			14
N. bilybara	N			N
N. burbidgeae	N			1
N. cavicola	Y			3
N. candelabra	N			N
N. debneyi	Y	0–5 (?)	Kostoff (1943)	28
N. eastii	Y			11
N. excelsior	Y			10
N. exigua	Y			13
N. fatuhivensis.	N			N
N. faucicola	N			N
N. fragrans	Y			5
N. gascoynica	N			N
N. goodspeedii	Y			7
N. gossei	Y	0–5 (1)	Takenaka (1962a)	20
N. hesperis	Y			1

N. heterantha	N			N
N. hoskingii	N			N
N. ingulba	Y			5
N. insecticida	N			N
N. karijini	N			N
N. maritima	Y			13
N. megalosiphon	Y	1–8 (3–4)	Takenaka (1962a)	19
N. monoschizocarpa	N			N
N. murchisonica	N			N
N. notha	N			N
N. occidentalis	Y	4–11 (?)	Wong (1975)	8
N. paulineana	N			N
N. pila	N			N
N. rosulata	Y			1
N. rotundifolia	Y			3
N. rupestris	N			N
N. salina	N			N
N. scopulorum	N			N
N. simulans	Y			4
N. stenocarpa	N			N
N. suaveolens	Y	0–3 0–4 (0)	Kostoff (after Goodspeed, 1954) Shinkareva (1979)	28
N. truncata	N			N
N. umbratica	Y			24
N. velutina	Y			18
N. walpa	N			N
N. wuttkei	Y	0–5 (0)	Laskowska and Berbeć (2012)	4
N. yandinga	N			N

(continued)

Table 3.1 (continued)

Species	Hybrid with *N. tabacum* Y = reported N = not reported	Chromosome pairing in F_1 hybrids with *N. tabacum*		Hybrids with species other than *N. tabacum* (number of species)[3]
		No. of bivalents: range and modal number (in parentheses)	Reported by	
Sylvestres				
N. sylvestris	Y	9–12 (12)	Goodspeed (1954)	22
Tomentosae				
N. kawakamii	Y			4
N. leguiana	N			N
N. otophora	Y	10–13 (12)	Goodspeed (1954), Takenaka (1957)	13
N. setchellii	Y	11-12 (12)	Goodspeed (1954)	6
N. tomentosa	Y	11-13 (12)	Goodspeed (1954)	13
N. tomentosiformis	Y	12 (12)	Goodspeed (1954)	19
Trigonophyllae				
N. obtusifolia	Y	2–10 (?) 0–11 (5–6)	Kostoff (1943) Takenaka (1953)	14
N. palmeri	Y	0–10 (4)	Goodspeed (1954)	8
Undulatae				
N. arentsii	Y			2
N. glutinosa	Y	0–11 (3) 2–6 (?)	Goodspeed (1954) Kehr and Smith (1952)	27
N. thyrsiflora	N			2
N. undulata	Y	0–8 (?) 0–8 (3–5)	Kostoff (1943) Takenaka (1953)	17
N. wigandioides	N			4

[1] data from the cross 4n *N. tabacum* x 2n *N. langsdorffii*

[2] data for F_1 *N. tabacum* x *N. glauca* (upper line) and its reciprocal (lower line)

[3] full list of interspecific hybrids (including those involving *N. tabacum*) is given in Chap. 7

N. pila, *N. rupestris, N. salina N. stenocarpa, N. truncata*, *N. yandinga, N. walpa*), were not found to have any hybridization record whatsoever.

3.2 Pre- and Post-fertilization Barriers to Production of Viable Hybrid Seeds in *Nicotiana*

3.2.1 Manifestations of Pre- and Postzygotic Barriers to Hybridization

The number of interspecific hybrids produced between various *Nicotiana* species, although impressive, is still only a small fraction of several thousand theoretically possible combinations. Not all hybrids are obtainable with equal ease. The process of speciation depends on the development of different blocks preventing plants of diverging taxa from crossing with each other and producing viable offspring. The barriers to hybridization can be roughly divided into prezygotic and postzygotic (He et al., 2019). Prezygotic barriers include inhibition of pollen adhesion to the stigma, inhibition of pollen germination, obstruction of pollen tube growth in the stigma and style, and failure of the pollen tube to penetrate the mycropyle. Postzygotic barriers manifest themselves as failure to produce a functional zygote, embryo abortion and failure of hybrid seeds to germinate, lethality of germinated seeds and seedlings, disturbed development of hybrid plants and their failure to develop flowers and, finally, failure of flowering hybrid plants to perpetuate themselves because of their inherent inability to produce offspring. In *Nicotiana*, blocks to hybridization have evolved relatively slowly, unlike gene and chromosome alterations, which have progressed more rapidly (Goodspeed, 1945). Barriers to hybridization were already encountered by early *Nicotiana* researchers. Numerous cases were recorded where upon crossing two different species, either no seeds were produced, the seeds were produced but they failed to germinate, the seeds germinated but the hybrid plants died, mostly at juvenile stages of growth (McCray, 1933; Kostoff, 1943) or mature flowering plants could not be perpetuated because of different forms of sterility (see Sect. 3.6).

3.2.2 Cross Incompatibility

The causes of cross incompatibility between *Nicotiana* species were discussed in detail by Kostoff (1943). Three major causes of the failure to obtain viable hybrid seeds were noted by him:

- failure of the pollen tube to reach the ovary
- failure of the sperm to fuse with the egg
- abortion of the hybrid embryo

The first of the causes may be attributed to various factors, such as differences in the length of styles between the maternal and paternal species (Swaminathan & Murthy, 1957; Stoyanova, 1979; Lee et al., 2008), physiological inhibition of pollen tube growth or mechanical obstruction to the growth of pollen tubes (Kostoff, 1943). Growth inhibition of the pollen tube (gametophytic incompatibility) was found to be conditioned genetically (Pandey, 1977). The growth-promoting substance (GPS) diffused in pollen walls becomes deactivated in incompatible stigmata by the genetically controlled system attached to GPS and prevents its normal function and pollen growth. Gametophytic incompatibility in the hybrid *N. tabacum* x *N. alata* was demonstrated to be controlled by differences in ribonuclease activities in self-compatible (SC) *N. tabacum* and self-incompatible (SI) *N. alata* (McClure et al., 1989; McClure, 1996). RNAase linked to the compatibility locus (S) resulting in the rejection of alien pollen was also implicated in the rejection of alien pollen in other intrasectional and intersectional crosses involving *N. alata* (Murfett et al., 1996). Mechanical obstruction may be caused by differences in cell size since somatic chromosome number is, at least within a certain range, correlated with cell size (Kostoff, 1943). By way of example, the thick pollen tubes of *N. tabacum* cannot make their way through the styles of *N. alata*, whereas reciprocal mating is possible (Kostoff, 1943). Another studied case in point was the cross between *N. tabacum* and *N. obtusifolia,* where the pollen tubes of the former species stopped growing in the styles of the latter (Chung et al., 1996). There are many other cases on record where a hybrid was successfully produced one way but not the other. It came to be known as unilateral incompatibility. Kostoff (1938a) found the hybrid *N. glauca* × *N. langsdorffii* to be produced with ease, whereas in reciprocal mating, the pollen tube of *N. glauca* was frequently arrested in the style of *N. langsdorffii*. Kuboyama et al. (1994) found that the pollen tubes of *N. repanda*, *N. obtusifolia*, and *N. rustica* were arrested at different phases of their growth through the styles of *N. tabacum.* The ability of *N. tabacum* pistils to inhibit foreign pollen tube elongation gradually increased as the flowers matured. A reverse situation was observed by Liao et al. (2017) in the hybrid *N. tabacum* cv. ‘K326’ x *N. alata*. Young flowers of *N. tabacum* inhibited the growth of *N. alata* pollen tubes and prevented them from reaching the ovary, but the inhibition was removed, and seeds could be obtained when senescent flowers were pollinated.

The Australian species of the section Suaveolentes are known to produce hybrids with *N. tabacum* when used as maternal parents, whereas reciprocal crosses are usually unsuccessful (Kostoff, 1943; Goodspeed, 1945). The hybrid of *N. tabacum* by the African species *N. africana* is a well-known exception to that rule (Gerstel et al., 1979; Doroszewska & Berbeć, 1996; Hancock et al., 2015), although successful reciprocal mating, *N. africana* × *N. tabacum*, was also reported (Nikova & Zagorska, 1990). Unilateral incompatibility is also absent when *N. fragrans* is crossed with *N. tabacum* (Tezuka et al., 2010). There are several cases on record when using the autotetraploid variant of a Suaveolentes species as the male parent resolved the problem of unilateral incompatibility with *N. tabacum* (see Sect. 3.4.1). Interestingly, Zaitlin and Mundell (2006) experienced no difficulty in obtaining viable hybrids by fertilizing female *N. tabacum* with pollen of *N. benthamiana,* whereas DeVerna et al. (1987) found that the mating was completely incompatible and they had to resort to ovule culture to overcome the incongruity.

Artificial pollination of ovules cultured *in vitro* was an effective approach to overcome unilateral incompatibility of *N. tabacum* crossed as females with several Suaveolentes species. In this way, viable hybrids of *N. tabacum* as the maternal parent were obtained with: *N. amplexicaulis* (Larkina, 1980), *N. benthamiana* (Subhashini et al., 1986; Mihaylova-Kroumova et al., 2020), *N. debneyi* (Butenko et al., 1970; Larkina, 2015, 2017), *N. excelsior* (DeVerna et al., 1987; Tezuka et al., 2010), *N. maritima* (Tezuka et al., 2010), *N. rosulata* (Ternovsky et al., 1976; Ternovsky & Larkina, 1978a; Larkina, 2015, 2017), N. *suaveolens* (Marubashi & Onosato, 2002, *N. velutina* (Tezuka et al., 2010) (for more information, see Sect. 3.4.3). Unilateral incompatibility between *N. tabacum* and the species of the section Suaveolentes also has important practical implications. Many of these species carry resistance to important diseases and pests of tobacco, and cytoplasmic factors from many of them produce cytoplasmic male sterility in cultivated tobacco. Since CMS is inherited maternally, it may obstruct the breeding process when the transfer of a desired trait from the Suaveolentes species to *N. tabacum* starts and proceeds in a matrilineal fashion (see Chap. 5).

The hybridization process between *N. tabacum* and another *Nicotiana* species that gets stalled at some stage, either pre- or postfertilization, cannot always be accurately classified in simple binary terms: cross compatible vs. cross-incompatible. There are many instances where success depends on whether the right genotype has been chosen within the parental species to be crossed. Kostoff (1943) cites several examples, e.g., *N. rustica* × *N. tabacum* or *N. tabacum* × N. *pauciflora*, where the wild species was successfully crossed with one *N. tabacum* variety but not with others. The crossability of *N. tabacum* with *N. alata*, generally very poor, was found to be largely affected by the genotype of *N. tabacum* (Kostoff, 1943), a phenomenon also confirmed by Ternovsky (1962) and Berbeć (1987b).

The ease with which hybrids of *N. tabacum* × *N. africana* could be obtained either directly from seeds or by resorting to an *in vitro* culture was substantially affected by the genotype of the *N. tabacum* parent in the study by Depta and Doroszewska (2019).

Different accessions of *N. suaveolens* also vary substantially in their ability to cross with *N. tabacum* (He et al., 2019).

The type of cytoplasm can also affect crossability. Liao et al. (2017) failed to obtain viable seeds by crossing regular male fertile *N. tabacum* cv. 'K326' with *N. alata,* but the mating was successful when a cytoplasmically male sterile lineage of K326 with the cytoplasm of *N. glauca* was used as the female parent.

3.2.3 Mechanisms of Pre- and Post Zygotic Incongruity

When the foreign pollen tube has reached the ovary but failed to fertilize the egg, the following phenomena may follow: cell division may occur in the nucellus without embryo or endosperm formation, resulting in no seeds, diploid endosperm may develop, resulting in seeds with no embryos, and induction of cell divisions in the egg may lead to the formation of parthenogenetic haploids or, rarely, diploid embryos. The phenomenon of haploid and diploid maternal soffspring of interspecific crosses will be addressed in one of the subsequent sections.

Various interspecific matings in *Nicotiana* result in the actual fusion of gametes, but the resulting zygote is dysfunctional, and the embryo is aborted soon after fertilization. An example case is the hybrid *N. obtusifolia* x *N. tabacum* (Chung et al., 1996), the hybrid embryo of which aborts soon after fertilization or develops poorly, resulting in inviable, nongerminating seeds. Disturbed and collapsed development of the hybrid embryo may result from the incongruity between the maternal endosperm and the "foreign" embryo as well as from the genetic factors inherent in the embryo itself. Brink and Cooper (1941) and Bannikova (1965a) studied seed development in the F_1 hybrid *N. rustica* × *N. tabacum*. The cross usually resulted in aborted seeds with less than 1% germination capability. Retarded growth of the endosperm, the failure of intergumentary cells to differentiate into conductive tissues and the overgrowth of the nucellus were the causes of the abortion of the hybrid seeds. Disturbed development of the embryo and the endosperm and deranged metabolic exchange between the embryo, endosperm and maternal tissue set in within a week after fertilization in the hybrid *N. rustica* × *N. paniculata* (Bannikova, 1965b). The development of the hybrid embryos was substantially slower compared to the embryo growth rate of self-fertilized *N. rustica.*

The nonviable seeds obtained from crossing *N. tabacum* with *N. alata* were void of both the embryo and the endosperm (Stoyanova, 1979). The embryo death by starvation in the hybrids *N. stocktonii* x *N. tabacum*, *N. nesophila* x *N. tabacum* and *N. repanda* x *N. tabacum* was attributed to the cessation of endosperm development (Reed & Collins, 1980a).

Recently, arrest of endosperm development leading to starvation and abortion of the embryo in the hybrid 4n *N. suaveolens* x 2n *N. tabacum* was attributed to disturbed EBN (Endosperm Balance Number) (He et al., 2020, 2022, 2023). According to the EBN hypothesis, the normal ratio of maternal vs. paternal ploidy level is approximately 2:1 due to the triploid genome of the endosperm. Disturbance of this ratio caused by different ploidy levels of parental genomes would lead to endosperm growth arrest. Specifically, excess maternal EBN in the cross 4n *N. suaveolens* x 2n *N. tabacum* led to precocious developmental transition and subsequent endosperm development failure accompanied by hypertrophy of the embryo arrested at the globular stage (He et al., 2020) or very early cessation of hybrid embryo growth, depending on the *N. suaveolens* accession used.

Chromosome elimination in postzygotic stages is yet another manifestation of postzygotic incongruity. Disturbed mitotic cell divisions in the hybrid embryo may lead to failure of some essential chromosomes to be included in the daughter cells and, consequently, result in embryo abortion. If uniparental chromosome elimination is the case, the affected embryos may develop into semiviable dysfunctional aneuploid hybrid plants or, in extreme cases, yield maternal or paternal haploids (see Sect. 3.5). Kramer and Reed (1988) and Hancock et al. (2015) reported the appearance of such irregular plants in the progeny of the hybrid *N. tabacum* x *N. africana*.

3.3 Seedling Lethality

3.3.1 *Occurrence of Premature Plant Death in* Nicotiana *Hybrids*

The incongruity between species of *Nicotiana* may extend beyond the survival of the embryos and affect the germinating seeds and newly emerged plantlets. Root necrosis, decay and death of juvenile plants are common among the hybrid offspring of different *Nicotianae.* In the first half of the twentieth century, East (1928) and Gentscheff (1931) listed *N. tabacum* × *N. alata*, *N. tabacum* × *N. langsdorffii*, *N. tabacum* × *N. longiflora*, and *N. tabacum* × *N. plumbaginifolia* among the hybrids that die at the cotyledonary stage or thereafter. McCray (1932) added *N. tabacum* × *N. glutinosa*, *N. nudicaulis* × *N. tabacum* and *N. suaveolens* × *N. tabacum* to the list of "weak" or prematurely dying hybrids. Kostoff (1943) also cited previously mentioned combinations and added *N. tabacum* × *N. pauciflora.* Foster (1943) was unable to raise the hybrid *N. repanda* x *N. tabacum* to maturity, and Clayton (1950) encountered that phenomenon in the hybrid *N. debneyi* × *N. tabacum.* Inviable hybrids of *N. eastii* × *N. tabacum* and *N. megalosiphon* × *N. tabacum* were reported by Chaplin and Mann (1961), and those of *N. gossei* × *N. tabacum* and *N. occidentalis* × *N. tabacum* were reported by Apparao and Ramavarma (1972) and Ternovsky et al. (1976), respectively. In later years, several other hybrid combinations were found to be lethal, especially those involving species of the section Suaveolentes and *N. tabacum* (Lloyd, 1975; Berbeć & Doroszewska, 1981; Nikova et al., 1991; Laskowska & Berbeć, 2012). There was one particular case in which the massive death of hybrid seedlings was advantageous. In the cross *N. tabacum* × *N. africana,* the seed set was good, and the seeds germinated well, but germination was followed by extensive root necrosis and death of the emerged seedlings (Gerstel et al., 1979). In that particular case, only ca. 1% of the plants survived beyond the cotyledonary stage, and the population of rare survivors was composed of true hybrids and occasional maternal haploids. The latter were used to produce completely homozygous lines in a one-step procedure that came to be deployed for both academic and practical purposes (Burk et al., 1979; Wernsman, 1992) as an alternative to the anther culture method (Nitsch & Nitsch, 1969).

3.3.2 *Types of Hybrid Lethality*

The mechanisms underlying the phenomenon of hybrid lethality remained obscure until the nineties of the last century. In-depth investigations into the causes of seedling unviability in interspecific hybrids of *Nicotiana* were undertaken primarily by Japanese researchers in the late 1980s. They were summed up by Tezuka et al. (2012) in an exhaustive review of the work that had been carried out by him and by his Japanese associates and colleagues.

According to the external symptoms exhibited by inviable seedlings, five types of hybrid lethality in *Nicotiana* have been distinguished to date:

- Type I: browning of shoot apex and root tip
- Type II: browning of hypocotyls and roots
- Type III: yellowing of true leaves
- Type IV: formation of multiple shoots
- Type V: fading of shoot color

Type II lethality seems to be prevalent in crosses involving *N. tabacum*. It is shown by 19 hybrids of *N. tabacum* with the species of the section Suaveolentes (Tezuka, 2012). The phenomenon occurred regardless of cross direction, with the exception of *N. wuttkei* × *N. tabacum*, the reciprocal of which was not successful (Laskowska & Berbeć, 2012). Further studies identified the causative factor of type II lethality in *N. debneyi* that interacts with the factors allegedly on chromosome Q in *N. tabacum* (Tezuka, 2012, see also Sect. 3.3.4).

Outside the hybrids with *N. tabacum*, Type II lethality was also observed in *N. paniculata* × *N. gossei*, *N. suaveolens* × *N. gossei* and *N. debneyi* × *N. repanda* (Tezuka, 2012). The lethality of the hybrid *N. tabacum* × *N. langsdorffii* (Watanabe & Marubashi, 2004) was not assigned to any of the lethality types. Based solely on the description by these authors but also from other accounts (Burk, 1972, Berbeć, unpublished), it could fit in with type II. However, from the account of Burk (1972), who used monosomics of *N. tabacum* to overcome the unviability of that hybrid, it appears that neither the use of Haplo H nor Haplo Q restored viability to the hybrid; some viable plants have been obtained from Haplos A, G, L and Z (see, however, also Sect. 3.3.4).

N. occidentalis produces type V lethal hybrids when crossed with *N. tabacum* (Tezuka, 2012).

Lethality types II and V are also peculiar in that the symptoms are temperature dependent, i.e. they develop at temperatures of 28 °C and below, are completely suppressed when the temperature is raised to 34–36 °C and recur once the temperature drops again below 30 °C. This phenomenon was first reported by Manabe et al. (1989) in the hybrid *N. suaveolens* × *N. tabacum*.

Of the other types of seedling lethality, type I was exhibited by *N. nudicaulis* × *N. tabacum*, type III by *N. repanda* × *N. tabacum,* (Iwai et al., 1984)*, N. paniculata* × *N. nudicaulis* and *N. glutinosa* × *N. repanda, and* type IV by *N. paniculata* × *N. alata* and *N. paniculata* × *N. glutinosa* (Tezuka, 2012).

According to Iizuka et al. (2012), *N. benthamiana* and *N. fragrans* produce only viable hybrids with *N. tabacum*. However, Krusteva et al. (2003) and Nikova et al. (2006) reported serious survival issues among the emerging seedlings of the F_1 hybrid *N. benthamiana* x *N. tabacum*. Subhashini et al. (1986) and DeVerna et al. (1987) failed to obtain any seeds from mating male *N. benthamiana* to female *N. tabacum*.

3.3.3 Causes of and Phenomena Associated with Hybrid Lethality

The temperature-dependent lethality of hybrid seedlings observed in types II and V is called programmed cell death (PCD) and is accompanied by apoptotic phenomena such as condensation of chromatin, fragmentation of nuclei, disintegration of DNA and aggregation of insoluble proteins in dying cells (Ueno et al., 2019). It is one of the mechanisms of reproductive isolation and occurs in other plant genera (Tezuka & Marubashi, 2004; Hancock et al., 2015). Bomblies (2009) suggested that PCD and apoptotic hybrid lethality are caused by the hyperactivation of plant defense responses and thus may share a common mechanism with resistance to some viral pathogens (e.g., TMV or TSWV), which is also based on eliciting a similar hypersensitive reaction (HR). Hancock et al. (2015) drew attention to the fact that the gene conferring the hypersensitive necrotic response to TMV and the gene controlling apoptotic hybrid lethality in *N. tabacum* are both located on the same chromosome H contributed by the ancestral form of *N. tomentosiformis*. The idea of the common genetic background of PCD and HR plant defense response was also discussed by Chen and Lin (2016). The two authors thought it very likely that the genes that cause necrosis and death in interspecific hybrids are also involved in immune responses and that the appearance of apoptotic phenomena in wide hybrids can be related to the activation of pathogenesis-related genes. It was argued that the activation of the PCD-related processes is due to the mismatch between the delicately configured immune systems of individual species that make up an interspecific hybrid. This interpretation became more precise when the gene Nt6549g30 was identified to encode a CC-NRL protein in *N. tabacum*, the CC-NRL group being the largest class of plant defense proteins that recognize effectors from a pathogen (Ma, 2017; Ma et al., 2020). The same Nt6549g30 gene was also demonstrated to control seedling lethality in the hybrid *N. tabacum* x *N. africana* (Ma, 2017, Ma et al., 2020). More recently, Katsuyama et al. (2021) suggested that both NRL proteins and the associated chaperone protein complexes that aid NRLs in unfolding were involved in developing seedling lethality in the hybrid *N. gossei* x *N. tabacum*. Hence, hybrid seedling death is essentially the effect of autoimmunity-based responses caused by incorrect interactions between parent-of origin NRLs and their associated proteins. As the result of those PCD-type autolytic processes, insoluble protein progressively aggregates in dying hybrid cells, a process demonstrated for the hybrid *N. suaveolens* x *N. tabacum* (Ueno et al., 2019).

Development of teratological changes is a peculiar phenomenon that may be involved in the failure of certain hybrid combinations to grow to maturity. Apparently, in most interspecific hybrids, genetic tumors usually appear in senescing plants (see Sect. 4.5.9), although Burk (1972) reported their occurrence before flowering in a plant of *N. tabacum* x *N. langsdorffii*. Likewise, in some crosses that did not involve *N. tabacum* (*N. rustica* × *N. alata* and *N. rustica* × *N. langsdorffii*), tumors and teratomata were observed in juvenile plants of those hybrids and in the hybrid *N. rustica* × *N. alata* (Takanashi & Marubashi, 2017). The onset of these abnormalities was temperature dependent. The hybrid *N. obtusifolia* × *N. tabacum* developed vitrification and tumors as early as the seedling stage, and these abnormalities were the main cause of seedling death in that hybrid (Liu & Marubashi, 2014). In the latter case, the abnormalities were probably of an origin different than genetic tumors since neither parental species belonged to the "plus" group as classified by Näf (1958).

Nicotiana species that were found to produce inviable hybrids with *N. tabacum* are listed in Table 3.2.

Table 3.2 Lethality of juvenile hybrid plants of *Nicotiana tabacum* with other *Nicotiana* species

Section	Species	Lethality L=lethal V=viable
Alatae	*N. alata*	V/L
	N. langsdorffii	L/V
	N. longiflora	L
	N. plumbaginifolia	L
Repandae	*N. nudicaulis*[2]	L type I
	N. repanda	L type III
Rusticae	*N. rustica*	V/L
Suaveolentes	*N. africana*	L Type II
	N. amplexicaulis	L Type II
	N. cavicola	L Type II
	N. debneyi	L Type II
	N. eastii[1]	L
	N. excelsior	L Type II
	N. exigua	L Type II
	N. goodspeedii	L Type II
	N. gossei	L Type II
	N. hesperis	L Type II
	N. ingulba	L Type II
	N. maritima	L Type II
	N. megalosiphon	L Type II
	N. occidentalis	L Type II
	N. rosulata	L Type II
	N. rotundifolia	L Type V
	N. simulans	L Type II
	N. suaveolens	L Type II
	N. umbratica	L Type II
	N. velutina	L Type II
	N. wuttkei	L Type II
Trigonophyllae	*N. obtusifolia*	L/V
Undulatae	*N. glutinosa*	V/L

Adapted after Tezuka (2012)

[1] Sesquidiploid hybrid from crossing *N. eastii* with autotetraploid *N. tabacum* (Chaplin & Mann, 1961)

[2] Vitrification and tumors developed by seedlings (Liu & Marubashi, 2014)

3.3.4 Location and Identification of Genetic Factors Responsible for Lethality of Hybrid Seedlings

Chromosomal Location of Heritable Factors in *N. tabacum* by Monosomic Analysis The first monosomic line of *N. tabacum* experimentally produced was probably the one derived from backcrossing the hybrid *N. sylvestris* x *N. tabacum* to *N. tabacum*. It was reported by Clausen and Goodspeed (1926) and named 'corrugated'. The original complete series of 24 lines of *N. tabacum*, each of them monosomic for one of the 24 chromosomes of the species, was developed by Prof. R. E. Clausen with his associates at the University of California, Berkeley, starting from the mid-twenties of the last century (Olmo, 1935; Clausen & Cameron, 1944; Lewis, 2011). Generally, four sources of monosomic lines in *N. tabacum* were indicated by Clausen and Cameron (1944):

(a) Monosomic variants in segregating populations from backcrossing F_1 (*N. tabacum* x *N. tomentosa*[1]) and F_1 (*N. tabacum* x *N. sylvestris*) to *N. tabacum*. The method may have taken advantage of the 'Drosera' scheme pairing in the gametogenesis of the initial hybrids (Goodspeed, 1954) and of other irregularities in homoeologous pairing between the 48-chromosome *N. tabacum* and its ancestral 24-chromosome progenitors. Thus, the backcross F_1 (*N. tabacum* x *N. sylvestris*) x *tabacum* could occasionally yield some true *N. tabacum* individuals and aneuploids, including those monosomic for the T subgenome chromosomes. In a similar fashion, individuals monosomic for the S subgenome chromosomes could be picked among the survivors of the cross F1 (*N. tabacum* x *N. tomentosa*) x *N. tabacum.* In those early studies, several monosomics were obtained in this manner but only from backcross populations that involved *N. sylvestris* (Olmo, 1935). More recently, monosomic individuals of *N. tabacum* were also identified in hybrid populations derived from the cross F_1 (*N. tabacum* x *N. tomentosiformis*) x *N. tabacum* using SSR markers for specific linkage groups of *N. tabacum* (Liu et al., 2017). These plants were monosomic for linkage groups 3 and 6 according to *the N. tabacum* genome map developed by Bindler et al. (2011);

(b) Spontaneous genomic mutations in normal disomic tobacco populations;

(c) Segregating offspring of *N. tabacum* lines that carried the asynaptic pale-sterile mutation discovered by Clausen (1931). Pale sterile plants produce microsporocytes with, on average, only 11 bivalents per cell, and the rest of the chromosomes remain unpaired. The cross pale sterile x normal type produces highly diversified offspring that consist of trisomics, single, double and triple monosomics and various monosomic-trisomic combinations. Monosomics for a single chromosome pair are bred into the normal type by repeated backcrossing accompanied by morphological and cytological selection;

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[1] At that time *N. tomentosa* was regarded as the progenitor species of *N. tabacum* and the contributor of its T subgenome

(d) Monosomics that arise spontaneously in the offspring of established ones due to the tendency of limited asynapsis in many monosomic types. Ultimately, those spontaneous variants proved to be the major source of monosomics developed in the genetic laboratory of the University of California in Berkeley (Clausen & Cameron, 1944).

The monosomics of *N. tabacum* and their morphological and developmental features in the background of the variety Red Russian were described by Prof. D. R. Cameron (Smith, 1968, 1979). At the same time, a labeling system of *N. tabacum* chromosomes was adopted in which chromosomes contributed by *N. tomentosiformis* are lettered from A through L and those by *N. sylvestris* originally from M through X (Olmo, 1935). The lettering was later changed from M through Z (Olmo, 1935; Smith, 1968) because the letters X and Y were traditionally assigned to the sex chromosomes.

Until recently, monosomic analysis was the only available tool with which the genes that control simply inherited traits could be assigned to particular chromosomes.

Location and Identification of the Lethality Factor in *N. tabacum* Responsible for Type II Hybrid Seedling Lethality Inoue et al. (1996) studied the survival of hybrids from crossing *N. suaveolens* with putative progenitor species of *N. tabacum,* i.e., *N. sylvestris* and *N. tomentosiformis*, which contributed subgenomes S and T, respectively, to the allopolyploid cultivated species. They found the hybrid plants *N. suaveolens* x *N. sylvestris* to develop typical lethality symptoms, whereas the plants from crossing *N. tomentosiformis* by *N. suaveolens* remained viable. The authors inferred from those results that it is the subgenome S of *N. tabacum* that carried the lethality factor. Based on an analogous study of the hybrids *N. debneyi* x *N. sylvestris* and *N. tomentosiformis* x *N. debneyi*, Tezuka et al. (2007) likewise concluded that the subgenome S of *N. tabacum* houses the factor/s that cause lethality in the hybrid *N. debneyi* x *N. tabacum* (Table 3.3,[2] column 4).

Based on the evidence discussed in the preceding paragraph, Marubashi and Onosato (2002) studied genetic causes of lethal hybrids by crossing ten monosomic *N. tabacum* lines for the sylvestroid chromosomes of *N. tabacum* (from Haplo N to Haplo Z except Haplo P and Haplo V) with *N. suaveolens*. When tested against *N. suaveolens* as the pollen parent, all but Haplo Q consistently yielded lethal hybrid offspring. In contrast, the progeny of Haplo Q x *N. suaveolens* segregated for viable and lethal seedlings, and this result was also confirmed in the study of Tezuka and Marubashi (2006a). Similarly, the progeny of the monosomic Haplo Q x *N. debneyi* segregated for lethal and viable individuals. Q chromosome-specific markers revealed the presence of the Q chromosome in lethal plants, whereas viable seedlings

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[2] In this Table, as in those to follow across this book, mating direction of sexual hybrids is generally not indicated. That information should be sought through references to the hybrid of interest either in the tables or in Chap. 7 of this review where all known *Nicotiana* hybrids, including those with *N. tabacum*, are tabulated. Cross directions in the hybrids and in their reciprocals, if reported, are given in column IV of Table 7.1.

Table 3.3 Chromosomal and genomic location of the *N. tabacum* factor controlling type II lethality in hybrid seedlings from crossing *N. tabacum* with several species of the section Suaveolentes on chromosome H (subgenome T) vs. chromosome Q (subgenome S) as established by different research teams

Species producing lethal hybrids in crosses with N. tabacum	Chromosome location of type II lethality factor		Involvement of S subgenome of *N. tabacum* in conferring type II lethality to hybrid progeny
	Chromosome H	Chromosome Q	
N. africana	Gerstel et al. (1979)[1]	Tezuka et al. (2010)[5]	
	Hancock et al. (2015)[2]		
	Ma et al. (2020)[3,4]		
N. debneyi	Ma et al. (2020)[4]	Tezuka et al. (2007)[5]	Tezuka et al. (2007)[7]
N. excelsior	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	
N. goodspeedii	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	
N. gossei	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	
N. ingulba		Tezuka (2012)[6]	
N. maritima	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	
N. megalosiphon	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	
N. simulans	Ma et al. (2020)[4]		
N. suaveolens		Marubashi and Onosato (2002)[5]	Inoue et al. (1996)[8]
N. umbratica	Ma et al. (2020)[4]		
N. velutina	Ma et al. (2020)[4]	Tezuka et al. (2010)[5]	

[1] determined by monosomic analysis of the progeny from mating *N. tabacum* to *N. africana* across the whole set of *N. tabacum* monosomics (from Haplo-A to Haplo Z); only the progeny Haplo H x *N. africana* segregated for lethal and viable individuals

[2] determined by demonstrating association of chromosome H with linkage group 11 of *N.* tabacum (Bindler et al., 2011) and by microsatellite marker genotyping

[3] determined by demonstrating that the gene *Nt6549g30* at the NtHL1 locus on chromosome H of *N. tabacum* controls hybrid lethality in *N. tabacum* x *N. africana*; the authors assigned chromosome H to the subgenome contributed by *N. sylvestris* but did not elaborate on this revision of the recognized classification of *N. tabacum* chromosomes

[4] demonstrated by obtaining viable progeny from interspecific crosses in which the *N. tabacum* parent carried the *Nt6549g30* allele whose ethality-conferring function was disabled via CRISP-Cas9 technology

[5] determined by monosomic analysis: hybrid progeny that involved Haplo Q as the *tabacum* parent segregated for lethal and viable individuals

[6] no details

[7] indirectly demonstrated by lethality reaction in *N. sylvestris* x *N. debneyi* vs. viable offspring of *N. tomentosiformis* x *N. debneyi*

[8] indirectly demonstrated by lethality reaction in *N. suaveolens* x *N. sylvestris* vs. viable offspring of *N. tomentosiformis* x *N. suaveolens*

were deficient for the Q chromosome (Tezuka et al., 2007). In another study conducted in that laboratory, Tezuka et al. (2010) found that in the offspring from crossing Haplo-Q monosomics of *N. tabacum* with *N. africana*, *N. excelsior*, *N. goodspeedii*, *N. gossei*, *N. maritima*, *N. megalosiphon* and *N. velutina,* the seedlings that carried chromosome Q were lethal, whereas those deficient for chromosome Q were viable. Hence, the authors concluded that the factor/s that triggered the seedling death response in those hybrid combinations must reside on chromosome Q of *N. tabacum*. Additionally, Tezuka et al. (2012) found that the SSR (simple sequence repeats) markers present in the inviable hybrid seedlings of *N. tabacum x N. africana* mapped to a linkage group that had been coded as linkage group 11 in subgenome S of *N. tabacum* according to Bindler et al. (2011).

Recent investigations have indicated that rare spontaneous survivors among the dying offspring of type II lethality hybrids may arise due to the loss of the distal part of the Q chromosome as a result of homoeologous translocations during spermatogenesis of the male *N. tabacum* parent (Nakata et al., 2021).

Controversy Regarding the Identitification of the *N. tabacum* Chromosome That Carries the Lethality-Controlling Factor The results discussed in the preceding section are at odds with what was reported by Gerstel et al. (1979), Hancock et al. (2015) and Ma et al. (2020). Gerstel et al. (1979) tested the whole range of *N. tabacum* monosomics (from Haplo A through Haplo Z) by crossing them with *N. africana*. It is only in the Haplo H x *N. africana* progeny that he found segregation for viable and lethal individuals. The remaining combinations, including Haplo Q x *N. africana,* yielded predominantly lethal offspring. Their conclusion was that chromosome H of the T subgenome of *N. tabacum* controlled the lethality of the hybrid *N. tabacum* x *N. africana*.

The more recent report by Hancock et al. (2015) supported those old findings. By using the same or similar set of SSR markers as those used by Tezuka et al. (2012), the authors recognized the association of the seedling lethality factor with linkage group 11 of the *N. tabacum* genome but were firm in associating linkage group 11 with chromosome H rather than with chromosome Q. They supported their claim by citing evidence mainly from earlier studies on the inheritance of TMV resistance in *N. tabacum* (Lewis et al., 2005, other unpublished data) but also because one of the markers for linkage group 11 was detected on chromosome H.

In his thesis, Ma (2017) modified the position on the controversy by conceding that chromosome H originated from the S genome. In a subsequent paper (Ma et al., 2020), the authors reiterated that chromosome H (linkage group 11) of *N. tabacum* was contributed by the ancestral form of *N. sylvestris,* thereby effectively moving chromosome H from subgenome T to subgenome S. They did so in recognition of the facts (see the final paragraph of this section) but in contradiction to the existing nomenclature and classification of monosomics (Clausen & Cameron, 1944; Smith, 1968, 1979; Tezuka et al., 2010; Lewis, 2011).

Table 3.3 presents the results of the genetic control of type II hybrid lethality in hybrids involving *N. tabacum* reported by Japanese and American scientists.

A very intriguing and unanswered question is how this controversy may have come about. The monosomic stocks used by both Gerstel et al. (1979) and Marubashi and Onosato (2002) were bred into Red Russian. In all probability, both Japanese and American stocks descended from those developed and described by Dr. Clausen and Dr. Cameron. This was explicitly stated by Marubashi and Onosato (2002), and it is the best guess for the monosomics used in the USA. The provenance of the Haplo-Q stock used by Tezuka et al. (2007, 2010) was not specified, although in both studies, the monosomics were apparently also ultimately derived from the same source in Berkeley, California. Both chromosome Q, as reported in Japanese studies (Tezuka et al., 2012), and chromosome H, as studied by the Americans (Hancock et al., 2015), showed structural instability and were prone to breakage in the hybrid *N. tabacum* x *N. africana*. Marubashi and Onosato (2002) and Tezuka et al. (2007, 2010) reported the necessity to use Haplo-Q as the maternal parent in crosses with the Australian species because of the failure of their Haplo-Q to produce viable pollen, a feature characteristic of both Haplo-H and Haplo-Q (Smith, 1968). Although Haplo-H and Haplo Q seem to be discernible from each other by some morphological and growth features (Smith, 1968, 1979), morphology is not a wholly dependable criterion in selection for monosomic types, as these differences are strongly influenced by the environment (Liu et al., 2017). Clausen and Cameron (1944) wrote at length on how difficult it was to maintain the identity and integrity of their monosomics and that despite their efforts, doubts remained as to the validity of some of them. All these things put together, it is obvious that at least some monosomics of *N. tabacum* are not unlikely to be confused with one another. Hence, it is probable that Haplo-H used earlier by Professor Gerstel and more lately by the team headed by Professor Lewis and Haplo-Q used by Professor Tezuka, his associates and by other research teams from Japan in reality represented the same monosome type. It was tacitly conceded by Ma (2017) when, in support of the assertion that the hybrid lethality factor locus, N. tab_HL1, resides near the end of chromosome H, he quoted both Hancock et al. (2015) and Tezuka et al. (2012), even though the Japanese researchers had reported on chromosome Q rather than H. The fact was communicated in the very title of their study (Tezuka et al., 2012). The same interpretation of the controversy was recently also advanced by Nakata et al. (2021) and summarized by Mino et al. (2022).

With what we know, it is not wholly unjustified to assume that while Clausen and Cameron's collection of monosomics was being developed and/or maintained in American laboratories, monosome H was at some point wrongly named and wrongly assigned to the T (tomentosoid) genome, whichever came first. A very puzzling part of the story, however, is how the "rectification" of both the misnaming (from H to Q) and the misplacing (from T to S subgenome) came about in the "Japanese chapter" of the collection. The Japanese researchers seem to have ignored or been unaware of the inconsistency of their results with those published in the US, at least in the early stages of their studies. They did not question the validity of any of the collection stocks, nor did they comment on the discrepancy or report on any corrections thereof. Other things put aside, the series of Japanese reports concerning the issue under discussion has turned out to be consistent and logical, by coincidence or otherwise.

Pivotal to the success in resoving this controversy is to establish the association of the linkage groups in the gene map of *N. tabacum* developed by Bindler et al. (2011) to the physical carriers of those genes i.e. the chromosomes. In Bindler's map there are 24 linkage groups corresponding to 24 chromosomes of the amphidiploid *N. tabacum*. Eleven of these were assigned to the tomentosoid (T) genome, nine to the sylvestroid (S) genome, and the remaining four were mixed and consisted of groups of either T or S genetic markers. Neither Bindler (Bindler et al., 2011) nor anyone else has made any attempts to link those linkage groups to particular physical units (chromosomes). Generally, reports on associating linkage groups with chromosomes of *N. tabacum* have been scant as yet and limited to chromosomes A, H and Q. Vontimitta and Lewis (2012) assigned chromosome A to Bindler's linkage group 4 but later corrected the association in favor of group 15 (Ma, 2017). It is not clear, however, if the assignment of chromosome A was changed accordingly. The assignments of chromosomes H and Q were discussed above. Mapping linkage groups to the corresponding chromosomes has barely started, and nearly all the work is still to be done. Most likely, it is not until linkage groups and chromosomes become mutually identifiable that controversies such as the one above will finally be prevented.

In a recent development that may be regarded as a *sui generis* sequel to the chromosome Q vs. H controversy, the transposon-tagging method combined with CRISP-Cas9 gene-editing technology was used to identify the gene in the genome of *N. tabacum* that controls the death of juvenile hybrid plants of *N. tabacum* x *N. africana* (Ma et al., 2020). To this end, a strategy was deployed that involved the use of a maize-derived binary transposable *Ac*/*Ds* system consisting of the autonomous immobile activator (Ac) and the *Ac*-controlled transposon (*Ds*) plus selectable markers linked to the *Ac* and *Ds* elements. As a result, the association was established between the DNA sequence identified as *Nt6549g30* and the *NtHL1* locus previously demonstrated to house the factor responsible for triggering the series of apoptotic events that cause the death of seedlings of that hybrid (Hancock et al., 2015, see also Sect. 3.4.3). The authors stated that its chromosomal location was on chromosome H, or linkage group 11, contributed by *N. sylvestris*. They found a high degree of similarity of *Nt6549g30* to several homologous genes in *N. sylvestris*.

3.3.5 *Genetic Control of Type V Lethality in* N. occidentalis *x* N. tabacum

First, it was suggested that Type V lethality in the hybrid *N. occidentalis* x *N. tabacum* was related to factors located on both subgenomes, S and T, of *N. tabacum* (Tezuka & Marubashi, 2012). Although type V was phenotypically observed in the hybrid, the type II genetic system must have also been functioning alongside because the hybrids of *N. occidentalis* with the progenitor species of *N. tabacum*, *N. sylvestris* and *N. tomentosiformis* showed type II and type V lethality, respectively. Using two new accessions of *N. occidentalis* (PI555541 and PI555690) alongside the old one (JT), Kawaguchi et al. (2021) demonstrated that the genetic control of type V hybrid lethality in the hybrid *N. occidentalis* x *N. tabacum* differed depending on the *N. occidentalis* accession involved: in JT x *N. tabacum,* it was controlled by alleles in both S and T subgenomes, whereas in the hybrids involving the new *N. occidentalis* accessions (PI555541 and PI555690), it was only subgenome T of *N. tabacum* that housed the factor/s controlling type V lethality. Both PI555541 and PI555690 yielded viable offspring when crossed with *N. sylvestris,* and the hybrid progeny of analogous crosses with *N. tomentosiformis* showed type V lethality.

3.4 Methods to Overcome Cross-Incompatibility, Embryo Abortion and Mortality of Juvenile Hybrid Plants

3.4.1 Managing Cross Incompatibility

Numerous approaches have been used to overcome cross incompatibility between species of *Nicotiana*. Swaminathan and Murthy (1957) bypassed the incompatibility between *N. debneyi* and *N. tabacum* that results from the difference in style length of parental species by cutting off the style of the maternal *N. tabacum* to a length of 2–3 mm and smearing the cut surface with a drop of sucrose agar. The pollen of *N. debneyi* was placed on the thus-prepared styles of the egg parent. The problem with this particular cross was more efficiently resolved by resorting to culturing excised ovules of *N. tabacum* fertilized *in vitro* with the pollen of *N. debneyi* (Butenko et al., 1970; Ternovsky et al., 1976). By resorting to the same technique, Marubashi et al. (1988) and Marubashi and Onosato (2002) produced seedlings of the hybrid *N. tabacum* x *N. suaveolens*. The same method was adopted to overcome cross-incompatibility between the long-styled *N. tabacum* used as the female parent and the relatively short-styled *N. knightiana*, *N. rustica*, *N. benthamiana* and *N. rosulata* as the pollen parent (Slusarkiewicz-Jarzina & Zenkteler, 1983, Larkina, 1980, DeVerna et al., 1987, Ternovsky et al., 1976).

The problem of unilateral incompatibility of *N. gossei* and *N. tabacum* was overcome by using the autotetraploid variant of *N. tabacum* as the pollen parent (Valleau, 1952). In some other hybrid combinations, the use of the autotetraploid variant of wild *Nicotiana* ensured success. The first attempt to cross 4n *N. repanda* as a female with 2n *N. tabacum* was only partly successful since the hybrid plants failed to reach flowering (Foster, 1943). The same cross was repeated with success by Pittarelli and Stavely (1975). Surviving hybrid plants were also obtained from crossing 4n *N. palmeri* with *N. tabacum* (Berbeć et al., 1982). However, an opposite effect was recently reported for the cross 4n *N. suaveolens* x 2n *N. tabacum,* which was incompatible due to collapsed endosperm development (He et al., 2020, 2022), whereas the regular combination (2n *N. suaveolens* x 2n *tabacum)* was compatible. For more details on manipulating ploidy levels to bypass the incompatibility of interspecific *Nicotiana* crosses and the underlying EBN (Endosperm Balance Number) theory, the reader is referred back to Sect. 3.2.3 of this chapter.

Senescing flowers of *N. tabacum* were found to be more receptive to fertilization by pollen of *N. alata* than freshly developed flowers (Liao et al., 2017).

In the hybrid *N. gossei* × *N. tabacum,* the incongruity between the two species was overcome by inducing structural changes in the parental chromosomes through the exposure of the paternal pollen to helium ions or gamma rays (Kitamura et al., 2003). Chromosome loss and chromosome rearrangements were observed in rare surviving hybrids, and their survival may have been the result of the deletion or disablement of the gene/s controlling apoptotic seedling death in that hybrid (see also Sect. 3.4.3).

3.4.2 Embryo Rescue by Culturing Ovules

Inviable embryos can be rescued, and hybrid plants can be regenerated by pollinating field- or greenhouse-grown plants *in situ* and culturing the excised fertilized ovules *in vitro*. Such an approach was adopted to overcome incongruity between *N. tabacum* and, e.g., *N. plumbaginifolia*, *N. sanderae*, *N. acuminata*, *N. nesophila*, and *N. stocktonii* (Nikova et al., 2006; Iwai et al., 1986; Reed & Collins, 1978).

Alternatively, *in vitro* cultured ovules can also be pollinated *in vitro*. Pollinating the cultured ovaries of *N. tabacum* with the pollen of *N. rustica* Marubashi & Nakajima, 1985 rescued the lethal hybrid *N. tabacum* × *N. rustica*. Liu et al. (2017) used this approach to obtain progeny from backcrossing the amphihaploid hybrid *N. tabacum* x *N. tomentosiformis* to *N. tabacum* used as the male parent.

In another successful embryo rescue experiment, the ovaries of *N. repanda* were X-irradiated prior to fertilization with pollen of *N. tabacum* (Shintaku et al., 1985). The two surviving hybrid plants were similar to *N. tabacum* in habit and growth type, but their flower morphology resembled that of *N. repanda*. Both showed chromosome deficiency (for 1 and 2 chromosomes, respectively) as judged by the expected amphihaploid chromosome number. This prompted the authors to surmise that in those plants, the lethality factor/s were eliminated with the missing chromosome/s. A similar approach based on partial chromosome elimination in the male parent through the use of irradiation was used by Shizukuda et al. (1983) to obtain "partial" or asymmetric hybrids of *N. tabacum* x *N. rustica*.

More examples of culturing fertilized ovules as a means to raise other incongruous interspecific hybrids to maturity are given in Table 3.4. Where embryo abortion is the result of a malfunctioning endosperm, the beneficial effect of aseptic culture is that it substitutes for the endosperm in providing nutrients to the embryo, thus assuring its continued development (Reed & Collins, 1980a).

Table 3.4 In vitro cultures used to overcome the death of embryos and juvenile plants in incongruous hybrids of *N. tabacum* with other *Nicotiana* species

Section	Species involved in the hybrid with *N. tabacum*[1]	Manifestation of incongruity	Method adopted	Reported by
Alatae	*N. alata* (M)	LS	OC *in vitro*	Papadopoulou et al. (1997)
	N. longiflora (M)	LS	OC *in situ*	Venkateswarlu et al. (1998)
	N. plumbaginifolia (F)	IS/ LS	SC OC *in situ*	Nikova et al. (2004) Nikova et al. (2006)
	N. plumbaginifolia (M)		OC *in vitro*	Prasad et al. (1985)
	N. sanderae (M)	IS	OC *in situ*	Nikova et al. (2003, 2006)
Paniculatae	*N. knightiana* (M)	NS	OC *in vitro*	Slusarkiewicz-Jarzina and Zenkteler (1983)
Petunioides	*N. acuminata* (M)	IS (embryo abortion)	OC *in situ*	Iwai et al. (1986)
Repandae	*N. nesophila* (F)	IS (embryo abortion)	OC *in situ*	Reed and Collins (1978)[1,2], Evans et al. (1982)
	N. nudicaulis (M)	LS	CC	Yamada et al. (1999)
	N. repanda (F)	LS type II	CL OC *in situ* OC *in vitro* + OI	Iwai et al. (1985) Iwai et al. (1985) Shintaku et al. (1985, 1986), Pontes et al. (2005)
	N. repanda (M)	IS, LS	OC *in vitro*, OC *in vitro* followed by treatment by IAA of germinating seedlings Culture of ovules pollinated in vitro + addition of IAA to potting medium to prevent dieback at later growth stages	Choi et al. (1998) Zhou et al. (1991)
	N. stocktonii (F)	IS (embryo abortion)	OC *in situ*	Reed and Collins (1978)[1,2], Muraida and Marubashi (2015)

(continued)

Table 3.4 (continued)

Section	Species involved in the hybrid with *N. tabacum* [1]	Manifestation of incongruity	Method adopted	Reported by
Rusticae	*N. rustica* (F)	NS	OC *in situ* OC *in vitro* by X-irradiated pollen OC *in vitro*	Douglas et al. (1983), Choi and Lee (1991), Sarala et al. (2023) Shizukuda and Nakajima (1982), Shizukuda et al. (1983) Choi and Hong (1992)
	N. rustica (M)	NS	OC *in vitro*	Marubashi and Nakajima (1985)
Suaveolentes	*N. africana* (F) *N. africana* (M) *N. africana* (M)	LS type II	GSC CC OC *in situ*	Nikova et al. (1988), Nikova and Zagorska (1990), Doroszewska and Berbeć (1990) Tezuka et al. (2010)
	N. amplexicaulis (M) *N. amplexicaulis* (F)	NS ?	OC *in vitro* OC *in situ*	Larkina (1980), DeVerna et al. (1987), Venkateswarlu et al. (1998), Sarala et al. (2023)
	N. benthamiana (M) *N. benthamiana* (F)	NS IS	OC *in situ*	Subhashini et al. (1986), DeVerna et al. (1987), Nikova et al. (2006)
	N. cavicola (F)	LS type II	OC *in situ*	Nikova et al. (2006)
	N. excelsior (M)	LS type II	OC *in situ*	Tezuka et al. (2010)
	N. debneyi (M)	NS	OC *in vitro* followed by CC OC *in situ*, OC *in vitro*	Butenko et al. (1970), Ternovsky et al., 1976, Tezuka et al. (2007)
	N. debneyi (M) *N. debneyi* (F)	LS	OC *in vitro* OC *in vitro* followed by CC OC *in situ*	Ternovsky and Larkina (1978b), Larkina (2015, 2017) Tezuka and Marubashi (2006b), Tezuka et al. (2010) Sarala et al. (2023)
	N. goodspeedii (F)	NS	OC *in vitro* followed by CC	Tezuka et al. (2010)

	N. gossei (F)	LS type II	OC *in situ* CC	Adachi and Inoue (1995), Sarala et al. (2023) Mino et al. (2002)
	N. ingulba	LS ? IS LS	GSC OC *in situ* OC *in vitro*	Butenko et al. (1970) Nikova, Palakarcheva, et al. (1998a) Tezuka et al. (2012)
	N. maritima (M)	NS	OC *in vitro*	Tezuka et al. (2010)
	N. megalosiphon (M) (F)	NS LS type II	OC *in situ* GSC OC *in situ*	Tezuka et al. (2010) García Cruz et al. (2008) Sarala et al. (2023)
	N. occidentalis (F)	LS type II	GSC	Butenko et al., 1970, Ternovsky et al. (1972), Semenova and Ivanova (1973)
	N. rosulata (M)	NS	OC *in vitro*	Ternovsky et al. (1976), Ternovsky and Larkina (1978a), Larkina (1980, 2015, 2017)
	N. suaveolens (M)	NS	OC *in vitro*	Marubashi and Onosato (2002), Tezuka and Marubashi (2004, 2006b)
	N. suaveolens (F)	LS	GSC, ISC	Lloyd (1975), Shinkareva (1979), Inoue et al. (1994)
	N. suaveolens (F)	LS type II	ISC in liquid media	Inoue et al. (1997)
	N. umbratica (M)	?	OC *in situ*	Sarala et al. (2023)
	N. velutina (F) *N. velutina* (M)	NS	OC *in vitro* OC *in vitro*	Tezuka et al. (2010)
	N. wuttkei (F)	LS type II	CC	Laskowska and Berbeć (2012)
Trigonophyllae	*N. obtusifolia* (F)	NS, IS	OC *in situ*	Chung et al. (1988), Choi and Lee (1991), Liu and Marubashi (2014)

Abbreviations used: *LS* lethal seedlings, *LS type II* seedling lethality type II, *IS* inviable seeds, *NS* no seeds, *OC in situ* culture of excised ovules from flowers pollinated in situ, *OC in vitro* culture of excised ovules pollinated in vitro, *CC* culture of cotyledons, *CL* culture of true leaf explants, *OI* irradiation of ovules, *GSC* culture of germinating seeds, *ISC* culture of intact seedlings; Footnotes marked by superscript numerals (arranged columnwise and downwards within columns); parent of the non-*tabacum* species in the hybrid: *F*= female, *M*=male

[1] Fertile amphidiploids recovered from culture

[2] Followed by cotyledon culture of ovule culture-raised seedlings

3.4.3 Managing Lethality of Juvenile Plants

Overcoming Hybrid Lethality Through Natural Processes Up to the mid-sixties of the last century and in some laboratories even much later, the *Nicotiana* investigators and breeders tried to manage the lethality of hybrid seedlings by the simple expedient of pollinating as many plants and flowers and sowing as many seeds as possible in the hope of obtaining rare phenotypes that would be able to survive to maturity. In quite a few cases, the policy bore fruit (e.g., Tsikov, 1966; Clayton et al., 1967; Gerstel et al., 1979; Berbeć & Doroszewska, 1981; Hancock et al., 2015). Since one of the major obstacles in obtaining hybrid seeds was premature flower drop after pollination, Burk and Chaplin (1979) gave a general recommendation to apply indole acetic acid (IAA) to the pedicels of pollinated flowers to prevent abscission. To the same end, Wark (1970) applied IAA to calyx sectors of maternal flowers. Recently, He et al. (2022), in a study on the causes of hybrid lethality in *Nicotiana,* confirmed the effectiveness of IAA in suppressing abscission in the interspecific cross *N. suaveolens* x *N. tabacum*, although the treatment *per se* had no effect on the lethality of hybrid seeds.

At least in the case of *N. tabacum* × *N. africana,* the survival of rare hybrid phenotypes was found to be related to the somatic instability of the hybrid embryos. As a result of chromosome loss or fragmentation, the specific loci contributed by *N. africana* and/or by *N. tabacum* and responsible for the development of apoptotic plant death syndrome in hybrid seedlings were eliminated, and rare hybrid genotypes deficient for lethality factors could be grown to maturity (Hancock et al., 2015). In another hybrid that showed PCD syndrome, *N. amplexicaulis* × *N. tabacum* (Berbeć & Doroszewska, 1981), different chromosomal constitutions were found among rare surviving hybrids, including an aneuploid deficient for 3 chromosomes, 6 aneuploids with two to four supernumerary chromosomes 12, and apparently regular amphihaploids ($2x = 42$) plus one apparent amphidiploid ($4x = 84$).

Overcoming Hybrid Lethality Through *In Vitro* Cultures The methods to overcome hybrid unviability that depended on fortuitous processes were inefficient, time and labor consuming and final success was a matter of luck. For plant breeders, another trade-off of this approach is the possibility that the surviving aneuploids may be deficient for the gene/genes of their specific interest.

Butenko and Luneva (1966) were probably the first to overcome hybrid lethality in *Nicotiana* by resorting to *in vitro* culture. They placed aseptically produced seeds of the nonsurviving hybrid *N. alata* × *N. glauca* on a nutrient medium supplemented with kinetin and produced viable hybrid plants. With time, different variants of *in vitro* cultures on solidified media to rescue dying hybrid seedlings became a widely used practice. A compilation of cases where interspecific hybrids involving *N. tabacum* were obtained by resorting to *in vitro* culture, regardless of method applied and cause of incongruity, is presented on a species-by-species basis in Table 3.4. By comparing Tables 3.4 and 3.10, it is notable that the majority of the hybrids listed in Table 3.4 were also obtained by earlier hybridizers in the natural way by direct pollination without the aid of *in vitro* techniques. Nonetheless, the gains of saved time and labor resulting from the improved seedling survival rate (Tezuka et al., 2010) caused the traditional approaches to have been all but displaced by aseptic cultures.

The role of tissue culture in overcoming lethality of *Nicotiana* hybrids is still not wholly understood, and the extent to which particular mechanisms are involved may differ among individual cases. The process of organ culture and plant regeneration is known to exacerbate somaclonal variation. According to Tezuka et al. (2012), the deletion of a chromosome or a chromosome fragment may lead to the elimination of the causative agent for interspecific lethality in hybrids rescued by tissue culture. The validity of that supposition was confirmed by Nakata et al. (2021). The authors demonstrated that the cultured hybrid seedlings of *N. suaveolens* x *N. tabacum* that had overcome lethality lacked the distal part of chromosome Q of *N. tabacum* that carries the Nt6549g30 gene responsible for the development of lethality syndrome (Ma et al., 2020). Such spontaneous chromosomal alterations that led to the cancellation of the PCD syndrome were convincingly demonstrated for the hybrid *N. tabacum* × *N. africana* by Hancock et al. (2015), see also Sect. 3.2.3). The preponderant class of lethal *N. africana* × *N. tabacum* hybrid plants obtained by Nikova and Zagorska (1990) that were rescued by tissue culture had fewer (44) mitotic chromosomes than the theoretically expected number (47). On the other hand, the *N. tabacum* × *N. africana* hybrid plants recovered from cotyledon culture by Doroszewska and Berbeć (1996) all had a regular number of 47 chromosomes. In yet another study of the same hybrid (Depta & Doroszewska, 2019), hybrid plants regenerated from cultured cotyledons were also classified as amphihaploids. In the latter case, flow cytometry was used to determine the ploidy level of viable regenerants. However, this relatively fast and convenient method lacks the resolution power to discriminate at the single chromosome level, as can be clearly seen from the data supplied by Hancock et al. (2015). Nonetheless, the chromosome counts of one of the first lethal hybrids rescued by cotyledon culture, *N. suaveolens* × *N. tabacum* (Lloyd, 1975), also revealed a regular amphihaploid number of 40.

Culture conditions and medium components may induce mutations that result in disabling the function of the lethality genes in otherwise chromosomally regular hybrid genotypes (Tezuka, 2012). What all those and some other tissue-culture rescued hybrids (Ternovsky et al., 1972, 1976; Yamada et al., 1999) had in common was that they were cultured and regenerated on media that included cytokinins in their composition. Inoue et al. (1994, 1997) demonstrated that the addition of cytokinins to the culture medium had a decisive effect on the survival of the lethal hybrid plants of *N. suaveolens* × *N. tabacum,* but the efficacy of the treatment varied with the type and concentration of the cytokinin used. Nakata et al. (2021) surmised that the increased rate of survival of *N. suaveolens* × *N. tabacum* hybrid seedlings on cytokinin-enriched medium was due to the presence of cytokinin-induced reactive oxygen species (ROS) in the studied hybrid explants. Since ROSs are known to cause chromosome breakage, they may have induced the observed fracture and loss of the distal part of chromosome Q that carried the Nt6549g30 gene essential for triggering the plant death response. This mechanism was assumed to account for the restoration of viability to at least a large part of the potentially apoptotic hybrid plants of *N. suaveolens* x *N. tabacum*. Hancock et al. (2015) also linked the rare survival of *N. tabacum* x *N. africana* seedlings grown *in vivo* to chromosome instability and to the deletion of the Nt6549g30 gene, but they associated the phenomenon with the innate behavior of chromosome H in that particular hybrid milieu rather than with the influence of any external factors (see, however, section 3.3.4 for comments on the identity of chromosome H and chromosome Q).

Temperature may be another factor related to overcoming hybrid lethality by tissue culture. High temperature-dependent suppression of hybrid lethality of types II and V is known to become ineffective once the hybrid plant is returned to normal growth conditions (Tezuka, 2012). However, in many cases, the temperatures prevailing inside culture containers are likely to be above the critical threshold of ca. 30 °C and thus may facilitate the operation of other, not temperature-related, factors in the *in vitro* environment. Incidentally, the survival *in vivo* of hybrid seedlings of *N. amplexicaulis* × *N. tabacum* was substantially improved when seed germination and seedling culture were moved from the greenhouse to a growth chamber fitted with fluorescent tubes that kept the ambient temperatures ca. 30 °C, and the process was conducted in covered glass containers (Berbeć & Doroszewska, 1981).

Shiragaki et al. (2020) reported that treatment of juvenile plants of the hybrid *N. suaveolens* x *N. tabacum* with L-2-aminooxy-3-phenylpropionic acid (AOPP) suppressed the development of type II lethality by inhibiting the phenylalanine ammonia-lyase (PAL) responsible for the production of phenolic compounds involved in seedling death.

Overcoming Hybrid Lethality Through the Use of *N. tabacum* Monosomics Burk (1972) was the first to resort to a monosomic parent to overcome hybrid lethality in an interspecific hybrid of *Nicotiana*. By crossing *N. langsdorffii* as the male parent to each of the 24 monosomies of *N. tabacum* as females, he was able to produce several viable hybrid plants, most of which had been obtained from Haplo A plus a few single survivors from Haplos G, L, and Z. A few years later, Gerstel et al. (1979) demonstrated the involvement of the *N. tabacum* H chromosome in the lethality of the hybrid N. *africana* x *N. tabacum* by producing viable and inviable plants in the progeny of *N. africana* x Haplo H. Tezuka et al. (2010) repeated the success of Gerstel et al. (1979) with the hybrid N. *afric*ana x *N. tabacum* and with hybrids of *N. tabacum* with several other species of the section Suaveolentes, reporting Haplo Q as the monosomic *N. tabacum* parent (see, however, Sect. 6.4.3 on the identity of chromosomes H and Q).

Even though the monosomic method seems to be easy and dependable for the production of viable hybrids, especially those exhibiting type II lethality, few, if any, researchers, apart from those cited above, have tried this approach. Most of those who did had goals in mind other than bypassing lethality obstacles. The most likely reason is that the monosomic stocks are relatively hard to access from external sources, and their in-house development and perpetuation are both costly and troublesome.

Overcoming Hybrid Lethality Through Gene Editing Ma et al. (2020) identified the gene of *N. tabacum* that controls the seedling death of *N. tabacum* x *N. africana* hybrid plants (see also Sect. 3.3.3). As the confirmation part of the study, the authors used CRISPR–Cas9 gene-editing technology to insert frameshift mutations within the *Nt6549g30* region. A transformant homozygous for a mutation of the *Nt6549g30* gene was found that conditioned nearly 100% survival of the hybrid seedlings that resulted from pollinating the engineered *N. tabacum* by *N. africana*. Thus, it was demonstrated that the mutation disabled the lethality-conferring function of *Nt6549g30,* thereby reconfirming the identity of *Nt6549g30* as the gene controlling hybrid lethality in *N. tabacum* x *N. africana*.

In yet another part of the same study (Ma et al., 2020), several crosses were made of the mutant line of *N. tabacum* with some other species of the section Suaveolentes (*N. amplexicaulis*, *N. debneyi*, *N. excelsior*, *N. gossei*, *N. megalosiphon*, *N. occidentalis*, *N. simulans*, *N. umbratica*, *N. velutina*) to determine whether the same factor was responsible for seedling death of those hybrids that previously had been known to exhibit type II seedling lethality (Tezuka, 2012). The viability of all but one of those hybrids was restored. The exception was the hybrid from crossing *N. occidentalis* with the Nt6549g30 mutant of *N. tabacum*. The results were consistent with lethality types exhibited by the studied hybrids. While all the surviving hybrids represented type II lethality, the hybrid *N. occidentalis* x *N. tabacum* that failed to respond to the Nt6549g30-mutant gene had been classified as type V (Tezuka, 2012). Obviously, a different mechanism of hybrid lethality is involved in the latter hybrid. The authors of the study (Ma et al., 2020) pointed out that their novel approach may expand the gene pool available for tobacco breeding. The current practical disadvantage of the method is that the CRISPR–Cas technology involves what in many countries is termed genetic modification and as such is subject to legal restrictions.

3.5 Maternal and Paternal Plants in the Offspring of Interspecific Crosses in *Nicotiana*

3.5.1 Gynogenic and Androgenetic Haploids

Both gynogenic and androgenetic haploids were reported in the offspring of interspecific crosses in *Nicotiana*. Gynogenic (maternal) haploids of several *Nicotiana* species (*N. alata, N. rustica, N. paniculata, N quadrivalvis*) stimulated by the pollen of an alien *Nicotiana* species were probably first reported by Wellington (1913). Single haploid plants of *N. rustica* were found among the progeny from crossing *N. tabacum* as the pollen parent with the former species as the female (Savelli cited by Kostoff, 1943) and with *N. gossei* (Apparao & Ramavarma, 1972). Clausen and

Mann (1924) discovered a haploid plant of *N. tabacum* among the offspring of the cross *N. tabacum* × *N. sylvestris*. Haploids of *N. tabacum* were also reported from crossing *N. tabacum* with *N. alata* and *N. quadrivalvis* (Wellington, 1913), *N. glutinosa* (McCray, 1932), and *N. longiflora* and *N. glauca* (Ternovsky, 1936a, 1936b). Haploid plants were induced in the offspring of *N. tabacum* plants whose stigmata were dusted with X-irradiated pollen of *N. alata* (Tanaka & Kurihara, 1968). The thus obtained gynogenic haploids were compared with those from anther culture (Kumashiro & Oinuma, 1985).

Androgenetic haploids among the offspring of interspecific crosses seem to occur much more rarely than their gynogenic counterparts. A supposedly androgenetic haploid of *N. tabacum* was found in the progeny from crossing maternal amphidiploid 4x (*N. glutinosa* × *N. tabacum*) with male *N. tabacum* (Clausen & Goodspeed, 1925; Clausen & Lammerts, 1929). A single androgenetic haploid of *N. africana* was detected among the offspring from mating *N. tabacum* with genetically transformed *N. africana* (Hancock et al., 2015). The divisions of the sperm of *N. tabacum* in the nucleus of *N. eastii* followed by chromosome doubling in the developing embryo may have accounted for the appearance of a cytoplasmically male sterile *N. tabacum* plant as a result of crossing maternal *N. eastii* with *N. tabacum* as the pollen parent (Berbeć & Berbeć, 1992). A case that bears a strong resemblance to the story of cms *eastii* was reported by Nikova et al. (1997). The authors attempted to transfer cms factors from *N. excelsior* to *N. tabacum*. Following the F_1 generation, the transfer seemed to proceed smoothly, but the very nature of the F_1 plants cannot be readily explained based on what the authors themselves reported. In the account by Nikova and her associates, their F_1 plants were similar to the male *N. tabacum* save for flower malformations, and they could be backcrossed with ease to the male recurrent parent. Based solely on morphological and fertility evidence supplied by the authors, it is verging on impossible that those F_1's were regular amphihaploid hybrids and were very unlikely to be spontaneous amphidiploids. Based on what the investigators themselves reported, it can be hypothesized that the male sterile *N. tabacum*-like phenotypes in the F_1 offspring may have originated as a result of unilateral elimination of *N. excelsior* chromosomes from the hybrid zygotes followed by chromosome doubling (compare the reports on gynogenic haploids by Chimoyo and Pupert (1988) and by Hancock et al. (2015) referred to in the paragraph next but one below.

Spontaneous androgenetic haploids in *N. tabacum* were proposed to be a convenient vehicle to transfer cytoplasmic male sterility from one variety to another in a single step by using a rootless mutation to discriminate between rare androgenetic haploids and true intervarietal hybrids (Horlow et al., 1993).

One of the interspecific crosses, *N. tabacum* × *N. africana*, produces maternal haploids very regularly, albeit normally at a very low rate. The phenomenon was deployed to develop a method by which haploid plants of *N. tabacum* can be generated for experimental and breeding purposes (Burk et al., 1979, Nielsen & Collins, 1989, see also Sect. 3.3.1). Gerstel and Wernsman (1979) assumed that gynogenic haploids arise spontaneously during the reproductive process without direct involvement of the pollinating species. The massive death of true interspecific hybrids at the cotyledonary stage gives a selective advantage to rare gynogenic haploids that are otherwise difficult to discern and pick up among regular progeny. One must note, however, that not necessarily all the haploids found in the progenies of mating *N. tabacum* to *N. africana* are purely gynogenic. At least some of them may have arisen as products of selective elimination of *N. africana* chromosomes from originally hybrid embryos (Chimoyo & Pupert, 1988; Hancock et al., 2015).

3.5.2 Maternal Diploids

Various Cases of Maternal Diploid Induction in *Nicotiana* Maternal diploid plants in the offspring of interspecific matings in *Nicotiana* have long been a controversial topic. At first, Goodspeed (1915) dismissed diploid maternals, apomictic or otherwise, as products of experimental errors in disagreement with East (1930) but later abandoned his former view, citing *N. paniculata* as the prime example of maternal diploidy following hybridization with other species (Goodspeed, 1954). Maternal plants continued to be reported as byproducts or even as sole products of mating maternal *N. tabacum* with *N. rustica* (Lehmann, 1936), *N. repanda* (Pittarelli & Stavely, 1975), *N. amplexicaulis* (Berbeć & Doroszewska, 1981), *N. wuttkei* (Laskowska et al., 2015), *N. africana* (Hancock et al., 2015), *N. sylvestris* (Eghis 1930) and various other interspecific matings (Murthy & Subbarao, 2004). Alleged apomictics were also reported in the progeny of *N. alata* and *N. forgetiana* as induced by pollination with X ray-irradiated pollen of *N. langsdorffii* (Pandey, 1974). Maternal plants were reported as the preponderant category of offspring when *N. tabacum* was mated to the amphidiploid 4x (*N. wuttkei* x *N. tabacum*) (Laskowska et al., 2015).

A singular case was reported by Kostoff (1935, 1938a), who described a parthenogenetically produced amphidiploid 4x (*N. glauca* × *N. langsdorffii*) obtained by fertilizing an amphihaploid plant 2x (*N. glauca* × *N. langsdorffii*) with pollen of *N. langsdorffii*. Kostoff explained the phenomenon by the presence of an unreduced monad cell with the doubled chromosome complement of the maternal hybrid that had been stimulated by pollen of *N. langsdorffii* to develop parthenogenetically into an amphidiploid embryo (Kostoff, 1935). Another case of that type that also involved *N. langsdorffii* was the production of amphidiploid seeds by the amphihaploid *N. knightiana x N. tabacum* upon pollination with pollen of F_1 *N. alata x N. langsdorffii* (Berbeć et al., 1982). In the latter case, along with maternal origin, another plausible course of events is the fusion of the unreduced egg cell produced by the amphihaploid with the sperm of *N. alata* x *N. langsdorffii* followed by selective elimination of the alatoid chromosomes during embryogenesis or/and embryo development. The plausibility of such an explanation is further supported by the fact that the same amphihaploid 2n (*N. knightiana* x *N. tabacum*) was prone to spontaneous seed setting (Berbeć et al., 1982). In a related phenomenon, Apparao et al. (1980) observed a massive selective loss of *N. gossei* chromosomes from the hybrid 4n *N. tabacum* × 2n *N. gossei,* resulting in F_1 progeny composed of plants phenotypically close to *N. tabacum*, each of which contained a full diploid complement of the maternal parent and two chromosomes from *N. gossei*.

Induction of Maternal Plants by Pollen of *N. alata* The mating of *N. tabacum* with *N. alata* is noteworthy, as it seems to have yielded maternal phenotypes more frequently than any other interspecific cross that involved *N. tabacum* as the female parent. Both maternal haploids and diploids of *N. tabacum* were induced by X-ray-irradiated pollen of *N. alata* (Tanaka & Kurihara, 1968; Pandey & Phung, 1982; Kumashiro & Oinuma, 1985). Spontaneous diploid maternal phenotypes were observed in the offspring of the cross *N. tabacum* × *N. alata* by Stoyanova (1979), Sarychev (1987), Berbeć (1987b), Naumenko (2012), and Liao et al. (2017). In the study of Stoyanova (1979), diploid maternals were practically the only class of progeny obtained from mating an unspecified Virginia variety of *N. tabacum* to *N. alata*, whereas crosses between other varieties of *N. tabacum* and *N. alata* yielded no maternal plants. In the latter instance, the author herself suspected experimental error, but she did not exclude other causes.

In one of his early experiments with interspecific hybrids, the author of this review studied the hybrid *N. tabacum* x *N. benavidesii* (Berbeć, 1978). In the course of his study, he obtained a number of subsesquidiploids (TTB) with chromosome numbers ranging from 55 to 58. Under open self-pollination, these sesquidiploids did not produce seeds by selfing but could be easily backcrossed to *N. tabacum*. A surprising part of the study was that they could also set seeds when their flowers were pollinated with pollen of *N. alata*. Generally, the offspring from regular backcrosses were morphologically similar to those resulting from pollinating the sesquidiploid with *N. alata*, with no traces of introgression from the pollinator species. Likewise, both classes of offspring showed a similar behavior in meiosis with approximately 24 bivalents and varying numbers of univalents, but the backcross products involving *N. alata,* univalents were generally higher in number. All these observations pointed to induction of parthenogenetic development of unreduced female gametes of the BC1 plants by pollen of *N. alata* without gametic fusion having actually taken place. Alternatively, fusion of gametes may have taken place, but the *N. alata* chromosomes were selectively eliminated from the developing embryo. However, another plausible but, under these circumstances, not very likely explanation is an experimental error, i.e. inadvertent spontaneous self-pollination in BC_1 plants. Interestingly, in the previously mentioned study on the hybrid *N. tabacum* x N. *alata* by the same author (Berbeć, 1987b), N. *tabacum* (TT) plants well protected against uncontrolled pollen contamination were mated to the sesquidiploid (TTA) as pollen parent. The mating resulted in 36 surviving plants, of which 29 closely resembled the maternal plants and the remaining seven, while departing from the maternal phenotype, did not show any traces of introgression from *N. alata*.

When emasculated but unprotected flowers of *N. tabacum* were fertilized with pollen of *N. alata* (Berbeć, 1987b), some genotypes regularly yielded viable and fertile *N. tabacum*-like phenotypes as the preponderant class of offspring, the rest being inviable or poorly viable hybrid seedlings. When carried out alongside and under the same conditions, interspecific matings of female *N. tabacum* with male *N. langsdorffii* and *N. benavidesii* only occasionally produced surviving maternal plants (unpublished observations of the author of this volume).

A phenomenon very similar to if not identical with those described in the preceding two paragraphs was reported by Naumenko (2010, 2012). Diploid maternals described as 'pseudogamic' and resulting from fertilizing intraspecific F_1 hybrids of *N. tabacum* with pollen of *N. alata* could be generated regularly and were not much different from the regular selfed progeny of those plants. Apomictic populations from crossing F_1 intervarietal hybrid plants showed segregation for plant height, leaf number and leaf size that was characteristic of the F_2 pulations obtained from the same plants by selfing. According to Naumenko, the difference between the alleged apomictics and the regular hybrids was that the former became stabilized already in the F_2 generation whereas the regular hybrids kept segregating in subsequent generations. This description recalls the behaviour of populations derived from gynogenic or androgenetic doubled haploid hybrid plants. In this particular instance, *N. alata* sperms may have induced chromosome doubling and embryo development in the maternal plants without actual gamete fusion. Alternatively, gamete fusion may have been followed by massive elimination of *N. alata* chromosomes and chromosome doubling, whichever is more likely. This way or the other, the most puzzling feature of Naumenko's account is the massive and regular incidence of the described phenomenon, the fact also reported earlier by Sarychev (1987).

Liao et al. (2017) recovered self-fertile maternal phenotypes along with apparently regular interspecific hybrids by fertilizing senescent flowers of cytoplasmically male sterile *N. tabacum* with pollen of *N. alata*. The puzzling part of the latter report was that the *N. tabacum*-like plants showed restored normal stamen morphology and self-fertility, and according to flow cytometry measurements, their genome was considerably smaller than that of their maternal parent. According to Liao et al. (2017), the maternal phenotypes in the offspring were actually hybrids rather than true maternals since *N. alata*-specific fragments, including the putative male fertility-restoring genes, were amplified in them with selected SSR probes. The two accounts, by Naumenko (2012) and by Liao et al. (2017), bear intriguing similarities, but Naumenko obviously used male fertile maternal plants in her study since she propagated her apomictics by selfing.

Some of the results described in this section are loosely reminiscent of the experiments reported by Pandey on what he called egg transformation without gametic fusion (Pandey, 1974, 1975, Pandey & Phung, 1982, see Sect. 4.4.4).

Overall, due to its elusive and inconsistent nature as well as different manifestations, the phenomenon of maternal genotypes in the progeny of some interspecific crosses is not readily amenable to systematic study and, therefore, is habitually dismissed as resulting from inadequate protection against inadvertent pollen contamination. This notwithstanding, maybe it deserves more attention than it has hitherto received.

3.6 Sterility of Interspecific Hybrids

3.6.1 *Causes of Sterility in Amphihaploid Hybrids of* Nicotiana

In *Nicotiana*, once all previously discussed pre- and postfertilization barriers have been successfully overcome or circumvented and the hybrid plant has been brought to flowering, it can be reasonably expected to be completely sterile. Actually, the very first interspecific *Nicotiana* hybrid ever reported, *N. paniculata* x *N. rustica*, was described by its creator J. G. Koelreuter as sterile, which was in stark contrast to the full fertility of the parental forms (Mayr, 1986).

In tobacco, just as in other organisms that perpetuate themselves by sexual reproduction, the fusion of two viable gametes, male and female, is a crucial event in the reproductive process. An orderly and undisturbed reductional division in meiocytes whereby the number of somatic chromosomes of an individual is reduced by half is the key element in assuring the formation of viable and functional gametes. In interspecific hybrids, this regular process can be upset because the chromosomes contributed by the two parental species are structurally different, which prevents their normal pairing, and/or the numbers of chromosomes are different, which leaves some of the chromosomes without a partner with which to pair.

The genus *Nicotiana* is unique among other plant genera in that its species show a high degree of cross-compatibility (approximately 450 hybrid combinations have been produced thus far (see Chap. 7 of this review), and at the same time, the vast majority of those hybrids are practically self- and cross-sterile, i.e., they are not capable of producing seeds either if pollinated by their own pollen or by the pollen of another species. In other genera, approximately 75% of successful interspecific hybrids show at least some degree of self-fertility (Stebbins, 1950). According to Goodspeed (1954), in the genus *Nicotiana,* chromosomal rearrangements and other structural changes accumulated faster than barriers to hybridization. This leads to deranged micro- and macrosporogenesis in hybrids, resulting in chromosomally imbalanced and thus inviable or dysfunctional gametes and, consequently, aborted or inviable ovules and pollen. Some intrasectional hybrids, e.g., in the sections Alatae, Trigonophyllae, Tomentosae and Suaveolentes, with equal numbers of chromosomes and chromosome homology high enough to ensure normal pairing regularly produce selfed offspring and can be backcrossed to their parental species. A puzzling exception to this rule was the intersectional hybrid between *N. alata* (section Alatae, chromosome number $n = 9$) and *N. amplexicaulis* (section Suaveolentes, chromosome number $n = 18$) reported by Gopinath et al. (1970). Although difficult to produce, once obtained, the amphihaploid hybrid (27 somatic chromosomes) was reportedly self- and cross-fertile. Unfortunately, no one else is known to have reproduced and studied that hybrid.

All known interspecific hybrids involving the cultivated tobacco *N. tabacum* with their number approaching 60 are sterile, although those originating from crossing it with its direct ancestors or their close relatives occasionally yield viable offspring when backcrossed to the *N. tabacum* parent (Clausen & Cameron, 1944, 1957). Upon backcrossing to *N. tabacum*, the hybrid *N. tabacum* × *N. tomentosiformis* produced offspring among which fertile 48-chromosome *N. tabacum*-like segregants were found (Brieger (1928). This is not surprising bearing in mind the 'Drosera' chromosome pairing in the hybrid parent of the cross (compare Sect. 3.3.4). Other exceptions and reservations concerning the sterility of interspecific hybrids with *N. tabacum* are indicated in subsequent sections. The sterility of an interspecific hybrid makes it of little use, especially if further generations are considered, e.g. for breeding purposes.

3.6.2 Bioconfinement

In some cases, hybrid sterility may be of potential advantage. Chambers et al. (2011), Ling et al. (2012), and Rice et al. (2013) considered the applicability of the hybrid *N. tabacum* × *N. glauca* for transgene-controlled production of pharmaceuticals because of the hybrid's capacity for biomass production and its other merits. They pointed to the sterility of the hybrid as the safeguard against unintended escape of the transgene to the environment, although they were aware that the sterility of the hybrid was not complete (Chambers et al., 2011; Rice et al., 2013), an issue that will be briefly discussed in the next paragraph of this section. The bioconfinement effect of hybrid sterility was also established for two closely related species, *Nicotiana tabacum* and *N. sylvestris* (Ahl-Ahmad et al., 2006). The hybrid of the two species was reported to produce no offspring either by selfing or by backcrossing to *N. sylvestris*. The authors concluded that the cultivation of a transgene-carrying *N. tabacum* in close proximity to native or ornamental *N. sylvestris* poses no significant risk of the unintended release of some novel genetically modified genes to the environment. Because of the high degree of self- and cross sterility plus the added benefits of perennial growth and the ease of clonal propagation, Lim et al. (2006) envisaged the usefulness of genetically modified first-generation 'synthetic tobacco' (4x (*N. sylvestris* × N. *tomentosiformis*)) for biopharmacy. However, the latter hybrid, although practically sterile, may not be entirely gene escape-proof (see Chap. 2), especially when commercial tobacco is grown nearby. Cost-effective production of seeding material may also be an issue.

3.6.3 Conversion of Sterile Amphihaploids to Fertile Alloploids by Making Use of Natural Processes in Hybrid Plants

Partial or Occasional Self- and Cross-Fertility of Otherwise Sterile Allohaploid Hybrids In most intended or actually implemented practical uses of interspecific *Nicotiana* hybrids, their sterility is a liability rather than an asset. The desired goal has been a hybrid that can be sexually perpetuated by selfing and, preferably, one that is also able to produce offspring upon backcrossing to the cultivated species. In their efforts to restore fertility to their interspecific hybrids, the early breeders were, consciously or otherwise, heavily dependent on the production of restitution gametes by their experimental materials. They thus made use of the fact that during the aberrant events of gametogenesis, one or both reductional divisions may fail, resulting in the formation of unreduced gametes that are usually chromosomally balanced and viable, as they retain the genomic integrity of their parental plants. The rate of formation of such restitution gametes may vary from negligible to quite substantial depending on parental genotypes, environment, plant age, etc. (Kostoff, 1943, Goodspeed, 1954, Doroszewska & Berbeć, 1996).

Kostoff (1936, 1938a) heavily relied on a high rate of restitution gametes in his attempt to develop an amphidiploid N. *sylvestris* x *N. tomentosiformis* (SSTT) by crossing the sterile F1 hybrid (ST) first to *N. sylvestris* and the resulting SST hybrid to N. *tomentosiformis*, the form that came to be known as “Kostoff’s hybrid” (see also Sect. 2.2.2). Kostoff (1943) observed that the production of restitution nuclei by *N. sylvestris* × *N. tomentosiformis* was further intensified when the hybrid was exposed to high temperatures accompanied by restricted water supply. Similarly, Rybin (1927) and Eghis (1927) crossed a tetraploid variant of *N. tabacum* (TTTT) with diploid *N. rustica* (RR). Upon backcrossing the resulting sesquidiploid (TTR) to the diploid parent (RR), they obtained occasional plants that had the amphidiploid (TTRR) genomic constitution. The above procedure did not always prove successful. In an attempt to produce amphidiploid 4x (*N. tabacum* x *N. benavidesii*), the author of this review backcrossed the sesquidiploid *N. tabacum*-*N. tabacum*-*N. benavidesii* to *N. benavidesii*. All but one of the offspring thus obtained were poorly viable amphihaploids with extra univalents, probably from *N. benavidesii,* and the remaining single plant was an unstable 68-chromosome subamphidiploid (Berbeć, 1978).

In a sterile amphihaploid, if an unreduced female gamete fuses with its unreduced male counterpart, a fertile amphidiploid may be produced by selfing. The best illustration of this fortuitous process is the case of the first artificial fertile *Nicotiana* amphidiploid ever made. After many years of unsuccessful attempts to self-pollinate the sterile hybrid 2n (*N. glutinosa* × *N. tabacum*), Clausen and Goodspeed (1925) obtained three seeds, out of which one germinated and grew to an amphidiploid. A few years later, that amphidiploid was used by Holmes (1938) to transfer resistance to tobacco mosaic virus (TMV) from *N. glutinosa* to *N. tabacum* (see Sect. 4.1). That resistance continues to be deployed in contemporary cultivars and thus, at least in several cases, its origin can be traced back to those remote but fateful events recorded by Clausen and Holmes many decades ago. There are several cases on record in which otherwise sterile hybrids of different *Nicotiana* species with *N. tabacum* yielded viable offspring through assiduous effort of selfing the amphihaploid plants. The key to success consisted in self-pollinating on a scale large enough to increase the small odds of an unreduced male and female gamete fusing together into an allopolyploid zygote. In exceptional cases, amphidiploids or sesquidiploids can be produced directly from mating two diploid species. Such a chance fusion of two restitution gametes produced by two parental species may give rise to a direct allopolyploid hybrid. Ternovsky (1962) reported spontaneous amphidiploids directly from the crosses *N. glutinosa* × *N. tabacum*, *N. tabacum* × *N. glauca* and *N. tabacum* × *N. sylvestris*. Another case of this kind is the appearance of the sesquidiploid plant in the hybrid progeny of the cross *N. tabacum* × *N. sylvestris* that appears to have arisen from the union of diploid restitution gametes of *N. tabacum* with a normal haploid gamete of *N. sylvestris* (Webber, 1930). If an amphihaploid was backcrossed to the *N. tabacum* parent, partly fertile sesquidiploids or near sesquidiploid plants were the usual outcome. Clayton (1954) observed a high rate of restitution gametes in the hybrid *N. debneyi* × *N. tabacum,* resulting in partially restored self-fertility and the production of allopolyploid progeny.

Low temperatures and excess moisture led to partially restored fertility in the hybrids *N. debneyi* × *N. tabacum* and *N. tabacum* × *N. glauca* (communicated to the author of this review by J. Berbeć). The production of restitution nuclei and viable pollen grains increased in the hybrid 2x (*N. tabacum* × *N. africana*) as the plants grew older (Doroszewska & Berbeć, 1996). In the latter case, the hybrid plants failed to yield any spontaneous seeds, notwirhstanding.

In some other reports (Berbeć & Opoka, 1966; Berbeć, 1971), the hybrid *N. tabacum* × *N. glauca* and its reciprocal retained some vestigial self-fertility, and the two hybrids could also be used both as pollen and egg parents to produce backcross offspring with *N. tabacum* from which further selfed generations were obtained. This simple expedient to overcome the sterility barrier by backcrossing the sterile hybrid to one of the parents was probably fairly frequently resorted to and in several cases proved successful (East, 1928; Ternovsky, 1936a, 1936b; Burk, 1967; Wichert-Kobus, 1967, 1971; Berbeć, 1980; Berbeć et al., 1982; Nikova et al., 1997).

Aneuploid plants having from 28 to 34 chromosomes obtained by culturing the anthers of the sterile hybrid *N. tabacum* × *N. sylvestris* probably also arose from the restitution gametes produced by that hybrid (Takahashi, 1973). Another plausible mechanism was the production of partly functional generative nuclei by the hybrid as a result of the 'Drosera' pairing process.

The citations of cases where spontaneous amphidiploids or sesquidiploids involving *N. tabacum* were produced are given in Table 3.5 on a species-by-species basis. The allopolyploids were obtained either directly from crossing diploid parental species or by selfing or cross-pollination of amphihaploid hybrids.

Use of Autotetraploid Forms of One or Both Parental Species Sterility barriers can also be circumvented if one or both parental species are used in the autotetraploid form. Clayton (1947) crossed autotetraploid *N. tabacum* with autotetraploid *N. longiflora* and obtained a partially fertile amphidiploid that was used in the interspecific transfer of a disease resistance factor. Similarly, an autotetraploid accession of *N. knightiana* was mated as the female to an autotetraploid variant of *N. tabacum* (4n = 96) to produce a fertile amphidiploid 4x (*N. knightiana* × *N. tabacum*) (Berbeć & Doroszewska, 1992). After a series of successful backcrosses to *N. tabacum* as the recurrent male parent, an alloplasmic lineage

Table 3.5 Spontaneous production of allopolyploid offspring by direct crosses of *N. tabacum* with an alien species or by amphihaploid hybrids involving *N. tabacum* as a result of self-fertilization (S) or cross-pollination (CP) with the *N. tabacum* parent

Species involved in the hybrid with *N. tabacum*	Cytological status of offspring	Fertilisation	Author
N. alata	TTAA	S	Ternovsky (1936), Stoyanova, 1979
	TTA	S, C_{TxA}	Stoyanova (1978, 1979)
	TTSanSan	S	Ternovsky (1936)
N. sanderae	TTSan	C_{TxA}	Ternovsky (1936, 1962)
	TTGlaGla	C_{TxA}	Ternovsky (1936)
N. glauca	TTGla	S, CP	Ternovsky, 1936a, 1936b, Stoyanova and Konotop (1975)
	GlaGlaTT, GlaTT	S, CP	Berbeć and Opoka (1966), Berbeć (1971)
N. rustica	TTR	S	Ternovsky (1936a, 1936b), Zhukov (1939)
	TTRR	CP	Eghis (1927)[2]
N. benavidesii	TTB	CP	Berbeć (1978, 1980)
N. knightiana	KKTT	S, CP	Berbeć et al. (1982)[3]
N. paniculata	PTT (?)	CP	Holmes (1937a, b)
N. glutinosa	GluGluTT	S, C_{TxA}	Clausen and Goodspeed (1925), Ternovsky (1962)
	TGluGlu	C_{TxA}	Ternovsky (1962)
N. sylvestris	TTSS	C_{TxA}	Ternovsky (1962)
	TTS	CP	East (1928)
	TTS (?)[1]	CP	Burk (1967)
N. amplexicaulis	AmAmTT AmTT	C_{AxT} CP	Berbeć and Doroszewska (1981) Nikova et al. (1997)
N. debneyi	DebDebTT (?)	S	Clayton (1950, 1954), Berbeć (1964)
N. exigua	ETT	CP, S	Wichert-Kobus (1971)
N. goodspeedii	GGTT	S	Palakarcheva et al. (1978)
N. megalosiphon	MMT (?)	CP	García Cruz et al. (2008)[4]
N. setchellii x *N. otophora*	TTSeO	C	Berbeć et al. (1982)
N. tomentosa	TTTom	CP	East (1928)
N. tomentosiformis	TTTmf	CP	East (1928)

Abbreviations and symbols used: haploid genomes: *T N. tabacum*, *A N. alata*, *Am N. amplexicaulis*, *San N. sanderae*, *Gla N. glauca*, *R N. rustica*, *B N. benavidesii*, *K N. knightiana*, *Glu N. glutinosa*, *S N. sylvestris*, *E N. exigua*, *G N. goodspeedii*, *M N. megalosiphon*, *Tom N. tomentosa*, *Tmf N. tomentosiformis*; *Se N.* setchellii; *O N.* otophora; TxA or AxT typed in subscript stand for T (*N.tabacum*) and A (alien species) and indicate the direction of crossing

[1] Allo-aneuploids of *N. sylvestris* x *N. tabacum* possessing full haploid genomes from both species plus unspecified number of extra chromosomes from *N. repanda* were backcrossed to *N. tabacum* (for more details see Sect. 4.4.1);

[2] Amphidiploids TTRR were obtained by crossing a sesquidiploid TTR with diploid N. tabacum;

[3] amphidiploids KKTT were obtained by spontaneous seed set by the amphihaploid KT and by induction of amphidiploid seeds by the amphihaploid KT with pollen of *N. alata* x *N. langsdorffii*;

[4] cytological status of the backcross hybrids not reported

N. tabacum cms knightiana was developed that showed vestigial self-fertility. The negative aspect of this "tetraploid-tetraploid" approach is that autotetraploids usually show a certain percentage of imbalanced gametes due to frequently occurring polyvalent associations in meiosis. The resulting offspring of such tetra-tetra crosses are thus very likely to contain aneuploids along with regular amphidiploids and may happen to be deficient for a desired genetic factor from the wild species.

More frequently, autotetraploid variants of *N. tabacum* were crossed with diploid *Nicotiana* species to produce sesquidiploids. Since sesquidiploids contain, at least in theory, a full diploid chromosome complement of the tetraploid parent, they are able to produce some chromosomally balanced, viable gametes and are usually at least partially fertile. The first sesquidiploids obtained in this manner were produced by crossing autotetraploid *N. tabacum* with *N. gossei* (Valleau, 1952) and with *N. plumbaginifolia* (Chaplin, 1954; Ar-Rushdi, 1957). Discussion on the use of autotetraploid forms of Nicotiana as components of interspecific hybrids is further expanded in part 4.4.3 of this discourse.

Known interspecific combinations involving autotetraploid *N. tabacum* are listed in Table 3.6.

3.6.4 Conversion of Sterile Hybrid Plants to Fertility by Means of External Agents

Use of Diverse Chemical or Physical Agents Since conversion to fertility that relies on the vagaries of nature was both time consuming and highly unreliable, external agents that might induce the chromosome doubling process were tried. Eghis (1930) applied chloroform to obtain the allopolyploid 4x (N. *tabacum* × *N. sylvestris*).[3] In another early attempt, Ternovsky (1939) restored fertility to the F_1 hybrid *N. tabacum* × *N. sylvestris* by exposing it to high temperature. Another approach to polyploidization was attempted by Kostoff (1937), who centrifuged the allohaploid germinating seeds of *N. tabacum* x *N. rustica* and, as a result, a fertile branch with the doubled chromosome complement was obtained.

Use of Antimitotic Drugs The search for efficient methods to induce chromosome doubling resulted in the identification of substances that act as antimitotic drugs. Such substances interfere with the formation of the spindle during cell division. The chromosomes lag at the equatorial plate of the dividing cell, and the newly synthetized nuclear membrane surrounds the chromosomes, which are now double in number. If the anti-mitotic agent is removed in a timely manner, the cells continue to divide, thus giving rise to polyploid tissues. Acenaphtene was probably the first antimitotic drug used by some *Nicotiana* investigators for its chromosome doubling

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[3] some authors consider that hybrid an allohexaploid (6x) on account of the amphidiploid origin of *N. tabacum* and *N. sylvestris* being one of the parental species of *N. tabacum*.

Table 3.6 Instances of sesquidiploid hybrids obtained by direct crossing autotetraploid forms of *N. tabacum* with other *Nicotiana* species

Species involved with the hybrid with *N. tabacum*	Author
N. alata	Kostoff (1930), Chaplin and Mann (1961), Chaplin (1962), Takenaka (1960, 1962b), Takenaka and Yoneda (1964), Ivancheva-Gabrovska and Manolov (1982), Berbeć (1987b), Laskowska and Berbeć (2005)
N. forgetiana	Burk (1972)
N. langsdorffii	Takenaka et al. (1955), Takenaka (1958, 1962b), Burk (1972)
N. longiflora	Takenaka (1962a)
N. plumbaginifolia	Clausen in 1952 (after Ar-Rushdi, 1957), Chaplin (1954), Moav (1958), Moav and Cameron (1960), Chaplin and Mann (1961), Chen (1971), Baalawy and Fox (1971), Dang et al. (2019)
N. sanderae	Ivancheva-Gabrovska and Manolov (1982)
N. glauca	Chaplin and Mann (1961), Chaplin (1962), Wichert-Kobus (1971), Kobus (1971)
N. benavidesii	Takenaka (1962b), Berbeć (1986)
N. knightiana	Chaplin and Mann (1961), Berbeć et al. (1982)
N. paniculata	Chaplin & Mann, 1961, Baalawy and Fox (1971)
N. raimondii	Berbeć et al., 1982
N. repanda	Valleau (1952)[1]
N. quadrivalvis	Chaplin and Mann (1961)
N. pauciflora	Chaplin & Mann, 1961
N. rustica	Chaplin and Mann (1961), Legg and Mann (1961), Pandeya and White (1981, 1984); Chaplin and Sisson (1984), Pittarelli and Sisson (1989), Nifong (2008)
N. africana	Doroszewska and Berbeć (1990)
N. amplexicaulis	Berbeć et al. (1982)
N. debneyi	Clayton (1950)
N. exigua	Wichert-Kobus (1967), Wichert-Kobus (1971), Kobus (1971)
N. gossei	Valleau (1952), Moav and Cameron (1960) Apparao et al. (1980)[2]
N. megalosiphon	Manolov et al. (1978)
N. suaveolens	Chaplin (1959), Chaplin and Mann (1961)
N. sylvestris	Ar-Rushdi (1955), Chaplin and Mann (1961), Wichert-Kobus (1971), Kobus (1971)
N. otophora	Ar-Rushdi (1955), Chaplin and Mann (1961)
N. setchellii	Ar-Rushdi (1955)[3]
N. tomentosa	Ar-Rushdi (1955)
N. tomentosa var. 'Acomayo'	Ar-Rushdi (1955)[3]
N. tomentosiformis	Ar-Rushdi (1955) , Chaplin and Mann (1961)
N. glutinosa	Clausen and Cameron (1957), Chaplin and Mann (1961), Baalawy and Fox (1971), Pirrie and Power (1986), Giddings and Rees (1992)[4]

[1] a slow growing hybrid plant that died before flowering;
[2] *N. gossei* used as male parent, selective loss of *N. gossei* chromosomes observed;
[3] female sterile and male fertile sesquidiploids;
[4] sesqudiploids were obtained by fusing tetrad protoplasts of *N. glutinosa* with mesophyll leaf protoplasts of *N. tabacum* (gametosomatic hybrids)

effects. Using that agent, Ternovsky (1962) obtained the amphidiploid 4x (*N. sylvestris* × *N. tomentosiformis*). Bolsunov used acenaphtene to produce amphidiploids from F_1 (*N. rustica* x *N. tabacum*) (Bolsunov, 1963) and from F_1 (*N. rustica* × *N. exigua*) (Bolsunov, 1970).

More recently, oryzalin, an herbicidal substance but also a very powerful antimitotic, was used to induce chromosome doubling in the amphihaploid *N. sylvestris* × *N. tomentosiformis* (Lim et al., 2006).

Starting with the first experiments by Warmke and Blakeslee (1939) that involved the hybrid *N. tabacum* × *N. glutinosa,* colchicine became the antimitotic drug of choice to restore fertility to sterile *Nicotiana* hybrids. Colchicine is used in various formulations, e.g., as water solutions of different strengths, water solutions with agar, mixtures with lanolin, etc. It was applied both *in situ*, on field- or greenhouse-grown plants and *in vitro* in various types of aseptic cultures. Chromosome doubling is induced at various growth stages and in different plant parts, in cultured embryos, germinating seeds, seedlings and growing plants. The dividing meristems to which the drug is applied include apices and ancillary buds. Some examples are given in Table 3.7.

3.6.5 Regeneration of Hybrid Plants from In Vivo *and* In Vitro *Cultures*

Regeneration from Callus *In Situ* This oldest and probably long-forgotten method made use of polyploid cells already present in the plant by inducing the growth of calli *in vivo*. Polyploid cells originate during the tissue differentiation process, mostly through endomitosis, which involves the mitotic division of chromosomes within an intact nuclear membrane. Using this approach, Protassenya (1935) obtained an allopolyploid hybrid from 2x (*N. rustica* × *N. tabacum* and Greenleaf (1938) doubled the chromosome complements of 2x (*N. sylvestris* × *N. tomentosiformis*), 2x (*N. sylvestris* × *N. setchellii*), 2x (*N. sylvestris* × *N. tomentosa*) and 2x (*N. glutinosa* × *N. sylvestris*). The callus growth in the amphidiploids produced by Greenleaf was induced by the application of heteroauxin (IAA).

Regeneration from Calli *In Vitro* As discussed in a previous section, aseptic cultures were found to be helpful in overcoming incongruity of certain interspecific combinations that resulted in premature death of hybrid seedlings. Since the regeneration of viable plants from the explants of lethal hybrids passes through the callus phase and sometimes requires several passages of culture to take effect, it also offers an opportunity for the preexisting endomitotic cells to develop into allopolyploid along with amphihaploid shoots. The reader is referred back to Table 3.4 for rare instances of hybrids involving *N. tabacum,* the culture of which resulted in restoration of both viability and fertility. More frequently, chromosome number was

Table 3.7 Induction of alloploidy in some interspecific hybrids involving *N. tabacum* by using colchicine

Species or hybrid involved with the hybrid with *N. tabacum*	Phase of treatment/treated organ	Author
N. alata	seedlings, apical meristem immature embryo	Gajos (1975, 1981) Patrascu et al. (1999)
N. longiflora	apical meristem	Venkateswarlu et al. (1998)
N. plumbaginifolia	seedlings	Moav and Cameron (1960)
N. glauca	germinating seeds	Smith (1939), Valleau (1952), Trojak-Goluch and Berbeć (2007)
N. raimondii	seedlings, germinating seeds	Berbeć (1988)
N. rustica[1]	apical meristem seedlings plant cuttings axillary buds	Smith (1939) Furusato (1960) Moav and Cameron (1961) Takenaka (1963), Marubashi and Nakajima (1985)
N. nudicaulis	axillary buds plus inflorescence	Burk and Neas (1964)
N. amplexicaulis	axillary buds	Wark (1970)
	germinating seeds, apical meristem	Berbeć and Doroszewska (1981) Berbeć and Doroszewska (1992)
N. benthamiana	immature embryo	Subhashini et al. (1986)
N. benthamiana × *N. glutinosa*	seedlings, apical meristem	Ramavarma et al. (1977)
N. debneyi	apical meristem	Smith (1941), Sand (1968), Ternovsky et al. (1976)
N. exigua	seedlings	Wichert-Kobus (1967)[3], Kobus (1971)[3]
N. goodspeedii	axillary buds	Wark (1970)
N. gossei	axillary buds	Burk and Dean (1975)
N. maritima	not reported	Wark (1970)
N. occidentalis	axillary buds	Ternovsky et al. (1972)
N. rosulata	apical meristem	Ternovsky and Larkina (1978a)
N. suaveolens[2]	apical buds	Lloyd (1975)
N. velutina	axillary buds	Wark (1970)
N. otophora	seedlings apical meristems	Gerstel (1960) Larkina (2015, 2017)
N. setchellii	apical meristems germinating seeds	Larkina (2015, 2017) Berbeć (unpublished)
N. setchellii x N. otophora	germinating seeds	Berbeć (1982)[4]
N. tomentosiformis	seedlings	Gerstel (1960)
N. sylvestris	apical meristem	Smith (1939)
N. obtusifolia	seedlings	Chung et al. (1988, 1996)

(continued)

Table 3.7 (continued)

Species or hybrid involved with the hybrid with *N. tabacum*	Phase of treatment/treated organ	Author
N. glutinosa	apical meristem, axillary buds	Blakeslee and Avery (1937), Warmke and Blakeslee (1939)
N. glutinosa x N. obtusifolia	seedlings	Appa Rao and Krishna Murthy (1963)

[1] amphidiploid *N. rustica* x *N. tabacum* also induced by acenaphtene (Bolsunov, 1963);
[2] amphidiploid also induced by treatment with acenaphtene by Izard and Hitier (1955);
[3] induction of polyploidy by joint action of colchicine and gibberellin;
[4] induction of chromosome doubling in trispecific allohaploid (allotriploid) *N. tabacum* x *N. setchellii* x *N. otophora*), the resulting allotriploid (allohexaploid) was male fertile but female sterile

doubled, and fertility was restored by taking explants from sterile hybrid plants that had grown past the stage critical for survival (Table 3.8).

3.6.6 'Synthetic Species'

Synthetized amphidiploids in *Nicotiana* are known to differ from one another in the extent of variation they exhibit in successive selfed generations. Some lineages of the new amphidiploid 4x (*N. wuttkei* × *N. tabacum*) (Laskowska et al., 2015) were stable enough to deserve the name of a 'synthetic species'. Such stable, self-perpetuating 'synthetic species' were previously developed within the section Suaveolentes by Krishnamurthy and Gopinath (1969): 4x (*N. velutina* × *N. amplexicaulis*), 4x (*N. occidentalis* × *N. amplexicaulis*), including the nullisomic lineage 4n = 76 of the latter amphidiploid. Each of those artificial amphidiploids was given a regular botanical description and treated, as the authors put it, "equal in rank with the existing *Nicotiana* species". Some of such 'synthetic species' were even given specific names: *N.* × *obtusiata* for 4x (*N. obtusifolia* × *N. attenuata*) (Anssour et al., 2009; Krügel, 2010; McCarthy et al., 2015) and *N.* × *mierata* for 4x (*N. miersii x N. attenuata*) (Pearse et al., 2006; Krügel, 2010), *N. edwardsonii* for 4x (*N. glutinosa* × *N. clevelandii*) (Christie, 1969), *N. vavilovii* for 4x (*N. glauca* × *N. langsdorffii*) (Kostoff, 1938b; Kostoff, 1939a, 1939b), *N.* × *diruex* for 4x (N *rustica* × *N. exigua*) (Bolsunov, 1970), *N.* × *didebta* for 4x (*N. debneyi* × *N. tabacum*) (Clayton et al., 1967, He et al., 2019, misnamed by the latter authors '*N.* x *didepta*), *N.* × *digluta* for 4x (*N. glutinosa* × *N. tabacum*) (Clausen & Goodspeed, 1925; Clausen, 1928); N. x *disualovii* for *N. suaveolens* x *N. quadrivalvis* (*bigelovii*) (Modilevsky, 1939); *N.* x *flindersiensis* for 4x (*N. suaveolens* x *N. glauca*) (Smith & Abashian, 1963); *N.* × *ditagla* for 4x (*N. tabacum* × *N. glauca*) (Ternovsky, 1934; Modilevsky, 1936). Other lineages

Table 3.8 Instances of restoring fertility to amphihaploid hybrids involving *N. tabacum* by means of culturing explants from viable hybrid plants at advanced growth stages

Section	Species involved in the amphihaploid hybrid with *N. tabacum*	Explant	Cytological status of regenerants	Reported by
Alatae	*N. alata*	ST ST	amphidiploids subamphidiploids, mixoploids[1]	Skucińska et al. (1977), Dorossiev et al. (1978) Nikova et al. (1999)
	N. plumbaginifolia	ST	aneuploids, mixoploids	Nikova et al. (2004)
	N. longiflora	ST	Allopolyploids[2]	Nikova et al. (2001)
	N. sanderae	ST	near-amphidiploids	Skucińska et al. (1977)
		ST	amphidiploids	Dorossiev et al. (1990)
			self-fertile regenerants of different ploidy level	Nikova et al. (2003, 2006)
Paniculatae	*N. paniculata*	ST	mixoploids	Nikova et al., 1991, Nikova and Vladova (2002)
Noctiflorae	*N. noctiflora*	ST	amphidiploids	Stanoeva and Petkova (1978), Dorossiev et al. (1978, 1990)
	N. glauca	ST	mixoploids	Raicu et al. (1978)
Suaveolentes	*N. africana*	ST C	amphidiploids amphidiploids	Keum et al. (1994), Nikova et al. (1988), Nikova and Zagorska (1990) Doroszewska and Berbeć (1990, 2000)
	N. amplexicaulis	LM	amphidiploids	DeVerna et al. (1987)
	N. benthamiana	LM ST	amphidiploids subamphidiploids/ mixoploids	DeVerna et al. (1987) Nikova et al. (1991), Krusteva et al. (2003)
	N. goodspeedii	ST	amphidiploids	Zagorska and Palakarcheva (1978) Dorossiev et al. (1990)
	N. gossei	ST	amphidiploids (?)	Dorossiev and Palakarcheva (1990)
			mixoploids	Nikova, Palakarcheva, et al. (1998a)
		ST	amphidiploids	Palakarcheva et al. (1995)
	N. ingulba	ST	amphidiploids	Nikova, Vladova, et al. (1998b)
	N. maritima	ST	amphidiploids	Dorossiev et al. (1978, 1990)
	N. velutina	ST	mixoploids	Nikova et al. (1991)

Abbreviations in column 3: *ST* stem pith, *LM* leaf midrib, *C* cotyledons/cotyledon segments

[1] Aneuploids of different ploidy level (44-93 chromosomes), mixoploids

[2] Male sterile, partly female fertile;

of the latter amphidiploid were found to be highly unstable (Szilagyi, 1975). Unstable amphidiploids will be discussed in Sect. 4.5.4.

The relatively recently produced synthetic species *N. excelsiana* for 4x (*N. excelsior* × *N. benthamiana*) (Fitzmaurice, 2002) shows that there is still a large unexploited potential, both academic and commercial, in interspecific combinations in *Nicotiana*. Due to its biological properties, *N. excelsiana* gained the status of a "proprietary species" protected by patent rights as a convenient tool in a newly developed protein production technology. In a recent study, *N. x excelsiana* was demonstrated as a valuable and agronomically exploitable source of griffithsin, an anti-HIV drug (Eapen et al., 2020) and was also studied for other secondary metabolites (Mihaylova-Kroumova et al., 2020).

3.7 Parasexual Hybrids

3.7.1 Parasexual Hybrids by Fusion of Isolated Somatic Protoplasts

The idea of circumventing prefertilization barriers to crossability that had evolved at the gametic level prompted the attempts to fuse the somatic cells that lacked such obstructions. It was also envisaged that fusion of unreduced somatic cells would directly produce fertile amphidiploids, thereby bypassing the sterility of sexually produced hybrids. In its basics, the procedure has remained unchanged from its inception and is divided into three stages:

- isolation of protoplasts by enzymatic degradation of cell walls
- inducing the naked protoplasts to fuse by the presence of chemical agents or by electric fields
- selective culture of fused protoplasts based on their physical properties, the presence of fluorescent markers or genetic complementation, e.g., resistance to antibiotics

Since spontaneous fusion of naked protoplasts is a rare phenomenon, several agents, both chemical and physical, were tested for fusion-inducing action. Among the physical agents, the electric-field mediated method, also called electrofusion, is most frequently used. In this method, the protoplasts are brought into close contact by the application of an alternating electric field (AC) followed by exposure to direct current (DC) pulses (Davey, 2017).

Despite the high efficiency of electrofusion, polyethylene glycol (PEG), a chemical agent, has been most popular in creating interspecific somatic hybrids in *Nicotiana*, although the compound is toxic to plant cells. Another frequently used approach is the combination of high pH and high concentration of Ca^{2+} cations (Ilcheva & San, 1997; Davey, 2017).

After the fusion treatment, the medium contains both fused heterokaryons and unfused parental protoplasts. Different methods were applied to discriminate against the latter in the selection process. They included simple visual identification (Bates, 1985; Nagao, 1978; Hamill et al., 1984) and the use of various genetic markers, such as chlorophyll mutations (Evans et al., 1981, 1982, 1983; Aviv & Galun, 1986, 1987), resistance to antibiotics, both spontaneous (Medgyesy et al., 1980) and transgenic (Bates, 1990; Pental et al., 1988, 1989; Lu & Yang, 1996). Transgenic resistance to two different antibiotics provided double complementary selective systems under which only heterokaryons could survive in a growth medium containing both markers (Sproule et al., 1991; Donaldson et al., 1993, 1995; Ilcheva et al., 2000, 2001).

Although Zheng et al. (2018) wrote that the cytoplasmically male sterile line of *N. tabacum* 'cms-sua' used in their study originated from the somatic fusion between *N. suaveolens* and *N. tabacum* made in the 1950s (sic!) the first well-documented interspecific somatic hybrid in *Nicotiana* was reported by Carlson et al. (1972). The hybrid *N. langsdorffii* + *N. glauca* was a regular fertile amphidiploid ($4x = 42$) and thus seemed to confirm the hopes attached to interspecific hybridization at the somatic level. Soon thereafter, however, it became apparent that the method had serious constraints. Over the years, the number of interspecific somatic combinations, mostly those involving *N. tabacum*, that yielded genetically stable, fertile amphidiploids was disappointingly small and limited to hybrids of *N. tabacum* with *N. glauca, N. nesophila, N. debneyi*, *N. megalosiphon*, and *N. otophora* (Table 3.9). The other hybrids synthesized by protoplast fusion that involved *N. tabacum* showed high variability in external morphology and mostly aneuploid chromosome numbers due to chromosome elimination, either random or preferential, resulting in asymmetric hybrids, i.e., those with predominance of genetic material from one parental species (e.g., Donaldson et al., 1995; Ilcheva et al., 1997, 2000). Nonetheless, the irregularity and imbalance of interspecific protoplast fusion products may carry inherent advantages, which is explained in the paragraph to follow.

Asymmetric hybrids have also been synthetized consciously using chemical agents (iodoacetate) or irradiation (gamma or X-rays) to inactivate all or part of the nuclear genome of one parent prior to fusion (e.g., Bates, 1990). Highly asymmetric hybrids to which the cytoplasmic DNA was contributed almost exclusively by the irradiated parent whereas the other parent donated both cytoplasmic and nuclear DNA (the so-called cybrids obtained by the donor-recipient method) proved to be a useful tool to produce interspecific mitochondrial recombinants and provided a fast method to transfer whole plasmons or selected cytoplasmic traits from one species to another (see also Sect. 5.3.1). Cytoplasmic recombinants are practically impossible to obtain by sexual hybridization since in *Nicotiana*, as in most other genera, the cytoplasmic DNA is inherited unilaterally through maternal lineage, save for some rare exceptions (Medgyesy et al., 1985; Horlow et al., 1990; Svab & Maliga, 2007). In this context, one may also note that such unilaterally incomplete or asymmetric hybrids can also be obtained from sexual matings using irradiated pollen (see Sect. 3.4.2). An extreme case of that latter approach was the highly contested "egg transformation" (see Sect. 4.4.4).

Table 3.9 Interspecific parasexual hybrids involving *N. tabacum*

Species involved in the hybrid with *N. tabacum*	Methods of fusion induction and selection of heterokaryons	Hybrid type/ Cytological status	Author	Notes
N. alata	PEG; visual	sterile hyper-aneuploids	Nagao (1979)	66-71 chromosomes
N. bonariensis	? ; visual	not determined	DeVerna (1984)	regenerated shoots
N. plumbaginifolia	EF; visual identification	Not determined	Bates (1985), Bates and Hasenkampf (1985)	
	EF, transgenic kanamycin resistance	Donor-recipient cybrids	Bates et al. (1987)	48 and 49-chromosome asymmetric male sterile *N. tabacum*-like hybrids, self- and cross-sterile aneuploid hybrids of intermediate morphology (58-70 chromosomes)
	Terbutryn resistance, X-irradiation	Donor-recipient cybrids	Menczel et al. (1986)	
	?, ?	not determined	Medgyesy et al. (1985)	morphology intermediate between parental species
	EF, ?	Not determined	Hamill et al. (1987)	
	PEG; elimination of non-fused protoplasts by inability to divide and form colonies	various types	Desprez et al. (1992)	sterile gametosomatic allotriploids and allotetraploids showing mixoploidy, chromosome numbers ranging from 44 to 68 including asymmetric cybrid (48 chromosomes)
N. sanderae	PEG; selective markers (kanamycin resistance and nitrate reductase deficiency)	sterile true amphidiploids	Dragoeva et al. (1977)	
N. glauca	PEG; chloroplast-deficient mutations	amphidiploids (fertile)	Evans et al. (1980), Gleba et al. (1984)	
	?, ?		Sun et al. (2007)	
			Fuentes et al. (2014)	vegetative hybrid obtained by grafting

(continued)

Table 3.9 (continued)

Species involved in the hybrid with *N. tabacum*	Methods of fusion induction and selection of heterokaryons	Hybrid type/ Cytological status	Author	Notes
		amphidiploids (fertile)		
N. knightiana	PEG; shoot inducibility and albino mutation	sterile mixoploids and aneuploids	Maliga et al. (1978)	44 to 126 chromosomes
	PEG; visual	amphidiploids (fertile) and aneuploids	Menczel et al. (1981)	68 to 115 chromosomes
N. paniculata	?; *Agrobacterium* T-DNA markers, nitrate reductase deficiency	cellular lines	Mueller-Gensert and Schieder (1987)	
N. quadrivalvis	PEG; chlorophyll mutations	fertile and sterile cybrids	Aviv and Galun (1986, 1987)	
N. nesophila	PEG; chlorophyll mutations	fertile amphidiploids	Evans et al. (1981, 1982)	
N. repanda	PEG; visual	sterile aneuploids	Nagao (1982)	58 to 64 chromosomes
	EF; nopaline gene, transgenic kanamycin resistance	aneuploids and mixoploids	Bates (1990)	obtained by gamma irradiation of *N. repanda* protoplasts
N. stocktonii	PEG; chlorophyll mutations	fertile hybrids	Evans et al. (1981)	amphidiploids (?)
	EF; ?		Chen et al. (2013)	partly fertile hybrids

N. rustica	PEG; visual	aneuploids	Nagao (1978)	60 to 91 chromosomes
	?; visual	aneuploids	Hamill et al. (1984)	63 to 87 chromosomes, poor to good pollen stainability accompanied by prevalence of self-sterile regenerants
	?; ?; PG, double transgenic markers (resistance to kanamycin and methotrexate)	rare amphidiploids and aneuploids	Naton et al. (1992), Donaldson et al. (1993)	56 to 96 chromosomes, massive chromosome elimination, PG as the fusing agent and selection of heterokaryons by double selective markers used by Donaldson et al. (1993)
	PEG; visual	fertile hybrids	Choi et al. (1993)	
	high pH/Ca^{++} method; transgenic resistance to kanamycin	gametosomatic hybrids	Pental et al. (1988, 1989)	unknown cytological status, transmission of mitochondria but not chloroplasts from the microspore parent (*N. tabacum*) found in fusion products
	high pH/Ca^{++} method; transgenic resistance to kanamycin		Lu and Yang (1996)	sesquidiploid chromosome number reported
N. africana	?; ?	donor-recipient cybrids	Kumashiro et al. (1988)	*N. tabacum* like cms phenotypes
N. benthamiana	?; ?	fertile amphidiploids (?)	Hagimori et al. (1993)	reported as ‘true hybrids’
N. debneyi	PG; double transgenic markers (resistance to kanamycin and methotrexate)	fertile amphidiploids and aneuploids	Sproule et al. (1991), Dijak et al. (1991)	
	PEG+ high pH/Ca^{++}; ?	donor-recipient cybrids	Kumashiro and Kubo (1986)	*N. tabacum* like cms phenotypes

(continued)

Table 3.9 (continued)

Species involved in the hybrid with *N. tabacum*	Methods of fusion induction and selection of heterokaryons	Hybrid type/ Cytological status	Author	Notes
N. megalosiphon	?;?	?	Chupeau (1987)	Self-sterile hybrids
	?; ?	amphidiploids and aneuploids (?)	Brandle et al. (1992)	variable intermediate morphology
	PEG; double transgenic markers (resistance to kanamycin and methotrexate)	amphidiploids and aneuploids (?)	Donaldson et al. (1995)	variable intermediate morphology
	PEG, EF; double transgenic markers (resistance to kanamycin and bialaphos)	aneuploids	Ilcheva et al. (1997, 2000)	highly variable morphology, highly asymmetric hybrids resulting from non-preferential loss of chromosomes of one or the other species
	: ?; X-irradiation followed by double transgenic markers (resistance to kanamycin and methotrexate)	donor recipient cybrids	Kasza and Kandra (1990)	cms forms characteristc of cytoplasm recipient and cytoplasmic recombinants
N. rotundifolia	PEG; double transgenic markers (resistance to kanamycin and bialaphos)	aneuploids	Ilcheva et al. (2001)	non-preferential chromosome loss
N. suaveolens	PEG; centrifugation using Percoll flotation protocol	Donor-recipient cybrids	Fitter et al. (2005)	Mitochondrial recombinants obtained with stigmatoid and carpeloid stamens characteristic of *N. tabacum* cms *suaveolens* but showing split corolla
	?	?	Zheng et al. (2018)	cms suaveolens alloplasmics derived from *N. tabacum* + *N. suaveolens* somatic hybrid reportedly obtained in the 1950s

N. sylvestris	PEG; no selection	donor-recipient cybrids	Zelcer et al. (1978), Aviv et al. (1980)	
	PEG; streptomycin resistance	amphidiploids and aneuploids	Medgyesy et al. (1980)	highly variable morphology
	PEG; Su mutation	amphidiploids	Evans et al. (1983)	
N. otophora	PEG; Su mutation	amphidiploids	Flick and Evans (1982), Evans et al. (1983)	
N. arentsii		not determined	DeVerna (1984)	regenerated non-flowering shoots
N. glutinosa	PEG; visual; PG; double transgenic markers (resistance to kanamycin and methotrexate)	amphidiploids and aneuploids	Nagao (1979), Donaldson et al. (1994)	selection of heterokaryons by double seletion markers by Donaldson et al. (1994)
	EF; chlorophyll mutation, nitrate reductase deficiency	not determined	Kim and Choi (1991)	reported as somatic hybrids based on morphological and biochemical evidence
	PEG; no selection	not determined	Uchimiya (1982)	hybrid character determined based on intermediate morphology and cms trait
		gametosomatic sesquidiploids (pentaploids)TTG	Pirrie and Power (1986), Giddings and Rees (1992)	
	dextran method; amino acid resistance and plant regeneration ability	aneuploids	Horn et al. (1983)	from 34 to 60 chromosomes, massive chromosome loss observed
N. undulata	PEG; no selection	not determined, intermediate morphology	Uchimiya (1982)	various mitochondrial recombinants expressing different manifestations of male sterility
	PEG; no selection	donor-recipient cybrids	Aviv and Galun (1986)	mitochondrial recombinants, including restored male fertility to fusion products
	PEG; ?	donor-recipient cybrids (aneuploids)	Liu-Bao et al. (1995)	highly asymmetric hybrids (51 to 66 chromosomes)

Column 2 contains abbreviated method of protoplast fusion followed by brief description of how heterokaryons were selected; question mark is put instead if the method applied is not known

Abbreviations: *PEG* polyethylene glycol, *EF* electrofusion

3.7.2 Gametosomatic Hybrids

A fusion of diploid mesophyll species of *N. tabacum* with haploid gametophyte protoplasts of an alien species was devised with an expectation to obtain sesquidiploid plants as a starting material for interspecific gene transfer equivalent to sesquidiploids from sexual matings (Davey et al., 1996, see also Sects. 3.6.3, 4.5.6). The method was experimentally tested by Pirrie and Power (1986) and by Giddings and Rees (1992). Their gametosomatic hybrids 2n *N. tabacum* + 1n *N. glutinosa* actually mimicked sesquidiploids (pentaploids) from sexual matings. In those experiments, haploid protoplasts were isolated at the tetrad stage. Alternatively, protoplasts isolated from mature pollen grains can be used for gametosomatic fusion (Desprez et al., 1992, Lu & Yang, 1996, Ping et al., 1996). Another benefit of gametosomatic fusion is that haploid gametophyte protoplasts fail to divide and do not form colonies in culture, which simplifies the selection of heterokaryons (Davey et al., 1996).

However, not all products of gametosomatic fusion represent true pentaploids. Gametosomatic regenerants (1n *N. tabacum* + 2n *N. plumbaginifolia*) made by Desprez et al. (1992) represented an array of sterile aneuploid forms that also showed mixoploidy plus a 48-chromosome asymmetric cybrid. According to those investigators, androgenetic regenerants obtained by asymmetric gametosomatic fusion can be used to transfer mitochondrial genomes separately from chloroplast genomes.

Despite their potential value, few interspecific gametosomatic hybrids have been reported. They include, apart from the previously mentioned 2n *N. tabacum* + 1n *N. glutinosa*, 1n *N. tabacum* + 2n *N. plumbaginifolia* (Desprez et al., 1992) and 1n *N. tabacum* + 2n *N rustica* (Pental et al., 1988, 1989; Mukhopadhyay et al., 1991; Lu & Yang, 1996; Ping et al., 1996).

A concise and informative review of parasexual hybridization by protoplast fusion in *Nicotiana* was prepared by Ilcheva and San (1997).

3.7.3 Graft Hybrids and Horizontal Gene Transfer

Grafting occurs when segments of two different plants come into close contact and fuse together into one separate and independent plant organism. In such a union of two plants or plant pieces, one of them provides the root of the new plant and is called rootstock or simply stock, while the other serves as the shoot with leaves and is referred to as 'scion'. Grafting as a fusion of two or more separate plant organisms occurs spontaneously in nature but has also been practiced by agriculturists.

In fruit farming practice, grafts have been used for two different purposes: for better growth and desirable performance and for genetic effects (Ohta, 1991).

The former function of grafting has been widely practiced by horticulturists from early antiquity, and its major objective is to improve the growth and performance of fruit plants or ornamentals by combining the superior qualities of the stock with those of the scion, e.g., winter hardiness with palatable fruit or other edible parts. The latter, much less popular and more controversial, was about to impose heritable changes in the scion by transferring genetic information from the stock. In the 1940s and 1950s, the concept gave rise to the so-called Michurinist genetics developed by the Soviet horticulturist I. V. Michurin, and the idea was officially proclaimed in the Soviet Union as an essential part of the so-called “revolutionary agrobiology”, the movement advanced by T. Lysenko and his followers throughout the Eastern bloc in deliberate and blatant opposition to the Mendelian principles of heredity (Goldschmidt, 2014; Zhou & Liu, 2015). The ‘graft hybrids’ theory was deeply mistrusted by scientists in the West and repeatedly proven to lack a scientific basis (Goldschmidt, 2014). Ultimately, it was abandoned both in the Soviet union and in other Eastern bloc countries. However, the recent decades have witnessed a revived interest in epigenetic and hereditary effects of grafting (Ohta, 1991; Goldschmidt, 2014).

Nicotiana species are easily amenable to grafting both among themselves or with other related solanaceous plants, but the technique has, until very recently, attracted little attention from Mendelian tobacco geneticists and breeders. It is only at the beginning of this century that R. Bock and his team from the Max Planck Institute came up with evidence that the entire chloroplast genomes could be transferred through the graft junction from *N. tabacum* to two other *Nicotiana* species: *N. glauca* and *N. benthamiana* (Stegemann et al., 2012). In their experiment, the authors demonstrated that the transfer was restricted entirely to chloroplast DNA and did not involve any nuclear DNA fragments.

However, in another experiment reported 2 years later (Fuentes et al., 2014), the same team demonstrated the interspecific fusion of the whole genomes of two grafting partners, *N. glauca* and *N. tabacum*. After the fusion of stock and scion had taken place, fragments of tissue of the fusion zone were excised and cultured *in vitro*. Callus culture and plant regeneration were performed by following the genetic complementation protocol based on double selectable markers, a technique routinely applied in somatic hybridization by protoplast fusion. As a result, a fully fertile, regular 72-chromosome amphidiploid 4x (*N. glauca* × *N. tabacum*) was obtained and given the name ‘*Nicotiana tabauca*’, a new species that arose by natural fusion of somatic cells.

A few observations can be made in connection with this unusual finding. The authors’ argument that their discovery supports the likelihood of spontaneous asexual hybridization taking place in nature seems to be essentially valid, although in their experiments they had to resort to artificial tools, unknown to nature, to make it happen. This notwithstanding, when put in their long-term evolutionary perspective, even extremely rare and least likely events can and most likely do occur. The authors also presented their discovery as a new tool for crop improvement mostly because, as they argued, grafting is technically less demanding than protoplast fusion. Indeed, it is, but the sexual method is even less demanding. Precisely the allopolyploids *N. tabacum* × *N. glauca* and their reciprocals have been repeatedly obtained by conventional crossing for nearly a century, and even the name for that ‘artificial species’ was invented (‘*Nicotiana* ditagla’) preceding ‘*N.* tabauca’ coined by the authors of the report by several decades (see Sect. 3.6.6). This notwithstanding, the significance of that discovery cannot be overestimated and fully deserves to be followed by other experiments involving other *Nicotiana* species, including those that show a high degree of mutual incompatibility. Regrettably, no new reports in that area, theoretical or practical, seem to have emerged thus far.

The demonstration of the feasibility of obtaining an interspecific hybrid by grafting is obviously reminiscent of the graft hybrids in the former Soviet Union mentioned in the introductory remarks to this section. The recent report by scientists from the Max Planck Institute may shed somewhat different light on the work of Mitschurin in Russia but also similar horticultural experiments by Burbank in the United States. The historical context to the achievement of Ignacia Fuentes and her colleagues was recalled by Zhou and Liu (2015).

One should also add that an analogous interspecific horizontal transfer through grafting was recently reported for mitochondrial genes from *N. sylvestris* responsible for restoring male fertility to the alloplasmic line of *N. tabacum* with the mitochondrial genome of *N. undulata* (Gurdon et al., 2016, see Sect. 5.3.2 on restoring male fertility to cms lines).

3.8 Ending Notes on Sexual and Asexual Interspecific Hybrids Involving *N. tabacum*

Somatic and gametosomatic hybrids that involve *N. tabacum* are listed in Table 3.9. A compilation of all interspecific hybrids involving cultivated tobacco, regardless of the method by which they were obtained, is presented in Table 3.10. Information was found on a total of 59 hybrids involving *N. tabacum*, and reciprocals were not included in the count.

In spite of various barriers to crossability discussed in the previous sections, the vast majority of those hybrids could be obtained by conventional crossing. Actually, only very few of those combinations may be considered to have been made possible owing to the use of advanced technologies. *N. nesophila* x *N. tabacum* (Reed & Collins, 1978), *N. occidentalis* x *N. tabacum* (Butenko et al., 1970), *N. rosulata* x *N. tabacum* (Ternovsky et al., 1976), *N. stocktonii* x *N. tabacum* (Reed & Collins, 1978) are known only from reports where hybridization was aided by tissue culture. *N. rotundifolia* + *N. tabacum* was reported as a somatic hybrid only (Ilcheva et al., 2001). Genetic engineering was deployed to obtain viable hybrids of *N. simulans* x *N. tabacum* and *N. umbratica* x *N. tabacum* (Ma et al., 2020). However, the latter two hybrids were also reported or hinted at by other authors (Kubo, 1985; Murthy et al., 2014), and *N. stocktonii* x *N. tabacum* was reported by Wong (1975). The reports on the latter three hybrids lacked details on how they were produced. Of two reports on *N. tabacum* x *N. bonariensis*, no details are known on the hybrid reported by Busconi et al. (2010).

Table 3.10 All interspecific hybrids involving the cultivated species *Nicotiana tabacum* (after Berbeć and Doroszewska 2020) with minor additions and modifications)

Section	Species involved in the hybrid with *N. tabacum*	F_1 hybrid (amphihaploid) reported by:[1]	Amphipolyploid (sexual or somatic) reported by:[1]
Alatae	*N. alata*	premendelian (Naudin (after Kostoff (1943)) East and Hayes (1912), East, 1928)	Ahuja (1962)[14], Gajos (1975, 1981), Dorossiev et al. (1978); Stoyanova (1978, 1980)[15]; Nikova et al. (1999)[16]; Nagao (1979)[17]
	N. bonariensis	Stavely (1979), Busconi et al. (2010)	Busconi et al. (2010) (?)[11] [18] triple hybrid 4x (*N. undulata-tabacum*) × *N. bonariensis* (Ahuja (1962))
	N. forgetiana	Takenaka (1963), Burk (1972)[2]	
	N. langsdorffii	East and Hayes (1912), East (1928), Hu (1956), Takenaka (1962b)[2], Burk (1972)[3]	Ahuja (1962)[14]
	N. longiflora	Malloch and Malloch (1924), Gentscheff (1931), Ternovsky (1936b)	Clayton (1947), Ahuja (1962), Morgan (1964), Smith et al. (1970), Sievert (1972a)
	N. plumbaginifolia	Gentscheff (1931), Pal and Nath (1936), Kincaid (1949), Chobanova (1977), Nikova et al. (2004)	Ar-Rushdi (1957), Moav and Cameron (1960), Apple (1962); Nikova et al. (2004)[20]
	N. sanderae	Christoff (1928), Kostoff (1930), Whitaker (1934), East (1935), Ternovsky (1962), Malecka (1977)	Ternovsky (1936), Skucińska et al. (1977), Malecka (1977), Dragoeva et al. (1977) (somatic),
Noctiflorae	*N. glauca*	premendelian (East (1928)): reported by Brongniart and Gris (1861) and Naudin in 1865 (according to Ternovsky (1936b)	Sarana (1934), Ternovsky (1934), Modilevsky (1936), etc.[19] many others, first ever interspecific graft amphidiploid reported by Fuentes et al. (2014)
	N. noctiflora	Palakarcheva (1975, 1992); Stanoeva and Petkova (1978), Dorossiev et al. (1978)	Dorossiev et al. (1978), Stanoeva and Petkova (1978)
	N. petunioides	Gisquet et al. (1940)	

(continued)

Table 3.10 (continued)

Section	Species involved in the hybrid with *N. tabacum*	F_1 hybrid (amphihaploid) reported by:[1]	Amphipolyploid (sexual or somatic) reported by:[1]
Paniculatae	*N. benavidesii*	Goodspeed (1945), Grebenkin (1968), Durbin and Uchytil (1977), Berbeć (1978a, 1980, 1987a)	Berbeć (1978a)[20], (1986)[20]
	N. cordifolia	Durbin and Uchytil (1977), Burk and Durbin (1978)	Burk and Durbin (1978)
	N. knightiana	Goodspeed (1945), Tanaka (1961), Takenaka (1962a), Slusarkiewicz-Jarzina and Zenkteler (1983), Berbeć (1987a)	Morgan (1964), Sievert (1972b), Berbeć et al. (1982)[21], Berbeć and Doroszewska (1992)
	N. paniculata	premendelian East (1928), Kostoff (1932), Holmes (1937a, b)	Nikova et al. (1991), Nikova and Vladova (2002)[22]
	N. raimondii	Kostoff (1943), Grebenkin (1968), Berbeć (1987a)	Burk et al. (1982), Berbeć (1988)
	N. solanifolia	Goodspeed (1945), Takenaka (1956b), Grebenkin (1968)	
Acuminatae	*N. acuminata*	Kostoff (1943), Iwai et al. (1986)	
	N. pauciflora	Gentscheff (1931), Kostoff (1943)[4], Goodspeed (1945)	
Polydicliae	*N. clevelandii*	Kehr and Smith (1952), Kaul (1988)	
	N. quadrivalvis	premendelian (East, 1928)	Fardy and Hitier (1945, 1947), Burk (1960), Calitz and Milne (1962), Morgan (1964), Smith et al. (1970), Gerstel and Burns (1983)
Repandae	*N. nesophila*	Reed and Collins (1978, 1980), Huesing et al. (1989)	Reed and Collins (1978),
	N. nudicaulis	Gentscheff (1931), Kostoff (1943), Burk and Neas (1964)	Burk and Neas (1964), Sievert (1972a)
	N. repanda	Foster (1943)[5], Kincaid (1949), Pittarelli and Stavely (1975), Nagao (1982[26]), Shintaku et al. (1985), Iwai et al. (1985), Choi et al. (1998)	Pittarelli and Stavely (1975), Zhou et al. (1991), Pontes et al. (2005)[23]
	N. stocktonii	Wong (1975), Reed and Collins (1978)	Reed and Collins (1978)

Rusticae	*N. rustica*	*N. rustica* × *N. tabacum*: premendelian (East (1928)), first reported by Koelreuter (Mayr, 1986) *N. tabacum* × *N. rustica*: (Eghis (1927))	Eghis (1927), Rybin (1927), Protassenya (1935), Ternovsky and Khudina (1938), Kostoff (1937, 1943), Furusato (1960), Moav and Cameron (1961), Takenaka (1963)
Suaveolentes	*N. africana*	Gerstel et al. (1979), Burk et al. (1979), Kandra (1984)[6] Keum et al. (1991, 1994), Doroszewska and Berbeć (1996)[7] Nikova and Zagorska (1990)[8], Ma et al. (2020)[9]	Doroszewska and Berbeć (1990, 1996)[7] Nikova and Zagorska (1990)[8] Keum et al. (1991, 1994)
	N. amplexicaulis	Wark (1970), Berbeć and Doroszewska (1981), DeVerna (1984), DeVerna et al. (1987)	Wark (1970), Berbeć and Doroszewska (1981), DeVerna et al. (1987)
	N. benthamiana	Subhashini et al. (1986), DeVerna (1984), DeVerna et al. (1987), Dorossiev et al. (1990), Nikova et al. (1991), Zaitlin and Mundell (2006), Iizuka et al. (2012), Mihaylova-Kroumova and Wagner (2016)	Subhashini et al. (1986), DeVerna et al. (1987), Dorossiev et al. (1990), Nikova et al. (1991), Krusteva et al. (2003)
	N. cavicola	Nikova et al. (2006)	Nikova et al. (2006) (?)
	N. debneyi	Kostoff (1943), Valleau (1952), Clayton (1958) (reciprocal), Grebenkin (1970), Larkina (2015) (reciprocal) Palakarcheva (1978) (reciprocal), Ma et al. (2020)[9]	Berbeć (1964), Bailov et al. (1964), Clayton (1968)[3], Grebenkin (1970) (4x (*N. tabacum* × *N. debneyi*))
	N. excelsior	Wark (1970), Gillham et al. (1977), Nikova (1986), Ma et al. (2020)[9]	Wark (1970)
	N. exigua	Wichert-Kobus (1967), Kobus (1971), Wark (1970), Manolov et al. (1978)	Wichert-Kobus (1967), Kobus (1971); Wark (1970), Manolov et al. (1978)
	N. fragrans	Durbin and Uchytil (1977), Tezuka et al. (2010)	
	N. goodspeedii	Butenko et al. (1970), Wark (1970), Palakarcheva (1974), Palakarcheva et al. (1978)	Wark (1970), Palakarcheva (1974), Zagorska and Palakarcheva (1978), Palakarcheva et al. (1978)
	N. gossei	Valleau (1952)[10] Takenaka (1962a), Parr and Thurston (1968), Dean (after Burk and Dean (1975)), Wark (1970), Ma et al. (2020)[9]	Wark (1970), Burk and Dean (1975), Tsikov and Tsikova (1986), Palakarcheva and Dorossiev (1992)
	N. hesperis	Kubo (1985)[11]	Kubo (1985)[11]

(continued)

Table 3.10 (continued)

Section	Species involved in the hybrid with *N. tabacum*	F_1 hybrid (amphihaploid) reported by:[1]	Amphipolyploid (sexual or somatic) reported by:[1]
	N. ingulba	Butenko et al. (1970), Nikova, Vladova, et al. (1998b), Tezuka et al. (2012)[12]	Nikova, Vladova, et al. (1998b)
	N. maritima	Wark (1970), Palakarcheva (1975), Dorossiev et al. (1978)	Wark (1970), Stanoeva and Petkova (1978), Dorossiev et al. (1978), Nikova et al. (1991)
	N. megalosiphon	Clayton (1950), Takenaka (1962a), Hranov (1970), Manolov et al. (1978), Ma et al. (2020)[9]	Palakarcheva and Bailov (1976), Manolov et al. (1978) (subamphidiploids)
	N. occidentalis	Butenko et al. (1970), Ternovsky et al. (1972), Wong (1975)	Ternovsky et al. (1972, 1973)
	N. rosulata	Ternovsky et al. (1976)	Ternovsky and Larkina (1978a)
	N. rotundifolia		Ilcheva et al. (2001)
	N. simulans	Kubo (1985)[11], Ma et al. (2020)[9]	Kubo (1985)[11]
	N. suaveolens	Premendelian (East (1928)), Izard and Hitier (1955), Lloyd (1975)	Izard and Hitier (1955), Morgan (1964)[24] Wark (1970), Lloyd (1975), Shinkareva (1979), Stavely (1979)
	N. umbratica	Murthy et al. (2014)	Murthy et al. (2014)[25]
	N. velutina	Wark (1970), Powell (1979), Ma et al. (2020)[9]	Wark (1970), Nikova et al. (1991) (fertile alloploids of undefined status)
	N. wuttkei	Laskowska and Berbeć (2012)	Laskowska et al. (2015)
	N. eastii	Chaplin and Mann (1961) (direct sesquidiploid EaTT)	
Sylvestres	*N. sylvestris*	East and Hayes (1912), Bellair (1913), Malinowski (1916), Goodspeed and Clausen (1917), Sachs-Skalińska (1917)	Rybin (1929), Eghis (1930), Ternovsky (1936a)

Tomentosae	*N. kawakamii*	triple hybrid only (*sylvestris* × *kawakamii*) × *tabacum* (Ohashi (1985))	Ohashi (1985)
	N. otophora	Goodspeed (1945), Ar-Rushdi (1955), Takenaka (1962a), Grebenkin (1968)	Goodspeed and Bradley (1942) Yang (1960), Gajos (1979)
	N. setchellii	Greenleaf (1941), Goodspeed (1945), Grebenkin, 1968	Larkina (1983), Berbeć (female sterile, unpublished)
	N. tomentosa	Goodspeed and Clausen (1928), McCray (1932)	Goodspeed and Bradley (1942)
	tomentosiformis	Brieger (1928), Breisser (1934), Lehmann (1936)	Fardy and Hitier (1945), Yang (1960), Gerstel (1960)
Trigonophyllae	*N. obtusifolia*	Takenaka (1956a), Tanaka (1961)	Chung et al. (1996)
	N. palmeri	Goodspeed (1945), Krishnamurthy et al. (1960), Berbeć et al. (1982)[13]	
Undulatae	*N. arentsii*		DeVerna (1984) (somatic)
	N. glutinosa	premendelian (East (1928)) first reported by Koelreuter (Mayr (1986))	Clausen and Goodspeed (1925), Clausen and Lammerts (1929), Ternovsky (after Ternovsky and Khudina (1938))
	N. undulata	Kehr and Smith (1952), Takenaka (1953), Takenaka (after Goodspeed and Thompson, 1959), Cameron (after Chaplin, 1964)	Kehr and Smith (1952), Ahuja (1962), Morgan (1964)

[1] only a maximum of six reports was referred to selected primarily by their order of appearance or by importance, in several cases many more investigators obtained or studied particular hybrid combinations;

[2] from crossing female autotetraploid *N. tabacum,* [2a]by mating *N. langsdorffii* to monosomic strains of *N. tabacum;*

[3] Clayton (1968) reported the amphidiploid 4x (*N. debneyi* × *N. tabacum*) to have existed already in 1938;

[4] crossability highly dependent on *N. tabacum* variety;

[5] F_1 hybrid 4n *N. repanda* x 2n *N. tabacum* died before flowering;

[6] F_1 hybrids between cms lines of *N. tabacum* and *N. africana* reported with restored male fertility;

[7] true amphihaploids (Doroszewska & Berbeć, 1996) or aneuploid near-amphihaploids (Gerstel et al. 1979) were obtained from mating female *N. tabacum* to male *N. Africana*;

[8] mixoploids, near-amphihaploids ($2n = 44$) and a near-amphidiploid obtained from mating female *N. africana* to male *N. tabacum;*

(continued)

[9] hybrid lethality was overcome and viable hybrid obtained by inactivating the gene present in the NtHL$_1$ locus of *N. tabacum* and responsible for the apoptotic death of hybrids with the species of the section Suaveolentes. The inactivation was achieved through editing of the NtHL$_1$ locus (Ma et al. 2020, see also sections 6.4.3 and 7.3.4);
[10] by using autotetraploid *N. tabacum* as the female parent (see Table 3.6);
[11] only circumstantial evidence exists for the hybrids *N. hesperis* × *N. tabacum* and *N. simulans* × *N. tabacum* having been synthesized based on the report on new cytoplasmically sterile lines cms *hesperis* and cms *simulans* (Kubo 1985);
[12] F$_1$ hybrids *N. tabacum* x *N. ingulba* obtained via ovule pollination in vitro by mating Haplo-Q monosomics of *N. tabacum* as females to *N. ingulba*;
[13] autotetraploid *N. palmeri* × *N. tabacum* (sesquidiploid PPT);
[14] failed to grow beyond seedling stage;
[15] apparently spontaneous amphidiploids derived from F$_1$ hybrids;
[16] male sterile subamphidiploids with partly restored female fertility;
[17] somatic hybrid;
[18] the account of converting several F1 hybrids, including *N. tabacum* × *N. bonariensis*, to fertile amphidiploids is ambiguous;
[19] subamphidiploids with partly restored male and female fertility;
[20] 68-chromosome allopolyploid obtained by backcrossing the sesquidiploid 3x (*N. tabacum* × *N. benavidesii*) (TTB) to *N. tabacum* plausibly by fusing an aneuploid unreduced gamete of TTB with a haploid gamete of *N. tabacum*;
[21] the amphidiploid was arrived at by three approaches: spontaneous seed set by amphihaploid *N. knightiana* x *N. tabacum*, direct crossing of tetraploid *N. tabacum* x tetraploid *N. knightiana*, induction of seeds by pollinating amphihaploid *N. knightiana* x *N. tabacum* with pollen of *N. langsdorffii* x *N. alata*;
[22] partly fertile unstable aneuploids;
[23] reported recovery of a fertile hybrid plant;
[24] the order of species in the listed amphidiploid suggests *N. suaveolens* as the maternal parent;
[25] there is a hint to fertile hybrids having been obtained but not explicit enough;
[26] somatic sterile aneuploids

Thirty-six *Nicotiana* amphihaploids that involved *N. tabacum* amphidiploids or near amphidiploids were produced by using various approaches. Most of those diploidized F_1 hybrids showed at least partially restored self-fertility: one was a female sterile amphidiploid (*N. tabacum* × *N. setchellii*), and the other was a nearly female sterile amphidiploid (*N. obtusifolia* × *N. tabacum*). Female sterility was also observed in the trigenomic allohexaploid 6x (*N. tabacum* × (*N. setchellii* × *N. otophora*) (Berbeć et al., 1982).

The author of this book found information on 26 *Nicotiana* species that were hybridized with *N. tabacum* by protoplast fusion, fewer than half the number of reported sexual hybrids (compare Tables 3.9 and 3.10). Among those 26 somatic hybrids, only a handful represented those in which whole genomes of both parents became united (*N. glauca* + *N. tabacum*, including a graft hybrid, *N. nesophila* + *N. tabacum*, *N. rustica* + *N. tabacum*, *N. debneyi* + *N. tabacum*, *N. otophora* + *N. tabacum*, *N. glutinosa* + *N. tabacum*). To the authors' knowledge, only two somatic hybrids with *N. tabacum* obtained by somatic fusion, *N. rotundifolia* + *N. tabacum* and possibly also *N. arentsii* + *N. tabacum,* have not been obtained by conventional sexual methods.

It appears that while the parasexual approach circumvents the prefertilization barriers that separate species from one another, its role in alleviating the incongruities existing between the fused genomes is of far less importance. The experience with somatic hybrids has confirmed an early observation by Zenkteler and Melchers (1978) that protoplast fusion contributes but little to expanding the crossability of different species within a genus. Chromosome loss, genome instability and other manifestations of intergenomic incongruities are among the common consequences of alloploidization and have been documented for both natural and synthetic allopolyploids that have arisen through the sexual process, a subject discussed in one of the previous sections. From numerous accounts, it appears that parasexual hybridization seems to exacerbate rather than mitigate those inherent incongruities since they are obviously far more numerous and more intense in somatic hybrids than those encountered in analogous hybrids synthesized via the sexual process. This said, asymmetry, a frequent phenomenon in most asexually produced hybrids, may actually facilitate gene flow between the fused genomes by bypassing the so-called bottle-necks of sexual introgression and by eliminating many deleterious linkage or epistatic effects that plague the sexual routes of gene transfer.

Last but not least, from the perspective of practical breeding issues to be discussed in the subsequent chapters, parasexual methods share the same disadvantage with genetic transformations at the molecular level. To wit, lawmakers of at least some countries have listed protoplast fusion been among the technologies used to generate genetically modified organisms (GMOs) thus effectively banning it in the development of commercially exploitable cultivars.

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