

## Cytological investigations of the interspecific hybrids of *Nicotiana tabacum* L. × *N. glauca* Grah.

Anna TROJAK-GOLUCH, Apoloniusz BERBEĆ

Institute of Soil Science and Plant Cultivation, Special Crops Department, Puławy, Poland

**Abstract.** Interspecific amphihaploid and amphidiploid hybrids between *Nicotiana glauca* Grah. ( $2n = 24$ ) and *N. tabacum* L. ( $2n = 48$ ) cultivars BY 103 and K 326 were analysed.  $F_1$  amphihaploids ( $2n = 36$ ) were viable and completely self- and cross-sterile, and mostly univalents were present during meiosis (with pairing range from 0 to 5). In some meiocytes, meiotic irregularities were observed, such as sporadic chromatin bridges and formation of restitution nuclei. The resultant  $F_1$  hybrids were easily converted to amphidiploids ( $2n = 72$ ) via colchicine treatment of seedlings. The number of univalents and the frequency of PMCs containing unpaired chromosomes indicated that amphidiploids *N. tabacum* cv. BY 103 or K 326 × *N. glauca* represented quite a high pairing category. However, they were male sterile because pollen mother cells were arrested at the tetrad stage. The termination of development of PMCs, and consequently male sterility, are very rare in this kind of tobacco hybrids.

**Key words:** amphihaploid, amphidiploid, interspecific hybridization, male sterility, *Nicotiana glauca*, *Nicotiana tabacum*.

### Introduction

The possibility of interspecific hybridization within the genus *Nicotiana* is a very important evolutionary issue. It can also provide new traits that are necessary in tobacco breeding. As described by Graham, the wild species of tobacco *N. glauca* belongs to genus *Nicotiana*, section *Paniculatae*. The importance of this species for tobacco breeders relies mostly on the fact that *N. glauca* shows full resistance to black root rot of tobacco caused by the soil-borne pathogenic fungus *Thielaviopsis basicola* Ferr. syn. *Chalara elegans* Nag. Raj et Kendrick

---

Received: October 7, 2002. Accepted: December 30, 2002.

Correspondence: A. TROJAK-GOLUCH, Institute of Soil Science and Plant Cultivation, Special Crops Department, ul. Czartoryskich 8, 24-100 Puławy, Poland, e-mail: anngol@iung.pulawy.pl

(ANONYMOUS 1990). Its also resists or tolerates potato virus Y (PVY), anthracnose, and powdery mildew.

However, the distant phylogenetic relationship between *N. tabacum* ( $2n = 48$ ) and *N. glauca* ( $2n = 24$ ), reflected in different numbers of mitotic chromosomes and a low degree of chromosome homology, often results in complete sterility of  $F_1$  hybrids obtained by crossing the two species (BERBEĆ, OPOKA 1971, SHILAGYI 1975, EVANS et al. 1980). The sterile amphihaploid  $F_1$  hybrids *N. tabacum*  $\times$  *N. glauca* were converted into amphidiploids by chromosome duplication. In spite of that, the plants obtained hereby were self-sterile as a result of incapability of producing pollen grains.

The main objective of this work was to achieve amphihaploid  $F_1$  hybrids and amphidiploid hybrids of *N. tabacum*  $\times$  *N. glauca*. We analysed the crossability of two selected tobacco cultivars with *N. glauca*, chromosome pairing during meiosis, as a probability of genetic exchange between two species, and disorders in pollen viability.

### Material and methods

A wild species of tobacco, *N. glauca*, originating from north-western Argentina and Bolivia, came from the seed collection of the Special Crops Department, Institute of Soil Science and Plant Cultivation, Poland. *N. glauca* ( $2n = 24$ ) was used as a male form in wide hybridization with two flue-cured cultivars of *N. tabacum* ( $2n = 48$ ): the Japanese cv. BY 103 and the American cv. K 326.

Hybridization between parental genotypes was performed under greenhouse conditions. The obtained  $F_1$  hybrids were grown in the greenhouse until they reached the generative phase. Independently, germinating seeds (when the seed coat opened and the radicle could be seen) of  $F_1$  generation were treated with 0.25% solution of colchicine for 4 hours at room temperature. Thoroughly rinsed with distilled water, germinating seeds were placed in a peat-based growing medium and seedlings were transplanted to the greenhouse.

Observations made on the resultant amphidiploids included measurements of the growth rate of flower buds in comparison to the parental cultivars BY 103 and K 326. Hybrids showing any morphological modifications in the development of their flower buds were additionally evaluated for delay in the course of microsporogenesis, in comparison to maternal cultivars of tobacco.

When the plants reached the generative phase, both hybrid generations were verified cytologically. This study included also both counting of mitotic chromosomes and analysis of meiosis in PMCs. Pollen viability was measured as a percentage of mature cells that could be stained with acetocarmine. To establish the number of mitotic chromosomes, corollas collected from young flower buds were soaked for 5 hours in 0.44% solution of oxychinoline with maltose added to obtain contraction of chromosomes and chromosome spreading. Additionally,

plant material was fixed in the Carnoy solution according to a procedure developed by BURNS (1964) and acetocarmine-stained preparations were prepared.

Material for the meiosis tests consisted of flower buds with anthers, and was kept for 12 hours at 4°C to prevent chromosome stickiness and to aid their spreading. The meiosis tests included observations of chromosome pairing at diakinesis and metaphase I in PMCs. On the basis of chromosome pairing data, the mean number of bivalents, modal numbers of bivalents and univalents per cell, variances and variation coefficients were calculated. The *N. tabacum* × *N. glauca* hybrids were also examined for meiotic irregularities in the following phases of meiosis.

## Results

The obtained amphihaploid hybrids presented a very satisfactory vitality and contained 36 chromosomes in their somatic cells (Figure 1). In both hybrid combinations the high number of univalents observed in metaphase I indicated that meiosis in PMCs was largely asynaptic. In more than half the tested meiocytes, chromosome pairing was not observed and the univalents were spread loosely in the cells (Figure 2a).

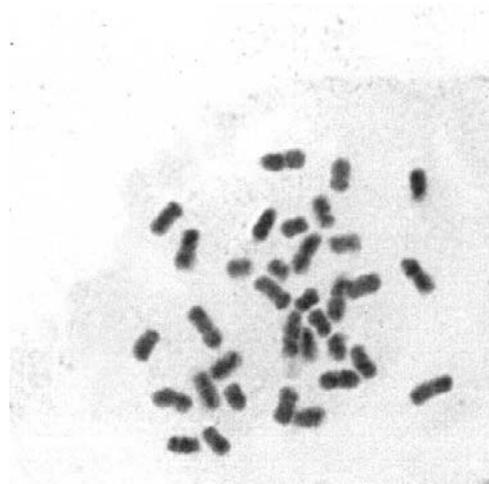


Figure 1. Mitotic chromosomes of amphihaploids *N. tabacum* cv. K326 × *N. glauca*

In some PMCs of the tested F<sub>1</sub> hybrids, mild chromosome pairing could be observed. Irrespective of the parental variety used, 1 to 3 bivalents were present in metaphase I (Figure 2b-c) and sporadically 5 bivalents could also be detected (Figure 2d). The bivalents observed were rod-shaped, with one terminal chiasma.

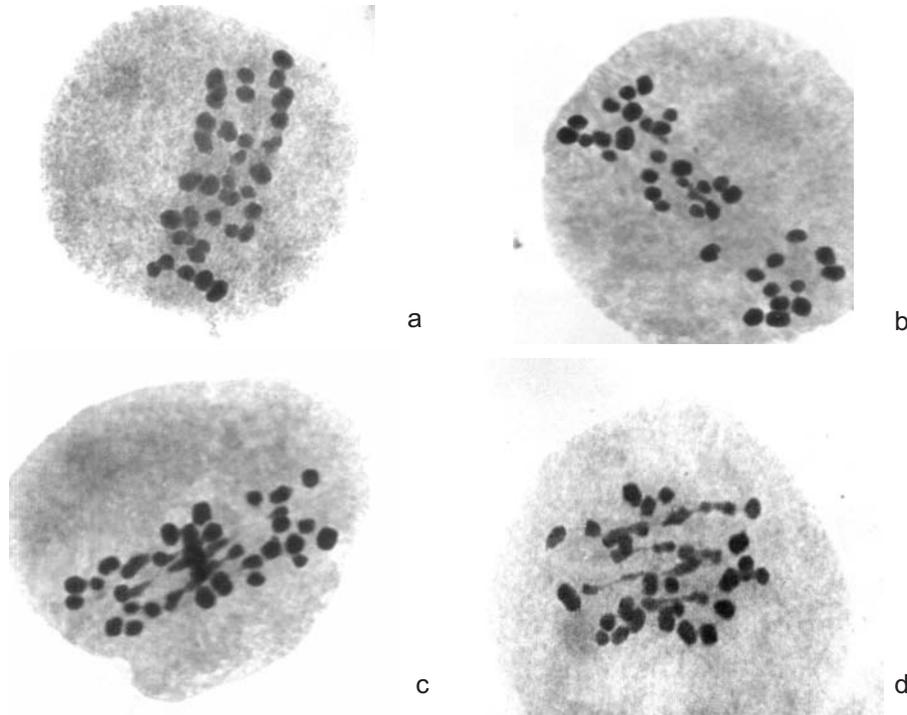


Figure 2. Metaphase I in pollen mother cells of amphihaploid hybrids *N. tabacum* cv. BY 103  $\times$  *N. glauca*: (a) 36 separated univalents, (b) one rod-shaped bivalent, (c) 3 bivalents

In some meiocytes (containing more than 4 bivalents) the chromosomes were aligned and looked as forms similar to metaphase plates. Chromosome associations other than bivalents could not be observed.

Statistical analysis of chromosome pairing at metaphase I did not show any significant differences in mean numbers of bivalents per cell among  $F_1$  hybrids (Table 1). The modal number of bivalents per cell was not found to be influenced by the constituent *N. tabacum* genome. Small differences were observed between variances and variation coefficient of observed bivalents.

In later meiotic stages (anaphase and telophase I), some irregularities could be observed, such as chromatid bridges (Figure 3a) and numerous lagging chromo-

**Table 1.** Chromosome pairing observed during metaphase I in pollen mother cells of amphihaploid  $F_1$  (*N. tabacum* cv. BY 103 or K326  $\times$  *N. glauca*)

Genotype	Number of tested cells	Mean bivalent number	Modal bivalent number	Pairing range	Variance	Variation coefficient
BY 103 $\times$ <i>N. glauca</i>	222	0.81	0	0 – 5	1.15	132%
K 326 $\times$ <i>N. glauca</i>	240	0.96	0	0 – 5	1.46	126%

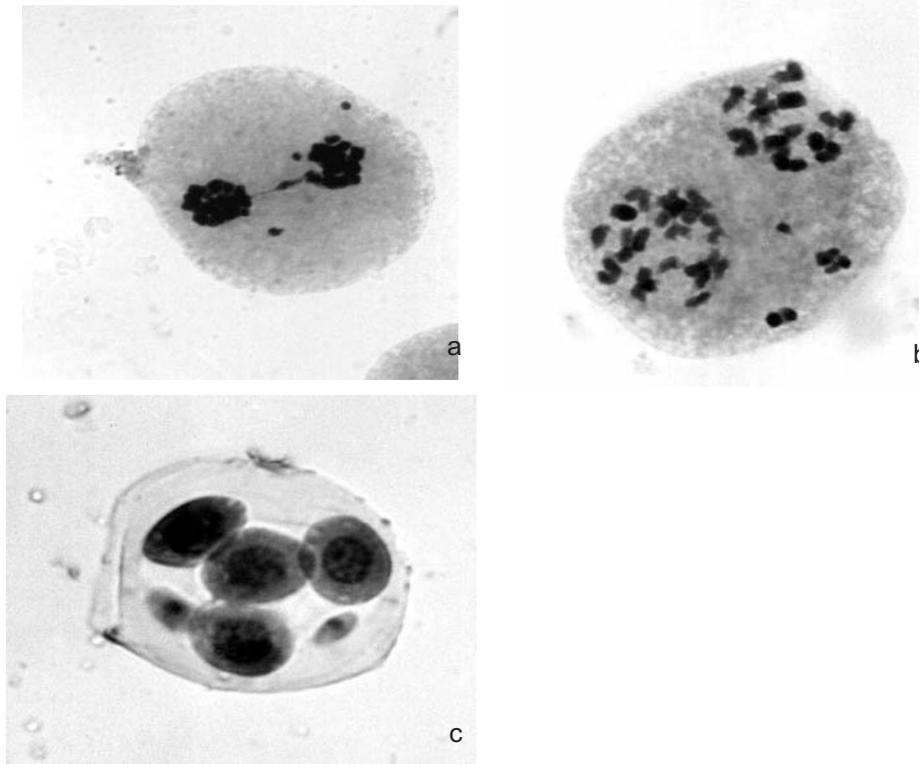


Figure 3. Irregularities observed during anaphase and telophase I of meiosis in amphihaploid hybrids *N. tabacum* cv. K 326 × *N. glauca*: (a) chromatid bridge, (b) chromosomes not included in daughter nuclei, (c) tetrad stage with 2 micronuclei

somes. Moreover, laggards not included in daughter nuclei (Figure 3b) often formed micronuclei (Figure 3c). A frequently observed phenomenon was a failure of one of the meiotic divisions, leading to formation of restitution nuclei and consequently of dyads, tryads or rarely monads. Frequencies of PMCs containing less than 4 microspores are presented in Table 2.

**Table 2.** Presence of pollen mother cells (PMCs) containing different numbers of microspores in amphihaploid  $F_1$  hybrids (*N. tabacum* cv. BY 103 or K326 × *N. glauca*) during tetrad stage

Hybrid combination	Monads	Dyads	Tryads	Tetrads	Total PMCs
	no.				
BY103 × <i>N. glauca</i>	16	163	20	79	278
% PMCs	5.8	58.6	7.2	28.4	100
K 326 × <i>N. glauca</i>	19	218	27	99	363
% PMCs	5.2	60	7.4	27.3	100

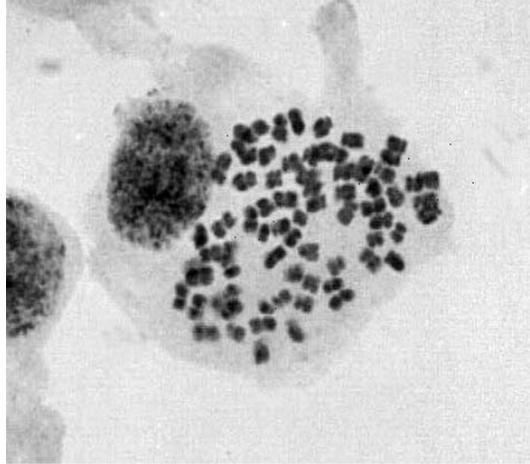


Figure 4. Mitotic chromosomes of an amphidiploid *N. tabacum* cv. BY 103 × *N. glauca*

Presence of unreduced gametes had no effect on hybrid fertility expressed as the level of pollen viability. Pollen produced by plants either would not stain with acetocarmine or was not produced at all. Morphological traits of plants obtained from  $F_1$  hybrid seedlings treated with colchicine, suggested their polyploid nature, and the presence of 72 mitotic chromosomes (Figure 4) confirmed the amphidiploid character of the plants.

In the case of hybrids *N. tabacum* cv. BY 103 × *N. glauca*, morphological investigations of flowers from amphidiploid plants showed delayed growth of corolla in relation to sepals, as compared with the maternal cultivar of tobacco.

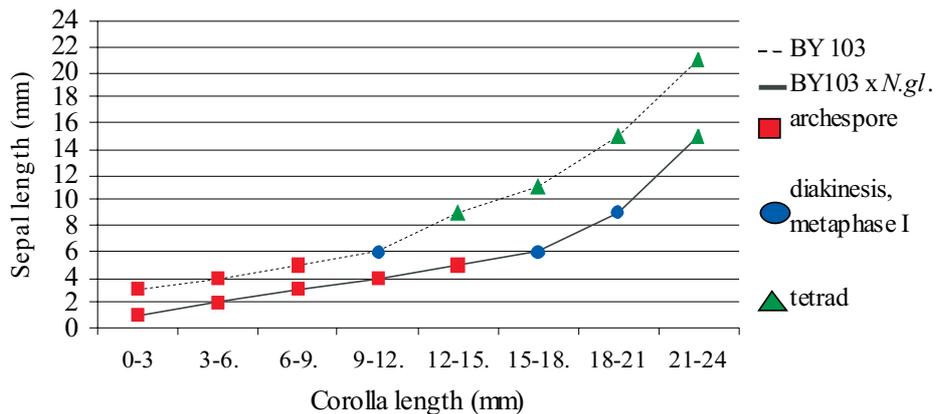


Figure 5. Comparison between microsporogenesis in pollen mother cells of amphidiploid hybrids *N. tabacum* cv. BY 103 × *N. glauca* and maternal cultivar of tobacco *N. tabacum* cv. BY 103

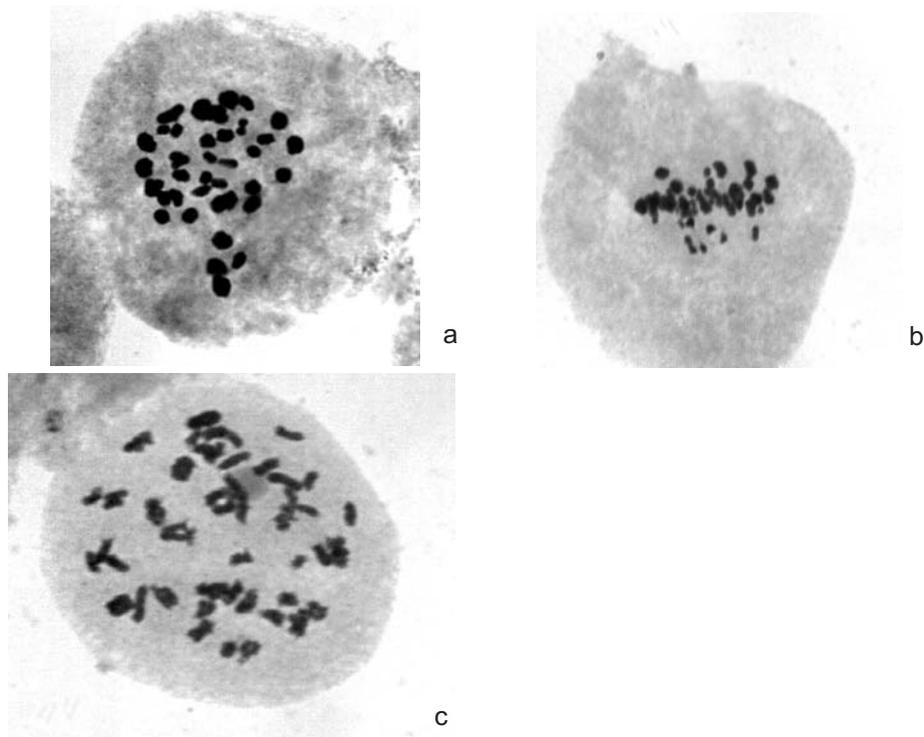


Figure 6. Meiotic configurations observed in pollen mother cells of amphidiploid *N. tabacum* cv. K 326 × *N. glauca* during metaphase: (a) 36 bivalents, (b) 8 univalents outside the metaphase plate; and during diakinesis: (c) 32 bivalents, 8 univalents, 2 trivalents

Additionally, meiosis was delayed and asynchronous in the PMCs (Figure 5). While in the anthers of *N. tabacum* cv. BY 103 mature microspores were easy to detect, in the PMCs of amphidiploid plants (*N. tabacum* cv. BY 103 × *N. glauca*) prophase and metaphase of I and II meiosis were still in process. Amphidiploid

**Table 3.** Frequency of univalents present during diakinesis in pollen mother cells (PMCs) of amphidiploids (*N. tabacum* cv. BY 103 or K 326 × *N. glauca*)

Hybrid combination	PMCs (no.)	PMCs with different numbers of univalents (no.)										
		0	1	2	3	4	5	6	7	8	9	10
BY103 × <i>N. glauca</i>	185	10	10	18	26	40	29	21	11	10	6	4
% PMCs	100	5.4	5.4	9.7	14	21.6	15.7	11.3	5.9	5.4	3.2	2.2
K 326 × <i>N. glauca</i>	231	16	22	40	38	45	23	19	13	10	3	2
% PMCs	100	6.9	9.5	17.3	16.4	19.5	9.9	8.2	5.6	4.3	1.3	0.9



Figure 7. Degradation of tetrad in amphidiploid hybrids *N. tabacum* cv. BY 103  $\times$  *N. glauca*

hybrids *N. tabacum* cv. K 326  $\times$  *N. glauca* produced flowers that did not differ morphologically from the maternal cultivar. Both corolla and stamens did not show any visible modifications.

Cytological analysis of chromosome pairing during diakinesis in all tested amphidiploid hybrids, showed a generally similar and regular course of meiosis irrespective of the maternal tobacco cultivar.

The majority of the 72 chromosomes were in bivalent associations (Figure 6a-b). The chromosomes that did not form bivalent pairs were observed as univalents, trivalents and tetravalents (Figure 6c). Univalents were much more frequent.

The data on PMCs analysed for univalent occurrence (Table 3) indicated that 94.6 and 93.1% (depending on the parental variety) of the PMCs contained 1 to 10 univalents. Some irregularities (such as laggards, chromosomes not included in daughter nuclei, and micronuclei present in high numbers) were detected in the PMCs during the late phase of meiosis. In the amphidiploid generation, termination of microsporogenesis and degeneration of PMCs were observed at the tetrad stage (Figure 7). This caused self-sterility of the plants.

## Discussion

As a result of direct crossing between *N. tabacum*  $\times$  *N. glauca*, viable amphihaploid  $F_1$  hybrids were obtained. Observation of meiosis revealed a low level of chromosome pairing. This indicates a minor phylogenetic relationship between *N. tabacum* and *N. glauca*, resulting in a low probability of genetic recombination.

The observed pairing range and the modal number of bivalents differ from results presented by GOODSPEED (1954). This may be caused by using different genotypes of *N. tabacum* and *N. glauca*. The great variation in the number of bivalents could also be a result of too early disjunction of bivalents. The amphihaploid hybrids obtained were completely sterile (self- and cross-sterile), which is typical for interspecific hybrids, for example *N. tabacum* × *N. glauca* (BERBEĆ, OPOKA 1971), *N. tabacum* × *N. gossei* (PALAKARCHEVA, DOROSSIEV 1992), and *N. tabacum* × *N. rustica* (KRUSTEVA 1995). The main reasons of this sterility, as some authors report, include disturbed pairing during meiosis, irregular and random chromosome distribution, and production of non-functional gametes.

In the case of amphihaploids studied in this work, the reasons of sterility were much more complex. On the one hand, lack of pairing, lagging chromosomes and production of micronuclei were observed. On the other hand, the tested hybrids produced a large number of restitution nuclei. The number and range of restitution nuclei confirmed results obtained by STOYANOVA (1970), and DOROSZEWSKA, BERBEĆ (1996, 2000) for other combinations of *Nicotiana* hybrids. In the case described here, however, the high number of unreduced gametes did not correlate with the ability to produce viable pollen grains.

The double number of chromosomes in F<sub>1</sub> hybrids permitted to obtain an amphidiploid generation. The fact that the resultant plants were self-sterile came as a surprise, especially that male sterility among studied and described amphidiploid forms has not been reported yet (SHILAGYI 1975, EVANS et al. 1980). According to those authors, self-fertility of *N. tabacum* × *N. glauca* allopolyploids is a result of total chromosome pairing.

The hybrids obtained in this work had incomplete chromosome pairing during diakinesis and metaphase I. The high frequency of univalents in meiosis confirmed a genetic unbalance and irregular meiosis in the hybrid cells. However, that could not have stopped microsporogenesis totally at the tetrad stage and resulted in male sterility.

The sterility in the amphidiploids among *Nicotiana* hybrids has not been reported yet, but cytoplasmic male sterility is rather common among *Nicotiana* amphidiploids (BERBEĆ 1994, 2000). It concerns mostly alloplasmic forms of tobacco and the most common symptom is anther discharge. Only in tobacco cultivars containing cytoplasm of *N. raimondii* it will cause termination of microsporogenesis at the tetrad stage similar to that in described amphidiploids.

## Conclusions

As a result of interspecific hybridization between *N. tabacum* cv. BY 103 or *N. tabacum* cv. K 326 and *N. glauca*, viable amphihaploid and amphidiploid hybrids were obtained. The amphihaploid hybrids obtained were completely sterile

and represented a low variable pairing category with 0 to 5 bivalents in meiosis. Morphological investigations of flowers from amphidiploid plants showed delayed growth of corolla in relation to sepals in hybrids *N. tabacum* cv. BY 103 × *N. glauca*. Amphidiploid hybrids were male sterile due to arrested PMCs development at the tetrad stage. Termination of microsporogenesis at the tetrad stage correlated with the lack of ability to produce viable pollen by the amphidiploid forms.

#### REFERENCES

- ANONYMOUS (1990). Illustrated Book of the Genus *Nicotiana*. Japan Tobacco Inc. Plant Breeding and Genetics Research Laboratory: 195.
- BERBEĆ J., OPOKA B. (1971). Badania nad F<sub>1</sub> zwrotnych mieszańców *N. tabacum* L. var. *Virginica* Comes × *N. glauca* Grah. (Research on reciprocal F<sub>1</sub> hybrids of *N. tabacum* L. var. *Virginica* Comes × *N. glauca* Grah.). *Pamięt. Puławski* 43: 5-38.
- BERBEĆ A. (1994). Cytologiczne, morfologiczne i użytkowe właściwości alloplazmatycznych form tytoniu uprawnego *N. tabacum* L. z cytoplazmą gatunków *N. knightiana* Goodspeed i *N. raimondii* Macbride (Cytologic, morphologic and economic traits of alloplasmic forms of cultivated tobacco *N. tabacum* L. with cytoplasm of *N. knightiana* Goodspeed and *N. raimondii* Macbride). Hab.PhD thesis. H (6) IUNG, Puławy .
- BERBEĆ A. (2000). Effect of sixteen different sources of cytoplasmic male sterility (cms) on some traits of flue-cured tobacco cultivar. *Inf. Bull. Spec. CORESTA Congres Lisbon*, abstr. 30: 79.
- BURNS J.A. (1964). A technique for making preparations of mitotic chromosomes from *Nicotiana* flowers. *Tobacco Science* 8: 1-2.
- DOROSZEWSKA T., BERBEĆ A. (1996). Chromosome pairing and microsporogenesis in interspecific F<sub>1</sub> hybrids of *N. africana* with different cultivars of *N. tabacum*. *J. Genet. Breed.* 50: 75-82.
- DOROSZEWSKA T., BERBEĆ A. (2000). Cytological investigations of polyploid interspecific hybrids of *N. africana* with different cultivars of *N. tabacum*. *J. Genet. Breed.* 54: 77-82
- EVANS D.A., WETTER L.R., GAMBORG O.L. (1980). Somatic hybrid plants of *N. glauca* and *N. tabacum* obtained by protoplast fusion. *Physiol. Plant.* 48: 225-230.
- GOODSPEED T.H. (1954). The genus *Nicotiana*. *Chronica Botanica*. Waltham, Mass. USA.
- KRUSTEVA D. (1995). Results from the hybridization between *N. rustica* L. var. *brasiliica* and *N. tabacum* L. *J. Genet. Breed.* 5(6): 226-233.
- PALAKARCHEVA M., DOROSSIJEV L. (1992). Results of hybridization between the species *N. gossei* D. and *N. tabacum* L. *Inf. Bull. Spec. CORESTA Congres Jerez de la Frontera*, abstr.: 163.
- SHILAGYI L. (1975). Elimination of chromosomes in an allopolyploid hybrid of *N. tabacum* × *N. glauca*. *Acta Bot. (Budapest)*: 28(1-2): 193-198.
- STOYANOVA M. (1970). Prouchvaniya na khibridi mezduvidovete *N. tabacum* i *N. glauca* Grah. *Otdalechena khibrizaczhiya na rastieniata*. Nauch. Sesiya, Sofia: 127-136.