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Development and cytometric evaluation of interspecific F_1 hybrids *Nicotiana tabacum* × *N. africana*

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Abstract. One of the most important threats to tobacco cultivation is the *Potato virus Y* (PVY). Sources of resistance to PVY are found both in *Nicotiana tabacum* cultivars and in wild *Nicotiana* species. The high variability of the virus and the ability to break the existing resistance make it necessary to search for new resistance sources or to combine the ones that are already known. The aim of the study was to combine *va* resistance of two cultivars VAM and Wiślica, showing resistance to most PVY isolates, with the wild *Nicotiana africana* species, which is immune to all known PVY isolates. Due to the significant genetic distance between these species, the seedlings of the obtained F_1 hybrids were not viable. In order to obtain viable hybrids, cotyledon *in vitro* cultures were used. Growth and development of the callus, as well as plant regeneration were differentiated depending on the maternal component used in the crossing with *N. africana*. The cotyledons of hybrids derived from cultivar VAM regenerated more efficiently, as 88 amphihaploid hybrid plants were obtain from this maternal parent. In contrast, only 27 amphihaploid hybrid plants were derived from cultivar Wiślica. Infertility of amphihaploid plants hampered their use in the breeding process. In order to restore fertility, the process of organogenesis from stem piths under *in vitro* conditions, was used. The degree of ploidy of regenerated plants was determined using flow cytometry. Despite the use of the same *in vitro* culture conditions for all the objects, a significant influence of the maternal component on the number of obtained regenerants and on the degree of their ploidy level was observed.

Keywords: *Potato virus Y*, *N. tabacum*, *N. africana*, *in vitro* culture, amphihaploid, amphidiploid

INTRODUCTION

Viral diseases cause large economic losses in tobacco cultivation, reducing crop yield and deteriorating leaf quality. *Potato virus Y* (PVY), belonging to the *Potyvirus* genus and the *Potyvirydae* family, is a big threat (Scholthof et al., 2011). It causes brown necrosis of the tobacco leaf veins, which inhibits the transport of water and mineral salts to leaf tissues, while chlorotic and necrotic spots on the leaf blade limit the surface as well as the assimilation capacity and gas exchange. The virus is transmitted by aphids in an unstable manner, which makes the chemical protection of plants difficult (Doroszewska, 2004). Due to the high variability of the virus (Przybyś et al., 2013) and its ability to break the existing resistance, it is necessary to search for new sources of resistance and combine the already known ones. The largest resource of genes determining resistance to pathogens, including PVY, are wild *Nicotiana* species (Doroszewska and Depta, 2011; Głażewska, 1977; Sievert, 1972). Sources of resistance are present among some *Nicotiana tabacum* cultivars. The German variety Virgin A Mutant (VAM), obtained as a result of exposure to X-rays (Koelle, 1958, 1961), exhibits resistance conditioned by a single recessive *va* gene located on the E chromosome (Gupton and Burk, 1973). The *va* gene is a deletion of the sequence segment responsible for susceptibility and has three allelic forms va^0, va^1, va^2 (Acosta-Leal and Xiong, 2008; Noguchi et al., 1999). This type of resistance conditioned by deletion was transferred by crossing crop varieties or it was obtained by mass selection (Carsten and Seehofer, 1960; Korbecka-Glinka et al., 2017b). However, resistance conditioned by the recessive *va* gene does not provide full protection against all PVY isolates. Within the *Nicotiana* genus, *Nicotiana africana* possesses resistance to all tested isolates (Doroszewska, 2004; Doroszewska and Czubacka, 2008; Doroszewska and Depta, 2011; Lucas et al., 1980).

Nevertheless, the use of wild species in crop breeding is difficult due to a number of barriers at different stages of interspecific introgression. Lethality of hybrids can manifest itself in different ways, which was the basis for the division into four types of inviability (Yamada et al., 1999).

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N. tabacum × *N. africana* hybrids were classified as type II, characterised by underdevelopment of the root system (Tezuka et al., 2010). Well germinated seeds of hybrid forms obtained by Doroszewska (1994), where five cultivars of cultivated tobacco were used as the parent form and the paternal component was the *N. africana* species, were characterised by a high degree of seedling mortality. The solution to the problem of inviability was the use of *in vitro* cotyledon cultures and regeneration of hybrid plants. The obtained amphihaploid forms were infertile due to the lack or very low chromosome pairing (Doroszewska and Berbeć, 1996). Restoration of fertility was possible by doubling the chromosomes, which occurred spontaneously during tissue culture (Doroszewska and Berbeć, 2000). Further cultivating work resulted in a BPA line derived from a cross between the PVY susceptible variety of tobacco BP-210 and the *N. africana* wild species. The BPA line is tolerant to all PVY strains, which means that disease symptoms are limited to chlorotic spots on the leaf blade and nerve patches, while nerve necrosis was never observed (Doroszewska, 2010).

The work on the use of *N. africana* was also conducted by Wernsman (1992), who obtained the NC152 addition line containing a pair of homologous chromosomes from *N. africana*. This line did not show full resistance to PVY; however, it provided partial protection against necrosis. Additional chromosomes derived from *N. africana* in the NC152 addition line were subsequently labelled with the mutated *dhfr* transgene conditioning the resistance to the antibiotic methotrexate (Campbell et al., 1994). The line obtained in this way was used by Lewis (2005) to assess the degree of gene transfer from *N. africana* both by classical cultivating by crossing with a susceptible Petite Havana variety, as well as utilising tissue cultures. The segregants resistant to the PVY NN isolate and having 48 chromosomes were selected from the BC_1F_1 generation and were crossed again with the susceptible K326 variant to transfer the *Nafr* fragment responsible for resistance. Based on the K326 variety, Lewis (2007) obtained nearly isogenic lines having various combinations of the PVY resistance or tolerance, including both the recessive *va* gene and the *Nafr* fragment. Obtaining the K326 *va*/*va* genotype required crossing with the resistant Greenville 136 strain. The lines obtained in this way displayed a varied level of resistance, which depended largely on the virulence of the PVY isolate used.

The full resistance of *N. africana* is probably a result of the presence of many resistance genes located on different chromosomes (Doroszewska, 2010). The lines obtained by Lewis (2005) contained a chromosome fragment originating from *N. africana* and this feature was partly dominant. In contrast, the BPA line resistance feature was recessive (Korbecka-Glinka et al., 2017a). Despite the difficulties in achieving full resistance to *Potato virus Y*, research indicates that the combination of factors from *N. africana* and *va* may increase the level of resistance or tolerance to PVY, also depending on the parent components used (Korbecka-Glinka et al., 2017b; Lewis, 2005, 2007).

The main purpose of this study was to combine resistance originating from the two sources. The German variety VAM and the Polish variety Wiślica containing different alleles of the *va* gene exhibiting resistance to most PVY isolates were crossed as females with *N. africana*, fully resistant to all known PVY isolates. The aim of the study was also to optimise the method of doubling the chromosomes of amphihaploid forms by culturing stem pith cells and determining the usefulness of cytometric approaches for rapid identification of amphidiploid forms.

MATERIALS AND METHODS

Crosses

Two tobacco cultivars (2n = 48): VAM and Wiślica, were used as females in crosses with the *N. africana* (2n = 46) that was the paternal component. Crosses were made in greenhouse and the female plants were protected against self-pollination as well as inadvertent cross-pollination. The obtained seeds were collected and purified, and then deposited in seed cabinets.

In vitro cultures

Due to the inviability of F_1 *N. tabacum* × *N. africana* hybrid seedlings, *in vitro* methods were used. Seeds were disinfected with 10% hydrogen peroxide for 20 minutes, after which they were washed three times in sterile distilled water, dried and plated on standard medium, as described by Linsmaier and Skoog (1965), with 2% sucrose, solidified on agar at pH 5.6. Plants that did not die and produced a healthy root system were transferred to a new growth medium and subsequently planted in soil. From the vast majority of seedlings exhibiting browning and dying roots, cotyledons were collected and cut into 2–3 fragments that were placed on a regeneration medium, as described by Lloyd (1975), which in addition to mineral substances and sucrose, contained 2.0 mg/l kinetin, 2.0 mg/l 3-indolylacetic acid (IAA) and 0.5 mg/l folic acid.

Cotyledon fragments plated on the nutrient medium formed callus and plant regeneration was observed. The shoots obtained by organogenesis were transferred onto a rooting medium with the addition of 0.2 mg/l IAA and 0.2 mg/l NAA. Subsequently, the rooted plants were transplanted into soil and maintained in greenhouse conditions.

In order to obtain fertile amphidiploid plants, the stem pith culture method was used. From the flowering amphihaploid plants of the F_1 generation, the middle part of the stem was cut off, divided into smaller parts and rinsed for several minutes with detergent water to preliminarily remove impurities. Sterilisation was carried out in 70% ethanol for 30 seconds, then in 10% sodium hypochlorite for 20 minutes and lastly the stem was rinsed three times. In-

ternal parts with the preserved stem pith were prepared from the stem fragments, which were then cultured on the medium, as described by Lloyd (1975). The regenerated plants were transferred onto the rooting medium and then transplanted into soil.

Morphological and cytological assessment

Morphological and cytological evaluation was carried out for all of the obtained plants. Morphological evaluation was conducted by comparing hybrid forms with the parental ones, as well as by comparing hybrid forms obtained as a result of regeneration from cotyledons with forms obtained from regeneration from pith shoots.

Cytological tests to confirm the hybridity status for plants regenerated from cotyledons were carried out by a microscopic method. Mitotic chromosomes were counted in corolla meristems (Burns, 1964), pretreated with hydroxyquinoline with maltose with the aim to accumulate metaphases as well as to shorten and scatter chromosomes.

Plants regenerated from stem pith were tested for ploidy level using a flow cytometer. The amphihaploid hybrid plant F_1 *N. tabacum* × *N. africana* was used as the standard. Samples for testing were obtained from young plants. Leaf tissue was chopped in isolation buffer to release the cell nuclei, which were subsequently stained with the DAPI dye (indole-4',6-diamidino-2-phenyloidine) and analysed by flow cytometer (Sysmex PartecCyFlow® Cube 8), where a UV lamp was utilised as the light source.

The following nomenclature was used to determine the cytogenetic status of plants:

– amphihaploid – a hybrid containing one haploid genome from each species,
– amphidiploid – a hybrid containing two haploid genomes from each species,
– mixoploid – a hybrid containing cells which have different ploidy.

RESULTS

As a result of hybridizing two cultivars of *Nicotiana tabacum* 'Wiślica' and 'VAM' with the wild species *N. africana*, a satisfactory amount of seeds was obtained, which were purified and intended for further breeding work. The tests demonstrated good germination ability of the seeds; however, seedling dying was observed, too. To prevent this, an attempt to obtain viable hybrid forms under *in vitro* culture conditions was undertaken. The seeds of both hybrids were disinfected according to the same procedure and were sown on the sowing medium. 7000 seeds obtained from crossing of *N. tabacum* 'Wiślica' × *N. africana* and 4000 seeds from crossing of *N. tabacum* 'VAM' × *N. africana* were sown. The difference in the number of seeds sown results from the fact that, despite good germination of both crossing forms, young seedlings from the crossing with Wiślica died more often. This made it challenging to obtain an appropriate number of seedlings, which were the material for culturing the cotyledon fragments onto the organogenesis medium. A varied ability for regeneration of cultured cotyledon fragments of young seedlings was also observed, depending on the maternal component used for crossing (Table 1). The fragments of cotyledons of hybrids, whose maternal parent was Wiślica, displayed considerably worse regeneration. For this reason, a greater number of cotyledon fragments were cultured for this hybrid combination. Much better growth was observed for the hybrid with VAM cultivar, in which the seedlings preserved green cotyledons even three weeks after sowing, despite the degeneration of the roots (Fig. 1a). At the same time, seedlings of hybrids with Wiślica cultivar turned yellow and died (Fig. 1b). Similarly, better condition of the cotyledon fragments cultivated on the callus medium was noted for hybrids with VAM cultivar in comparison to Wiślica (Fig. 2). Furthermore, the process of shoots regeneration was more efficient (Fig. 3), which resulted in obtaining 88 amphihaploid hybrid plants. The process of regeneration was entirely different in the case of hybrids with Wiślica cultivar. Despite using nearly twice as many seeds, only 27 regenerates were obtained, which were generated from the fragments of cotyledons during the organogenesis process.

In addition, for both hybrid combination, where either VAM or Wiślica was the maternal component, plants characterised by well-developed roots and good growth were obtained. Morphological and cytological evaluation revealed that in both cases maternal haploids were the prevalent class (Table 1). The haploids arose from haploid embryos that developed from gametophyte cells with a reduced number of chromosomes, without fertilisation. Along with the maternal haploids, in combinations where the maternal form was VAM, spontaneous interspecific hybrids containing 47 chromosomes in somatic cells were observed.

The obtained interspecific hybrids *N. tabacum* 'VAM' × *N. africana* and *N. tabacum* 'Wiślica' × *N. africana* sho-

Table 1. Effectiveness of obtaining interspecific hybrids *Nicotiana tabacum* × N. *africana*.

Hybrid	Number of seeds sown	The number of cultured cotyledons	Maternal haploids	Spontaneous hybrids[#]	Hybrids obtained as a result of organogenesis
N. tabacum 'Wiślica' × *N africana*	7000	1100	4	0	27
N. tabacum 'VAM' × *N. africana*	4000	880	5	6	88

[#] true hybrids surviving without the aid of tissue culture

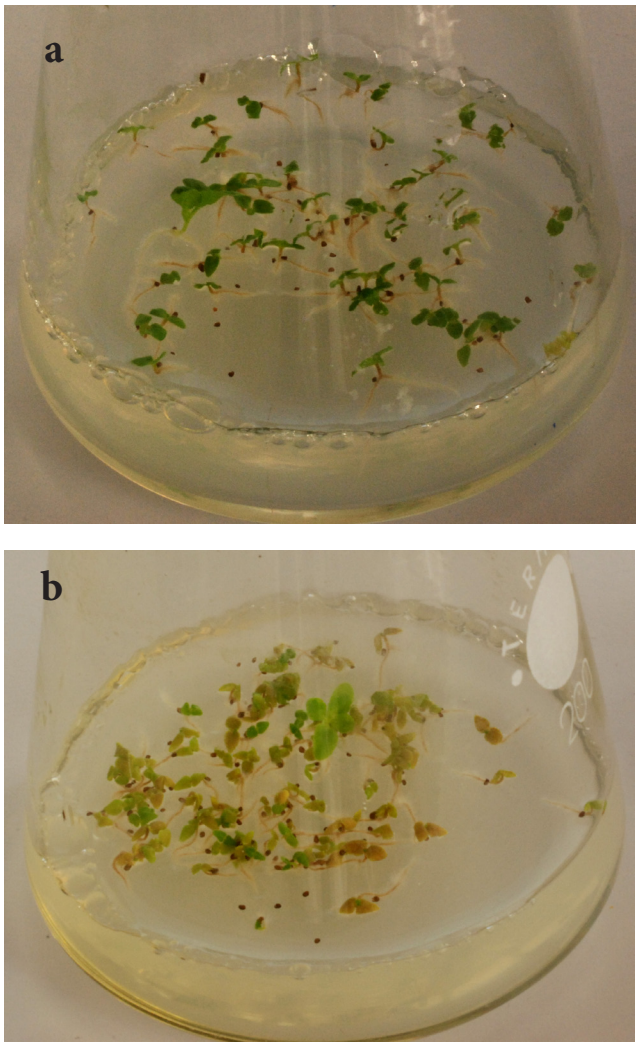

Figure 1. Seedlings of hybrids *N. tabacum* 'VAM' × *N. africana* (a) and *N. tabacum* 'Wiślica' × *N. africana* (b) after 3 weeks after sowing.

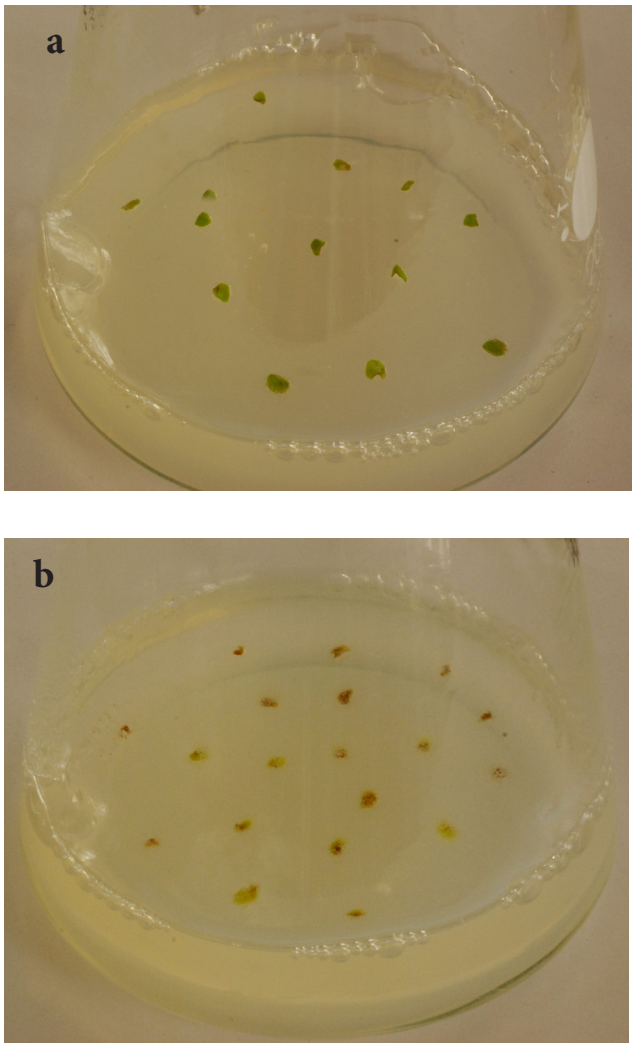

Figure 2. Cotyledons of hybrids *N. tabacum* 'VAM' × *N. africana* (a) and *N. tabacum* 'Wiślica' × *N. africana* (b) on organogenesis medium.

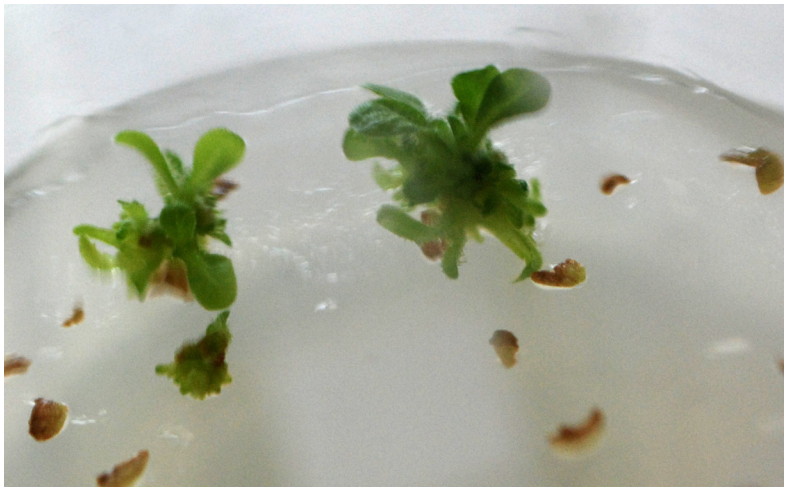

Figure 3. Regeneration of F_1 hybrid *N. tabacum* 'VAM' × *N. africana* from cotyledons.

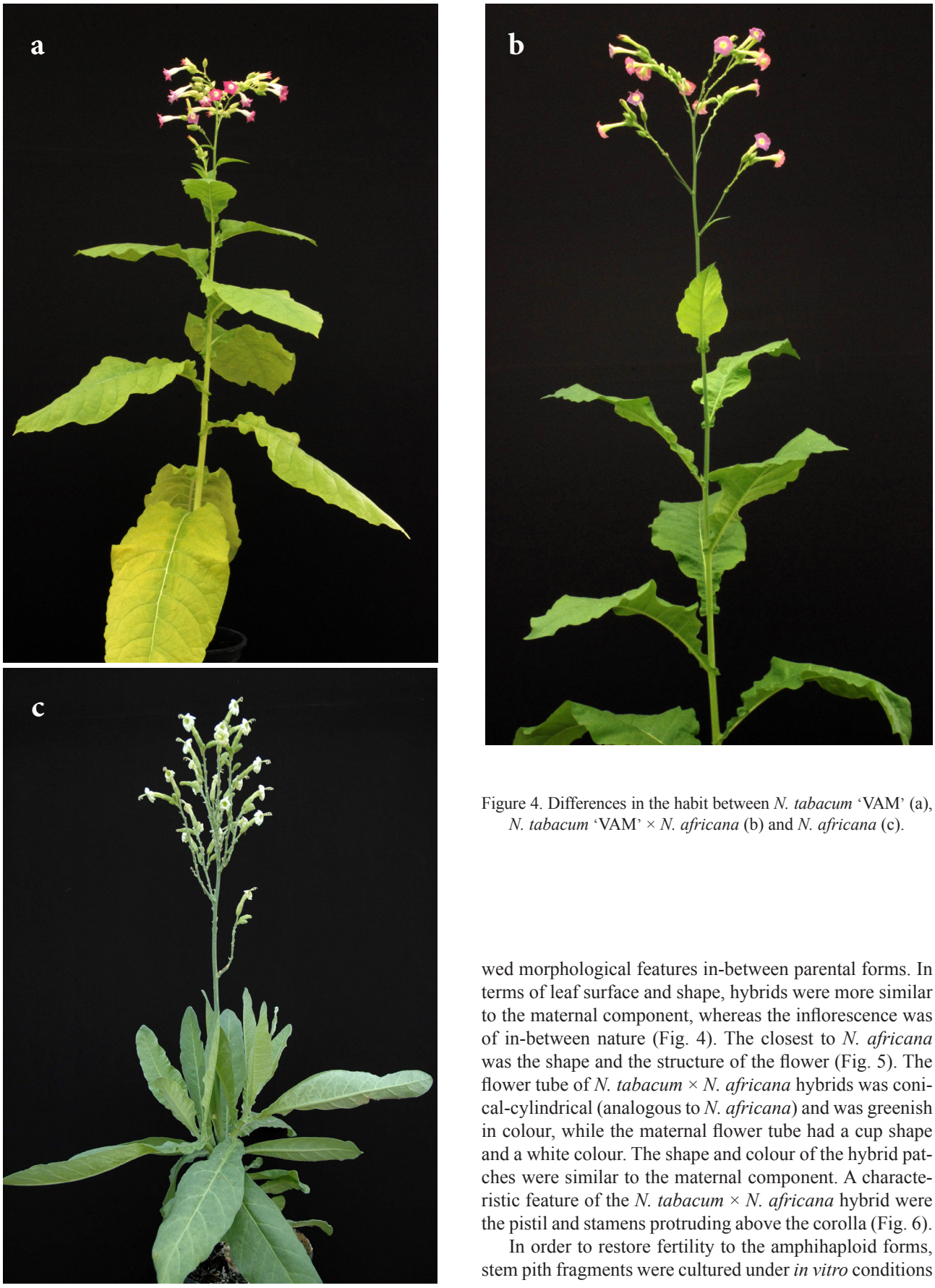

Figure 4. Differences in the habit between *N. tabacum* 'VAM' (a), *N. tabacum* 'VAM' × *N. africana* (b) and *N. africana* (c).

wed morphological features in-between parental forms. In terms of leaf surface and shape, hybrids were more similar to the maternal component, whereas the inflorescence was of in-between nature (Fig. 4). The closest to *N. africana* was the shape and the structure of the flower (Fig. 5). The flower tube of *N. tabacum* × *N. africana* hybrids was conical-cylindrical (analogous to *N. africana*) and was greenish in colour, while the maternal flower tube had a cup shape and a white colour. The shape and colour of the hybrid patches were similar to the maternal component. A characteristic feature of the *N. tabacum* × *N. africana* hybrid were the pistil and stamens protruding above the corolla (Fig. 6).

In order to restore fertility to the amphihaploid forms, stem pith fragments were cultured under *in vitro* conditions

Figure 5. Differences in flower morphology between *N. tabacum* 'VAM' (a), *N. tabacum* 'VAM' × *N. africana* (b) and *N. africana* (c).

Figure 6. Pistil and stamens protrude above corolla of *N. tabacum* 'VAM' × *N. africana* hybrid.

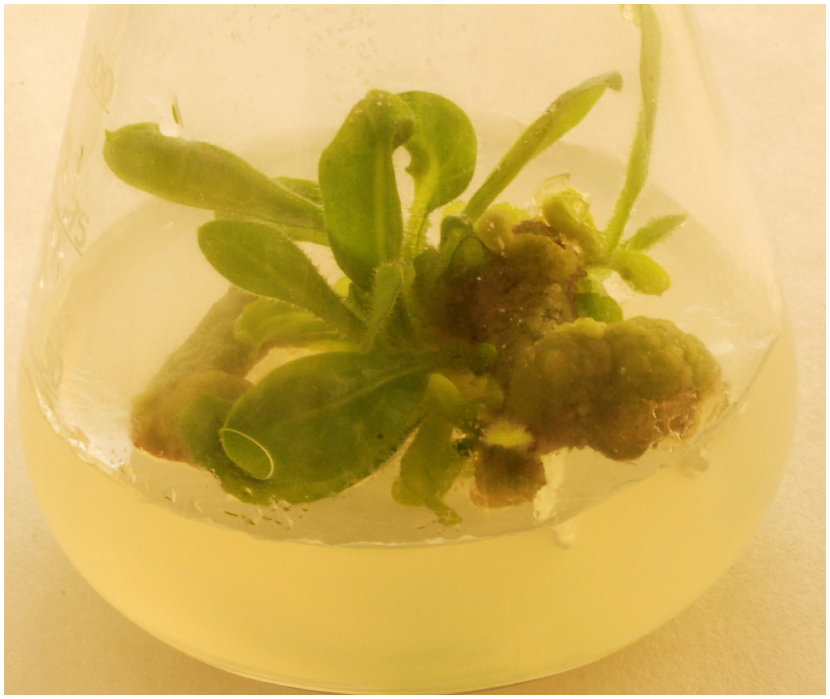

Figure 7. Regeneration of F_1 *N. tabacum* 'VAM' × *N. africana* hybrid from stem piths.

(Fig. 7). This process was associated with the presence of polyploid cells formed by endomitosis in the stem pith. Despite employing identical *in vitro* culture conditions for all objects, the results varied both in terms of the number of regenerates and their ploidy. Mitotic chromosome counts in flower corollas revealed the presence of amphihaploid plants with 47 chromosomes and amphidiploid plants with 94 chromosomes (Fig. 8). One of the amphihaploid plants provided the standard for the cytometric analysis (Fig. 9). The tests were carried out for the remaining 360 young plants. The use of flow cytometry significantly accelerated the process of assessing ploidy of the regenerants.

In the case of *N. tabacum* 'VAM' × *N. africana*, 5 hybrid plants that had grown to maturity unaided by tissue culture (F_1VS1 – S5), and one hybrid was obtained in the process of organogenesis from cotyledons (F_1VO1). All these six hybrids provided the explants for the process of regeneration from the stem pith. The number of obtained amphihaploids and amphidiploids varied notably (Table 2).

The highest number of regenerants was obtained for the F_1VS2 hybrid – 129 plants, whereas the lowest for the F_1VO1 hybrid – 14 plants. Nevertheless, only 20 amphidiploids were obtained from the F_1VS2 plant, which accounted for 15.5% of the total plants obtained. For F_1VS1 and

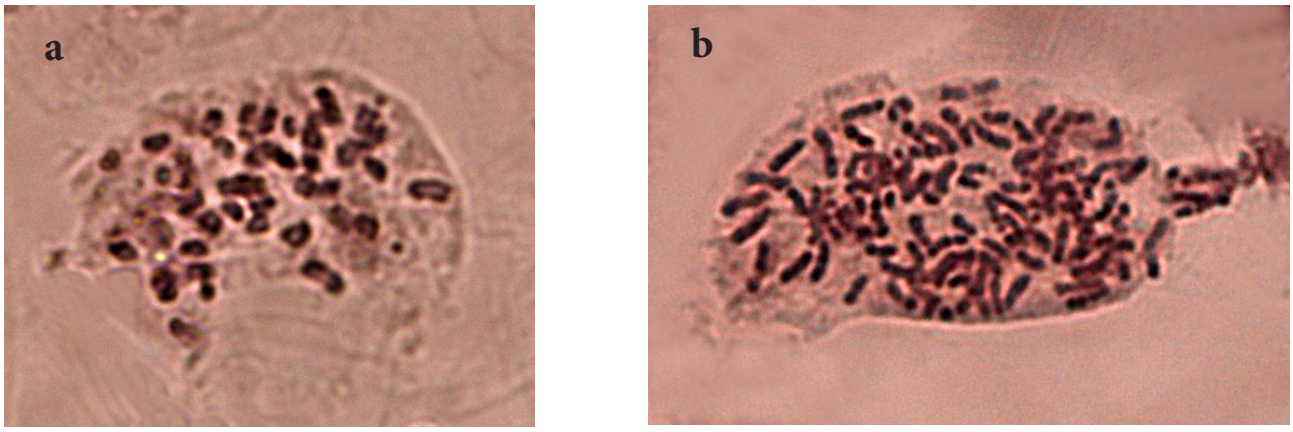

Figure 8. Amphihaploid F_1 *N. tabacum* 'VAM' × *N. africana* hybrid with 47 chromosomes (a) and amphidiploid F_1 *N. tabacum* 'VAM' × *N. africana* hybrid with 94 chromosomes (b) in the somatic cells.

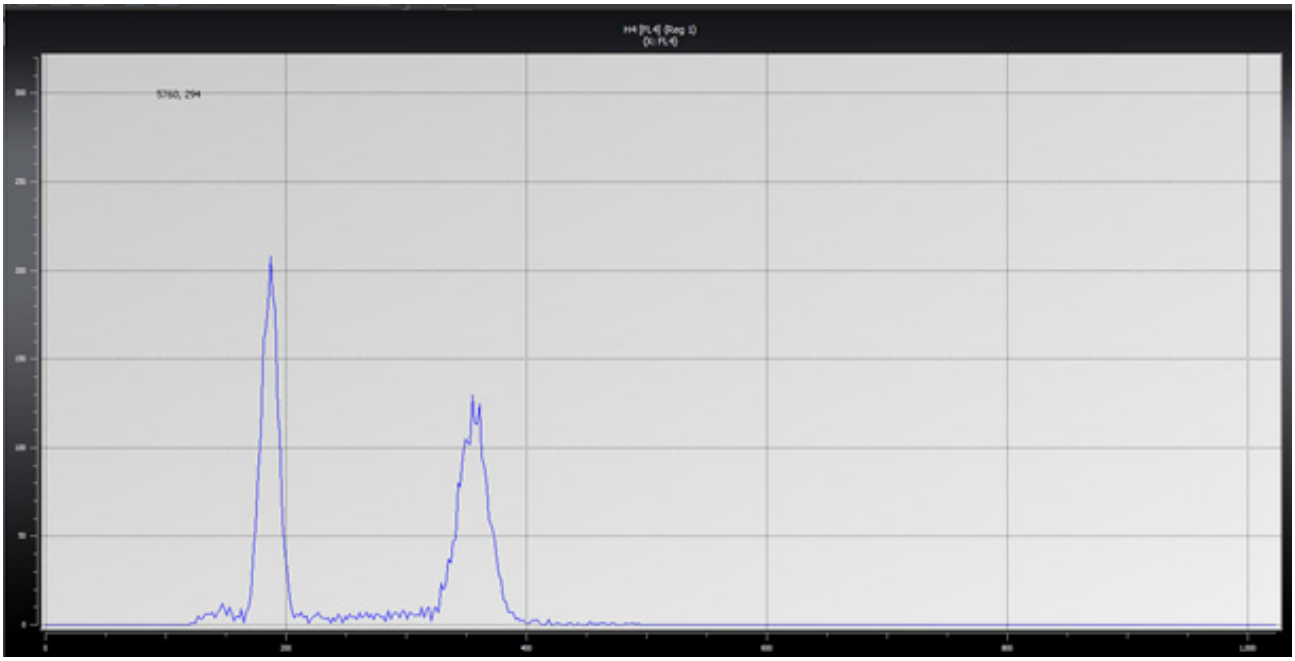

Figure 9. Evaluation of the ploidy level of interspecific hybrids *N. tabacum* 'VAM' × *N. africana* by flow cytometry.

Table 2. The number of hybrid plants *Nicotiana tabacum* 'VAM' × *N. africana* obtained by organogenesis stem piths *in vitro* culture.

Ploidy	Hybrids											
	F_1VS1		F_1VS2		F_1VS3		F_1VS4		F_1VS5		F_1VO1	
	number	%	number	%	number	%	number	%	number	%	number	%
Amphihaploid	**24**	88.9	**109**	84.5	**20**	74.1	**17**	42.5	**5**	23.8	**10**	71.4
Amphidiploid	**1**	3.7	**20**	15.5	**7**	25.9	**22**	55.0	**16**	76.2	**3**	21.4
Mixoploid	**2**	7.4	**0**	0.0	**0**	0.0	**1**	2.5	**0**	0.0	**1**	7.2
Total	**27**	100.0	**129**	100.0	**27**	100.0	**40**	100.0	**21**	100.0	**14**	100.0

F_1VS1–5 – hybrids *N. tabacum* 'VAM' × *N. africana* obtained spontaneously
F_1VO1 – hybrid *N. tabacum* 'VAM' × *N. africana* obtained as a result of organogenesis

Table 3. The number of hybrid plants *Nicotiana tabacum* 'Wiślica' × *N. africana* obtained by organogenesis stem piths *in vitro* culture.

Ploidy	Hybrids							
	F_1WO1		F_1WO2		F_1WO3		F_1WO4	
	number	%	number	%	number	%	number	%
Amphiploid	**29**	54.7	**46**	66.7	**6**	66.7	**25**	96.2
Amphidiploid	**24**	45.3	**23**	33.3	**3**	33.3	**1**	3.8
Mixoploid	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Total	**53**	100.0	**69**	100.0	**9**	100.0	**26**	100.0

F_1WO1-4 – the number of hybrids *N. tabacum* 'Wiślica' × *N. africana* obtained by organogenesis pith stems *in vitro* culture

Table 4. Effectiveness of doubling the chromosomes by organogenesis stem piths *in vitro* culture.

Ploidy	*Nicotiana tabacum* 'VAM' × *N. africana*		*N. tabacum* 'Wiślica' × *N. africana*	
	number	%	number	%
Amphihaploid	**185**	71.7	**106**	67.5
Amphidiploid	**69**	26.7	**51**	32.5
Mixoploid	**4**	1.6	**0**	0.0

F_1VS3 hybrids, 27 plants, mostly amphihaploids, regenerated. More amphidiploid than amphihaploid plants were obtained for hybrids F_1VS4 (22/40) and F_1VS5 (16/21), which accounted for 55% and 76.2% of the total plants obtained, respectively. Four mixoploid plants were obtained in the polyploidisation process. Overall, for all explants, 185 amphihaploid plants, 69 amphidiploid plants and 4 mixoploids were obtained. The efficiency of doubling the chromosomes by the method of organogenesis from stem piths under *in vitro* conditions in the case of *N. tabacum* 'VAM' × *N. africana* hybrid was 26.7% (Table 4).

In order to double the number of chromosomes of the *N. tabacum* 'Wiślica' × *N. africana* hybrid, 4 hybrid plants regenerated *in vitro* were utilised (Table 3). The highest number of regenerants was obtained for the F_1WO2 hybrid – 69 plants, while the lowest for F_1WO3 – 9 plants. In each case, more amphihaploid than amphidiploid plants were obtained, the former ranging from 54.7 to 96.2% of the total number of plants. The largest number of amphidiploid plants were obtained for the F_1WO1 hybrid (24/53), whereas the lowest for the F_1WO4 hybrid (1/26). No mixoploid plants were obtained. In total, 106 amphihaploid plants and 51 amphidiploid plants were obtained from all cultured explants. The efficiency of the process of doubling the chromosomes by the method of regeneration under *in vitro* conditions in the case of the *N. tabacum* 'Wiślica' × *N. africana* hybrid was 32.5% (Table 4).

DISCUSSION

In the present study, considerable amounts of seeds of *Nicotiana tabacum* × *N. africana* hybrid species were obtained, both in the case of the VAM and Wiślica cultivars. Similarly to the studies by Doroszewska (1994), as well as by Tezuka et al. (2010), obtaining seeds for hybrids of cultivated tobacco from *N. africana* was not problematic. On the other hand, underdevelopment of the root system at the seedling stage, 5–7 days after germination, was a challenge. Overcoming this crossing barrier required the use of tissue cultures. For this purpose, a cotyledon culture was employed (Lloyd, 1975), which induced shoot development through the callus phase. The regenerated plants were transferred to a rooting medium and then acclimated in the greenhouse. Differences in the number of regenerated plants depending on the maternal component were observed. Despite the use of identical *in vitro* culture conditions, more plants were obtained when the maternal form was VAM cultivar than when Wiślica cultivar was. In the work described by Doroszewska (1994), where a similar medium composition was used, there were no observed differences in the ability to regenerate individual types of hybrids depending on the maternal component.

Another issue was the infertility of the amphihaploid generation, whose plants contained one haploid genome from each parent. One of the methods for restoring fertility includes the use of colchicine. The compound belongs to the so-called mitotic poisons that interfere with the mitosis process. This approach was utilised to double the number of chromosomes of the amphihaploid form of the *Nicotiana occidentalis* × *N. tabacum* hybrid (Ternovskii et al., 1974) and the *Nicotiana suaveolens* × *N. tabacum* hybrid (Lloyd, 1975). The safer way to obtain amphidiploid forms is to use *in vitro* cultures. For this purpose, the phenomenon of spontaneous chromosome doubling during the extended cotyledon culture can be used. Plants obtained this way (Doroszewska and Berbeć, 2000) were characterised by

a changed phenotype, and cytological examination performed in mitotic corolla preparations revealed the number of chromosomes typical for amphidiploids. Another approach for doubling the number of chromosomes employed in the current study was the use of the endomitosis process, which naturally occurs in the stem pith. This allowed for obtaining a large number of plants with a various number of chromosomes. In the case of the hybrid, whose maternal form was VAM cultivar, the stem pith explants of five plants were cultured and 14 to 129 regenerants were obtained. For hybrids with Wiślica cultivar, the minimum number of regenerants was 9, while the maximum was 69. Regeneration from stem pith explants was utilised by Nikova and Zagorska (1990) for the *N. africana* × *N. tabacum* hybrid that also resulted in regenerants of high morphological and cytological diversity, which was confirmed by counting chromosomes in the root tips. Furthermore, Hamada et al. (2001) used stem pith culture to double the haploid chromosomes of *Nicotiana tabacum* mutants obtained from anther cultures treated with helium ion beams. The composition of the medium varied slightly from the one used in our studies mainly in terms of the used hormones, and the cytological identification was made from the meristematic tissue of young roots.

Distinguishing between amphihaploid and amphidiploid forms is an important step in the breeding process. Determination of ploidy can be carried out by means of cytological analysis by counting the number of chromosomes made in corolla lobes (Doroszewska, 2004) or in root tips (Hamada et al., 2001; Tezuka et al., 2010). However, it is a tedious and time-consuming process, and the plants must be in the right phase. Flow cytometry is an alternative to cytological analysis. In the present study, cytometric analysis of young plants obtained from culture of stem pith explants was performed. In total, 185 amphihaploid plants, 69 amphidiploid plants and 4 mixoploids were obtained for the *N. tabacum* 'VAM' × *N. africana* hybrid. On the other hand, for the *N. tabacum* 'Wiślica' × *N. africana* hybrid, 106 amphihaploid plants and 51 amphidiploid plants were obtained. The use of this method allowed for a quick and precise assessment of the cytological status of plants. Identification of maternal haploids and aneuploids of the *Nicotiana tabacum* × *N. africana* hybrid using a flow cytometer was used by Hancock et al. (2015). However, they used *Glycine max* as the standard for the analysis, and the utilised dye was propidium iodide, for which the reading was made by using an argon laser. In the current study, a cytologically tested amphihaploid plant was used, while DAPI was used as the dye, for which the reading was made by using a UV lamp. This method is faster and does not require the addition of RNAse and a long incubation period, which is necessary when using propidium iodide.

Obtaining fertile hybrid forms whose parental forms contain various sources of PVY resistance is a very important stage in the breeding process that was initiated. The *va*-type resistance present in the varieties used for hybridisation is conditioned by the deletion of the susceptibility gene. This resistance is also present in many other cultivars; however, its degree varies. Many years of research indicate (Doroszewska and Czubacka, 2008; Korbecka-Glinka et al., 2017b) that VAM cultivar containing the allelic form va^0 displays the highest resistance, while the va^2 form found in the VSCR variety is the weakest. Wiślica cultivar with the va^1 allele used for crosses exhibits intermediate resistance. Unfortunately, no variety of *Nicotiana tabacum* is resistant to all *Potato virus Y* isolates. The ability of PVY to recombine causes the breaking of *va*-type resistance and makes it necessary to search for new sources of resistance or to combine existing ones. The wild species *Nicotiana africana* possesses resistance to all tested PVY isolates (Doroszewska, 2004; Doroszewska and Czubacka, 2008; Doroszewska and Depta, 2011; Lucas et al., 1980). For this reason, this species has been used in numerous breeding efforts. Many studies (Doroszewska, 2010; Lewis, 2007; Wernsman, 1992) indicate that it is difficult to transfer full resistance response from *N. africana* to a hybrid with *N. tabacum*. However, it is important that there was an increase in the resistance of hybrid forms to *Potato virus Y*, which is usually characterised by tolerance. A different mechanism of inheritance of the PVY resistance factor has also been observed (Korbecka-Glinka et al., 2017b; Lewis, 2005). It is significant that susceptible varieties were used as the breeding component in the aforementioned studies (Doroszewska, 2010) or the *va* resistance factor was combined with resistance from *N. africana* indirectly (Korbecka-Glinka et al., 2017b; Lewis, 2007). For this reason, the hybrid forms *N. tabacum* 'VAM' × *N. africana* and *N. tabacum* 'Wiślica' × *N. africana* obtained in this work, directly connecting the PVY resistance factors, are valuable breeding material. Restoration of fertility allows for obtaining successive hybrid generations from self-pollination, as well as backcrossing with the maternal parent. The degree of resistance of the obtained hybrids *N. tabacum* 'VAM' × *N. africana* and *N. tabacum* 'Wiślica' × *N. africana* to the *Potato virus Y* is the subject of ongoing research.

CONCLUSIONS

1. As a result of crossing parental forms bearing various sources of resistance to PVY, valuable interspecific hybrids necessary to create resistant breeding lines were obtained.
2. The use of the endomitosis process to double the number of chromosomes contributed to improved efficiency in obtaining fertile amphidiploids useful for research and for further stages of breeding.
3. The use of flow cytometry shortened the process of assessing the ploidy state of regenerated hybrid forms.

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