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Assessment of Resistance to PVY in Interspecific Hybrids Obtained by Combining Type *va* Resistance from *Nicotiana tabacum* with the Resistance from PVY-Immune Species *Nicotiana africana*

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Abstract: Tobacco veinal necrosis caused by the potato virus Y (PVY) substantially affects yields and crop quality of tobacco. PVY shows high variability in virulence due to numerous mutations and recombinations. The types of resistance available for tobacco include the *va* type, effective against some PVY isolates but succumbing to others, and the *Nicotiana africana*-derived type, fully effective in its native genetic milieu but limited to varying degrees of tolerance when transferred to *N. tabacum*. The objective of this study was to evaluate the pyramiding potential of these two sources of resistance in alloploid hybrids of *Nicotiana tabacum* x *N. africana*. To this end, amphidiploids involving *N. tabacum* cvs. VAM and Wiślica, each being the carrier of a different variant of the *va* gene, were created and advanced from F_1 to two subsequent selfed generations and to BC_1 generation with either VAM or Wiślica as the recurrent *N. tabacum* parent. The hybrid populations thus produced were challenged with two PVY necrotic isolates, IUNG 23 and IUNG 20, mild and severe isolate belonging to the $PVY^{N\text{-}Wi}$ and PVY^{NTN} groups, respectively. The mild isolate failed to infect any of the hybrids or parental species. The hybrids varied in their response to IUNG 20. The F_1 and F_2 populations were composed of asymptomatic hosts to PVY and of those showing mild vein clearing but no necrotic plants. There was a progressive increase in ability to resist PVY in successive amphidiploid generations, with 30 and 10% of fully resistant plants appearing in the F_3 amphidiploids involving VAM and Wiślica. Further research is needed to account for the appearance of segregants completely resistant to PVY, since neither *N. africana* factor is fully expressed in *N. tabacum*, nor do the *va* alleles confer resistance against PVY^{NTN} isolates.

Keywords: *Nicotiana tabacum*; *Nicotiana africana*; *va* gene; PVY resistance; PVY isolates; interspecific hybrids

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1. Introduction

Nicotiana tabacum is an important non-food crop used in the manufacture of tobacco products and it is grown in nearly 100 countries across the world. Poland ranks 27th among the world's tobacco producers and is the second tobacco producer in Europe. In Poland, tobacco production is an important source of income for farms in the areas where poor soils predominate [1]. The use of tobacco to produce biopharmaceuticals is also gaining in importance [2,3].

Tobacco veinal necrosis caused by potato virus Y (PVY) is a major threat to tobacco production. The disease affects leaf yields but also deteriorates crop quality. The symptoms include chlorotic spots of the leaf blade but also clearing and necrosis of leaf veins. Those changes restrict the transport of water and minerals and also negatively affect the uptake of nutrients and gaseous exchange. PVY infests many solanaceous species including potatoes, tomatoes and peppers [4–6].

PVY is spread from plant to plant by aphids [7,8]. According to Crosslin [9], it is the green peach aphid (*Myzus persicae*) that is the most effective PVY insect vector, but many other aphids also transmit the virus. In Poland, many years of research have provided evidence pointing to *Aphis fabae* and *Brachycaudus helichrysi* as the major PVY-transmitting aphids [10]. It is noteworthy that PVY is transmitted by aphids in a non-persistent manner [5]. This spread mechanism consists in that the virus particles become attached to the epithelium of the aphid stylet and do not circulate in the insect. The virus is released during successive feeding events, and in this manner, the aphids can spread the virus to a large number of plants during a short time [5,11]. The use of chemical treatments as a means of controlling PVY is largely ineffective, with the only discernible benefit being the reduction of aphid populations.

PVY is a member of the genus Potyvirus of the family Potyviridae. The PVY genome consists of 9700 nucleotides forming a single strand of sense RNA, the 5′ end of which is covalently linked to the viral protein genome (VPg) and the 3′ end of which is adenylated [12,13]. The virus is surrounded by the coat protein (CP). The infection of tobacco by PVY occurs when the virus protein VPg binds to the eukaryotic translation initiation factor eIF4E and to its isomeric forms. The eIF4E protein is a product of the *Va* gene [14,15].

Substantial variation arising from point mutations and also from recombination among isolates has resulted in the occurrence of many PVY strains [16–19]. Due to a high diversity among PVY strains, their classification was developed based on the data from biological, serological and molecular tests. Biological tests allowed five groups, PVY^{O}, PVY^{C}, PVY^{N}, PVY^{E} and PVY^{Z}, to be distinguished. The grouping was related to the capability of PVY strains to induce veinal necrosis in potato varieties carrying resistance genes *Ny*, *Nc* and *Nz*. Another criterion was the ability of those strains to induce veinal necrosis or leaf mottling in tobacco [5,20]. Four strains (PVY^{O}, PVY^{C}, PVY^{E} and PVY^{Z}) do not produce necrotic symptoms in tobacco and have varied responses in potatoes depending on the presence of resistance genes in particular varieties [11,20–23]. It is only the PVY^{N} strain that produces vein necrosis in tobacco. Two subgroups, $PVY^{N\text{-}Wi}$ and PVY^{NTN}, were distinguished within that strain [24]. The $PVY^{N\text{-}Wi}$ was found for the first time in the potato cultivar Wilga. The $PVY^{N\text{-}Wi}$ isolates produce mild mottling in potatoes [25], whereas the PVY^{NTN} isolates cause the potato tuber necrotic ringspot disease (PTNRD) [26]. Those two subgroups arose as a result of recombination between the genomes of PVY^{O} and PVY^{N} [5]. The distinction between the two subgroups is feasible by performing serological tests using antibodies manufactured by Bioreba AG [27]. It exploits the fact that the protein coat of $PNY^{N\text{-}Wi}$ is the same as that of PVY^{O} [28]. In recent years, there has been a significant increase in the population of recombinant strains, particularly PVY^{NTN} and $PVY^{N\text{-}Wi}$. Furthermore, PVY^{NTN} isolate shows the ability to break the resistance of the sources existing within *N. tabacum* [5,10,29–31].

The development of genetic resistance is crucial for effective management of the PVY disease. To this end, it is imperative to search for new sources of resistance and to experiment with combining the available ones. The resistance to PVY in some varieties of *Nicotiana tabacum* is controlled by a deletion within the *Va* susceptibility gene [32] and is referred to as *va*. Furthermore, the recessive *va* gene has three allelic variants, va^{0}, va^{1} and va^{2}. The recessive *va* gene is currently the main source of resistance to PVY in present-day commercial tobacco cultivars [33,34]. It is precisely this deletion that confers resistance to PVY to the varieties VAM and Wiślica, the maternal *N. tabacum* parents of the interspecific hybrids and their derivatives investigated in this study.

The *NtTPN1* gene that confers tolerance to PVY is another available source [35]. The investigations by Depta et al. [36] allowed the identification of five *N. tabacum* varieties that were tolerant: Złotolistny IHAR, LB Koro, Virginia Gold Dollar, Virginia 278 and Zamojska 4.

In the genus *Nicotiana*, *Nicotiana africana* is a species that carries complete resistance to PVY. It has been confirmed by exposing *N. africana* to a large number of PVY isolates of diverse virulence [11,37]. The species was used in numerous breeding studies aimed at

developing tobacco that is resistant to PVY [38–42]. Complete resistance to PVY was also found in the species *N. raimondii* and *N. knightiana* [43,44]. The deployment of those species for breeding purposes was difficult because of the recessive character of the resistance factor from *N. raimondii* [45]. A number of *Nicotiana* species showed resistance only to some PVY isolates. In addition, 27 species were tolerant of all PVY isolates to which they were exposed [46].

Breeding for resistance to PVY in tobacco is also possible using genetic transformation with the *Agrobacterium tumefaciens* [11,47]. Four tobacco varieties were transformed using a pROKY construct housing a modified PVY polymerase gene, as well as by using an LMVCP construction, the carrier of the lettuce mosaic virus coat protein gene [11]. Another example is the use of the PVY-replicase coding cDNA to transform *N. tabacum* cv. Burley 21 resulting in a high proportion of resistant transformants [47].

Another method available is the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), a gene-editing technology that allows precise single gene-targeted genome modification. Using that technology, the *Va* susceptibility gene in the LJ911 cultivar was switched off, and breeding lines resistant to PVY were obtained [48]. However, genetically engineered tobacco varieties are not being commercialized because of legal restrictions concerning GMOs.

The aim of this study was to evaluate the resistance to PVY in hybrid derivatives combining type *va* resistance from two varieties of *N. tabacum* with resistance from the PVY-immune species *N. africana.*

2. Materials and Methods

2.1. Plant Material

Interspecific hybrids and their derivatives were developed and studied at the Institute of Soil Science and Plant Cultivation, Puławy, Poland. The hybrids obtained from crossing two *N. tabacum* varieties, VAM (Figure S1a) and Wiślica (Figure S5a), with the wild species *N. africana* (Figure S1b) were used for the purpose of this study. Previously, in vitro cultures were used to develop the hybrids and their derivatives. Lloyd's medium [49] was deployed to overcome the mortality of seedlings obtained from crossing *N. tabacum* with *N. africana* [42]. Viable amphihaploids F_1 2x (*N. tabacum* cv. VAM x *N. africana* (Figure 1a and Figure S2a) and 2x (*N. tabacum* cv. Wiślica x *N. africana*) (Figure S6a) were thus obtained possessing 47 somatic chromosomes. Fertility was restored to the female and male sterile amphihaploids through the use of stem pith culture [42].

In this study, the following generations of the *N. tabacum* x *N. africana* hybrids of different cytogenetic status were tested for PVY response:

- Amphidiploids F_1 4x (*N. tabacum* cv. VAM x *N. africana (*Figure 1b and Figure S2b); 4x (*N. tabacum* cv. Wiślica x *N. africana*) (Figure S6b)—somatic chromosome number = 94, each hybrid having two haploid genomes from *N. tabacum* and *N. africana* as a result of chromosome doubling [42];
- Amphidiploids F_2 4x (*N. tabacum* cv. VAM x *N. africana* (Figure S3a); *N. tabacum* cv. Wiślica x *N. africana*) (Figure S7a)—somatic chromosome number = 94, obtained by self-fertilization of an amphidiploid plant of the F_1 generation that developed no disease symptoms after having been exposed to PVY (Figure 1c);
- Amphidiploids F_3 4x (*N. tabacum* cv. VAM x *N. africana* (Figure S3b); *N. tabacum* cv. Wiślica x *N. africana*) (Figure S7b)—somatic chromosome number = 94, obtained by self-fertilization of PVY-symptomless amphidiploid plants of the F_2 generation (Figure 1d);
- Sesquidiploids BC_1 3x (*N. tabacum* cv. VAM x *N. africana)* x *N. tabacum* cv. VAM (Figure 1e and Figure S4) and 3x (*N. tabacum* cv. Wiślica x *N. africana)* x *N. tabacum* cv. Wiślica (Figure S8)—somatic chromosome number = 71, obtained by mating symptomless F_1 amphidiploid plants to the maternal parent (*N. tabacum*).

Parental forms showing different responses to PVY were also included in the study:

- *Nicotiana tabacum* cv. VAM (chromosome number 2n = 48) (Figure 1f)—flue-cured market type cultivar of German origin, resistance to PVY controlled by the va^0 allele [50];
- *Nicotiana tabacum* cv. Wiślica (chromosome number 2n = 48)—flue-cured market type cultivar of Polish origin, resistance to PVY controlled by the va^1 allele [51];
- *Nicotiana africana* (chromosome number 2n = 46) (Figure 1g)—wild *Nicotiana* species indigenous to Africa; shows resistance to all PVY isolates to which it was exposed [37].

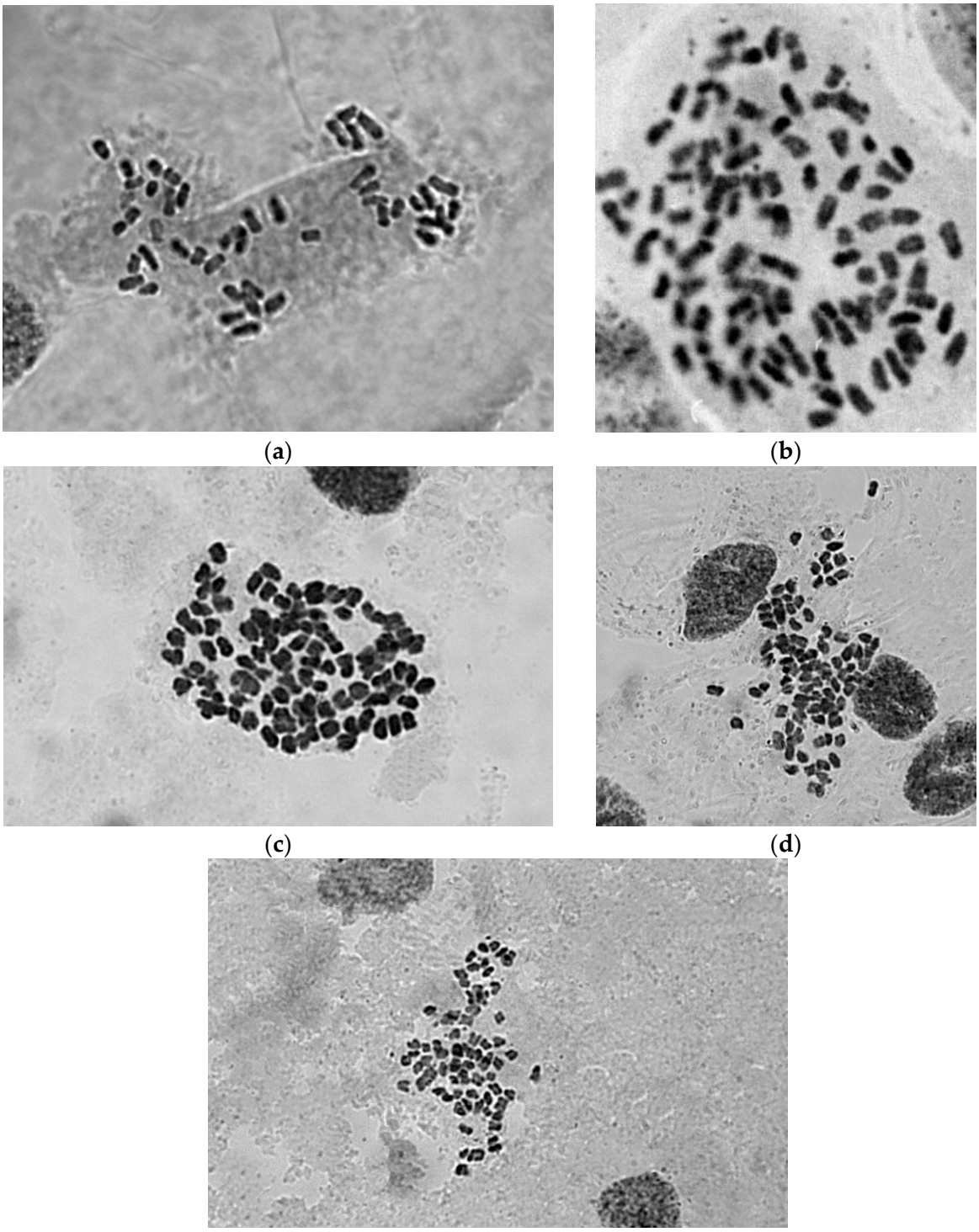
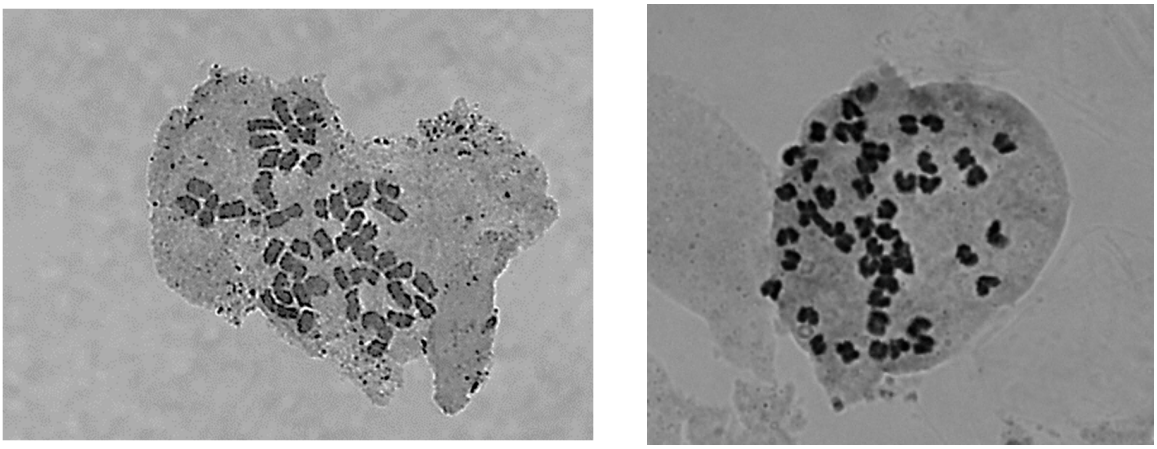


Figure 1. *Cont.*



(**f**) (**g**)

Figure 1. Mitotic chromosomes in the hybrid *N. tabacum* cv. VAM x *N. africana* and parental form: (**a**) F_1 amphihaploid 2n = 47; (**b**) F_1 amphidiploid 2n = 94; (**c**) F_2 amphidiploid 2n = 94; (**d**) F_3 amphidiploid 2n = 94; (**e**) BC_1 sesquidiploid 2n = 71; (**f**) *N. tabacum* cv. VAM 2n = 48; (**g**) *N. africana* 2n = 46.

2.2. Biological Tests

The degree of resistance to PVY shown by different generations of the *N. tabacum* x *N. africana* hybrids and their parents was evaluated by PVY inoculations performed in the greenhouse. Disinfected seeds were sown and individual seedlings were subsequently picked and replanted, each to a single pot. Testing for resistance involved 50 plants of each hybrid generation (F_1, F_2, F_3 and BC_1). Ten plants of each parental species, VAM, Wiślica and *N. africana*, were tested alongside. Additionally, ten plants of each of two other *N. tabacum* cultivars, Samsun H and Burley 21, highly susceptible to PVY, were used as inoculation efficiency checks.

The studied hybrids, their parents, and controls were challenged with two PVY isolates, differing in the degree of virulence:

- IUNG 23—referred to as a mild PVY isolate that fails to break the type *va* resistance in VAM, Wiślica and V. SCR, the three varieties each carrying a different *va* gene allele. The isolate is included in the $PVY^{N\text{-}Wi}$ group [26]. Another name for the $PVY^{N\text{-}Wi}$ is the $PVY^{N}W$ [25]. It is detectable by the cocktail of PVY monoclonal antibodies manufactured by Bioreba AG, Reinach, Switzerland that recognizes PVY isolates belonging to different strain groups but is not detected using the kit of monoclonal antibodies directed against the necrotic PVY serotype manufactured by Bioreba AG [27,52].
- IUNG 20—referred to as a severe PVY isolate since it breaks the *va* type resistance present in VAM, Wiślica and V. SCR, causing veinal necrosis. It is classified in the PVY^{NTN} isolate group and is detectable using either the Bioreba AG kit: PVY monoclonal cocktail or the monoclonal antibodies directed against the necrotic serotype [27,52].

A PVY-susceptible tobacco variety, Samsun H, was used in the tests to obtain the appropriate inoculum. Samsun H also carries the gene controlling resistance to tobacco mosaic (TMV), thereby ensuring that a pure, uncontaminated PVY isolate is obtained. Leaves of Samsun H, infected with an appropriate isolate, were ground in a mortar with a small amount of water. The extracted sap was rubbed into the surface of carborundum-dusted leaves of the tested plants. After inoculation, the plants were protected against direct sunlight for 48 h. Observations of disease symptoms were carried out two and four weeks after inoculation and scored as follows:

(a) Number of plants exhibiting no symptoms (ns);
(b) Number of plants displaying only vein clearing (VC);
(c) Number of plants exhibiting vein necrosis (VN).

The data obtained are presented as a percentage of each group of plants displaying a specific symptom in relation to the total number of plants in the generation under examination.

2.3. Serological Tests

To confirm the presence of the virus in the tested plants, DAS-ELISA immune-enzymatic tests were performed following 4 weeks after inoculation [53]. A mix of monoclonal antibodies manufactured by Bioreba AG directed against different PVY strains (PVY monoclonal cocktail) was used in the tests. Leaf fragments were sampled and homogenized with extraction buffer. The homogenisate was transferred to an antibody-precoated plate and incubated overnight at 4 °C. After rinsing the plate with washing buffer, a conjugate of the antibody linked to alkaline phosphatase was applied to the plate. The pNPP (p-Nitro Phenyl Phosphate) substrate was used to visualize the samples. The absorbance was measured at a 405 nm wavelength after 45 min following substrate application using a Tecan Sunrise reader. Both the negative control supplied by the manufacturer and the positive control (PVY-susceptible variety Samsun H) were used as inoculation checks.

Additional immune-enzymatic assays were performed to detect the presence of the virus in different parts of inoculated plants. After 6 weeks following the inoculation, from each of the two BC_1 populations, 10 plants showing veinal necrosis of their lower leaves were selected. The plants were assayed for the presence of the PVY virus in the 5th–6th leaf (the lower leaf) and the 7th–8th leaf (the upper leaf).

3. Results

The F_1, F_2 and F_3 generations of the amphidiploids *N. tabacum* cv. VAM x *N. africana* and *N. tabacum* cv. Wiślica x *N. africana* and their respective sesquidiploid (BC_1) derivatives involving either VAM or Wiślica as their recurrent *N. tabacum* parent differed among themselves and from the control varieties in terms of degree of resistance to the PVY virus. These differences were related both to the type of PVY isolate used as well as to how the parental forms responded when challenged with those isolates.

3.1. Response to Inoculation with IUNG 23 Isolate

None of the tested hybrid populations developed disease symptoms following the inoculation with IUNG 23, which qualified as a mild isolate (Table 1). This finding applied equally to the hybrid combinations propagated by selfing (F_1, F_2 and F_3 generations) or by backcrossing to the cultivated parent (BC_1) regardless of which *N. tabacum* parent was used (VAM or Wiślica). Two and four weeks after inoculation, in no case did the plants develop PVY symptoms, and no presence of PVY was detected in the sap of the tested plants by DAS-ELISA.

Table 1. Response of the hybrids *N. tabacum* x *N. africana*, their parental forms and inoculation checks when challenged with the IUNG 23 isolate.

Hybrid/Variety	Numer of Tested Plants	Symptoms After 2 Weeks (%) *			Symptoms After 4 Weeks * (%)			ELISA Assay ** (%)
		ns	VC	VN	ns	VC	VN	
N. tabacum cv. VAM x *N. africana*—F_1 (amphidiploid)	50	100	0	0	100	0	0	–
N. tabacum cv. VAM x *N. africana*—F_2 (amphidiploid)	50	100	0	0	100	0	0	–
N. tabacum cv. VAM x *N. africana*—F_3 (amphidiploid)	50	100	0	0	100	0	0	–
(*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM—BC_1 (sesquidiploid)	50	100	0	0	100	0	0	–

Table 1. *Cont.*

Hybrid/Variety	Numer of Tested Plants	Symptoms After 2 Weeks (%) *			Symptoms After 4 Weeks * (%)			ELISA Assay ** (%)
N. tabacum cv. Wiślica x *N. africana*—F_1 (amphidiploid)	50	100	0	0	100	0	0	–
N. tabacum cv. Wiślica x *N. africana*—F_2 (amphidiploid)	50	100	0	0	100	0	0	–
N. tabacum cv. Wiślica x *N. africana*—F_3 (amphidiploid)	50	100	0	0	100	0	0	–
(*N. tabacum* cv. Wiślica x *N. africana*) x *N. tabacum* cv. Wiślica—BC_1 (sesquidiploid)	50	100	0	0	100	0	0	–
N. tabacum cv. VAM	10	100	0	0	100	0	0	–
N. tabacum cv. Wiślica	10	100	0	0	100	0	0	–
N. africana	10	100	0	0	100	0	0	–
N. tabacum cv. Samsun H	10	0	0	100	0	0	100	+
N. tabacum cv. Burley 21	10	0	0	100	0	0	100	+

Explanations: ns (no symptoms), VC (vein clearing), VN (vein necrosis); *—percentage of plants showing a given resistance response (ns/VC/VN) ** DAS-ELISA assay was performed 4 weeks after inoculation; + virus present in sap; – virus not present in sap.

3.2. Response to IUNG 20 Isolate

Depending on the hybrid generation tested, a varied resistance response to PVY was recorded after inoculation with the IUNG 20 isolate referred to as a severe or virulent isolate (Table 2). The amphidiploids of the F_1, F_2 and F_3 generations of *N. tabacum* x *N. africana* involving both VAM and Wiślica did not develop veinal necrosis on either date of observation.

Within two weeks after inoculation, 70% of the F_1 amphidiploid plants involving VAM as the *N. tabacum* parent did not develop any disease symptoms and 30% developed vein clearing. Four weeks after inoculation, the number of symptomless plants went down to 20%, whereas the remaining 80% developed vein clearing. Similarly, in the F_1 amphidiploid involving Wiślica as the *N. tabacum* parent, none of the plants developed infection symptoms within two weeks after inoculation, but after 4 weeks, the percentage of symptomless plants dropped to 30%, and the remaining 70% developing vein clearing. However, when assayed with DAS-ELISA, all F_1 amphidiploid plants involving either VAM or Wiślica as the *N. tabacum* parent were found to contain the virus in their sap.

In the subsequent F_2 generation of the amphidiploid involving VAM, within 2 weeks following inoculation, 80% of plants remained symptomless and 20% developed vein clearing. Within the next two weeks, the counts remained unchanged. The F_2 amphidiploid involving Wiślica responded differently: the percentage of symptomless plants decreased from 90% observed two weeks after inoculation to 80% on the second observation date (four weeks after inoculation). The remaining plants developed vein clearing symptoms. This notwithstanding, DAS-ELISA confirmed the presence of the virus in the sap of both asymptomatic and symptomatic plants.

In the third generation of the amphidiploid (F_3), the exposure to the severe IUNG 20 isolate produced an identical response, regardless of whether VAM or Wiślica was involved in the pedigree of the hybrid. The plants developed no disease symptoms on either observation date, 2 or 4 weeks following inoculation. Furthermore, some of the plants tested negative for the presence of PVY in the sap: 30% of the F_3 plant population derived from the amphidiploid involving VAM and 10% of the F_3 plants having Wiślica as the *N. tabacum* parent. In both populations, the remaining asymptomatic plants tested positive for the presence of PVY in the sap.

Table 2. Disease symptoms in the hybrids, parental varieties and controls and the results of DAS-ELISA assay following inoculation with the IUNG 20 isolate.

Hybrid/Variety	Numer of Tested Plants	Symptoms After 2 Weeks (%) *			Symptoms After 4 Weeks * (%)			ELISA Assay ** (%)
		ns	VC	VN	ns	VC	VN	
N. tabacum cv. VAM x *N. africana*—F_1 (amphidiploid)	50	70	30	0	20	80	0	+
N. tabacum cv. VAM x *N. africana*—F_2 (amphidiploid)	50	80	20	0	80	20	0	+
N. tabacum cv. VAM x *N. africana*—F_3 (amphidiploid)	50	100	0	0	100	0	0	−/+ (30/70)
(*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM—BC_1 (sesquidiploid)	50	68	32	0	14	10	76	+
N. tabacum cv. Wiślica x *N. africana*—F_1 (amphidiploid)	50	100	0	0	30	70	0	+
N. tabacum cv. Wiślica x *N. africana*—F_2 (amphidiploid)	50	90	10	0	80	20	0	+
N. tabacum cv. Wiślica x *N. africana*—F_3 (amphidiploid)	50	100	0	0	100	0	0	−/+ (10/90)
(*N. tabacum* cv. Wiślica x *N. africana*) x *N. tabacum* cv. Wiślica—BC_1 (sesquidiploid)	50	48	52	0	12	18	70	+
N. tabacum cv. VAM	10	0	100	0	0	0	100	+
N. tabacum cv. Wiślica	10	0	100	0	0	0	100	+
N. africana	10	100	0	0	100	0	0	−
N. tabacum cv. Samsun H	10	0	0	100	0	0	100	+
N. tabacum cv. Burley 21	10	0	0	100	0	0	100	+

Explanations: ns (no symptoms), VC (vein clearing), VN (vein necrosis); *—percentage of plants showing a given resistance response (ns/VC/VN); ** DAS-ELISA assay was performed 4 weeks after inoculation; + virus present in sap; − virus not present in sap.

A different situation was observed in the BC_1 populations obtained from backcrossing the F_1 amphidiploids to their respective *N. tabacum* parents (VAM and Wiślica). In the sesquidiploids (*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM, 68% of plants developed no symptoms within two weeks after inoculation (Figure 2a); the remaining 32% developing vein clearing. No vein necrosis was recorded on that date. However, after another two weeks, only 14% of the plants remained symptomless, 10% developing vein clearing (Figure 2b) and 76% veinal necrosis (Figure 2c) on lower leaves. A different pattern of PVY symptom development was observed in the sesquidiploid involving Wiślica as the recurrent *N. tabacum* parent. Following two weeks after inoculation, 48% of the plants remained symptomless, 52% developing vein clearing symptoms. Four weeks after inoculation 12% of the plants remained symptomless, 18% developed vein clearing, and 70% developed veinal necrosis of lower leaves. In no case was necrosis found on the upper leaves. All plants of the two sesquidiploid populations tested positive for the presence of the virus regardless of whether they developed PVY infection symptoms or remained symptomless.

The varieties of *N. tabacum* used in the study showed visible signs of infection with the virulent PVY isolate. However, in Samsun H and in Burley 21, the necrotic response occurred as soon as 2 weeks after inoculation, whereas in VAM and in Wiślica, the plants initially developed vein clearing, the necrotic symptoms appearing on a later date. The

presence of the virus in those varieties was confirmed by DAS-ELISA. It is only the species *N. africana* that resisted the infection by IUNG 20, the fact confirmed by a negative result of the serological test.

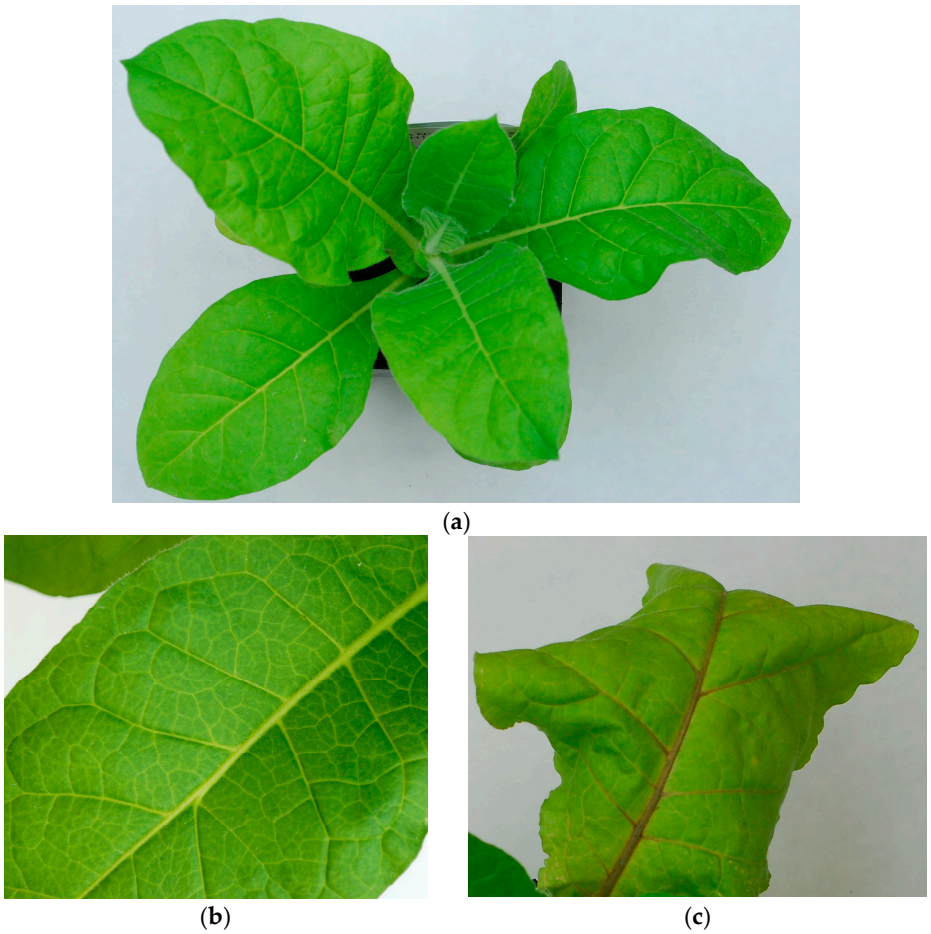


Figure 2. Resistance response of the sesquidiploid (*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM to the severe PVY isolate IUNG 20: (**a**) symptomless plant two weeks after inoculation (**b**) vein clearing on leaves four weeks after inoculation (**c**) vein necrosis on leaves four weeks after inoculation.

The observations on how the virus moved to the upper parts of the plant revealed certain differences in this movement depending on the hybrid involved (Table 3). In the BC_1 hybrid involving VAM as the maternal parent in two out of ten plants, the DAS-ELISA assay showed the presence of PVY in upper leaves. Likewise, in the sesquidiploid population with Wiślica as the recurrent maternal parent, the presence of the virus was found in the upper leaves of three plants only. In the remaining plants of that population, only the lower leaves tested positive for presence of the virus.

Table 3. DAS-ELISA performed on selected BC_1 plants of the hybrid *N. tabacum* x *N. africana* having either VAM or Wiślica as the recurrent *N. tabacum* parent.

Hybrid	Number of Tested Plants	Positive Result of ELISA Assay	
		Lower Leaves *	Upper Leaves **
(*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM—BC_1	10	10	2
(*N. tabacum* cv. Wiślica x *N. africana*) x *N. tabacum* cv. Wiślica—BC_1	10	10	3

Explanations: * 5th–6th leaf; ** 7th–8th leaf.

4. Discussion

The core finding of this study is the identification of individuals fully resistant to a severe PVY^{NTN} isolate within advanced selfed populations of the amphidiploid *N. tabacum* x *N. africana* created to combine *va*-controlled resistance of the *N. tabacum* parent with that contributed by *N. africana*. Previously, based on near-isogenic genotypes of *N. tabacum* cv. K 326, Lewis [40] demonstrated an improved effect of combining *va*-type resistance to PVY with *N. africana*-derived factor (*Nafr*) vs. the effect of the *va* gene and *Nafr* acting alone. However, in Lewis's study, no fully resistant combinations of *va* x *Nafr* were reported. An implication for future strategies of interspecific gene transfer ensuing from this report is the importance of the amphidiploid stage for inducing novel and desirable genetic rearrangements [54], especially when simple backcrossing fails to produce the desired results.

There are many and often unpredictable barriers that the interspecific breeders have to surmount to achieve their specific goals. These difficulties stem from a variety of incongruities between species that include pre-fertilization barriers such as different manifestations of cross incompatibility and post-fertilization incongruities such as embryo abortion and mortality of hybrid plants at different development stages [55–58]. The premature death of juvenile hybrid seedlings is the chief obstacle in hybridizing *N. tabacum* with *N. africana* [55,56]. As part of the preparatory stage of this study, a culture of cotyledon explants was used to bypass that barrier and amphihaploids 2x Nicotiana *tabacum* x *N. africana* were successfully grown to maturity [42]. In turn, the sterility of amphihaploid hybrids, another manifestation of interspecific incongruity, was managed by doubling their chromosomes in stem pith cultures [42,58].

The expression of *va* type resistance to PVY present in *N. tabacum* varies greatly depending on which *va* variant is interacting with which PVY strain [36,51]. On the other hand, the complete resistance present in *N. africana* undergoes significant reduction in efficacy once the resistance factor is transferred to *N. tabacum* [39,40]. Resistance to PVY of the *N. tabacum* parents involved in the hybrids is conferred by the recessive *va* gene, its va^0 allele carried by VAM and the va^1 allele by Wiślica [59]. The two varieties express different levels of resistance to the virus. The German variety VAM (Virgin A Mutant) arose by irradiation with the mutagenic X-rays [50]. In this manner, a deletion on chromosome 21 [59], ca. 1 Mb in size [60], was obtained. However, the sizes of deletion within the *Va* region may differ [32], resulting in different levels of susceptibility to PVY [61]. Wiślica, a Polish-bred variety was derived by conventional pedigree selection from the cross between another Polish breeding line and an American cultivar. Its deletion within the *Va* region is shorter than that of VAM's [51]. The superiority of the resistance present in VAM to that conferred by the other variants of the *va* gene was established in a number of studies [36,51,62]. The observed superiority may be attributed to the fact that the va^0 allele is actually composed of two distinct alleles, namely, va^1 and va^2. The va^2 allele is responsible for obstructing virus movement from cell to cell, whereas the va^1 allele constrains the build-up of the virus in plant cells. [63]. Julio et al. [32] screened 168 accessions of *N. tabacum* for resistance to PVY using the PVY^N isolate and identified 45 accessions as resistant. Based on molecular analysis, the resistant accessions were divided into four groups that differed for the size of the deletion within the *Va* gene, controlling susceptibility to PVY. The degree of resistance shown by accessions in those groups was found to be closely related to how large was the deleted part of the *elF4E-1* gene and of other genes. The deletion of the entire *elF4E-1* gene that occurs in VAM confers the highest degree of resistance but does not make the plant immune to all PVY isolates since the virus is able to bind to another protein coded by the genes of the *elF4E* group [61].The amphidiploids and sesquidiploids of the hybrid *N. tabacum* x *N. africana* were evaluated for resistance to PVY using two isolates, $PVY^{N\text{-}Wi}$ and PVY^{NTN}, currently the most dangerous strains attacking tobacco fields [5,10,29]. Of these two strains, PVY^{NTN} isolates are particularly hazardous as they are capable of breaking *va* type resistance, most widely used in present-day tobacco cultivars [10].

None of the hybrid populations tested in this study became infected upon exposure to the IUNG 23 PVY isolate (PVY^{N-Wi} group). It applied equally both to the F_1, F_2 and F_3 amphidiploids and to the BC_1 sesquidiploids. The absence of the virus in plant tissues was corroborated by DAS-ELISA analysis. Additionally, the PVY^{N-Wi} isolate was unable to infect the maternal forms: *N. tabacum* cvs. VAM and Wiślica. Likewise, Korbecka-Glinka et al. [51] demonstrated that six isolates belonging to the $PVY^{N}W$ group failed to overcome the resistance of VAM and Wiślica.

The other isolate (IUNG 20) used in this study belongs to the PVY^{NTN} strain termed as severe. It broke the type *va* resistance of VAM and Wiślica in a previous study by Depta et al. [36]. Similarly, Korbecka-Glinka et al. [51] reported that both VAM and Wiślica developed veinal necrosis upon exposure to the PVY^{NTN} isolates that they used in their study. In this study, in none of the amphidiploid populations (F_1, F_2 or F_3) was veinal necrosis observed upon challenging them with IUNG 20. Furthermore, there was a progressive overall improvement in the degree of resisting PVY^{NTN} infection among the amphidiploid plants as their populations were advanced from the 1st to the 3rd generation. The difference between the F_1 vs. F_2 populations was chiefly in terms of how fast mild infection symptoms would manifest themselves, the PVY symptoms developing generally slower in the F_2 populations. Another conspicuous tendency was the decreasing percentage of plants finally developing mild infection symptoms (vein clearing) as the selfed populations exposed to IUNG 23 were advanced from F_1 to F_3. Mildly infected plants, prevalent in the F_1 populations, depending on the amphidiploid formula, dropped to 10 or 20% in the F_2 and to nil in both F_3 populations. Not present in the F_1 and F_2 amphidiploid populations, a novel category of plants appeared in the F_3 generation. Those plants did not develop external symptoms of infection and there was no virus in their tissues upon exposure to IUNG 20, and therefore, they could be considered fully resistant. They accounted for 30% of the F_3 population involving VAM as the *N. tabacum* parent and for 10% of F_3 plants derived from crossing Wiślica with *N. africana*. The remaining and preponderant class in the F_3 generation were symptomless hosts of the virus with none of them developing visible infection symptoms.

The majority of plants in the successive amphidiploid generations exposed to the PVY^{NTN} isolate contained PVY virus in their tissues either developed mild infection symptoms or remained asymptomatic. Such forms are usually termed tolerant [41,64,65]. Some authors also recognize mild vein necrosis as a tolerant response [66]. However, tolerance in this sense is a broad and somewhat ambiguous term as it comprises two or more distinct categories and, for clarity's sake, was deliberately avoided in accounting for the results of this study.

It must be noted in the context of this discussion that unlike the F_1 amphidiploids studied here, a hybrid of *N. tabacum* x *N. africana* involving the weak (va^2) variant of the *va* gene and exposed to a necrotic PVY^{NZ} isolate studied by Doroszewska [67] expressed near-full resistance of the *N. africana* parent, developing no visible symptoms of infection and reduced levels of the virus in the sap. This said, it is difficult to account for the increasing proportion of asymptomatic vs. symptomatic plants in successive selfed generations of the amphidiploids reported here. It could have been the effect of exerting pressure towards improved resistance by picking symptomless plants to produce consecutive generations of the amphidiploid. As mentioned previously, the amphidiploid stage offers the best opportunity for interspecific chromosomal interchange. The direct evidence for such an interchange in the amphidiploid *N. wuttkei* x *N. tabacum* was furnished by Laskowska et al. [68], where the presence of recombinant chromosomes was revealed by genomic in situ hybridization. Amphidiploids of *N. tabacum* x *N. africana*, although relatively stable, formed trivalents and, occasionally, associations of higher valencies, indicating that the flow of genes between the two species and gene rearrangements via translocation or segmental substitution are entirely possible in that gene transfer stage [41,69].

Up to now, attempts to recover full resistance of *N. africana* in the genetic milieu of *N. tabacum* have failed [40,41,51,70] and only lineages differing in the degree of tolerating

the infection were developed. In those lines, the tolerant response was obtained either through the intermediary stage involving an alien addition line [40,70] or by backcrossing, resulting in *N. tabacum* recovering *N. tabacum* phenotypes in which the tolerance-conferring factor from *N. africana* was present either as translocation or segmental substitution [41]. The appearance of apparently fully resistant individuals in the 3rd generation of the amphidiploid under study may indicate some novel gene rearrangement resulting, e.g., in the loss or switching off of the *va* locus [48] or in some new epistatic interactions that possibly can be passed on to and selected in further backcross generations. These results also provide a clue that it may be worthwhile to screen carefully selfed amphidiploid populations for individuals with complete or improved ability to resist PVY to be used as starting material in future attempts to transfer resistance from *N. africana*. The compromised expression of resistance to PVY from *N. africana* in currently available introgressions is probably related to polygenic inheritance (unspecified accessory genes) or to unwelcome gene interactions [40,41,70].

Like in the amphidiploids, in the sesquidiploid (BC_1) generation, the response to infection by the PVY^{NTN} strain followed a very similar pattern, regardless of the *N. tabacum* parent involved. The preponderance of necrotic individuals in the BC_1 population would corroborate the recessive character of the major resistance gene from *N. africana*. A digenic pattern of inheritance is another explanation, but it must assume a unidirectional loss of *N. africana* chromosomes in the BC_1 generation. The mitotic chromosome number of 71 unequivocally points to regular sesquidiploid status of those populations, thereby disproving that assumption. Actually, the presence of both symptomless hosts and plants developing vein clearing in the BC_1 populations points to a more complex inheritance where more genes from both species are involved, in this particular case, most probably also including the *va* alleles contributed by the N *tabacum* parent. The latter possibility is indirectly supported by the final counts of plants developing vein clearing in the BC_1 populations involving VAM (10%) and Wiślica (18%). It appears that the incidence of vein clearing in the presence of the stronger va^0 allele from VAM was reduced nearly by half compared to that recorded in the presence of the weaker va^1 allele from Wiślica.

5. Conclusions

This manuscript presents an evaluation of PVY resistance in interspecific hybrids combining *va* type of resistance from two *Nicotiana tabaccum* cultivars (VAM and Wislica) with that of the PVY-immune species *Nicotiana africana*. The most significant achievement of our research is the generation of plants showing resistance to the highly virulent PVY^{NTN} isolate in amphidiploid F_3 populations of *N. tabaccum* x *N. africana* hybrids. These plants represent a valuable source of PVY resistance, which will be the subject of further investigation. Furthermore, research will concentrate on eliminating unfavorable traits derived from *N. africana* in order to obtain resistant forms with good performance characteristics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14122284/s1, Figure S1. The plants of parental forms: (a) *Nicotiana tabacum* cv. VAM 2n = 48; (b) *Nicotiana africana* 2n = 46; Figure S2. The hybrid forms: (a) amphihaploid F_1 2x (*N. tabacum* cv. VAM x *N. africana*) 2n = 47; (b) amphidiploid F_1 4x (*N. tabacum* cv. VAM x *N. africana*) 2n = 94; Figure S3. Hybrids forms: (a) amphidiploid F_2 4x (*N. tabacum* cv. VAM x *N. africana*) 2n = 94; (b) amphidiploid F_3 4x (*N. tabacum* cv. VAM x *N. africana*) 2n = 94; Figure S4. Sesquidiploid BC_1 3x (*N. tabacum* cv. VAM x *N. africana*) x *N. tabacum* cv. VAM 2n = 71; Figure S5. The plants of parental forms: (a) *Nicotiana tabacum* cv. Wiślica 2n = 48; (b) *Nicotiana africana* 2n = 46; Figure S6. The hybrid forms: (a) amphihaploid F_1 2x (*N. tabacum* cv. Wiślica x *N. africana*) 2n = 47; (b) amphidiploid F_1 4x (*N. tabacum* cv. Wiślica x *N. africana*) 2n = 94; Figure S7. Hybrids forms: (a) amphidiploid F2 4x (*N. tabacum* cv. Wiślica x *N. africana*) 2n = 94; (b) amphidiploid F3 4x (*N. tabacum* cv. Wiślica x *N. africana*) 2n = 94; Figure S8. Sesquidiploid BC_1 3x (*N. tabacum* cv. Wiślica x *N. africana*) x *N. tabacum* cv. Wiślica 2n = 71.

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