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Review

The Use of the Polish Germplasm Collection of *Nicotiana tabacum* in Research and Tobacco Breeding for Disease Resistance

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Abstract: The Polish germplasm collection of *Nicotiana tabacum* was started in the 1920s. Up to now, more than eight hundred accessions originating from different regions of the world have been gathered in the collection. It includes valuable breeding lines and obsolete cultivars, among them cytoplasmic male-sterile lines. Numerous cultivars are rich sources of features desired in tobacco breeding. Therefore, the accessions are continually characterised in terms of their various features, one of the most important of which is disease resistance. Much research is being done to explain the nature of resistance and its genetic basis. Moreover, cultivars with good agronomic characteristics are used in wide hybridisation, being recipients of resistance genes from wild species or are genetically modified with transgenes conditioning resistance. The biological diversity of cultivars also allows a proper selection of plant material for pathogen studies, while the large number of the accessions facilitates research into the conditions for long seed storage. Numerous examples of the use of Polish tobacco germplasm in research and breeding, specifically in disease resistance, have been presented in this paper.

Keywords: tobacco breeding; resistance; potato virus Y; tomato spotted wilt virus; tobacco mosaic virus; black root rot; blue mould; seed storing; cytoplasmic male sterility

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1. Introduction

The Polish collection of *Nicotiana tabacum* cultivars initiated in the 1920s was started by Prof. Lucjan Kaznowski. He started to collect cultivars imported from abroad for use in domestic breeding. The first accessions included in the collection were Bulgarian cultivars Enidże Stary and Kaba-Kułak. By 1930, the collection consisted of 25 tobacco cultivars coming from different European countries. In the next decade, the collection was expanded to include 150 more cultivars. In subsequent years, it was gradually enriched with cultivars and valuable breeding lines, and currently there are 803 accessions in the collection. The latest ones were added in 2019.

One third of accessions are cultivars or breeding lines obtained in Polish plant breeding centres. Many have been derived as a result of breeding programs conducted for several decades at the Institute of Soil Science and Plant Cultivation (IUNG), Puławy, Poland. Over seventy accessions originate from the USA and a similar number were obtained from the former USSR, mostly from its European part, the Krasnodar Region. Numerous accessions come from France, Germany, Romania, Italy, Hungary, Canada and Australia. There are also cultivars obtained from other European countries, as well as from South America, Asia and Africa, totalling 30 countries in all. The gathered tobacco germplasm is diverse and represents all known tobacco types. There we can find light, dark, flue-cured, air-cured, oriental, broadleaf and cigar tobacco. Moreover, besides standard cultivars, there are alloplasmic lineages and mutants. Many cultivars have resistance to common tobacco threatening diseases and are therefore a valuable material that can be used in breeding.

Collection accessions are stored as seed samples in the storage room at a temperature of 4 °C at IUNG. They are regenerated every few years. For this purpose, seeds are sown and seedlings are planted in the field of the agricultural experimental station near Puławy. Duplicate seed samples are preserved in the storage of the National Centre for Plant Genetic Resources: Polish Genebank, located in The Plant Breeding and Acclimatization Institute (IHAR), Radzików, Poland. The accessions are distributed to researchers, breeders, farmers and hobbyists all over the world.

2. Characterisation of Tobacco Accessions in Terms of Resistance to Diseases

One of the aims of a plant collection is to maintain biodiversity. Tobacco cultivars and breeding lines are sources of various traits that can be used for further breeding. An essential goal is to obtain cultivars resistant to diseases. Therefore, it is important to characterise accessions in respect of resistance sources. For the purpose of resistance breeding in tobacco, genotypes are being screened for resistance to diseases (Table 1).

Table 1. Sources of resistance to the most important diseases of tobacco within accessions maintained in the Polish Genebank of *N. tabacum*.

Cultivar	Origin of Resistance	Inheritance	References
	blue mould (*Peronospora tabacina*)		
Sirogo, Sirone	*N. goodspeedii*	unknown	[1]
Criollo Correntino, Chileno Correntino	unknown	-	[2]
Hicks Resistant, Bel 61-9, Bel 61-10	*N. debneyi*	oligogenic, partially dominant	[2–4]
Chemical Mutant	induced mutation of cv. Virginia Gold	monogenic, partially dominant	[2]
GA 955	*N. excelsior*	unknown	[2]
Wiślica	*N. tabacum* cv. Ovens 62	unknown	[5]
	powdery mildew (*Erysiphe cichoracearum*)		
Kokubu	mutation within genes *NtMLO1* and *NtMLO2*	digenic, recessive	[6]
	black root rot (*Berkeleyomyces sp.*)		
AC Gayed	*N. debneyi*	monogenic, dominant	[7]
TN 86, TN 90	*N. debneyi*	monogenic, dominant	[2,8]
	tobacco mosaic virus (TMV)		
Samsun H, Buley 21, Kutsaga Mammoth, Vamorr 50	*N. glutinosa*	monogenic, dominant	[4,7,9,10]
Ambalema	unknown	digenic, ressessive	[4,10]
	potato virus Y (PVY)		
VAM (Virginia A Mutant)	gene *va* (mutation within susceptibility gene)	monogenic, recessive	[11]
TN 86, TN 90	gene *va* from VAM	monogenic, recessive	[2,12]
Havana IIC, V.SCR (Virginia SCR), Bursan, Wiecha, Bachus, Wiktoria, Weneda	gene *va*	monogenic, recessive	[5,13]
Virginia Kaznowskiego, Wilia, Wisła, Złotolistny IHAR	probably from oriental cultivars	-	[2]
Wiślica, Elka 245, Wika	gene *va*	monogenic, recessive	[14]
Węgierski Ogrodowy	unknown	-	[13]
Lechia A, Zamojska 4	gene *NtTPN1*	monogenic, recessive	[15]
	tomato spotted wilt virus (TSWV)		
Polalta, Wiktoria	*N. alata*	monogenic, dominant	[16]

The table contains an updated description presented by Laskowska [2].

One of the most economically important viral diseases is tobacco veinal necrosis, caused by some strains of potato virus Y (PVY). Isolates belonging to strain groups PVY^O and PVY^C induce only mosaic symptoms on tobacco while these from groups PVY^N and PVY^{NTN} cause veinal necrosis [17]. There are numerous tobacco cultivars with recessive resistance of *va* type, which is the result of a deletion within the gene of translation initiation factor 4E (eIF4E) [14]. The effectiveness of *va* type of resistance present at different cultivars gathered in the Polish Genebank was tested in many studies. Depta et al. [18] observed the development of PVY infection on four tobacco cultivars: susceptible Samsun H and Burley 21, as well as resistant VAM and Wiślica. They showed that highly virulent isolates (PVY^N and PVY^{NTN}) can break the resistance of VAM and Wiślica but symptoms developed more slowly compared to susceptible cultivars.

Korbecka-Glinka et al. [19] widened these studies using nine cultivars and one breeding line in inoculation tests with ten diverse PVY isolates belonging to PVY^{NTN} and PVY^N groups. They selected resistant cultivars: V.SCR, PBD6, TN 86, VAM and Wiślica and susceptible ones: BP-210, K 326, NC 95 and Samsun H. Both VAM and Wiślica proved to be the most effective sources of resistance as they developed no symptoms and had no virus detected in a serological test of four PVY^NW isolates. In the case of cultivars TN 86 and V.SCR the virus was not found in the tissues of plants inoculated with three isolates and one isolate, respectively. In contrast, the tested breeding line BPA, which was derived from a cross *N. tabacum* cv. BP-210 × *N. africana* [20], showed tolerance to all ten PVY isolates, which was manifested by only mild symptoms of vein clearing, chlorotic spots and absence of veinal necrosis despite the presence of the virus in plant tissues.

Depta et al. [13] determined the presence of the *va* type of resistance in the case of 25 cultivars from the genebank resources and tested its effectiveness by inoculating plants with four PVY isolates. Their studies confirmed the resistance of VAM, Wiślica and V.SCR and showed PVY resistance of light flue-cured cultivars Wiecha, Wiktoria, Weneda and light air-cured cv. Bachus. An ineffectiveness of the *va* gene was revealed for a few other accessions which developed necrotic symptoms. Some tested materials showed PVY tolerance manifested by only mild symptoms without necrosis in spite of the lack of a resistance gene as was the case for the above-mentioned BPA line. Interestingly, the authors found that Hungarian dark air-cured cv. Węgierski Ogrodowy, which amplified markers associated with PVY susceptibility, remained symptomless after inoculation with a mild PVY isolate. Such results indicate a different genetic basis of resistance of this cultivar.

As the cultivars vary in their degree of resistance, researchers are looking for reasons for these differences. VAM is supposed to carry two recessive alleles va^1 (limiting virus cell-to-cell movement) and va^2 (limiting virus accumulation) [14,21] which contribute to the high effectiveness of its resistance. Michel et al. [14] studied durability of PVY resistance depending on the type of deletion and, among other things, they included Polish cultivars Wiślica, Wika and Elka 245. They indicated that Wiślica has large deletion of over 1 Mb at *eIF4E-1* gene-like cultivars regarded as the most resistant, such as VAM, TN 86 and PBD6. However, the durability of its resistance is a little weaker compared to TN 86 and VAM because of a lower expression level of the *eIF4E-2* gene (being an ortholog of the *eIF4E-1* gene) which was proved through RNAseq and qRT-PCR analyses. In turn, the resistance of cvs Wika and Elka 245 is even weaker as they have a smaller deletion.

Julio et al. [5] tested 92 *N. tabacum* accessions coming from different research facilities including cultivars Wiślica and Bursan from the Polish Genebank. The authors identified polymorphic fragments associated with sources of resistance to three pathogens. It was demonstrated that Wiślica did not show resistance to *Thielaviopsis basicola* (currently *Berkeleyomyces basicola*, syn. *Chalara elegans*) but it had resistance to two pathogens: potato virus Y (*va* type of resistance) and *Peronospora tabacina* (inherited from Australian cv. Ovens 62) while Bursan had the same type of resistance to PVY. In fact, the neighbour joining method indicated close relationship between both cultivars.

Some cultivars show a tolerance to the virus that is only mild symptoms without necrosis, in spite of the lack of a resistance gene. French researchers studied the genetic basis

of PVY tolerance of several accessions from the Imperial Tobacco germplasm collection [15]. Among them there were cultivars originally from the Polish gene resources such as Lechia A, Lechia LB, Zamoyska and Zamojska 4. The locus responsible for tolerance was mapped in F_2 populations coming from crossing two tolerant cultivars Lechia A and Zamojska 4 with susceptible Virginia 115 and Yellow Special, respectively. Using the previously developed SSR (Simple Sequence Repeat) markers [22,23], the researchers found the gene conferring tolerance on chromosome 13 and named it as *NtTPN1* [15]. Lechia (syn. LB-838) is a flue–cured cultivar bred at IUNG, derived from crossing Polish cv. LB-Koro with Australian cv. Hicks Resistant, carrying resistance to blue mould. PVY tolerance of LB-Koro, Zamojska 4 and a few other cultivars was also shown by Depta et al. [13].

The interesting accessions maintained in the Polish Genebank are cultivars resistant to tomato spotted wilt virus (TSWV). One of them is Polalta: a Polish cultivar with resistance originating from *N. alata* [24]. Polalta belongs to the group of dark-cured tobacco similar to the Puławski type [25]. The introgression from the wild species has been located in linkage group 7 in the region between 0 and 40 cM, which was indicated by comparing the genome sequence of Polalta with those of *N. tabacum* and *N. alata* [26]. Resistance of Polalta is conditioned by a single dominant gene: *RTSV-al* [27]. Unfortunately, it is associated with other genes responsible for morphological deformations such as thickened, abnormal ribbon-shaped leaves as well as the tendency to form tumours [16,24]. Moreover, deformations are more severe in hybrid forms obtained, which hinder the use of Polalta in breeding. Therefore, AFLP (Amplified Fragment Length Polymorphism) markers and then, on their basis, SCAR (Sequence Characterised Amplified Region) markers have been developed for TSWV resistance which could be helpful in reducing the size of *N. alata* introgression in backcrossed hybrids. The markers were tested also in cv. Wiktoria originating from the same interspecific hybrid as cv. Polalta [24]. Wiktoria comes from crossing this hybrid with cvs Virginia Skroniowska 78, V.SCR and Wiślica [28]. As a result of the morphological deformations, neither Polalta nor Wiktoria are commercially cultivated, but they have been included into the genebank as a source of valuable resistance to an important tobacco disease. Their resistance is the hypersensitive type which was proved by Laskowska et al. [16] in tests including artificial inoculation with TSWV, whereby all tested plants belonging to Polalta and only 4 of 15 plants within Wiktoria showed resistance. The rest of Wiktoria plants did not amplify SCAR markers and remained susceptible.

Tobacco collection accessions have been also tested for resistance to black root rot caused by fungal pathogen *Thielaviopsis basicola*. Berbeć and Trojak-Goluch [29] tested six flue-cured tobacco cultivars originating from Germany cv. V.SCR (syn. VD), Polish cultivars Wilia and Wiślica (accessions in the Polish Genebank) and Hungarian ones: Hevesi 9, VJ 1 and VJ 17. Moreover, they tested two breeding lines TB-570N and PTU-1098. On the basis of the results, they described VJ 17 as very resistant, VJ 1 and line PTU-1098 as moderately resistant, Wilia, Wiślica and Hevesi 9 as moderately susceptible and line TB-570N as susceptible.

There are some cultivars demonstrating resistance to tobacco mosaic virus (TMV) among tobacco accessions in the Polish Genebank. Sixty-two cultivars were tested by Depta et al. [10] in respect of reaction to TMV inoculation. One of them was Samsun H, a cultivar bred in 1938 by Holmes, who transferred resistance to *N. tabacum* cv. Samsun from *N. glutinosa* which possessed a single dominant *N* gene responsible for hypersensitive reaction to TMV infection [30,31]. Samsun H and other oriental cultivars such as Diubek 556, Newrokop 261 and Samsun 155 showed hypersensitivity [10]. Similar reactions were noticed in the case of a few other cultivars belonging to dark-cured, Burley and Virginia types. The presence of an *N* gene was detected in all these cultivars. In turn, dark-cured cv. Ambalema, in spite of the lack of an *N* gene, showed tolerance in that its mosaic symptoms were not observed in the infected plants but the presence of the virus was detected with serological tests. However, both resistance and tolerance of tested accessions were broken after increasing the temperature above 28 °C, causing systemic necrotic response.

3. Tobacco Breeding

Changing climatic conditions, increasing pathogen pressure and the growing demands of the tobacco industry are contributing to continual progress in breeding new cultivars. The Polish Genebank is rich resource of *N. tabacum* cultivars which have been used in tobacco breeding (Table 2).

Table 2. The use of tobacco cultivars from the Polish germplasm collection in breeding.

Cultivar	Tobacco Type	Origin	Source of Resistance	Resistance for	Transfer Method	Effect	References
AC Gayed	flue-cured	Canada	transgene with PVY replicase gene	PVY	*Agrobacterium* transformation	resistant breeding lines	[32–35]
BP-210	flue-cured	Poland	*N. africana*	PVY	interspecific hybridisation	tolerant breeding line	[20,32]
			N. benavidesii	PVY	interspecific hybridisation	resistant hybrids BC_1F_3	[36,37]
BY 103	air-cured	Japan	*N. glauca*	black root rot	interspecific hybridisation	amphidiploids, post-sesquidiploids with diverse resistance	[38,39]
			transgene with PVY replicase gene; transgene with LMV coat protein gene	PVY	*Agrobacterium* transformation	resistant breeding lines, DH lines	[32–34,40]
Izyda	flue-cured	Poland	*N. knightiana*	PVY	interspecific hybridisation	amphihaploids	[36]
K236	flue-cured	USA	*N. debneyi*	black root rot	crossing with cv. Wentura	resistant doubled haploids	[41]
			N. glauca	black root rot	interspecific hybridisation	post-sesquidiploids with diverse resistance	[38,39]
			transgene with PVY replicase gene; transgene with LMV coat protein gene	PVY	*Agrobacterium* transformation	resistant breeding lines	[32–34]
Mc Nair 944 (MN 944)	flue-cured	USA	transgene with PVY replicase gene; transgene with LMV coat protein gene	PVY	*Agrobacterium* transformation	resistant breeding lines	[32–34]
Nadwiślański Mały	dark air-cured	Poland	*N. africana*	PVY	interspecific hybridisation	amphihaploids	[42,43]
Nadwiślański Mały tetraploid	dark air-cured	Poland	*N. alata*	TSWV	interspesific hybridisation	sesquidiploids	[44]
Puławski 66	dark-cured	Poland	*N. wuttkei*	blue mould	interspesific hybridisation	amphihaploids	[45]
TB 566 tetraploid	flue-cured	Poland	*N. alata*	TSWV	interspecific hybridisation	sesquidiploids	[46]
TN 90	air-cured	USA	*N. wuttkei*	blue mould	interspecific hybridisation	non-viable hybrids	[45]
VAM	flue-cured	Germany	*N. africana*	PVY	interspecific hybridisation	amphidiploids	[47]
Virginia 278	flue-cured	Germany	*N. africana*	PVY	interspecific hybridisation	sesquidiploids	[42,43]
Virginia Gold Dollar	flue-cured	USA	*N. africana*	PVY	interspecific hybridisation	amphidiploids	[42,43]
Virginia SCR	flue-cured	Germany	*N. africana*	PVY	interspecific hybridisation	sesquidiploids	[42,43]
Virginia Skroniowska 78, V.SCR, Wiślica	flue-cured	Poland	*N. alata*	TSWV	interspecific hybridisation	resistant cv. Wiktoria	[28]

Table 2. *Cont.*

Cultivar	Tobacco Type	Origin	Source of Resistance	Resistance for	Transfer Method	Effect	References
Wiślica	flue-cured	Poland	*N. alata*	TSWV	intercultivar hybridisation with cv. Polalta	resistant DH lines	[48]
			N. africana	PVY	interspecific hybridisation	amphidiploids	[47]
			N. wuttkei	blue mould	interspecific hybridisation	sesquidiploids	[45,49]
			N. glauca	black root rot	interspecific hybridisation	resistant breeding lines WGL	[50,51]
Zamojska 4	flue-cured	Poland	*N. raimondii*	PVY	interspecific hybridisation	amphihaploids	[36]

3.1. Breeding for PVY Resistance

When tobacco veinal necrosis became a serious problem in Poland in the late 1950s and then the early 1960s [37], the breeders were faced with the need to breed resistant cultivars that were also adapted to the country's climatic conditions. Since then, there has been an ongoing search for sources of PVY resistance within *Nicotiana* species that could be used in the breeding of new tobacco cultivars [52].

At that time, cultivar BP-210 became popular with breeders. It is a good quality flue-cured cultivar bred at IUNG by selection from an Australian breeding line. It is resistant to blue mould but because of a high susceptibility to PVY it has been withdrawn from cultivation [53]. As a result of crossing with *N. benavidesii*, carrying resistance to PVY, the interspecific hybrids have been obtained [36]. Moreover, the tetraploid form of BP-210 was fertilised with pollen of *N. benavidesii* to obtain sesquidiploids which then were backcrossed with diploid BP-210, resulting in obtaining resistant plants [37].

A promising source of resistance has been transferred to *N. tabacum* from a wild species by Doroszewska and Berbeć [42,43], who crossed *N. africana* with five tobacco cultivars selected from among Polish Genebank accessions. They used flue-cured cultivars: V.SCR, Virginia 278 and BP-210 (Polish cultivars) and American cv. Gold Dollar and dark-cured Polish cv. Nadwiślański Mały. Next, hybrids resulting from crossing BP-210 and *N. africana* served to obtain breeding line BPA which showed tolerance to all tested PVY isolates [20,32,54]. A further attempt to transfer resistance from *N. africana* was crossing this species with VAM and Wiślica in order to enhance the resistance of these cultivars [47].

In turn, Polish flue-cured cultivar Izyda was applied in an attempt to transfer resistance from a wild species *N. knightiana* [36]. Another Polish cv. Zamojska 4 was crossed first with cv. LB-838 (syn. Lechia) and the hybrids showed tolerance to PVY; that is, they developed only mild vein clearing after inoculation but no systemic symptoms. Then the hybrids were used as the male parent in crossing with *N. raimondii* and, in the case of the amphidiploid of the hybrid and segregating backcross populations, an interesting phenomenon was observed. Both factors, i.e., tolerance from the tabacum parent (both Lechia and Zamojska 4 have tolerance to PVY) and resistance from *N. raimondii*, stopped each other resulting in strong susceptibility [36,55].

Resistance to PVY has been also obtained by genetic transformation of four flue-cured cultivars; two American ones MN 944 and K 326 and Japanese BY 103 as well as one coming from Canada, AC Gayed [32]. All of them are accessions of the Polish Genebank. They were transformed with three constructs containing modified PVY replicase gene with in sense (ROKY1) and antisense (ROKY2) orientation as well as coat protein gene of lettuce mosaic virus (LMV CP). Analysis of the resistance in successive generations of transgenic plants and their agronomic traits has allowed for selection of the two most promising lines: cv. MN 944 with transgene LMV CP and cv. AC Gayed with transgene ROKY2 [33,34]. Next, they have been crossed to obtain advanced generations [56] as well as stable double transgenic hybrid lines with high resistance [57]. Moreover, transgenic lines MN 944 LMV CP and AC Gayed ROKY2 were crossed with cultivars with resistance of *va* type, VAM

and Wiślica, which resulted in obtaining hybrid lines carrying two different sources of resistance to PVY. Of these VAM × MN 944 LMV CP and Wiślica × MN 944 LMV CP proved to be resistant even to a strong PVY isolate [58]. Moreover, a hybrid line originating from crossing MN 944 LMV CP and cv. Wiślica served as a material for obtaining double haploids [35]. The tobacco transgenic lines with resistance to PVY, and enhancing it by combining with *va* resistance, widened variability within the species *N. tabacum*.

3.2. Breeding for TSWV Resistance

The attempt to transfer TSWV resistance to *N. tabacum* was made by Berbeć [44], who crossed one of the cultivars maintained in the Polish Genebank, Nadwiślański Mały, with *N. alata*, which is a source of resistance to the virus. However, he obtained hybrids with low survival rates and complete sterility, which made continuation of the breeding process impossible. It was only by using a tetraploid form of *N. tabacum*, cv. Nadwiślański Mały or cv. TB 566, that viable hybrids could be obtained [44,46]. However, backcrossing to *N. tabacum* failed [46]. The use of a bridging species proved to be key to breeding success. The first cultivar resistant to TSWV was obtained by Gajos who used *N. otophora* as bridging species and thereby managed to transfer the resistance from *N. alata* to *N. tabacum* [7,25]. Unfortunately, these breeding efforts were not sufficiently documented, as it is not clear to which tobacco cultivar. Nevertheless, this way cv. Polalta was obtained [24,25,59]. An interspecies hybrid, which was used to obtain Polata, was also crossed with cultivars in the Polish collection such as Virginia Skroniowska 78, V.SCR and Wiślica. The results of these efforts was cultivar Wiktoria [28]. Both Polalta and Wiktoria have been included into the Polish Genebank as a source of valuable germplasm because they represent very few sources of TSWV resistance available today within *N. tabacum*. So far, attempts to use Polalta in crossbreeding with other tobacco cultivars have been unsuccessful because the hybrids show a number of morphological malformations. Laskowska and Berbeć [48] tried to break this phenomenon by anther culture obtaining haploid plants, originating from crossing Polalta with Wiślica and then doubled haploids. As a result, they bred three double haploid lines PW-833, PW-834 and PW-900 resistant to TSWV and simultaneously showing morphological deformations milder than those characteristic for Polalta.

3.3. Breeding for Resistance to Fungal Diseases

Resistance to black root rot has been transferred to *N. tabacum* from a wild species *N. glauca*. From the Polish germplasm collection, two cultivars, BY103 and K326, have been selected for interspecific hybridisation [38]. The obtained amphihaploids showed high resistance while the level of resistance of the sesquidiploids and post-sesquidiploids varied [39]. The resistance from *N. glauca* was also transferred to cv. Wiślica [50] which resulted in the obtention of resistant breeding lines WGL [51], and finally a resistant commercial cultivar [60]. It is noteworthy that breeding line WGL was also crossed with doubled haploid line PW-834 carrying resistance from Polalta in order to obtain first haploids and then doubled haploid lines DH3 and DH6 with resistance to both black root rot and TSWV [26,61,62]. Next, the lines were crossed with a high-quality flue-cured cultivar WAC 121D7 renamed as Wentura (with resistance to both black root rot and PVY) and the obtained plants of F_4 generation carrying *N. alata* introgression showed resistance to TSWV and simultaneously had morphology without deformations characteristic for Polalta [63]. One of the WGL lines was also crossed with line BPA carrying resistance to PVY [64]. Moreover, cv. K 326 served also as a good quality component in crossing with cv. Wentura carrying resistance to black root rot transferred from *N. debneyi*. Next, 24 doubled haploid lines were produced from the hybrids and five of them were completely resistant [41].

Tobacco cultivars were also crossed with *N. wuttkei* carrying resistance to another fungal disease dangerous for tobacco plantations: blue mould caused by *Peronospora tabacina*. The initial studies on the possibility of crossing *N. tabacum* with the wild species were made with the use of three cultivars from the Polish Genebank: Wiślica, Puławski

and TN 90. All of them are somewhat resistant to blue mould. The seedlings F_1 were not able to survive but the authors managed to obtain amphihaploids originating from crossing *N. wuttkei* × *N. tabacum* cv. Wiślica and *N. wuttkei* × *N. tabacum* cv. Puławski from cotyledons under in vitro conditions [45]. Next, the amphidiploids from hybrids *N. wuttkei* × *N. tabacum* cv. Wiślica were produced by in vitro chromosome doubling and backcrossed with Wiślica giving a single sesquidiploid plant [49]. Unfortunately, the amphidiploid plants could not be backcrossed, as male parents to *N. tabacum* and sesquidiploids would not cross as male components with *N. tabacum*. Therefore, the cytoplasmic inheritance could not be reversed to that of *N. tabacum*. Hence, further breeding progress was not possible because of the massive onset of cytoplasmic male sterility in further backcrossed populations [45,49]. Nevertheless, the obtained hybrids can be regarded as a new synthetic species carrying resistance to blue mould.

The use of cultivars from the Polish Genebank in breeding to combine sources of resistance to different diseases in a single genome is presented in the diagram (Figure 1).

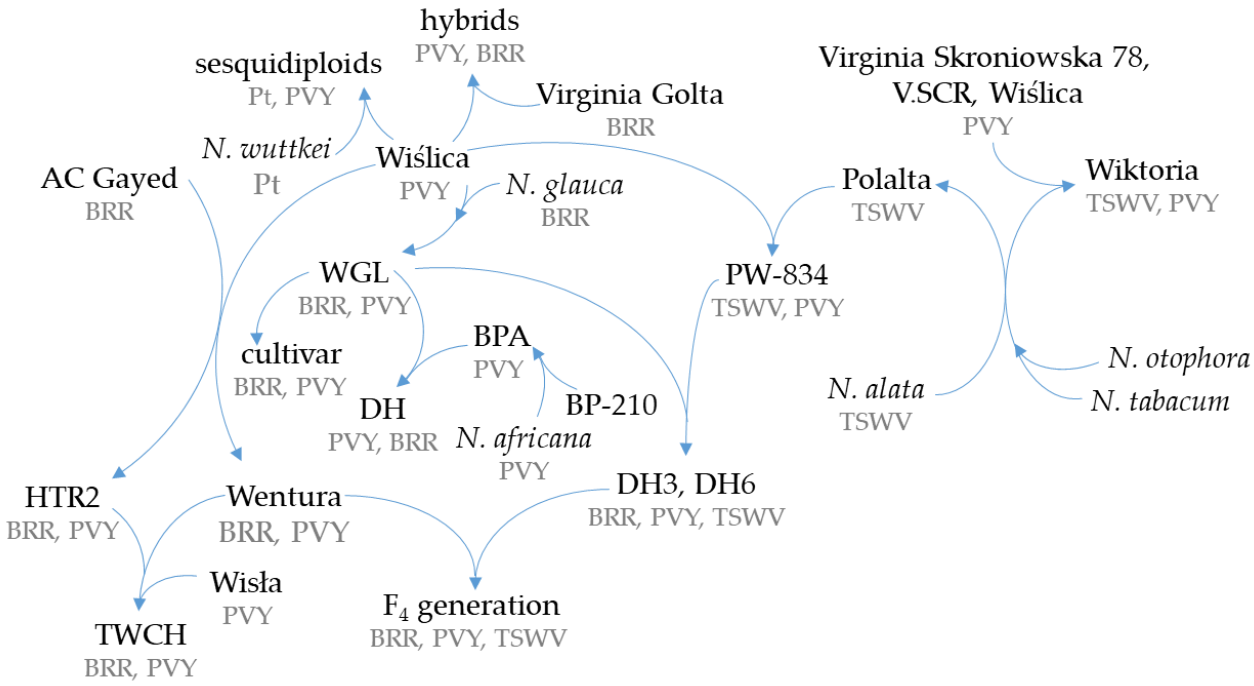

Figure 1. The use of cultivars from the Polish germplasm collection in tobacco breeding leading to combining resistance sources to different diseases. Breeding materials: *Nicotiana* species: *N. tabacum*, *N. alata*, *N. africana*, *N. othophora*, *N. wuttkei*; cultivars: AC Gayed, Wiślica, Virginia Golta, Virginia Skroniowska 78, V.SCR, Polalta, Wiktoria, BP-210, HTR2, Wentura, Wisła; breeding lines: WGL, BPA, PW-834, TWCH (three-way-cross hybrid), DH, DH3, DH6 (doubled haploid lines). BRR, PVY, TSWV, Pt–resistance to black root rot, PVY, TSWV and *Peronospora tabacina*, respectively, carrying in breeding materials.

4. Cultivars with Cytoplasmic Male Sterility (cms) and Their Application in Breeding for Disease Resistance

Cytoplasmic male sterility, which is the result of incompatibility between the genome of the cell nucleus and the cytoplasm, is used in plant breeding to obtain forms incapable of self-pollination which makes it easier for a breeder to carry out controlled production of hybrids for commercial use and prevents unauthorised reproduction of seeds [65]. Cytoplasmic male-sterility is mostly obtained as a result of the substitution of cytoplasm from a different species in place of the native cytoplasm and, in autogamic plants, less frequently may be the result of a spontaneous mutation [66,67].

The Polish collection of *N. tabacum* includes over fifty accessions which are cytoplasmic male sterile. Most of them are the result of the work of scientists from IUNG, obtained on

the base of Polish cultivars BP-210, BP-Koro, Lechia, Nadwiślański and Puławski. Moreover, there are several accessions of Zamojska 4 and Wiślica with cytoplasm originating from different wild *Nicotiana* species, and Wiślica with cytoplasm from an *N. tabacum* mutant. However, some alloplasmic forms such as Zamojska 4 cms *goodspeedii*, Zamojska 4 cms *bigelovii* and Zamojska 4 cms *suaveolens* were received from the Tobacco Research Board in Zimbabwe while other ones with cytoplasm replaced from *N. glutinosa*, *N. megalosiphon*, *N. occidentalis*, *N. plumbaginifolia* and *N. undulata* were obtained from the Russian research facility WITIM in Krasnodar [67]. A few more alloplasmic forms were obtained from other foreign breeding centres such as cv. Erzegowina cms from Italy or a breeding line from USA.

Alloplasmic forms develop modified, non-functional male generative organs or none at all. The morphological changes may also affect other parts of the flower and sometimes the entire plant, for example the habit of forms cms with cytoplasm from *N. goodspeedii* and *N. megalosiphon* differ significantly from the initial cv. Zamojska 4 [67]. Moreover, alloplasmic forms of that cultivar developed no stamens, malformed ones or petaloid, stigmatoid structures in their place [66,67]. In the case of Wiślica, most of the alloplasmic forms develop abnormal or no stamens. A few cms forms differed in leaf area and plant height relative to their fertile counterpart and, for example, Wiślica cms *bigelovii* provided higher yield [68]. Among the many alloplasmic forms gathered in the tobacco germplasm collection, Zamojska 4 *knightiana* stands out because it has retained partial fertility in contrast to the others which are completely sterile. Therefore, this particular cms form was used by Berbeć [69] to conduct studies to determine whether the presence of alien cytoplasm caused changes in the nuclear genome. For this purpose, he compared offspring of Zamojska 4 cms *knightiana* obtained by self-pollination and that backcrossed with Zamojska 4. The selfed progeny showed depressive agronomic performance; therefore, the author concluded that it may be a result of mutations within the nuclear genome which accumulated over generations while such a phenomenon did not occur in the case of the use of pollen of autoplasmic Zamojska 4.

The presence of alien cytoplasm can also affect other characteristics, including the plant resistance to pathogens [65,66]. That is why cms forms must be well characterised in order to determine their real suitability for breeding. Forms of Zamojska 4 with cytoplasm substituted from *N. eastii* and *N. plumbaginifolia* species were severely infected with powdery mildew under field conditions in a season characterised by a high incidence of the pathogen, while the other cms forms showed tolerance [67].

As far as the response to PVY infection was tested, under conditions of artificial inoculation it was found that the use of cytoplasm from the species *N. amplexicaulis*, *N. debneyi*, *N. exigua*, *N. glauca*, *N. glutinosa*, *N. knightiana* and *N. raimondii* did not contribute to changes in the level of tolerance responses of alloplasmic male-sterile forms of Zamojska 4. On the contrary, the use of cytoplasm from the species *N. goodspeedii*, *N. megalosiphon* and *N. undulata* resulted in an increased tolerance of the cms forms and delayed symptoms, whereas the presence of cytoplasm from the species *N. eastii*, *N. occidentalis* and *N. suaveolens* resulted in decreased tolerance. Changes in the resistance response also affected the cms form with cytoplasm of the *N. tabacum* mutant [66]. The type of cytoplasm also differentiated the occurrence of symptoms of PVY infection and cercosporiosis on male-sterile forms of Wiślica under natural pressure of the pathogens causing these diseases. Alloplasmic forms with cytoplasm substituted from *N. bigelovii*, *N. occidentalis*, *N. undulata*, *N. exigua* and *N. suaveolens* showed increased symptoms of *Cercospora* sp. infection, with the last two showing an additional decreased resistance to PVY compared to cv. Wiślica [68].

Hybrids originating from crossing Wiślica and Virginia Golta (maintained in the Polish Genebank German cultivar carrying resistance to black root rot) with four kinds of alien cytoplasm also varied in respect of susceptibility to PVY, black root rot, cercosporiosis, as well as in respect of agronomic performance, which demonstrates one more time that the choice of cytoplasm source is important in cultivar breeding [70].

Cytoplasmically male-sterile cvs HTR2 and Wentura were used by Berbeć [71] in comparing three-way cross hybrids of tobacco to single-cross ones. The cultivars originated from crossing of two cultivars maintained in the Polish Genebank: Wiślica and AC Gayed (Figure 1). The hybrid F_1 resulting from crossing of HTR2 cms × Wentura was crossed with another cultivar from the Polish collection, Wisła, or with the local cvs VP 06 and VB 08. The resulting three-way cross hybrids were compared in respect of agronomic performance to single-cross ones in which HTR2 cms and Wentura cms were used as maternal parents in crossing with Wisła, VP 06 or VB 08. Currently, at least two three-way cross hybrids are grown as commercial cultivars in Poland (VRG 5 TL and VRG 10 TL) [72].

5. Studies on Viral Pathogens

Some collection accessions are used in research, of which they are not the subject but play an important role in the preparation of the experiment proper. This is because the collection accessions are well characterised in terms of their numerous features, so the researcher can choose from among them the one that best meets his/her expectations. An example of a cultivar often used in numerous studies on viral pathogens or plant resistance, is the oriental cv. Samsun H. It is in the plants of Samsun H that the multiplication of viruses is carried out. Indeed, Samsun H shows hypersensitivity-type resistance to tobacco mosaic virus–the pathogen which is very common in tobacco crops and often accompanies infection with less common viruses. Therefore, the multiplication of the virus in Samsun H makes it possible to purify a viral isolate from TMV.

Moreover, cv. Samsun was used in the identification of a virus that had been noticed in tobacco crops for the first time in 2004 in Poland, Germany and Hungary [73]. Artificial inoculation of Samsun plants resulted in chlorotic lesions and severe necrosis of leaves as well as stunting of plants. The molecular and serological studies led to the identification of the virus as Colombian Datura virus.

In turn, other tobacco cultivars, whose resistance to the PVY virus was already well established, were used in the studies of this virus. Three cultivars Wiślica, VAM and Samsun H differing in resistance to PVY were the test set in studies identifying molecular point mutations in several viral isolates coming from Poland, Germany and Croatia [74]. The use of three cultivars allowed formation of a characteristic of isolates in terms of symptom severity depending on the kind of source of resistance. Samsun H reacted with susceptibility to all isolates while Wiślica and VAM showed disease symptoms as a result of inoculation with 8 of 15 PVY isolates. Sequencing viral genomes revealed that point mutations within their VPg region were responsible for the ability to break resistance in VAM and Wiślica. The authors deduced that there has been a change in the amino acid sequence by substitution of lysine with threonine in position 105 in resistance-breaking isolates. They also conferred another changes previously found by other researchers [75,76].

6. Seed Response for Long-Term Preservation

When plant gene resources are stored in the form of seeds, it is extremely important to optimise the conditions of the storage facility in such a way that the seed samples will remain viable for as long as possible. The longer the seeds remain viable, the longer the periods between their regeneration can be. The lower the frequency of regeneration, the lower the cost of maintaining the collection and labour intensity, but most importantly the lower the risk of genetic erosion of accessions.

Therefore, it is important to conduct research to determine the viability of seeds maintained in storage facilities. Such kinds of test should be made for each plant species. Accessions from the tobacco collection served as research material in studies of the viability of seeds stored under conditions of different humidity and ambient temperature [77]. The authors showed that the storage in reduced relative humidity (RH = 50.5 ± 6.3 %) was essential for maintenance of the seeds in a viable state. A proportion of 0.4 of seed lots stored under these conditions for 12 years still were able to spawn the normal seedlings from at least 75% of seeds, while none of those maintained in the conditions with higher

humidity (RH of about 77%) retained that ability. In turn, germination tests on *N. tabacum* seeds belonging to 227 collection accessions stored at 0 °C for 33 years indicated that only 10% of accessions retained the ability to germinate in no less than 75% of seeds [78]. In comparison, seeds stored in the Genebank of the Leibniz-Institut (IPK, Gatersleben, Germany) at a temperature of −15/−18 °C maintained a longer vitality at this level; that is, more than half of tested *Nicotiana* accessions had a high capacity of germination even after 40 years. The seeds stored in IPK had an approximate moisture content of 6% while the content of those in the Polish Genebank was 4%. Therefore, one should bear in mind that although temperature proved to be a key factor in seed viability, seed moisture may also be essential.

7. Conclusions

Maintaining the Polish germplasm collection of tobacco is primarily done to protect valuable breeding materials and obsolete cultivars which are no longer commercially grown. The need for long-term storage of seeds makes it possible to study the impact of external conditions on preserving their viability. However, caring for a collection is not limited to keeping accessions in a viable condition and genetically pure, but also presents a challenge in that one is obliged to constantly learn about them. This is especially true, as *N. tabacum* accessions come from many regions of the world and therefore represent a huge genetic diversity. Research aims are dictated, on the one hand, by the development of modern research methods, and on the other hand, by the needs of farmers and the tobacco product market. That is why many researchers focus on the study of resistance sources and the possibility of using them to breed new cultivars that will be used in current commercial cultivation. It seems that the direction of further research on *N. tabacum* should be to search for accessions resistant to biotic and abiotic stresses resulting from climate change.

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