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Evaluation of Responses of Potato Cultivars to Potato Spindle Tuber Viroid and to Mixed Viroid/Viral Infection

Olga S. Afanasenko [1,*], Nina M. Lashina [1], Nina V. Mironenko [1], Elena I. Kyrova [1], Elena V. Rogozina [2], Natalia G. Zubko [1] and Aleksander V. Khiutti [1]

[1] All-Russian Institute of Plant Protection, 196608 St. Petersburg, Russia
[2] N. I. Vavilov All-Russian Institute of Plant Genetic Resources, 190031 St. Petersburg, Russia
* Correspondence: olga.s.afan@gmail.com

Abstract: Potato spindle tuber viroid (PSTVd) is a harmful quarantine disease with wide geographic distribution. To date, experimentally proved resistance or tolerance of potato cultivars to PSTVd has not been reported. The aim of this study was to evaluate responses to four PSTVd strains of 39 modern potato cultivars of different origin. Four PSTVd strains of different origin, the intermediate VP35, VP87, and two sever strains FP10-13 and NicTr-3, deposited in GenBank, were used. Transcripts of these strains were used to inoculate tomato plants of the cv. Rutgers. Before PSTVd inoculation with tomato sap, all plants were tested for viral infection by ELISA. The presence of PSTVd in infected plants was verified by RT-PCR as well as by RT-qPCR at sixty days post-inoculation (dpi). The strain-specificity in the response of cultivars to viroid infection was revealed. Five cultivars were identified in which, after the first inoculation of plants with all PSTVd strains, normal in shape tubers were formed. All plants of the next generation derived from infected but normally shaped tubers showed strong symptoms of disease. PSTVd and mixed viroid/viral infection (PVY + PSTVd, PVM + PSTVd, and PVY + PVS + PSTVd) led to a significant decrease in the number and weight of tubers in most of the cultivars studied.

Keywords: potato cultivars; potato spindle tuber viroid (PSTVd); symptoms; tolerance; mixed viroid/viral infection

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1. Introduction

The potato spindle tuber viroid (PSTVd) is a single-stranded, highly structured ring RNA about 360 nucleotides long that lacks any protein-coding sequences [1]. PSTVd belongs to the family *Pospiviroidae* and the genus *Pospiviroid*. In vivo infection with PSTVd besides potatoes has been found mainly in plants of the family *Solanaceae*, such as pepino, tomato [2], and petunia [3]. A wide range of hosts has been found in artificial infestation, with at least 138 species from ten families. Most of them were symptomless carriers of PSTVd [4].

Symptoms of potato plants infected with PSTVd are stunted, with thinning stems. Yellowing, necrosis, and various leaf deformations occur, and infected tubers become small and deformed. The degree of PSTVd-induced symptoms depends on the strain and of the host cultivars' susceptibility [5]. Additionally, the decrease in potato productivity depends on the duration of cultivation of infected tubers and agrotechnics and may vary from 20–30% to 90–100% [6]. At the same time, Mackie et al. [7] determined that yield loss from PSTVd was significant regardless of variety and crop: 50–90% for potato tubers and 52–89% for tomato fruit.

Although PSTVd is subject to external quarantine, the disease has a wide geographic distribution. PSTVd on either potatoes or tomatoes has been detected on all continents (EPPO/CABI, accessed on 21 August 2022) [7]. In Russia between the years 1960–1970, PSTVd was widespread in the Volga region and was listed as a major potato disease in

this region [8]. In the 1990s, the ubiquitous spread of PSTVd in Russia, including the Far East [9,10] and northwest Russia [6], was noted. Recent studies, using modern detection methods, have shown that the problem of viroid infection in Russia remains: PSTVd was detected in 10.3% of randomly sampled potato tubers from the Volga Federal District and in 3.5% from the Far Eastern Federal District by RT-PCR [11]. Malko et al. [12] also identified 2% PSTVd infected potato tubers/leaves out of 197 samples studied in the Nizhny Novgorod Region.

To date, experimentally proved resistance or tolerance of potato cultivars to PSTVd has not been reported. Singh [13] found two clones of *Solanum berthauitii* Hawkes from seventeen tested plants which were resistant to the mild and severe strains of PSTVd. Such studies have also been conducted on tomatoes, in which tolerance even to lethal strains of PSTVd was found in two wild tomato species: *S. pimpinellifolium* L. LA0373 and *S. chmielewskii* (C.M. Rick et al.) D.M. Spooner et al. (SOLAN_CHM) LA1028 [14].

The aim of this study was to evaluate responses to four PSTVd strains (intermediate and severe to tomato cv. Rutgers) of 39 modern potato cultivars of different origins, most of which were registered in the Russian State Register of Breeding Achievements and symptoms of mixed viroid/viral infection.

2. Materials and Methods

2.1. Plant Materials

The research material is represented by 39 potato cultivars: 19 from Russia (Avrora, Ariel, Armada, Bizon, Elizaveta, Krepysh, Meteor, Mirazh, Navigator, Nevskiy, Sapsan, Sineglazka 2016, Sprinter, Siurpriz, Udacha, Favorit, Phioletovyi, Flagman, Charoit), 15 from Western Europe (Arizona, Bellarosa, Bernina, Vineta, Gala, Donata, Impala, Colomba, Queen Anna, Labadia, Madeira, Nandina, Riviera, Sanibel, Sorentina), one cultivar from Kazakhstan (Berkut), two cultivars from Belarus (Briz and Manifest), and two breeding lines from A.G. Lorch Federal Research Center for Potato (1923-3 and 4434-1) (Table S1).

Pre-basic (mini-tubers and super-super elite) and basic (super elite and elite) categories of the studied cultivars tubers were obtained from the seed farm Agricultural consumer supply and market cooperative "Ustyuzhenskiy potato" (Ustyuzhna, Vologda region). Most of the studied cultivars are registered in the Russian State Register of Breeding Achievements. Six cultivars (Armada, Bizon, Mirazh, Navigator, Sapsan, Flagman) and two advanced breeding lines (1923-3 and 4434-1) are not yet included in the Register (Table S1).

2.2. PSTVd Strains

Four PSTVd strains were used to evaluate the tolerance of potato varieties: VP35 (LC523658), VP87 (LC523667), FP10-13 (LC523676), and NicTr-3 (LC654171). These strains were chosen based on their different geographic origin and their different aggressiveness against a highly susceptible tomato cv. Rutgers. Strains VP87 and VP35 were isolated from potato leaves collected in the Volga Federal District, strain FP10-13 from potato tubers from the Far Eastern Federal District in 2019 [11]. The PSTVd strain NicTr-3 was isolated from potato tubers of cv. Nikulinskiy from Novosibirsk Region in 2021. All strains are deposited in the international information database DDBJ (DNA Data Bank of Japan, Data set "Viruses" http://blast.ddbj.nig.ac.jp/blastn?lang=ja, accessed on 21 August 2022).

The aggressiveness of these strains was assessed by symptoms on the tomato cv. Rutgers (*Solanum lycopersicum*): VP35 and VP87 were characterized as intermediate and FP10-13 as severe [11]. The strain NicTr-3 also showed severe symptoms on the tomato cultivar Rutgers (unpublished data).

Viroid RNAs were transcribed from viroid cDNAs and used as inoculums. cDNA of four PSTVd strains was synthesized (4 μg of each strain) in LLC "BioPharmExpert" (St. Petersburg, Russia) using plasmid pUC57 (https://www.genscript.com/vector/SD1176-pUC57_plasmid_DNA.html (accessed on 1 August 2022). It was given to us on paper disks for subsequent elution. Viroid RNAs synthesis was performed using the T7 RiboMAX TM Express Large Scale RNA production System (Promega) transcription mix according

to the manufacturer's protocol and used as an inoculum for indicator tomato plants of cv. Rutgers, according to the method reported by Matsushita and Penmetcha [15].

2.3. Plant Inoculation and Tolerance Assessment

Potato and tomato plants were grown in a growth room at a temperature of 25 ± 2 °C with a photoperiod of 16 h of light and 8 h of dark under Phytolamps (TL-FITO L1517 88 VR, LED Brand: OSRAM OSLON® SSL) and adequately watered. Each tuber was planted in 2000 cm^3 plastic pots filled with "Terra vita" soil. Seven-day germination potato plants and 14-day tomato plants were used for inoculation by PSTVd.

To prepare the inoculum, 0.1 g of fresh tomato leaf tissue of cv. Rutgers—60 days post-inoculation (dpi) with PSTVd transcribed strains was ground in 1 mL sodium phosphate buffer (pH 7.0) and filtered through cheesecloth.

For mechanical inoculation, the cotyledons of tomato cv. Rutgers were dusted with carborundum and gently rubbed over the surface of the leaves with a plastic pestle. Ten microliters of inoculum were placed on the injured leaf surface and rubbed several times with a sterile plastic pestle. At 60 dpi, the presence of viroid in the inoculated tomato plants was determined by RT- PCR.

PSTVd-infected tomato sap was used to inoculate potato cultivars [7]. To inoculate 7-day potato plants, a 0.5–1.0 cm longitudinal stem incision was performed with a sterile razor on a stem apex, and 10 µL of the PSTVd strain suspension—obtained as described above—was applied. For inoculation with each PSTVd strain, three plants of each cultivar were used in three replications, with nine plants in total. Two plants of each cultivar in each replication (total six plants) were inoculated with sterile water (mock-inoculation). In 60 dpi, the presence of PSTVd in the inoculated plants of potato cultivars was determined by RT-PCR and RT-qPCR.

After 90 days, the degree of lesion formed on tubers (presence of deformation), their number, and their weight were evaluated.

2.4. Detection of PSTVd in Potato and Tomato Plants by RT-PCR and RT-qPCR

Top leaves of inoculated and control mock-inoculated potato or tomato plants were collected at 60 and 90 dpi. Total RNA was extracted from 0.1 g of leaf tissue of each plant using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and subsequently used for one-step RT-PCR to detect PSTVd with primer sets P3/P4 [16] and P1/P2 [17] or 68PV-R + 87PV-F [18]. RT-PCR was performed using PrimerScript One Step RT-PCR ver 2 kit reagents (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions in 10 µL.

RT-PCR was performed on a MyCycler Thermal Cycler (Bio Rad, CA, USA) at 50 °C for 30 min, 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. An additional synthesis step was performed at 72 °C for 5 min, followed by storage at 12 °C. The size of the PSTVD diagnostic fragment is about 360 bp.

RT-qPCR detection of PSTVd was performed using the Potato spindle tuber viroid-RT kit (Syntol, Moscow, cat. no. PV-004) according to the kit manufacturer's recommendations. RNA was isolated using a CytoSorb kit (Sintol, Moscow, Russia, cat. no. EW-001) according to the kit manufacturer's recommendations. The reaction was performed on a CFX-96 Touch Real-Time PCR detection system amplifier (BioRad, Hercules, CA, USA).

2.5. Detection of viral infection in potato plants by ELISA (enzyme-linked immunosorbent assay) and RT-qPCR

All plants of potato cultivars were tested for the presence of the most widely-occurring viruses in Russia. These are potato virus X (PVX), potato virus Y (PVY), potato virus S (PVS), and potato virus M (PVM), which were detected frequently in a survey of 11 regions of Russia [12]. Double antibody sandwich ELISA procedures are used with commercial diagnostic kits produced by the "Biotechnology" Scientific Production Association at the A.G. Lorch Potato Research Institute (Moscow, Russia).

Total RNA was extracted from 0.1 g of leaf tissue of each plant using the CytoSorb kit (Sintol, Moscow, cat. no. EW-001) according to the manufacturer's recommendations.

RT-qPCR detection of PVX, PVY, PVS, PVM, PLV, and PVA was performed using Potato Virus X and Potato Virus Y-RT kit (Syntol, Moscow, cat. no. PV-001), Potato Virus M and Potato Leafroll Virus-RT kit (Syntol, Moscow, cat. no. PV-002), Potato Virus S, and Potato Virus A-RT kit (Syntol, Moscow, cat. no. PV-003) according to the kit manufacturer's recommendations. The reaction was performed on a CFX-96 Touch Real-Time PCR detection system amplifier (BioRad, Hercules, CA, USA).

2.6. Statistical Analysis

Statistical processing of the data was performed using the program Statistics 12. Fisher's exact test was used to compare the number of plants with different types of tuber deformation after the first inoculation with four PSTVd strains and Student's *t*-test with Bonferroni correction when comparing mean weight of tubers per plant. When the *p* value is 0.05 or less, it was judged that there was a significant difference.

3. Results

3.1. Assessment of Symptoms on Tomato cv. Rutgers after Inoculation by Four PSTVd Strains

Previously, three PSTVd strains, at primary isolation, were divided into the intermediate (VP35, VP87) and severe (FP10-13) according to their response to inoculation of tomato cv. Rutgers [11]. Strain NicTr-3 was also determined in our studies as severe (unpublished data). Under our conditions, strains VP35 and VP87, as well as FP10-13 and NicTr-3, caused severe damage to Rutgers tomato plants (Figure 1).

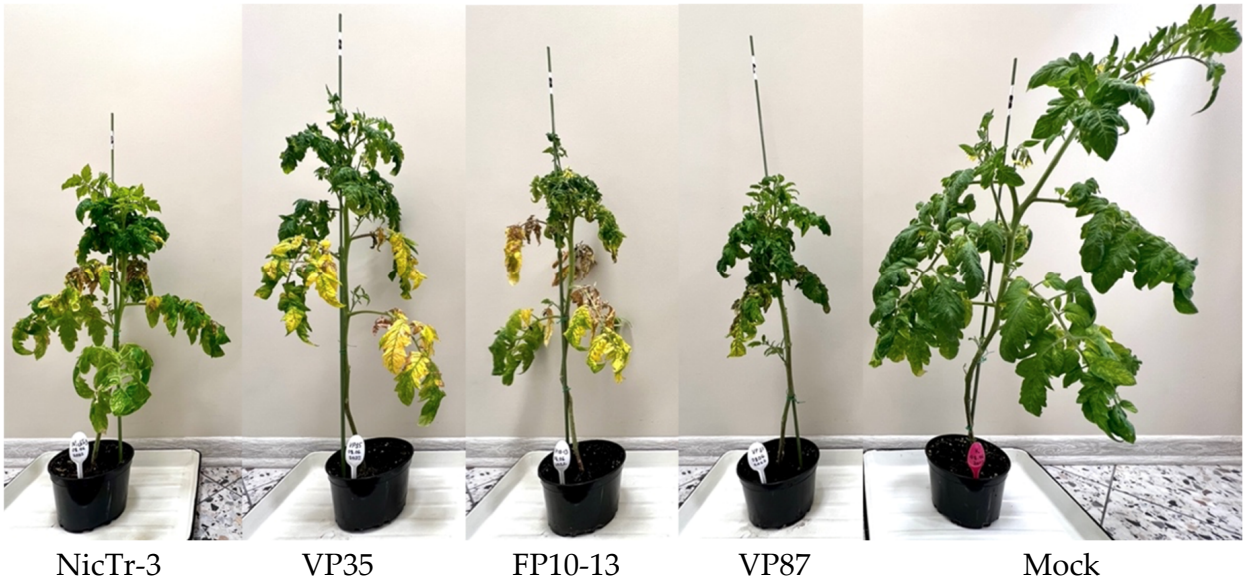


Figure 1. Symptoms in Rutgers tomatoes infected with four strains of PSTVd at 30 dpi. All PSTVd strains showed stunting, severe leaf curling, epinasty, stem and leaf necrosis; VP87 caused leaf mosaic and small necrosis (Figure S1).

Cultivar Rutgers infected by four PSTVd strains showed stunting, severe leaf curling, epinasty, stem, and vein necrosis at 30 and 60 dpi. Strains VP35, NicTr-3, and FP10-13 caused leaf, stem, and vein necrosis, and strain VP87 caused severe curling and wrinkling of leaves, especially of the plant apex, as well as vein necrosis (Figures 1 and S1).

The results of PSTVd detection in tomato sap used to inoculate potato plants are shown in Figure 2.

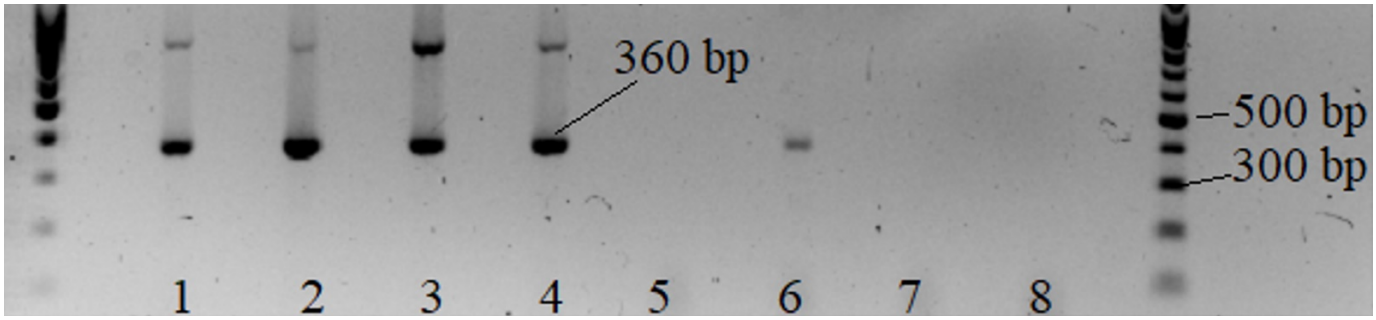


Figure 2. RT-PCR detection of PSTVd with the P3/P4 primers in Rutgers tomato plants inoculated with different PSTVd strains: 1—NicTr-3; 2 and 6—VP35; 3—VP87; 4—FP10-13; 5—Mock; 7 and 8—negative control (distilled water). On the sides of the gel are molecular weight markers 100 bp (Gene Ruller, Fermentas).

3.2. Disease Symptoms

Disease symptoms in potato haulm of PSTVd-infected plants at 60 dpi were not observed in all cultivars. The phenotype of most plants did not differ from the mock-inoculated plants. Some cultivars showed symptoms of thinning of stems and leaf malformation (cv. Arizona), necrosis of veins and leaves (cv. Sanibel), chlorosis and bronzing of leaves (cv. Sprinter), wrinkling, curling of leaves, deformation of leaf tips (cv. Favorit), and deformation of the leaf apex (cv. Gala) (Figure 3).

Figure 3. Symptoms of viroid infection on the haulm of different potato cultivars: (**A**)—cv. Arizona, strain NicTr-3; (**B**)—cv. Sanibel, strain VP35; (**C**)—cv. Sprinter, strain NicTr-3; (**D**)—cv. Favorit, strain NicTr-3; (**E**)—cv. Gala, strain VP87.

For most cultivars, PSTVd-infected plants did not show visible symptoms. The presence of tube malformation was considered the main criteria for sensitivity to PSTVd of cultivars. Five types of symptoms on tubers were identified at 90 dpi (Figure 4):

(I) Typical spindle-shaped, elongated, with varying degrees of deformation, often with overextension in the middle of the tuber;

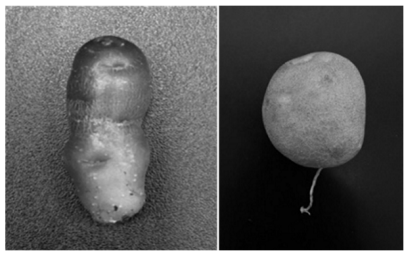
(II) Tuber malformation—deep tuber constriction, formation of large and small swellings, tuber budding;

(III) Pressed eyes on potato tubers and deep stroking on the tuber;

(IV) Pear-shaped and pointed end of tubers;

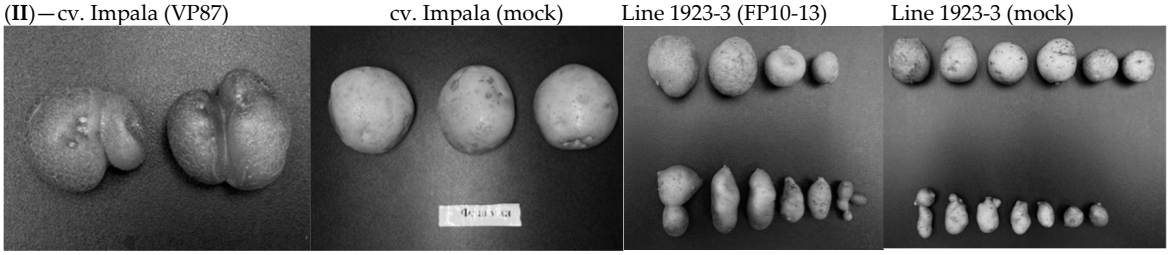
(V) Significant reduction in tuber size (pea) compared to healthy plants.

To confirm the presence of viroid infection, RT-PCR and RT-qPCR detection were performed for plants of all cultivars (Table 1, Figures 5 and S2).

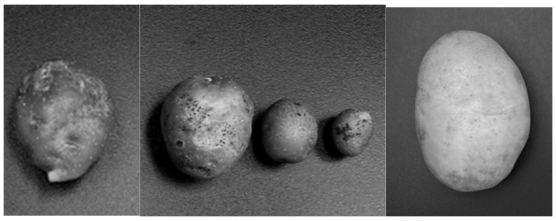


(**I**)—cv. Nevskiy, (VP35) cv. Nevskiy (mock)

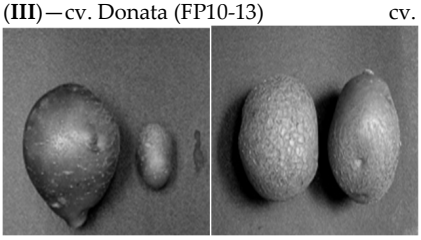
(**II**)—cv. Impala (VP87) cv. Impala (mock) Line 1923-3 (FP10-13) Line 1923-3 (mock)



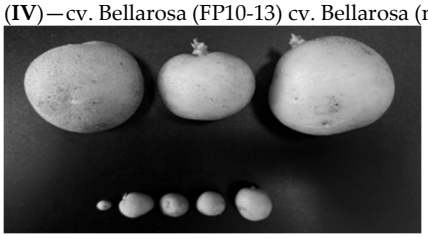
(**II**)—cv. Flagman (NicTr-3) cv. Flagman (mock) cv. Udacha (VP87) top row—mock cv. Avrora (VP87) top row—mock



(**III**)—cv. Donata (FP10-13) cv. Donata (mock)



(**IV**)—cv. Bellarosa (FP10-13) cv. Bellarosa (mock)



(**V**)—cv. Colomba (NicTr-3) top row—mock

Figure 4. Symptoms on tubers of PSTVd-infected plants at 90 dpi: (**I**)—typical spindle-shaped; (**II**)—tubers malformation; (**III**)—pressed eyes on a potato tubers and deep stroking on the tuber; (**IV**)—pear-shaped and pointed end of tubers; (**V**)—significant reduction in tuber size (pea) compared to healthy plants.

Table 1. Symptoms in potato tubers at 90 dpi with four PSTVd strains and results of viroid RT-PCR and RT-qPCR detection.

No	Cultivar	The First Inoculation by Strains:							
		VP87		VP35		FP10-13		NicTr-3	
		RT-PCR	Form	RT-PCR	Form	RT-PCR	Form	RT-PCR	Form
1	Avrora	−/+	D	No data			N	No data	
2	Arizona	+/+*	N	+	N	+/+*	N	+	S
3	Ariel	+	N	+	N/S	+/+*	N	+	N
4	Armada	+	N	No data		No data		+	M
5	Bellarosa	+	N	+	N	+/−*/+*	N\P	+	N
6	Berkut	+	N	+	N/P	+	N	No data	
7	Bernina	+	N	+/+*	N	+/+*	N	+/+*	N/P
8	Bizon	+	N	+	M	No data		No data	
9	Briz	+	N/D	+/+*	N	+/+*	N	No data	
10	Charoit	+	N	+	N	+/+*	N	+/+*	N/P
11	Colomba	+	N	+	N/D	+/+*	N	+/+*	N/D
12	Donata	+/+*	N	+	N	+/+*	N/P	+	N
13	Elizaveta	−/+	N	+	N	No data		No data	
14	Favorit	−/+	N/P/M	+	N/P	+	N/P	+/+	N
15	Flagman	+/+*	N	+/+*	M/D	No data		+/+*	N/D
16	Gala	+	N/M	No data		+/+*	N	+/+*	P
17	Impala	+	D	+	N/D	+	N/D	+/+*	N
18	Krepysh	+/+*	N/P/M	+	N	+	N/P	No data	
19	Labadia	+	N	+/+*	N	+/+*	N	+/+*	N
20	Madeira	+	N	+	N	+/+*	N	+	N/S
21	Manifest	+	N	+	N	No data		+	N
22	Meteor	No data		No data		+	N\D	No data	
23	Mirazh		N	+	N/S	+	N	+	N
24	Nandina	+	N/P	+/+*	N	+/+*	N	+/+*	N
25	Navigator	+	D	+	S	+/+*	N	+/+*	N/S
26	Nevskiy	+/+*	N	+/+*	N/S	No data		+/+*	N
27	Phioletovyi	+/−/+*	N/P	No data		+	N/D	+/+*	N/P
28	Queen Anna	+	N	+/+*	N	+/+*	N/P	+	N/S/M
29	Riviera		N	+	N	+	N	No data	
30	Sanibel	+	N/P	+	S	+/+*	N/P	No data	
31	Sapsan	-	N		N	+	M	+	N
32	Sineglazka2016	No data		+	N	+	N	No data	
33	Siurpriz	+	N	+	N	+	N	+/+*	N
34	Sorentina	+/+*	N	No data		+	N	+/+*	N
35	Sprinter	+/+*	N	+	N	+/+	N/D	+	N
36	Udacha	+	D	+	N	+	N	+/+*	N
37	Vineta	+	N	+/+*	N/P	+/+*	N	+/−*	N/D
38	1923-3	+	N	+	N/S	+	N/D	+	D
39	4434-1	+	N	+	N/D	+	N/D	+	N
Total cultivars with viroid symptoms			**11**		**14**		**13**		**13**

The symbols "+" and "−"—indicate positive and negative results of PSTVd RT-PCR detection with primer set—P3/P4; "+*"and "−*"—positive and negative results of PSTVD RT-qPCR detection; N—asymptomatic (normal tuber shape); S—spindle-shaped, elongated tubers, D—different types of tubers malformation (deformation); P—pear-shaped tubers; M—small tubers; N/D —both normal tuber shape and malformation; N/P—both normal tuber shape and pear-shaped tubers.

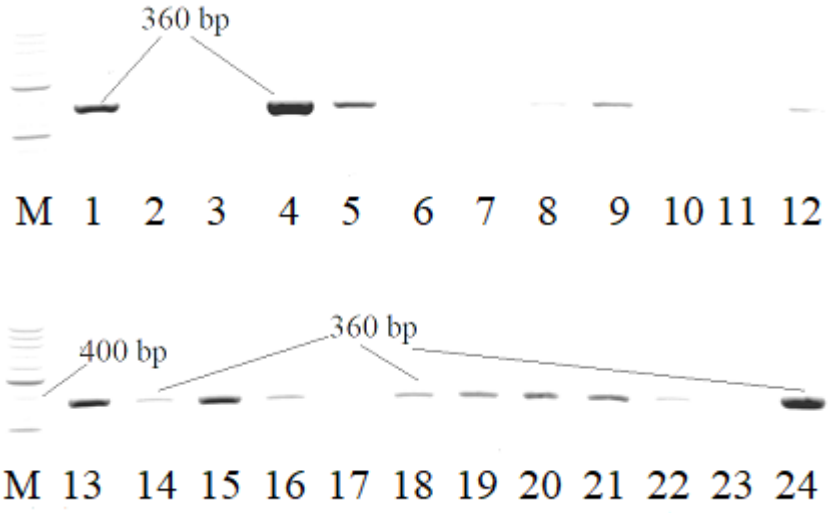


Figure 5. Detection of PSTVd strain VP87 at 60 dpi in potato cultivars: 1—Udacha, 2—Avrora, 3—Elizaveta, 4—Gala, 5—Manifest, 6—Queen Anna, 7—Bernina, 8—Riviera, 9—Colomba, 10—Favorit, 11—Sapsan, 12—Bizon, 13—Berkut, 14—Nandina, 15—Siurpriz, 16—Labadia, 17—Mirazh, 18—Ariel, 19—Arizona, 20—Armada, 21—Madeira, 22—Krepysh, 23—mock, 24—PSTVd-infected tomato cv. Rutgers.

Positive detection, indicating replication of viroid strains in the plants, was obtained for all cultivars inoculated with four strains. In the case of detection of a weak product of amplification or its absence in individual plants, repeated PCR tests were carried out, which confirmed the presence of a viroid.

The expression of clear symptoms of one of the five types of disease manifestation was observed for eight cultivars when inoculated with strain VP87 (Aurora, Briz, Impala, Navigator, Nandina, Sanibel, Udacha and Phioletovyi), for fourteen with strain VP35 (Ariel, Berkut, Bizon, Vineta, Impala, Colomba, Mirazh, Navigator, Nevskiy, Sanibel, Favorit, Flagman, 1923-1 and 4434-1), for thirteen with strain FP 10-13 (Bellarosa, Donata, Impala, Queen Anne, Krepysh, Meteor, Sanibel, Sapsan, Sprinter, Favorit, Phioletovyi, 1923-3 and 4434-1), and for thirteen with strain NicTr-3 (Arizona, Armada, Bernina, Vineta, Gala, Colomba, Queen Anne, Madeira, Navigator, Phioletovyi, Flagman, Charoit, and 1923-3). The number of plants with a normal tuber shape after the first inoculation was significantly higher than the number of plants with different types of tuber deformation (Fisher exact one-tailed—0.0001, $p < 0.05$). The same number of cultivars had symptoms of deformation and pear-shaped symptoms when infected with FP10-13 strain. Deformation symptoms were significantly more frequent than pear-shaped symptoms when infected with strains VP35 ($p = 0.02$), VP87 ($p = 0.01$), and NicTr-3 ($p = 0.0006$) (Figure 6).

Average tuber weight for PSTVd- infected plants was more than two and a half times less compared to mock-inoculated plants, and these differences were significant according Student's *t*-test with Bonferroni correction, $p < 0.05$ (Figure 7). PSTVd symptoms were found in none of the cases in the cultivars of all four strains. Cultivars Navigator, Sanibel, Phioletovyi, and line 1923-3 showed symptoms of viroid infection simultaneously to three strains: Queen Anne, Colomba, Favorit, Flagman, and line 4434-2 to two strains. All the listed cultivars, as well as those for which severe symptoms are noted for at least one PSTVd strain after the first inoculation, can be classified as susceptible. Five cultivars, Labadia, Siurpriz, Manifest, Riviera, and Sorentina, were found to be tolerant to the first inoculation by four PSTVd strains, and they formed normal in-shape tubers.

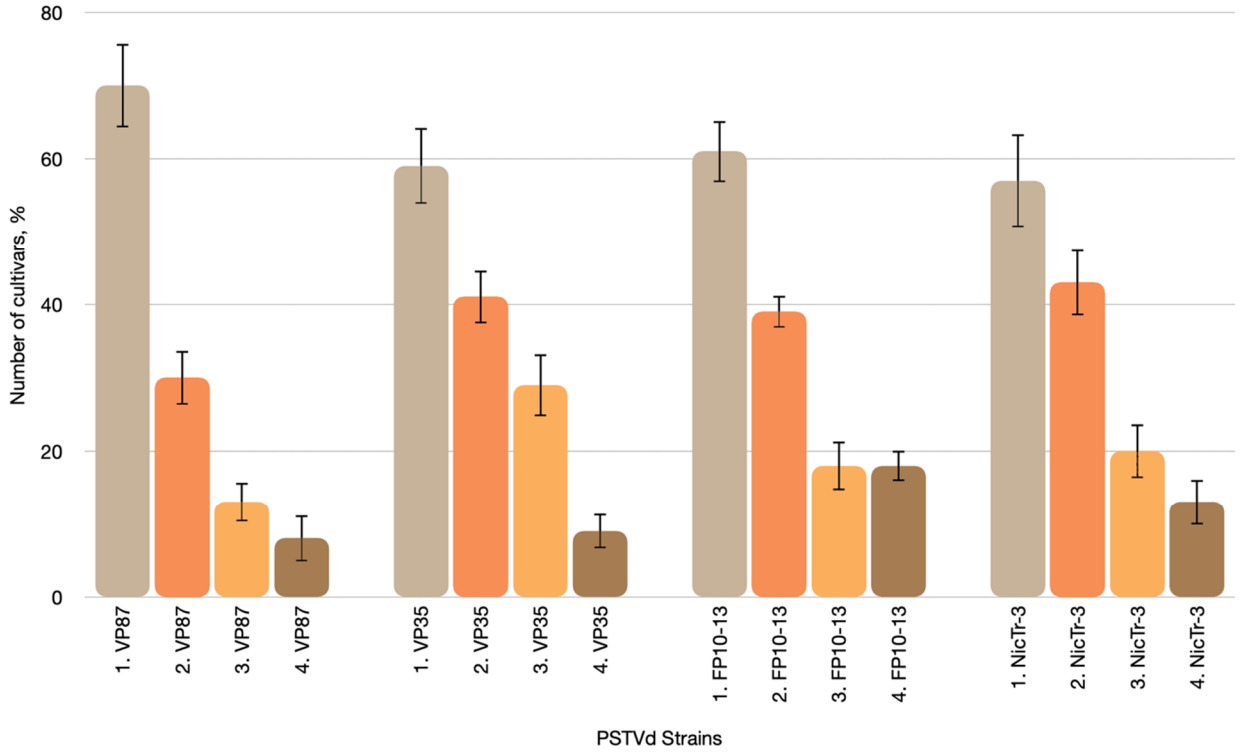


Figure 6. Number of potato cultivars (%) with symptoms of viroid infection of tubers at 90 dpi with four strains of PSTVd: 1—The number of cultivars with normal tuber shape; 2—with all five types of symptoms on tubers; 3—with symptoms of different deformations (except for pear-shaped); 4—with symptoms of pear shape. Differences are significant (Fisher exact one-tailed—0.0001, $p < 0.05$) between the frequency of cultivars with normal tuber shape (1) and all types of tuber deformation (2, 3, and 4) and between ddeformation symptoms and pear-shaped symptoms when infected with strains VP35, VP87, and NicTr-3.

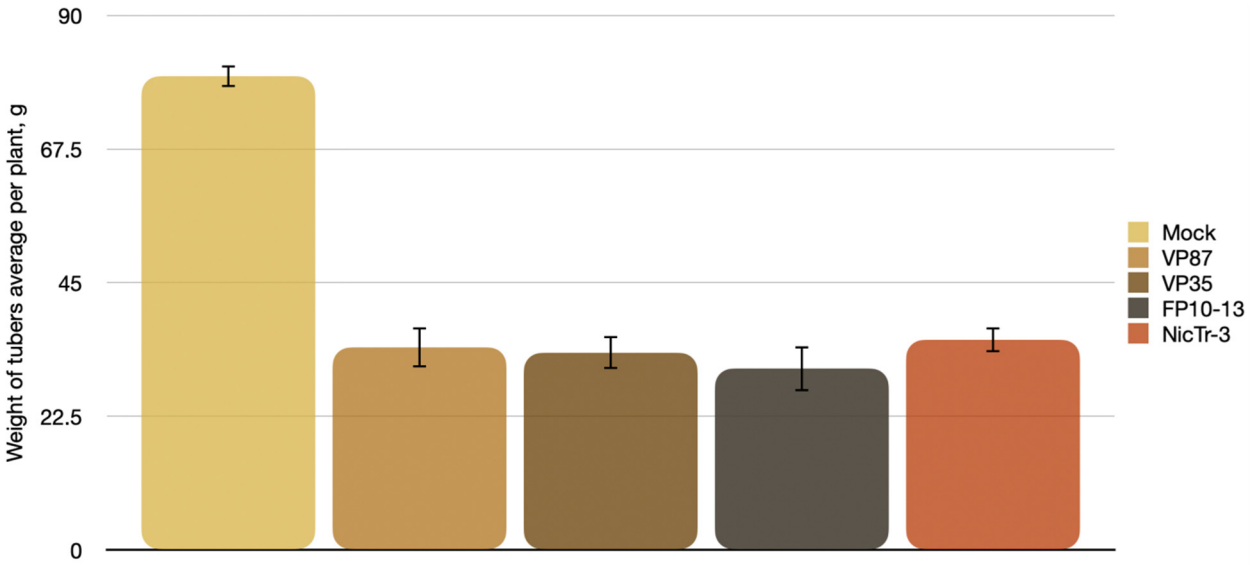


Figure 7. Mean value for the weight of tubers per plant for PSTVd-infected and mock-inoculated plants of 39 potato cultivars. Differences between mock- and PSTVd-inoculated plants are significant according to Student's *t*-test with Bonferroni correction, $p < 0.05$.

3.3. Symptoms on Second Generation Plants Derived from PSTVd-Infected but Normal in Shape Tubers

Twenty-two plants of sixteen PSTVd-infected potato cultivars that had formed after the first inoculation tubers of normal shape were planted for observation of symptoms. Only 32% of plants emerged simultaneously with control healthy plants 12 days after planting (Figure 8, Table S2). The delay in sprouting in the remaining PSTVd-infected plants extended for up to 48 days (Figures 8 and S4). Infected tubers of two cvs Riviera and Donata failed to germinate (Table S2).

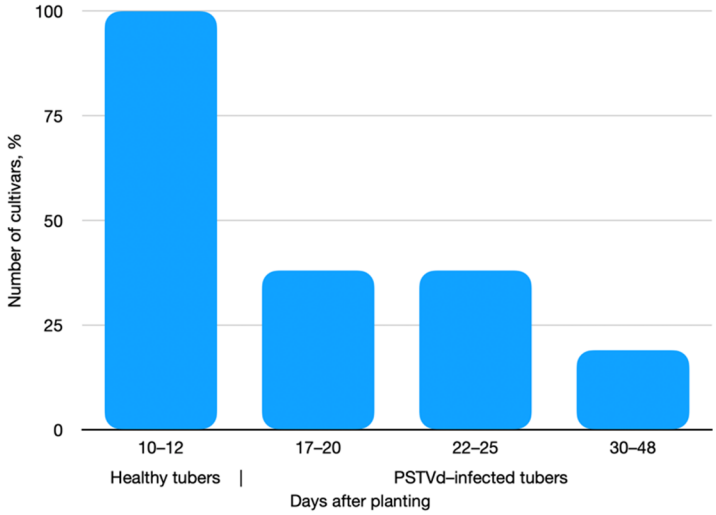


Figure 8. Period of emergence from healthy and PSTVd-infected tubers of second generation.

Tubers from PSTVd-infected plants of one cultivar germinated irregularly and depended on the viroid strain. For example, sprouts of cv. Bernina appeared 16, 29, 48 (strain FP10-13), and 24 (strain NicTr-3) days after planting. At 48 days after planting, sprouts of cv. Favorit and Sapsan (infested by strain NicTr-3), Nevskiy (VP87), Bernina, and Sorentina (FP10-13) appeared.

Most infected tubers had one, maximum three, sprouts (Figure S3). Stems were thinning, leaves were small, and some cultivars exhibited severe necrotization of leaves and stems (Colomba, Nevskiy et al.) (Figures 9 and 10).

Figure 9. Potato plants 30 days after planting with a healthy tuber (**left**) and tubers from PSTVd infected plants (**right**): 1—cv. Arizona, strain PSTVd VP87; 2—cv. Labadia, strain VP35.

1 2 3

Figure 10. Symptoms of viroid infection at 90 days after planting on cultivars of the second reproduction: 1—Colomba, strain NicTr-3, 2—Nevskiy, strain VP87, 3—Elizaveta, strain VP35.

Sprouts that appeared with a delay of 48 days, compared with mock-inoculated, had filamentous stems and were non-viable (Figure 9, Table S2). On average, for 22 plants of 16 cultivars, the number of tubers per infected plant was 37% and the weight of one infected tuber was 1.6% relative to healthy plants in the mock (Table S2).

Plants of six cultivars—Arizona, Bernina, Krepysh, Sapsan, Sorentina, and Favorit—did not form tubers (Table S2).

3.4. Symptoms of Viroid/Viral Mixed Infection

Before inoculation with different PSTVd strains, all plants were tested for viral infection by ELISA and RT-qPCR. All viral plants were also inoculated with PSTVd strains to determine the effect of viral/viroid mixed infection on the manifestation of disease symptoms. The most severe symptoms after the first inoculation of PSTVd were observed in plants of cv Navigator with mixed infection of PSTVd strain NicTr-3 + PVY + PVS + PVM; strain VP87 + PVM (Figure 11), and of cv. Impala with mixed infection PSTVd + PVY and PSTVd + PVX + PVS + PVM (Figure 12). However, severe tuber deformation was also observed for cv. Navigator (Figure 11) and Impala during primary PSTVd inoculation of virus-free plants (Figure 12).

PSTVd NicTr-3 + PVY+ PVS+ PVM PSTVd VP87+ PVM PSTVd NicTr-3

Figure 11. Tubers of cv. Navigator at 90 dpi with PSTVd and viral/viroid mixed infection.

In most cases, plants with mixed viroid/viral infection formed normal tubers after the first PSTVd inoculation (Table S4), but with a reduced number and average weight compared to healthy ones (Table S4, Figure 12). For three cultivars in which the number of plants infected by certain viruses was more than three, the average weight per tuber of PSTVd-infected, viral-infected, and mixed-infected plants was compared. In this small sample of tested plants, significant differences in average tuber weight per plant were obtained for the three cultivars when comparing PSTVd-infected with mock-inoculated plants (Figures 13 and S4, Table S5). For the cv. Colomba, a significant decrease in tubers weight was noted in PSTVd-infected and mixed PSTVd + PVY infection compared with mock, while the differences between tuber weight in mock- and PVY-infected plants were not

significant (Figure 13, Table S5). For the cv. Impala, only viral infection (PVX + PVS + PVM) did not lead to a significant decrease in tuber weight compared with mock, while in those infected only with PSTVd and PSTVd + PVX + PVS + PVM, the decrease in tuber weight was significant compared with mock. For the cv. Sanibel, significant differences in the studied trait were obtained only when comparing the productivity of plants infected with PSTVd and mock, while the tuber weight of PSTVd-infected and PSTVd + PVM-infected plants had no significant differences (Figures 13 and S4, Table S5).

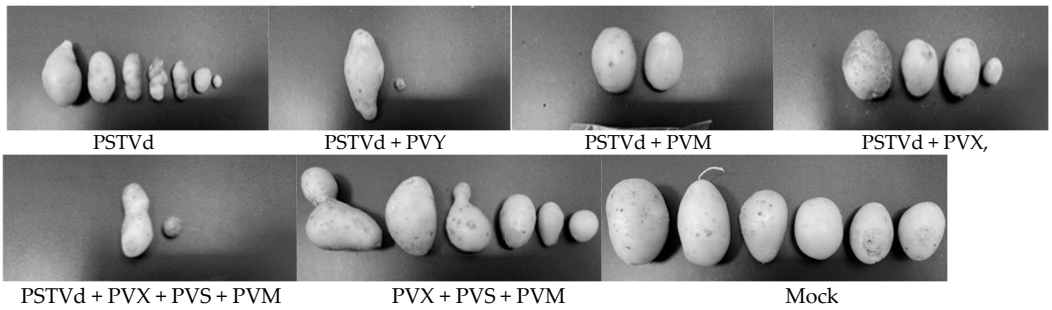


Figure 12. Tubers of cv. Impala at 90 dpi with PSTVd strain NicTr-3, viral, and viral/viroid mixed infection.

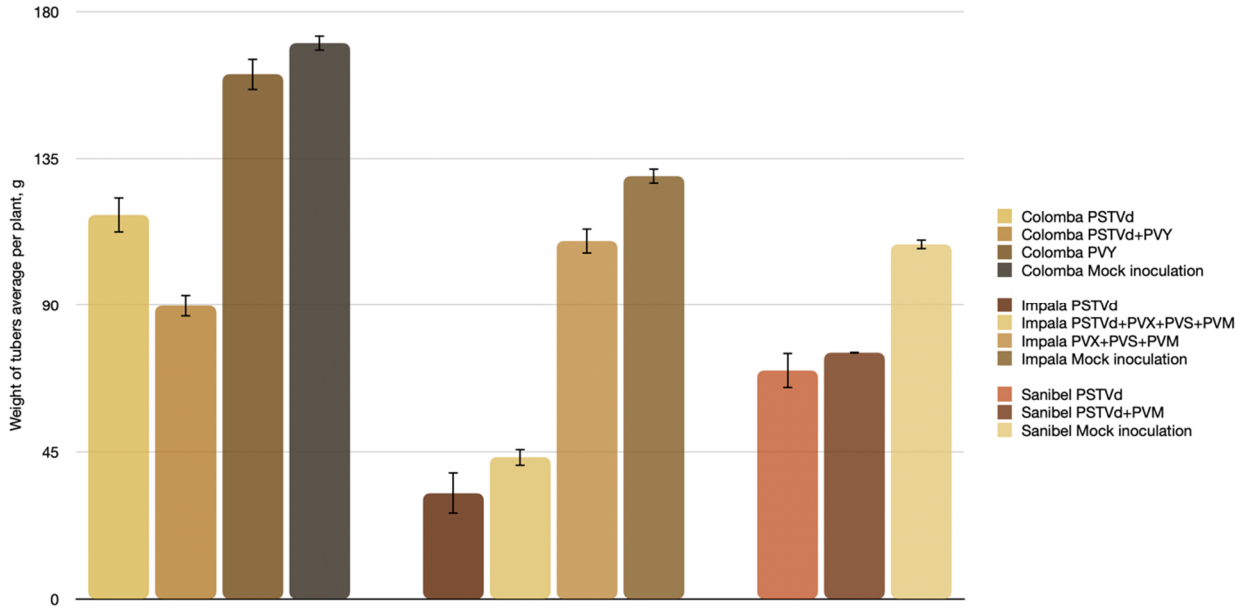


Figure 13. Mean weight of tubers per plant infected with PSTVd (strain NickTr-3), viral-infected, and viroid/viral-mixed infected of three potato cultivars. According to Student's *t*-test with Bonferroni correction ($p < 0.05$) for the cv. Colomba differences significant between mock- and PSTVd-infected and mixed PSTVd + PVY-infected plan ts for the cv. Impala between mock-, PSTVd-, and PSTVd + PVX + PVS + PVM-infected plants for the cv. Sanibel between mock- and PSTVd-infected plants.

When comparing weight of tubers per plant of PSTVd-infected, viral-infected, and viroid/viral-mixed infected plants, regardless of cultivar, significant differences were found between mock- and PSTVd-infected and viroid/viral-mixed infected plants (PVY + PSTVd, PVM + PSTVd, and PVY + PVS + PSTVd). The weight of tubers infected only with PVY did not differ from the weight of tubers of healthy plants (Figure 14; Table S6).

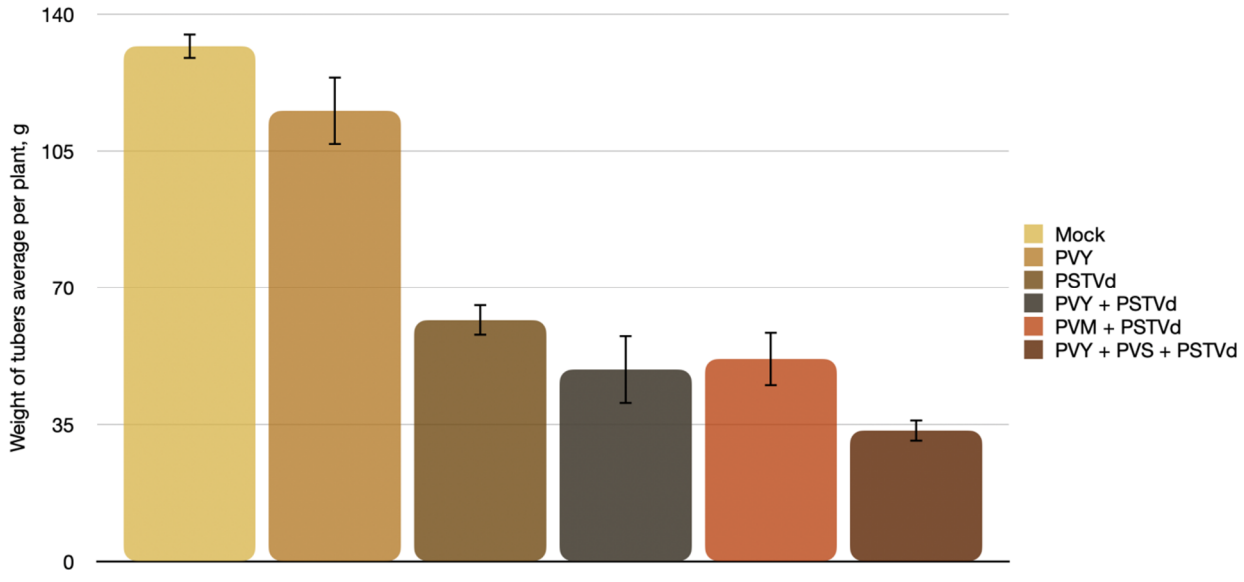


Figure 14. Weight of tubers per plant of PSTVd-infected, viral-infected, and viroid/viral-mixed infected plants regardless of cultivar, mean value for: Mock—11 cv., 66 plants; PVY—2 cv., 4 plants; PSTVd—11 cv., 35 plants; PVY + PSTVd—6 cv., 9 plants; PVM + PSTVd—6 cv., 8 plants; PVY + PVS + PSTVd—3 cv., 5 plants. According to Student's *t*-test with Bonferroni correction ($p < 0.05$), differences significant between mock and PSTVd, PVY + PSTVd, PVM + PSTVd, and PVY + PVS + PSTVd.

4. Discussion

PSTVd are single-stranded highly structured 360-nucleotide ring RNA particles lacking any protein-coding sequences [1]. It autonomously replicates depending on host plants' transcriptional machinery [19].

The control system for PSTVd, including preventing its introduction, monitoring for the pathogen, and its containment and eradication (OEPP/EPPO, Bulletin OEPP/EPPO Bulletin 41, 394-399, accessed on 21 August 2022), has led to a significant reduction in its prevalence in North America and Europe [20] (EPPO Reporting Service no. 03—2022 Num. article: 2022/068, accessed on 21 August 2022). However, outbreaks of PSTVd have increased in various regions of the world in tomato crops because of its spread with seed [21]. Mackie et al. [7] found that PSTVd from tomato plants can cause serious yield and tuber quality losses in potatoes.

In Russia, the lack of a mandatory test for PSTVd infection during obtaining virus-free seed potatoes has contributed to the spread of infection in potato cuttings and micropropagation. According to Kastalieva et al. [22], PSTVd was detected in about 50–70% of potato plants in vitro in Russia.

For the first time, the symptomatology in 39 modern potato cultivars after inoculation with four strains of PSTVd—intermediate VP35 and VP87 and severe FP10-13 and NicTr-3—was assessed. The success of infection was verified by RT-PCR and RT-qPCR.

The visible symptoms of viroid infection on potato plants resemble those associated with potato virus diseases, and include stunting, epinasty, leaf distortion, vein discoloration or necrosis, vein clearing, chlorotic or necrotic spots, mottling and necrosis of leaves, and (rarely) death of the entire plant [23]. The disease symptoms may result from RNA interference or gene silencing induced by viroid-derived siRNA. We also found all these symptoms, but after the first inoculation, potato haulm of most cultivars was symptomless. At 90 dpi, the leaves of most infected potato cultivars remained green, while the leaves of most mock-inoculated plants began to shrivel. Additionally, PSTVd-inoculated tomato plants remained green for more than 8 months. Apparently, viroid infection reprograms the expression of apoptosis-inhibiting genes, thus providing conditions for the preservation and replication of viroid molecules.

Among the 132 viroid strain/potato cultivar combinations, 61.4% of the cultivars after the first inoculation were found to be symptomless carriers of PSTVd. They formed tubers that were normal in shape but smaller and sometimes not different in size from the mock-inoculated. The least number of potato cultivars with symptoms of viroid infection on tubers was observed after the first inoculation with VP87 strain (21.6%), and for the remaining strains, approximately the same number of cultivars had symptoms of viroid infection (Figure 5).

Originally determined and deposited in 2020 as intermediate against the tomato variety Rutgers [11], the VP35 strain under our conditions caused severe damage to Rutgers tomato plants. In relation to potatoes, this strain was at the level of the two sever strains FP10-13 and NicTr-3 and even exceeded them in the number of cultivars with severe tuber deformation after initial inoculation (Figure 6). This may be due to mutation variability in this strain.

Upon infection, viroid forms a population of sequence variants in their host plant, called quasi-species [24]. Polymorphic RNA molecules of PSTVd, in accordance with the concept of quasi-species, are a source of adaptation to new hosts and new life cycle conditions.

The PSTVd ring molecule consists of five structural domains: terminal left, pathogenicity, central, variable, and terminal right [23]. In a viroid-infected cell, changes in the nucleotide sequence of viroid progeny occur during viroid replication. The rate of these mutations is affected by the nuclear RNA polymerases that mediate viroid replication in host cells. Nucleotide sequence changes in the pathogenicity domain play an important role in determining strain virulence [25]. Mild, intermediate, severe, and lethal PSTVd strains differ in only a few nucleotide changes located in the "virulence modulating region (VM)" in the pathogenicity domain [26,27]. Mutations of only 3 to 4 nucleotides transform an intermediate isolate into a lethal one against the cv. Rutgers [14] and, conversely, mutations in the PSTVd-I^{wt} strain at positions 42 and 64, amplified by other nucleotide changes at positions 43, 310, and 311\312, resulted in the attenuation of disease symptoms on tomatoes due to reduced viroid accumulation [25]. In addition, changes in the central domain of PSTVd have also been shown to lead to changes in pathogenicity. For example, replacement of a nucleotide at position 257 in the central domain of strain $PSTVd^{Int}$ from uracil (U) to adenine (A) resulted in increased pathogenicity on tomato up to lethality [28].

It was shown that one week after PSTVd inoculation, only 25% of the nucleotide sequence variants were the same as in the strain used for infection. After two weeks, the sequence frequency of the original strain increased to 70% and then stabilized after 3–4 weeks [24].

Thirty-four cultivars in which plants with tubers malformation were detected after initial inoculation with at least one strain can be characterized as susceptible. However, it should be noted that pear-shaped symptoms, if they are not accompanied by additional signs, such as tuber cracking, necrotic reticulum, and pressed eyelets, can be attributed to weak manifestation of the disease. Such tubers may not be subjected to culling during potato seed production and, therefore, will be sources of the infection (cvs Krepysh, Sanibel). There were five cultivars in which, after the first inoculation of plants with all PSTVd strains, normal tuber shapes were formed (cvs Labadia, Siurpriz, Manifest, Riviera, and Sorentina). The same results were obtained by Singh [13]: most clones of *S. berthaultii* after the first inoculation were symptomless.

The emergence of sprouts from the normal in shape PSTVd-infected tubers of the next generation, regardless of cultivar and PSTVd strain, was stretched in time and reached 30–48 days after planting. All plants obtained from infected tubers had symptoms of viroid infection: delayed sprouting, thinning of stems, shallowness, and necrosis of leaves and stems. Some PSTVd-infected tubers did not germinate (9.1%); 36.3% of plants from infected tubers did not form new tubers 90 days after planting, and 54.5% of plants had small and deformed tubers (Table S3). These data indicate natural elimination of diseased plants and self-healing of agrocenosis from PSTVd noted earlier by Mozhayeva [29]. In

terms of epidemiology, vegetative propagation of latently infected plant material poses a great danger to potato seed production because it dramatically increases the number of viroid-infected plants, thereby increasing the opportunity for accidental transfer to other plants and sensitive species growing nearby [23].

Studies of wild potato species resistance/tolerance to PSTVd are currently known [13,30]. Eight species (one accession of each species) were found with different response to PSTVd based on disease severity and tomato cv. Rutgers index: *S. etuberosum* Lindl. was immune while *S. demissum* Lindl. was susceptible; *S. sucrense* Hawkes and *S. chacoense* Bitt. were resistant and *S. stoloniferum* Schlechtd. et Bché., *S. berthaultii, S. acaule* Bitt. and *S. guerreroense* Corr. were tolerant [30]. Additionally, Singh [13] found that two clones of *S. berthaultii* were resistant to the mild and severe strains of PSTVd. Since we found no tolerance among the modern potato cultivars, it seems reasonable to look for sources of resistance to PSTVd among wild potato species.

Mixed viral infections in plants are common and can result in synergistic or antagonistic interactions [31]. There are no data in the literature on possible synergistic interactions between viral and viroid infections in potato. In our studies, most plants with mixed viral and viroid infection formed normal-shape tubers, but with a reduced number and average weight compared to healthy ones. For some cultivars (Navigator, Colomba, and Impala), a strong manifestation of tuber deformation was noted in viroid/viral mixed infection. However, on these cultivars, symptoms of tuber deformation were also noted during the first inoculation with PSTVd. PSTVd-infected plants did not differ from plants with mixed viroid/viral infection in terms of average weight of one tuber, thus we did not find synergism in mixed viroid/viral infection.

Thus, as a result of tolerance assessment of 39 modern potato cultivars, five cultivars were symptomless carriers of PSTVd after the first inoculation with four strains was identified. The strain specificity of the majority of cultivars in terms of the degree of symptom development after the first inoculation was established. Strain VP87 was the least aggressive with respect to potato cultivars. The strains FP10-13, NicTr-3, and VP35 were characterized as severe, but had specific aggressiveness in relation to potato cultivars. Cultivars that were symptomless after the first inoculation were found to have severe viroid symptoms in plants obtained from PSTVd-infected tubers. Mixed viroid/viral infection lead to an increase manifestation of tuber deformation only for certain cultivars. No synergism in the manifestation of mixed viroid/viral infection symptoms on tubers was found.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12122916/s1, Table S1: Potato cultivars and breeding lines used to assess tolerance to PSTVd; Figure S1: Symptoms on the leaves of tomato cv. Rutgers at 60 dpi with four PSTVd strains; Figure S2: The FAM channel of real time PCR for detection of potato spindle tuber viroid in different potato cultivars; Table S2: Symptoms on second generation plants derived from PSTVd-infected but normal in shape tubers; Figure S3: Potato cultivars 90 days after planting with PSTVd-infected tubers that appeared 30-48 days later; Table S3: Average tuber weight per plant of PSTVd-infected (strain NicTr-3), viral-infected, and mixed viroid (strain NicTr-3)/viral infected plants; Table S4: Symptoms on tubers of three potato cultivars when infected with PSTV strain NicTr-3 and mixed viral/viroid infection; Figure S4: Mean error when comparing weight of tubers per plant infected with PSTVd (strain NicTr-3), viral-infected and viroid/viral-mixed infected of three potato cultivars; Table S5: Student's *t*-test with Bonferroni correction when comparing mean weight of tubers per plant infected with PSTVd (strain NicTr-3), viral-infected and viroid/viral-mixed infected of three potato cultivars; Table S6: Student's *t*-test with Bonferroni correction when comparing weight of tubers per plant of PSTVd-infected, viral-infected and viroid/viral-mixed infected plants regardless of cultivar.

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