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Phenotypic and DNA Marker-Assisted Characterization of Russian Potato Cultivars for Resistance to Potato Cyst Nematodes

Tatjana A. Gavrilenko [1,*], Aleksander V. Khiutti [2], Natalia S. Klimenko [1], Olga Y. Antonova [1], Natalia A. Fomina [1] and Olga S. Afanasenko [2]

[1] N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), 190000 Saint-Petersburg, Russia; ns-klimenko@mail.ru (N.S.K.); olgaant326@mail.ru (O.Y.A.); n.fomina@vir.nw.ru (N.A.F.)
[2] All Russian Institute of Plant Protection (FSBSI VIZR), 196608 Saint-Petersburg, Russia; alexanderkhyutti@mail.ru (A.V.K.); olga.s.afan@gmail.com (O.S.A.)
* Correspondence: tatjana9972@yandex.ru

Abstract: Potato is one of the most important food crops in the world and also in the Russian Federation. Among harmful organisms reducing potato yield potential, the potato cyst nematodes (PCN) are considered to be ones of the most damaging pests. Information on PCN resistant cultivars is important for potato breeding and production. Russian potato cultivars are characterized in the state-bio-test program for resistance to only one PCN species *Globodera rostochiensis* and one pathotype Ro1 which is reported to be present in the country. This study aimed to find domestic cultivars with multiple resistances to different PCN species and different pathotypes using phenotyping coupled with molecular marker analysis due to the risk of the occasional introduction of new pests. The phenotypic response was determined by the inoculation of plants with pathotypes Ro5 of *G. rostochiensis* and Pa3 of *G. pallida*. The obtained results were supplemented by the state-bio-test data on resistance to Ro1 of *G. rostochiensis*. Nine of 26 Russian cultivars were resistant both to Ro5 and Ro1 pathotypes and two cultivars possess multiple resistances to both PCN species. Most tested molecular markers associated with the *Gpa2*, $GpaV_{vrn}$, $GpaV^{s}_{spl}$, *Grp1* loci showed discrepancies with phenotyping. However, a predictive haplotype and epistatic effect were detected.

Keywords: potato (*Solanum tuberosum*); nematode resistance; *Globodera rostochiensis*; *Globodera pallida*; bioassays; marker-assisted selection

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1. Introduction

Potato is one of the most important food crops in the world and also in the Russian Federation which is the third largest producer of the crop and fourth by potato harvested area [1]. Many harmful organisms reduce the potato yield potential. Among them, the potato cyst nematodes (PCN) are considered to one of the most damaging worldwide-recognized quarantine pests of potato [2–5]. PCN originated in South America and since the 19th century they were introduced in North and Central America, Europe, Asia, Africa and Australia [3,4]. There are two economically important PCN species–the golden cyst nematode *G. rostochiensis* (Wollenweber) and the pale cyst nematode *G. pallida* (Stone) Behrens causing 19% to 80% potato yield losses depending on the cultivar, seasonal conditions and the pathogen populations in the fields [6,7]. Besides yield losses, the quarantine status of the PCN implies the high expense for monitoring and regulatory activities. Nematode eggs can survive within the cysts for several decades in the absence of a suitable host removing infested lands for a long time [8]. Many nematicides are ineffective and toxic to the environment. Moreover chemical control might be ineffective [9,10].

At present, the golden cyst nematode is recorded in all European countries and *G. pallida* is not present in only eight European countries [2–4,11,12]. These two PCN

species are differentiated into eight pathotypes–five (Ro1–Ro5) for *G. rostochiensis* and three pathotypes (Pa1–Pa3) for *G. pallida* [13]. It was later suggested to rename the pathotypes of golden nematode and to recognize only three reliably recognizable pathotypes (Ro1/Ro4, Ro2/Ro3 and Ro5) [14]. Among them Ro1 is the most widely distributed pathotype of the golden nematode [15]. For *G. pallida*, there is no current information on the prevalence of a particular pathotype in European countries.

In the Russian Federation, *G. rostochiensis* was first reported in 1948 in the North-West part of the country and since then it has spread in the European part of the country, as well as in Southern Siberia and in the Russian Far East [16]. Ro1 of *G. rostochiensis* is the only one pathotype recorded in the Russian Federation [17,18]. According to the data given in the official sources, "The National Reports on the quarantine phytosanitary status of the territory of the Russian Federation", for the last three years (2018–2020), the area of established quarantine phytosanitary zones for *G. rostochiensis* has decreased more than 1.5 times (from 1 120 413.27 hectares in 2018 to 733 421.09 hectares in 2020).

This is due to the increasing trend of growing cultivars which are resistant to pathotype Ro1 of *G. rostochiensis* [19–21]. In Russia, the state bio-testing of potato cultivars for nematode resistance is performed only for Ro1 of *G. rostochiensis*. At present, 57.1% of 490 cultivars included in the State Register of Breeding Achievements are resistant to Ro1 of golden potato nematode but only 31.4% of resistant cultivars were bred in Russia [22].

Pathotypes Ro2/Ro3 and Ro5 of golden nematode have never been reported for the Russian Federation. At the same time, they were detected in some regions of neighboring countries: in Finland—pathotype Ro2 [23], in Norway—pathotypes Ro2 and Ro3 [24] and in Poland—pathotype Ro5 [25].

G. pallida has never been reported for the Russian Federation. Pale cyst nematode was detected in a few territories of Finland [26] and Estonia [27]; restricted distribution was reported for Norway where all three pathotypes of *G. pallida* were identified [24,28]. *G. pallida* was also reported for specific region of Ukraine [29] and Poland [30]. However, currently, the pale cyst nematode is no longer present in these two countries [5].

The emergence of new pathotypes is possible due to both adaptation processes in PCN populations and the invasion from overseas. These risks make it necessary to search for cultivars, breeding clones, germplasm accessions with resistance to new pathotypes of PCN. More than 50 potato species show resistance to at least one pathotype of *G. rostochiensis* and/or *G. pallida*, in at least one accession [15]. The resistance against PCN was transferred into potato cultivars from Andean species (*Solanum tuberosum* ssp. *andigena* Hawkes, *S. vernei* Bitter & Wittm., *S. sparsipilum* (Bitter) Juz. & Bukasov, *S. spegazzini* Bitter, *S. multidissectum* Hawkes, others). More than 20 genes and *QTLs* involved in control of resistance to different PCN pathotypes and species have been identified and mapped; many of them are clustered in the potato genome, especially on the chromosome V (see the latest review of [15,31]). *R*-genes and QTLs conferring multiple resistance to different PCN species and PCN pathotypes are particularly valuable for breeding, for example *Grp1_QTL* conferring resistance to *G. pallida* Pa2 and Pa3, and also to *G. rostochiensis* pathotype Ro5 [32,33]. The *Grp1_QTL* was transferred into breeding lines from interspecific hybrid between *S. tuberosum, S. vernei, S. oplocense* Hawkes, *S. tuberosum* ssp. *andigena* [32]. The use of bioassays for selection of nematode resistant genotypes is time consuming, laborious and costly. In the last decades, in addition to phenotypic evaluation, marker-assisted selection (MAS) has increasingly been used for identification of PCN resistant cultivars and advanced breeding lines [34–45] and for assessment of genetic diversity for PCN resistance in potato germplasm collections [17,46–48]. The results of molecular screening are not influenced by environmental conditions and the plant developmental stage. At the same time MAS does not always give certain results when compared with bio-test data what can be related to the recombination between the linked marker and the resistance locus, the quantitative inheritance of resistance traits controlled by multiple QTLs with different effects and the insufficient number of reliable markers associated with them, the allelic effects and the epistatic interactions [49–51].

A number of molecular markers of major *R*-genes and QTLs involved in the quantitatively inherited nematode resistance were developed for breeding of the PCN resistant potatoes [15,52]. According to results of molecular marker analysis the majority of commercial potato cultivars which are highly resistant to the most wide-spread Ro1 pathotype of *G. rostochiensis* have the major *H1* gene from *S. tuberosum* ssp. *andigena* while the *Gro1-4* gene from *S. spegazzini* is much less common [36–41,43,53,54].

In the case of Russian cultivars, molecular screening for *H1* resistance was first applied by V. Birukova and colleagues [55] who screened with marker TG689 a set of 109 cultivars of different origin (Western European, Ukrainian, Belarusian) including 36 cultivars bred in Russia. Later in a series of our research, a bigger subset of 211 cultivars bred in the Russian Federation were screened with several markers of the *H1* and *Gro1-4* genes [42,56–59]. These subsets included cultivars with declared resistance/susceptibility to Ro1 of golden nematode [22]. The obtained results indicated high (87%) predictive association between the presence of two markers of the *H1* gene-57R and TG689- and phenotypic resistance to Ro1. The diagnostic value of these markers can be increased up to 90% in the case of counting moderately resistant cultivars as resistant genotypes [42,56–59]. This study also demonstrated the extremely low frequency of *Gro1-4* resistance-predictive allele within the subset of Russian potato cultivars.

In the case of resistance to pathotype Ro5 of golden nematode and to Pa2/Pa3 pathotypes of *G. pallida*, MAS is less effective due to quantitative inheritance of these traits [15,37,52,60] in comparison with molecular screening for the *H1* resistance.

Bio-testing of Russian cultivars for resistance to pathotypes other than Ro1 pathotype of *G. rostochiensis* and to *G. pallida* has never been performed. Information about the presence of cultivars potentially resistant to the pale nematode pathotypes was obtained in molecular screening with the intragenic marker Gpa2-2 of the *Gpa2* gene which is involved in control of resistance to the pathotypes Pa2/3 of *G. pallida* [57–59,61]. However, for cultivars which were selected in molecular screening and had marker Gpa2-2, resistance was not validated in bio-testing.

The aim of this study was to search for potato cultivars bred in Russia with resistance to pathotype Ro5 of *G. rostochiensis* and to Pa3 of *G. pallida* using bioassays and molecular marker analysis with DNA markers associated with four loci: *Gpa2*, $GpaV_{vrn}$*_QTL*, $GpaV^{s}_{spl}$*_QTL*, *Grp1_QTL*. The results obtained were supplemented by the official data of state tests on resistance to Ro1 of *G. rostochiensis* [22] for the same cultivars to select genotypes with multiple resistance to Ro1, Ro5 pathotypes of golden nematode and to Pa3 of *G. pallida.*

2. Materials and Methods

2.1. Plant Material

The research material is represented by 26 popular potato cultivars bred in five Russian breeding centers which are well adapted to different regions of the Russian Federation (Alyj parus, Antonina, Čaroit, Danaâ, Evraziâ, Grand, Gusar, Holmogorskij, Il'inskij, Kolobok, Krasavčik, Krepyš, Liga, Lomonosovskij, Lûbava, Meteor, Naâda, Nakra, Nevskij, Plamâ, Samba, Sirenevyj tuman, Tango, Utro, Varâg, Vympel). ISO 9: 1995 transliteration standard was used for transliteration of the Russian names (epithets) of cultivars from Cyrillic script into a Roman alphabetic script according to Recommendation 33A of the international code of nomenclature for cultivated plants [62]. Plant material for this study was obtained from collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR). In our previous research, all these cultivars were genotyped in SSR analysis and tested using molecular markers of the *H1* and *Gro1-4* genes conferring resistance to pathotype Ro1 of *G. rostochiensis* as well as with DNA markers associated with *R*-genes of extreme resistance to PVY, PVX and resistance to late blight [57–59]. Data about their resistance to Ro1 of golden nematode were obtained from the results of bioassays recorded in the State Register of Breeding Achievements [22].

Seven Western European cultivars (Alouette, Damaris, Estrella, Labella, Queen Anne, Red Lady, Red Scarlett) with a known PCN resistance score were used as controls in evaluation of nematode resistance.

2.2. PCN Inoculation and Resistance Evaluation

2.2.1. Pathotypes of *G. rostochiensis* and *G. pallida*

Pathotype Ro5 of *G. rostochiensis* and pathotype Pa3 of *G. pallida* were used as infectious material for evaluation of potato cultivars resistance. These pathotypes were kindly provided by Dr. Anna Podlewska-Przetakiewicz from the Institute Hodowli i Aklimatyzacji Roślin (IHAR, Poland) for laboratory biotests.

Reproduction of *G. rostochiensis* and *G. pallida* pathotypes was performed on the universally susceptible potato cultivar Nevskij according to the methodology recommended by the European and Mediterranean Plant Protection Organization (EPPO) [63], following to all rules of work with quarantine objects. After four months, sufficient for the development of *G. rostochiensis* and *G. pallida*, cysts were extracted from the soil by flotation [63] and transferred for storage in a refrigerator at +4°C until using to inoculate potato genotypes.

2.2.2. Evaluation for PCN-Resistance in Bio-Tests

Potato cultivars were evaluated for resistance to *G. rostochiensis* and *G. pallida* using the methods recommended by the EPPO [63].

Each tuber was planted in 500 cm^3 plastic pots half filled with soil (250 cm^3). *G. rostochiensis* and *G. pallida* inoculum was added to each pot at the rate of 1500 eggs and larvae per 100 cm^3 of soil. Eggs and larvae were obtained by crushing cysts in a drop of water. After plant inoculation, additional soil was added to the top of the pot. Plants were left in controlled conditions at 22 °C with a photoperiod of 16 h of light and 8 h of dark and adequately watered.

After three months, extraction of cysts from soil was performed by flotation [63]. The extracted cysts were transferred to microscopy slides in a drop of water, crushed, and the number of eggs and larvae were counted. Relative susceptibility was determined using the formula: number of eggs and larvae on the roots of the tested cultivar/number of eggs and larvae on the roots of the control susceptible cultivar × 100%. For determination of relative susceptibility to the pathotype Ro5 of *G. rostochiensis* and Pa3 of *G. pallida* cv. Nevskij were used. The cv. Nevskij, which was used earlier in our studies as a susceptible control in the assessment of resistance to pathotype Ro1 [17] was found to be susceptible to pathotypes Ro5 and Pa3 as well as in preliminary experiments. Infestation results were evaluated according to the 9-point EPPO scale [63] (Table 1).

Table 1. EPPO scale for accounting the infestation rate of potato cultivars by *G. rostochiensis* and *G. pallida*.

Relative Susceptibility (% of Eggs and Larvae to Susceptible Control)	Score	Resistance
<1	9	Very high
1.1–3	8	High/very high
3.1–5	7	High
5.1–10	6	Moderate/high
10.1–15	5	Moderate
15.1–25	4	Moderate/low
25.1–50	3	Low
50.1–100	2	Low/very low
>100	1	Very low

Two cultivars Damaris and Estrella were used as resistant control for pathotype Ro5 of *G. rostochiensis*. In addition, three cultivars, Labella, Queen Anne, Red Lady, were used as susceptible control for pathotype Ro5; three cultivars Alouette, Labella, Queen Anne were used as susceptible controls for pathotype Pa3 of *G. pallida* (Table 2). The status of PCN resistance of these control cultivars was obtained from the variety lists of the Julius Kuhn-Institute, Gross Lüsewitz, Germany [64] and from Potato Variety Database [65].

Table 2. Potato cultivars used as controls in PCN resistance evaluation.

Cultivar	Ro5	Pa3	Ro1
Alouette	–	S	R
Damaris	R	–	R
Estrella	R	–	R
Labella	S	S	R
Queen Anne	S	S	R
Red Lady	S	S	R
Red Scarlett	–	–	R

"–" no data.

Besides German cv. Red Scarlett [66] which is in demand on the market in Russia has been also involved in phenotyping.

Each potato cultivar was evaluated in three replications, with three to five plants tested in each replication.

2.3. Marker Assisted Selection

2.3.1. DNA Isolation and PCR Amplification

Genomic DNA was isolated from leaf samples following the CTAB-extraction method with some modification [67]. Quality and quantity of the isolated DNA samples was checked using agarose gel electrophoresis (0.8% agarose gel) and spectrophotometer (Implen NanoPhotometer N60 Touch).

The CAPS- and SCAR-markers associated with the *R*-genes (QTLs) conferring PCN resistance used in this study are shown in Table 3.

Table 3. DNA markers associated with genes (QTLs) conferring PCN resistance used in this study.

Gene/QTL	Chromosome	Source of Introgressed Gene (QTL) [1]	Pathotype	Marker	Primer Sequence	Annealing Temperature	References for Primer- and Marker-Developers
Gpa2	XII	*adg*	Pa2,3	Gpa2-1	F: TTTAGCACGGAATGTGGGGA R: GTTTCCCCATCAAAACTCAC	60	[39]
				GP34/TaqI	F: CGTTGCTAGGTAAGCATGAAGAAG R: GTTATCGTTGATTTCTCGTTCCG	62	[68,69]
				77R/HaeIII	F: CTCGAGGGATTGAATCCAAATTAT R: GGAAGCAGAATACTCCTGACTACT	57	[70]
$GpaV^{s}_{spl\_}$ *QTL*	V	*spl*	Pa2,3	TG432/DraI	F: GGACAGTCATCAGATTGTGG R: GTACTCCTGCTTGAGCCATT	66	[60]
				GP179/EcoRV	F: GGTTTTAGTGATTGTGCTGC R: AATTTCAGACGAGTAGGCACT	55	[60,71]
Grp1_QTL	V	*vrn*, *opl*, *adg*, *tub*	Ro5; Pa2,3	TG432/RsaI	F: GGACAGTCATCAGATTGTGG R: GTACTCCTGCTTGAGCCATT	66	[33]
Grp1_QTL	V	*vrn*, *opl*, *adg*, *tbr*	Ro5; Pa2,3	GP21/DraI	F: GGTTGGTGGCCTATTAGCCA R: GCTCCAACACGGAAGGTTTTC	55	[32,71]
Gpa5_QTL	V	*vrn*	Pa2,3				
Grp1_QTL	V	*vrn*, *opl*, *adg*, *tub*	Ro5; Pa2,3	GP179/RsaI	F: GGTTTTAGTGATTGTGCTGC R: AATTTCAGACGAGTAGGCACT	55	[32,71,72]
Gpa5_QTL	V	*vrn*	Pa2,3				
Gpa5_QTL	V	*vrn*	Pa2,3	SPUD1636	F: GTGCGCACAGGGTAAAACC R: ACCTTAGCGGATGAAAGCC	65 → 60	[46]
$GpaV_{vrn}$*_QTL*	V	*vrn*	Pa2,3	HC	F: ACACCACCTGTTTGATAAAAAACT R: GCCTTACTTCCCTGCTGAAG	65 → 60	[34]

[1] *adg*—*S. tuberosum* ssp. *andigena*, *vrn*—*S. vernei*, *spl*—*S. sparsipilum*, *opl*—*S. oplocense*, *tbr*—*S. tuberosum* ssp. *tuberosum*.

PCR reactions were carried out in a total volume of 20 µL containing 10 ng DNA template, 1 × PCR reaction buffer ('Dialat', [73]), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP ('Dialat'), 0.5 µM of forward/reverse primer (synthesized by 'Evrogen', [74]) and 1U Taq polymerase ('Dialat').

PCR was carried out according to the standard procedures. The annealing temperatures and cycling conditions corresponded to those indicated by primer-developers (references see in the Table 3). Amplification with TG432 primer pairs was conducted following PCR-conditions proposed by A. Finkers-Tomczak with colleagues (2009) [33] with the annealing temperature 66 °C to reduce the number of extra fragments.

All reactions were conducted in at least three replicates.

The cultivar Cara was used as positive control for markers GP34/TaqI and 77R/HaeIII [68,69] and cultivar Innovator–for marker HC [34].

2.3.2. Restriction

For CAPS-markers, enzymes TaqI, HaeIII, DraI, EcoRV, RsaI (from 'SibEnzyme' LLC [75]) were used. The restriction was performed overnight according to the manufacturer's protocols.

2.3.3. Electrophoresis

The PCR products were separated by electrophoresis in 2.0% agarose gels in a TBE buffer followed by ethidium bromide staining and visualization in UV light. Documentation was done using GelDoc XR gel system (BIO-RAD).

2.3.4. Diagnostic Bands of Molecular Markers

The sizes of the amplification products corresponded to published by authors for STS-marker Gpa2-1—1120 bp [39], for CAPS-marker TG432 digested with RsaI—1900 bp [33] and for ASA-marker HC—276 bp [34]. The data about presence/absence of these diagnostic bands (Supplementary Figures S1a, S2a and S3) were registered in molecular marker analysis of the subset of Russian cultivars in present research.

The sizes of diagnostic bands for CAPS-markers GP179/RsaI and GP21/DraI were not indicated by the authors, however, they showed the electrophoreograms with digested PCR products both for resistant and susceptible genotypes [32]. Therefore, it was possible to select polymorphic bands which were present in resistant and absent in susceptible genotypes in the electrophoreograms. Corresponding bands were registered in our research (Supplementary Figures S2b and S4).

In the case of the CAPS-marker 77R/HaeIII, the size of the diagnostic band was not indicated by authors, however, they showed the target restriction fragment in the electrophoreograms by an arrow for the analyzed genotypes including cultivar Cara [70]. That's why cv. Cara was included in our study as control in molecular marker analysis. We recorded an approximately 900 bp polymorphic fragment of cv. Cara as diagnostic. D. Milczarek and co-authors (2011) tested 77R/HaeIII on PCN resistant and susceptible cultivars and designated the 750 bp fragment as target for their plant material. However, in our subset the 750 bp band was not detected (Supplementary Figure S1b).

The marker GP34/TaqI was developed on the cultivar Cara [69], but the information about size of diagnostic bands and the electrophoreograms are absent in this paper. Later, D. Milczarek with co-authors (2011) involved marker GP34/TaqI in molecular screening and indicated the 495 bp band as diagnostic. However, we did not reveal any fragment of such size in the subset of tested Russian cultivars. In our subset several polymorphic bands of GP34/TaqI were detected. Among them, two bands approximately 520 bp and 550 bp-long were registered as possible diagnostic fragments (Supplementary Figure S5) as these bands were detected in cv. Cara.

As for two CAPS-markers GP179/EcoRV and TG432/DraI [60], there is no data about target fragment size, about control genotypes and there are no images of the amplification products digested with restrictases. In case of CAPS-marker GP179/EcoRV, an approximately 150 bp and 400 bp bands resulting from the digestion of the PCR product GP179

with restrictase EcoRV were considered as diagnostic fragments (Supplementary Figure S6). The restriction profiles of the CAPS-marker TG432/DraI consisted of several polymorphic fragments for each cultivar (Supplementary Figure S7) that complicated identification of the target fragment. Due to the complexity of fragment detection, we had to abandon this marker.

2.4. Statistical Analysis

Fisher's exact test was used to determine if there is the relationship between these markers and bio-test data.

3. Results

3.1. Bio-Test for Resistance to Pathotype Ro5 of G. rostochiensis and Screening of Potato Cultivars with Molecular Markers of the Grp1_QTL Involved in Control of Resistance to Pathotype Ro5 of Golden Nematode

The results of evaluation for resistance to pathotype Ro5 of *G. rostochiensis* of 33 potato cultivars (including controls) are shown in Table 4. The bio-test revealed differences among cultivars, but not between replications. Cultivars Damaris and Estrella used as resistant controls and cvs. Labella, Queen Anne and Red Lady used as susceptible controls showed the expected results in assessments (Table 4).

No cysts were detected on the roots of 10 tested cultivars (Alyj parus, Danaâ, Evraziâ, Gusar, Holmogorskij, Krepyš, Liga, Meteor, Naâda, Red Scarlet) and two resistant controls Damaris and Estrella. On the roots of four cultivars: Il'inskij, Sirenevyj tuman, Utro, Vympel a small number of cysts and larvae were formed, which corresponded to resistance. Eleven cultivars including controls were susceptible (score 1–4) to pathotype Ro5. Six cultivars (five Russian cvs. Antonina, Grand, Kolobok, Nakra, Samba and one foreign cv. Alouette) showed moderate level of resistance (score 5–6) in bio-test.

In total 16 of the 33 evaluated cultivars were characterized to be very highly resistant (score 9), six were moderately resistant (score 5–6) and 11 were susceptible (score 1–4) to pathotype Ro5 of *G. rostochiensis* (Table 4).

Three CAPS-markers associated with *Grp1_QTL* involved in the control of resistance to pathotype Ro5 of *G. rostochiensis* were tested on 33 cultivars (Table 4 and Supplementary Figures S2 and S4). The results showed that most cultivars that are highly resistant (score 9) to the Ro5 pathotype of *G. rostochiensis* exhibit the marker allele(s). Most (81,3%) of highly resistant cultivars possessed positive bands of markers TG432/RsaI and GP179/RsaI and 68,8% of resistant cultivars were positive for marker GP21/DraI (Table 4). At the same time positive bands of at least one of the three CAPS-markers were detected in many susceptible (score 1–4) cultivars (Table 4). Only one of 11 susceptible cultivars–Čaroit–was negative for all three markers of the *Grp1_QTL*. The Fisher's exact test showed the absence of relations at significant level between the bioassay data and the presence/absence of the markers associated with the *Grp1_QTL* in the subset of 27 cultivars (16 highly resistant and 11 susceptible) when six moderately resistant cultivars were not counted.

Table 4. Resistance of potato cultivars to *G. rostochiensis* pathotype Ro5 and data of molecular marker analysis with three markers of the Grp1_QTL. Resistance group: R—resistant, MR—moderately resistant, S—susceptible. "+"—fragment presents, "−"—fragment absents.

№	Cultivar	Average Number of Cysts in 500 cm^3 of Soil	Average Number of Eggs and Larvae per 500 cm^3 of Soil	Relative Susceptibility	Cultivar Response (1–9 EPPO Scale)	Resistance Group for Pathotype Ro5 of Golden Nematode	Results of Molecular Screening with Markers			Declared Resistance Group for Pathotype Ro1 of Golden Nematode	Presence of the Gro1-4 Marker **
							TG432/ RsaI	GP179/ RsaI	GP21/ DraI		
Domestic cultivars											
1	Alyj parus	0.0	0.0	0.0	9	R	+	+	−	− *	−
2	Antonina	9.0	7683.4	7.7	6	MR	−	+	+		−
3	Čaroit	16.6	15,530.1	15.7	4	S	−	−	−	S	−
4	Danaâ	0.0	0.0	0.0	9	R	+	−	+	S	−
5	Evraziâ	0.0	0.0	0.0	9	R	−	+	+	R	−
6	Grand	7.8	10,536.0	10.6	5	MR	−	+	+	R	+
7	Gusar	0.0	0.0	0.0	9	R	−	+	+	R	−
8	Holmogorskij	0.5	0.0	0.0	9	R	+	+	+	R	−
9	Il'inskij	2.1	1060.6	1.0	9	R	+	+	+	S	−
10	Kolobok	13.6	11,772.8	11.9	5	MR	−	+	+	S	−
11	Krasavčik	58.5	69,024.0	69.8	2	S	+	+	+	S	−
12	Krepyš	0.0	0.0	0.0	9	R	+	+	+	R	−
13	Liga	0.0	0.0	0.0	9	R	+	+	+	R	−
14	Lomonosovskij	32.8	23,557.8	23.8	4	S	+	+	+	S	−
15	Lûbava	98.9	59,435.5	60.1	2	S	+	+	+	S	−
16	Meteor	0.0	0.0	0.0	9	R	+	+	+	R	−
17	Naâda	0.0	0.0	0.0	9	R	−	+	+	R	−
18	Nakra	10.0	5294.5	5.3	6	MR	+	+	+	S	−
19	Nevskij	80.8	98,775.1	100.0	1	S	+	−	−	S	−
20	Plamâ	50.6	64,727.8	65.5	2	S	+	+	+	R	+
21	Samba	9.0	10,544.5	10.6	5	MR	+	+	+	S	+
22	Sirenevyj tuman	4.0	540.2	0.5	9	R	+	+	−	S	−
23	Tango	22.8	28,137.7	28.4	3	S	−	+	+	− *	−
24	Utro	6.5	789.4	0.7	9	R	+	−	+	S	−
25	Varâg	19.9	19,098.3	19.3	4	S	+	+	+	*S*	−
26	Vympel	1.5	259.8	0.2	9	R	+	+	+	*R*	−

Table 4. *Cont.*

№	Cultivar	Average Number of Cysts in 500 cm^3 of Soil	Average Number of Eggs and Larvae per 500 cm^3 of Soil	Relative Susceptibility	Cultivar Response (1–9 EPPO Scale)	Resistance Group for Pathotype Ro5 of Golden Nematode	Results of Molecular Screening with Markers			Declared Resistance Group for Pathotype Ro1 of Golden Nematode	Presence of the Gro1-4 Marker **
							TG432/ RsaI	GP179/ RsaI	GP21/ DraI		
Domestic cultivars											
Foreign cultivars											
27	Alouette	20.4	24,383.2	24.6	5	MR	+	+	–	R	–
28	Red Scarlett	0.0	0.0	0.0	9	R	+	+	–	R	–
Controls											
29	Damaris	0.0	0.0	0.0	9	R	+	+	–	R	+
30	Estrella	0.0	0.0	0.0	9	R	+	–	–	R	+
31	Labella	19.0	23,524.6	23.8	4	S	+	–	–	R	–
32	Queen Anne	59.0	77,158.5	78.1	2	S	+	–	–	R	–
33	Red Lady	41.1	45,225.0	45.7	3	S	+	+	+	R	–
Totally estimated cultivars (N) including: N = 33: 16 R (score 9), 6 MR (score 5–6), 11 S (score 1–4)											
No. (%) of highly resistant (score 9) cultivars with the marker							13 of 16 (81.3%)	13 of 16 (81.3%)	11 of 16 (68.8%)		
No. (%) of highly resistant (score 9) cultivars without the marker							3 of 16 (18.7%)	3 of 16 (18.7%)	5 of 16 (31.2%)		
No. (%) of susceptible (score 1–4) cultivars with marker							9 of 11 (81.8%)	7 of 11 (63.6%)	7 of 11 (63.6%)		
No. (%) of susceptible (score 1–4) cultivars without the marker							2 of 11 (18.2%)	4 of 11 (36.4%)	4 of 11 (36.4%)		
No. of moderately resistant (score 5–6) cultivars with marker							3 of 6	6 of 6	5 of 6		
No. of moderately resistant (score 5–6) cultivars without marker							3 of 6	0 of 6	1 of 6		

* These two cultivars not yet included in the National List, so data of the State bio-testing for resistance to Ro1 are not yet available; ** data about presence of the Gro 1–4 marker were obtained from our previous research [42,56–58].

A comparison of the results obtained for all three markers together allowed identifying six haplotypes in the studied subset. However, none of them were associated with phenotypic data for resistance to pathotype Ro5 of *G. rostochiensis* (Table 4).

The results of bioassay of 26 domestic cultivars for the pathotype Ro5 resistance were complemented with the official data [24] of state tests on resistance to Ro1 of *G. rostochiensis* and with declared resistance to Ro1 for foreign cultivars. Nine Russian cultivars (Danaâ, Evraziâ, Gusar, Holmogorskij, Krepyš, Liga, Meteor, Naâda, Vympel) and three foreign cultivars (Damaris, Estrella, Red Scarlet) possess multiple resistances to pathotypes Ro5 and Ro1 of golden nematode (Table 4).

3.2. Bio-Test for Resistance to Pathotype Pa3 of Pale Nematode (G. pallida)

Inoculation with pathotype Pa3 of pale nematode was performed on the smaller set of 19 cultivars. Only two of 16 tested cultivars Danaâ and Vympel showed high resistance (score 9) to the pathotype Pa3 of *G. pallida*. One cv. Samba was moderately resistant (score 6). All remaining cultivars including two susceptible controls were strongly affected by the pathotype Pa3 of pale nematode (Table 5).

Table 5. Resistance of potato cultivars to *G. pallida* pathotype Pa3.

№	Cultivars	Average Number of Cysts in 500 cm^3 of Soil	Average Number of Eggs and Larvae per 500 cm^3 of Soil	Relative Susceptibility	Cultivar Response (1–9 Scale)
			Domestic cultivars		
1	Alyj parus	15.5	21,142.6	66.0	2
2	Antonina	23.5	24,157.1	75.4	2
3	Čaroit	14.7	20,856.0	65.1	2
4	Danaâ	0.0	0.0	0.0	9
5	Grand	7.5	5060.4	15.8	4
6	Gusar	81.4	117,851.5	367.9	1
7	Krasavčik	7.5	6370.8	19.8	4
8	Krepyš	52.1	66,204.5	206.7	1
9	Lomonosovskij	123.0	129,119.7	403.1	1
10	Naâda	74.2	59,727.8	186.5	1
11	Nevskij	27.7	32,025.0	100.0	1
12	Plamâ	44.6	56,282.6	175.7	1
13	Samba	7.2	2705.8	8.4	6
14	Tango	131.0	32,517.1	101.5	1
15	Utro	24.1	18,682.5	58.3	2
16	Vympel	0.0	0.0	0.0	9
			Foreign cultivar		
17	Red Scarlett	19.0	16,810.3	52.4	2
			Controls		
18	Alouette	33.6	38,369.6	119.8	1
19	Red Lady	32.3	40,028.1	124.9	1

Two cultivars resistant to pathotype Pa3 of pale nematode - Danaâ and Vympel (Table 5) were also resistant to Ro5 and Ro1 pathotypes of golden nematodes (Table 4). Cultivar Samba was moderately resistant to pathotype Pa3 of pale cyst nematode and pathotype of Ro5 of golden nematode and susceptible to pathotype Ro1 of *G. rostochiensis* (Tables 4 and 5). Four domestic cultivars (Čaroit, Krasavčik, Lomonosovskij, Nevskij) showed susceptibility to all three PCN pathotypes–Pa3, Ro5, Ro1.

3.3. MAS with DNA Markers of Genes (QTLs) Conferring Resistance to Pathotype Pa3 of Pale Nematode (G. pallida)

Nine DNA markers associated with four loci involved in the control of resistance to Pa3 pathotype of *G. pallida* (*Gpa2, Grp1_QTL*, $GpaV^{s}_{spl}$*_QTL* and $GpaV_{vrn}$*_QTL*) were used in molecular marker analysis of the whole set of 33 cultivars including controls (Supplementary Figures S1–S7). Data for 19 cultivars that took part in phenotyping are shown in Table 6.

Most markers did not have association with the phenotypic data. Thus, *GpaVSspl_QTL* defined by the CAPS-marker GP179/EcoRV was found in many susceptible cultivars and in one of the two resistant to pale cyst nematodes genotypes. Marker HC of the $GpaV_{vrn}$*_QTL* was detected both in resistant and in susceptible cultivars; this marker was not taken in the total calculation of diagnostic value because the target band was unclear for the score in four cvs.: Antonina, Krasavčik, Naâda, Vympel (Table 6, Supplementary Figure S3).

Two markers Gpa2-1 and 77R/HaeIII of the *Gpa2* gene were completely coinciding and showed relatively high but not significant concordance with bio-test data (a *p*-value lower than 10% according to the Fisher's exact test). Although the subset of 19 cultivars participated in phenotyping and marker assay was small for statistical evaluation, both were highly resistant to pathotype Pa3 of *G. pallida* cultivars Danaâ and Vympel have two markers Gpa2-1 and 77R/HaeIII and 13 of 16 Pa3-susceptible cultivars were band-negative. At the same time diagnostic bands of these two markers were detected in three susceptible cvs. Alyj parus, Čaroit, Utro (Table 6).

The combination of four resistance-predictive alleles (three of the *Gpa2* gene: Gpa2-1, 77R/HaeIII, GP34/TaqI-520 and one marker GP21/DraI of the *Grp1_QTL*) was detected only in two highly resistant to pathotype Pa3 cultivars Danaâ and Vympel (Table 6).

Table 6. Molecular marker analysis of potato cultivars with DNA markers associated with QTLs conferring resistance to *G. pallida* pathotype Pa3. "+"—diagnostic fragments present, "−"—diagnostic fragments absent. Resistance group: R—resistant, MR—moderately resistant, S—susceptible.

№	Cultivar	Cultivar Response (1–9 Scale) for Pa3	Resistance Group	QTLs/Markers								
				Gpa2				*Grp1_QTL*			$GpaV^{S}_{spl}$_QTL	$GpaV_{vrn}$_QTL
				Gpa2-1	77R/ HaeIII	GP34/ TaqI-520 bp	GP34/ TaqI-550 bp	TG432/ RsaI	GP21/ DraI	GP179/ RsaI	GP179/ EcoRV	HC
1	Alyj parus	2	S	+	+	+	+	+	−	+	+	−
2	Antonina	2	S	−	−	+	+	−	+	+	+	*
3	Čaroit	2	S	+	+	−	−	−	−	−	−	−
4	Danaâ	9	R	+	+	+	−	+	+	−	−	+
5	Grand	4	S	−	−	+	+	−	+	+	+	−
6	Gusar	1	S	−	−	−	+	−	+	+	+	−
7	Krasavčik	4	S	−	−	−	+	+	+	+	+	*
8	Krepyš	1	S	−	−	+	+	+	+	+	+	−
9	Lomonosovskij	1	S	−	−	+	+	+	+	+	+	+
10	Naâda	1	S	−	−	+	−	−	+	+	+	*
11	Nevskij	1	S	−	−	+	−	+	−	−	−	−
12	Plamâ	1	S	−	−	+	+	+	+	+	+	−
13	Samba	6	MR	−	−	+	−	+	+	+	+	−
14	Tango	1	S	−	−	−	−	−	+	+	+	+
15	Utro	2	S	+	+	−	−	+	+	−	−	−
16	Vympel	9	R	+	+	+	+	+	+	+	+	*
				Controls								
17	Alouette	1	S	−	−	+	−	+	−	+	+	−
18	Red Lady	1	S	−	−	−	−	+	+	+	+	+
19	Red Scarlett	2	S	−	−	+	+	+	−	+	+	+

Table 6. *Cont.*

№	Cultivar	Cultivar Response (1–9 Scale) for Pa3	Resistance Group	QTLs/Markers								
				Gpa2			*Grp1_QTL*				$GpaV^{S}_{spl}$_QTL	$GpaV_{vrn}$_QTL
				Gpa2-1	**77R/ HaeIII**	**GP34/ TaqI-520 bp**	**GP34/ TaqI-550 bp**	**TG432/ RsaI**	**GP21/ DraI**	**GP179/ RsaI**	**GP179/ EcoRV**	**HC**
Totally estimated cultivars (N) including: N = 19: 2 R (score 9), 1 MR (score 6), 16 S (score 1–4)												
No. of resistant (score 9) cultivars with the marker				2 of 2	2 of 2	2 of 2	1 of 2	2 of 2	2 of 2	1 of 2	1 of 2	
No. of resistant (score 9) cultivars without the marker				0	0	0	1 of 2	0	0	1 of 2	1 of 2	
No. of susceptible (score 1–4) cultivars with marker				3 of 16	3 of 16	10 of 16	9 of 16	10 of 16	11 of 16	13 of 16	13 of 16	
No. (%) of susceptible (score 1–4) cultivars without the marker				13 of 16 (81.3%)	13 of 16 (81.3%)	6 of 16 (37.5%)	7 of 16 (43.8%)	6 of 16 (37.5%)	5 of 16 (31.3%)	3 of 16 (18.8%)	3 of 16 (18.8%)	

* unclear to score.

4. Discussion

Currently, most of the nematode-resistant commercial cultivars grown in different countries are protected by the major *H1* gene from clone CPC1673 of *S. tuberosum* ssp. *andigena* which provides for over 50 years durable resistance to the most widespread pathotype Ro1 of *G. rostochiensis* [15,76]. At the same time cultivation of varieties with resistance to only one pathotype and one PCN species should be done carefully to prevent emergence of new virulent pathotypes and the increase of *G. pallida* populations, which are more difficult to be controlled [77,78]. Several examples confirm the likelihood of such risks. A propagating population of *G. rostochiensis* was detected on cultivars carrying the *H1* gene which has been remaining effective for many years against golden nematode in the state of New York, U.S., where populations of *G. rostochiensis* were considered to consist solely of pathotype Ro1; later it was identified as Ro2 [79,80]. The *H1* gene is not effective against the pale potato cyst nematode. Widespread planting of cultivars resistant to only *G. rostochiensis* has led to an increase in *G. pallida* infestations which currently is the predominant species in the U.K. [77]. Practically, the best economically and environmentally friendly approach to prevent the spread of PCN is the planting of cultivars with multiple resistance against different pathotypes of both species—*G. rostochiensis* and *G. pallida* through pyramiding the *R* genes/QTLs in a single cultivar [15]. Therefore, information about the resistance loci presented within the cultivar gene pool is the basis for the development of new breeding material with multiple resistance to PCN.

In some countries, for example, in Russia, *G. rostochiensis*, pathotype Ro1 is the only detected species and pathotype. Therefore, new sources of resistance to PCN species may be in demand in the coming future. In the present study we report the results of bio-tests for resistance to pathotype Ro5 of *G. rostochiensis* and to *G. pallida* pathotype Pa3 which were conducted for the first time for Russian cultivars and successful finding of several domestic cultivars with multiple PCN resistance. Phenotypic assays revealed 13 domestic cultivars which are highly resistant to the pathotype Ro5 of golden nematode, nine of them (cvs. Danaâ, Evraziâ, Gusar, Holmogorskij, Krepyš, Liga, Meteor, Naâda, Vympel) are also resistant to pathotype Ro1 and two of them (cvs. Danaâ and Vympel) possess resistance both to the pathotypes Ro5 and Ro1 of *G. rostochiensis* and to *G. pallida*, pathotype Pa3.

The main sources of resistance to the pathotype Ro5 of golden nematode and to the Pa2/Pa3 of pale nematode are *S. vernei* [15,34,52] and *S. tuberosum* ssp. *andigena* [15,35,52], wherein most of the European resistant varieties are protected against *G. pallida* by the major $GpaV_{vrn}$*_QTL* from *S. vernei* [34]. For Russian cultivars the sources of introgression of resistance to the pathotype Ro5 of golden nematode and to Pa3 of pale nematode are not clear because of their complex origin. Five of 13 highly resistant to Ro5 cultivars (Alyj parus, Liga, Naâda, Sirenevyj tuman, and Danaâ) have the same interspecific hybrids with *S. tuberosum* ssp. *andigena* and with *S. vernei* in their pedigrees [57]; these six cultivars were developed by the same breeders who probably used the common source of the *Grp1* resistance. Two Russian cultivars Gusar and Evraziâ originated from crosses with resistant to Ro5 German cultivars Arosa and Barbara respectively. Source of PCN resistance of cv. Vympel is unknown. In the pedigrees of the rest six highly resistant to Ro5 cultivars there are indications about the involvement of *S. tuberosum* ssp. *andigena* and/or *S. vernei* in their origin.

Cultivars Danaâ and Vympel showing multiple PCN resistance are particularly interesting for national breeding programs directed on marker-assisted-gene pyramading of the *R*-genes/QRLs to different pests and diseases. For potatoes a successful stacking of the major *R*-genes involved in monogenic control of resistance to pathogens was reported in a number of papers [39,44,50,53,81]. Several studies demonstrate the potential of MAS for stacking of QTLs involved in the control of quantitative resistance to *G. pallida*, pathotype Pa2/Pa3 [45,82,83] as well as for pyramiding of QTLs conferring resistance to both PCN species—*G. pallida*, pathotype Pa2/3 and *G. rostochiensis*, pathotype Ro5 [41,43–54]. The

efficiency of such breeding programs can be significantly improved using marker-assisted selection.

However, unlike the success with MAS for resistance to Ro1 conferring the major *H1* gene, marker-assisted selection for durable resistance to the other pathotypes of *G. rostochiensis* as well as to *G. pallida* is much more complicated. Two aspects can be considered with regards to the efficiency of MAS for multiple PCN resistance: (1) diagnostic value of molecular markers which is dependent on the genetic background of the tested germplasms; (2) different scores for moderate resistant genotypes and stability of phenotyping data.

Many DNA markers associated with QRLs of PCN resistance have been developed but they are pedigree-specific since these molecular markers were designed using specific segregating populations. For example, CAPS-marker TG432/RsaI of the *Grp1* locus conferring resistance to Ro5 pathotype of *G. rostochiensis* and HC marker linked to the $GpaV_{vrn}$ conferring resistance to *G. pallida*, Pa2/Pa3 demonstrated the high diagnostic value in Spanish [81] and Indian breeding programs [41,43,44]. At the same time D. Milczarek with colleagues [37] showed low predictive value of these markers for the detection of resistant and susceptible cultivars. The low diagnostic value of the HC marker linked with the $GpaV_{vrn}$*_QTL* was shown by K. Asano with colleagues [39]. Our results demonstrated that in the genetic background of tested Russian cultivars, markers of the *Gpa2*, *Grp1_QTL*, $GpaV^{S}{}_{spl}$*_QTL* and the $GpaV_{vrn}$ lost their predictive value-none of the used markers was associated with the phenotypic data of resistance to pathotype Ro5 of *G. rostochiensis* or to Pa3 of *G. pallida*.

According to A. Barone with colleagues (1990) [84], a major dominant gene *Gro1* confers resistance to all pathotypes of *G. rostochiensis* including Ro5. In our previous research it was shown the extremely low frequency of the Gro1-4 marker in the Russian cultivar genepool [42,56–59]. A few Gro1-4-positive cultivars were involved in the present study, however, any association with resistance to Ro5 pathotype was not detected. Similar results were obtained by D. Milczarek with colleagues (2011) for West-European potato cultivars [37]. It is also possible that selected in present study Russian cultivars originated from unknown sources of resistance to pathotype Ro5.

It should be mentioned that the diagnostic value of molecular marker can be dependent on by different scores. In our case, the diagnostic value of molecular marker was calculated for highly resistant genotypes (nine scores according to the EPPO scale). In Indian breeding programs, both highly resistant and moderately resistant genotypes are considered to have a desirable level of resistance [43]. In the case of counting moderately resistant cultivars as resistant genotypes the diagnostic value of the GP179/RsaI marker of the *GrpI_QTL* may increase significantly (Table 4). In the present study we selected six moderately resistant to Ro5 cultivars and one with a moderate level of resistance to pathotype Pa3 of *G. pallida*. At the same time, it should be kept in mind that the cultivation of moderately resistant varieties can lead to a rapid loss of resistance due to the selection of more adapted and productive populations of nematodes [85].

Results of the present study indicate that the combination of the marker-resistance alleles of the three loci—*Gpa2*, *Grp1* and $GpaV_{vrn}$*_QTL* can be more efficient against the pathotype Pa3 of *G. pallida* than a single locus. The most effective combination of five resistance-predictive alleles (Gpa2-1, 77R/HaeIII, GP34/TaqI-520–GP21/DraI–HC) improved resistance prediction. This haplotype was found only in two highly resistant cultivars Danaâ and Vympel and it was not detected in 17 cultivars which are susceptible to pathotype Pa3 of *G. pallida*. Three other Gpa2-1-positive cultivars (Alyj parus, Čaroit, Utro) are susceptible to *G. pallida* pathotype Pa3, they all are HC-negative, in addition cv. Čaroit lost marker-predictive allele of the *Grp1*_QTL. On the other side, several susceptible to pale cyst nematode cultivars were HC-positive and at the same time–Gpa2-1-negative. This relationship indicates the influence of epistatic effects between three QTLs (*Gpa2, Grp1,* $GpaV_{vrn}$) on resistance to *G. pallida.* Nevertheless, the presence-predictive-haplotype should be verified on the bigger subsets and in segregating populations.

In conclusion, examples of successful application of molecular breeding for selection of genotypes which are resistant to both PCN species (*G. pallida* and *G. rostochiensis*) and to their different pathotypes (except Ro1) are still very limited. For today, selection of cultivars for multiple PCN resistance still needs the further development of more reliable and repeatable molecular markers for QTLs with different effects on resistance which could be applied in different genetic background. Recent progress in SNPs genotyping helped to improve the PCR markers [45] and to increase their diagnostic values. Our future studies will focus on the involvement of resistant cultivars selected in this study into the breeding and on the application of bio-tests and improved DNA markers in large scales of genotypes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11122400/s1, Figure S1: Diagnostic bands of markers (indicated by an arrow) for identification of *Gpa2* gene, Figure S2: Diagnostic bands (indicated by an arrow) of TG432/RsaI (A) and GP179/RsaI (B) markers for identification of *Grp1_QTL*, Figure S3: Diagnostic bands of HC marker for identification of $GpaV_{vrn}$*_QTL* locus, Figure S4: Diagnostic band of GP21/DraI marker (indicated by the arrows) for identification of *Grp1_QTL*, Figure S5: Diagnostic bands of markers GP34/TaqI (indicated by the arrows) for identification of *Gpa2* gene, Figure S6: Diagnostic bands (indicated by the arrows) of markers GP179/EcoRV for locus $GpaVs_{spl}$*_QTL*, Figure S7: Restricted DraI- fragments of PCR-products amplified from potato cultivars' DNA used primers TG432.

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