RESISTANCE OF *HELMINTHOSPORIUM SOLANI* STRAINS TO SELECTED FUNGICIDES APPLIED FOR TUBER TREATMENT

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SUMMARY

Helminthosporium solani strains were isolated from potato tubers collected in Russia or taken from imported German and Dutch seed tubers. Sequences of the nuclear ribosomal genes and internal transcribed spacers (ITS) for all 24 tested strains were identical and had 100% similarity to the sequences from GenBank identified as *Helminthosporium solani*. The obtained molecular data confirmed the morphological identification based on the width and length of conidia, the shape of conidiophores and the colony morphology. Screening for resistance to the fungicides Score 250 SC (active ingredient difenoconazole 250 g/l), Quadris (azoxystrobin 250 g/l), Tecto 500 SC (thiabendazole 500g/l), Zeroxxe [colloidal silver particles (3 g/l) stabilized with amphoteric surfactant] was done. Agar blocks with pure cultures of the fungal strains were placed in the centre of Petri dishes containing malt agar amended with fungicide concentrations of 0.1, 1, 10, 100 and 1000 mg/l (accounted for the concentration of the active ingredient). Malt agar free of fungicide was used as the control. Growth inhibition of 50% (EC_{50}) compared to the control was detected based on the dose-response curves. Difenoconazole ($EC_{50} < 0.12$mg/l) and colloidal silver ($EC_{50} < 76$ mg/l) were the most effective fungicides. No strains resistant to the aforementioned fungicides were found. In most cases, azoxystrobin was effective against *H. solani* ($EC_{50} < 7$ mg/l), but there were several strains with high resistance to this fungicide ($EC_{50} > 100$ mg/l). Thiabendazole appears to be effective against the sensitive strains of *H. solani* ($EC_{50} < 7.3$ mg/l); however, six studied strains from Russia and the Netherlands were found to be extremely resistant to it ($EC_{50} > 1000$ mg/l). The sequence of their β-tubulin gene contained a SNP mutation in the 198 codon or 200 codon, translating to Gln (CAG) instead of Glu (GAG) or Tyr (TAC) instead of Phe (TTC), respectively. Thus, the resistance to thiabendazole of the Russian, European and American strains had the same genetic background and was conferred by the same mutations.

Keywords: potato silver scurf, *Helminthosporium solani*, potato tuber diseases, fungal potato pathogens, modified silver nanoparticles.

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INTRODUCTION

Silver scurf of potato (*Solanum tuberosum* L.), caused by the fungus *Helminthosporium solani* Durieu & Montagne, is a surface-blemishing disease of potato tubers (Read and Hide, 1984; Gore, 2017). At harvest time, the infected tubers have grey lesions on the periderm that appear silvery when moist. Under favorable storage conditions, the fungal sporulation makes tubers black and sooty. In the case of red skinned potato cultivars, the silver scurf can cause a complete loss of skin pigmentation. It does not cause yield losses at the time of harvest, but causes weight loss of stored potatoes due to increased water loss, resulting in excess shrinkage and flabbiness (Secor and Gudmestad, 1999). Portions of the periderm may eventually slough off. Infection often takes place during the growing season and lesions may be visible at the time of harvest. Disease severity increases greatly during long-term storage of tubers. The disease does not affect any other part of the potato plant except the tubers (Secor and Gudmestad, 1999). Initially considered a disease of minor importance, the silver scurf is now becoming a disease of high economic consequence because of the increasing consumer demand for washed potato.

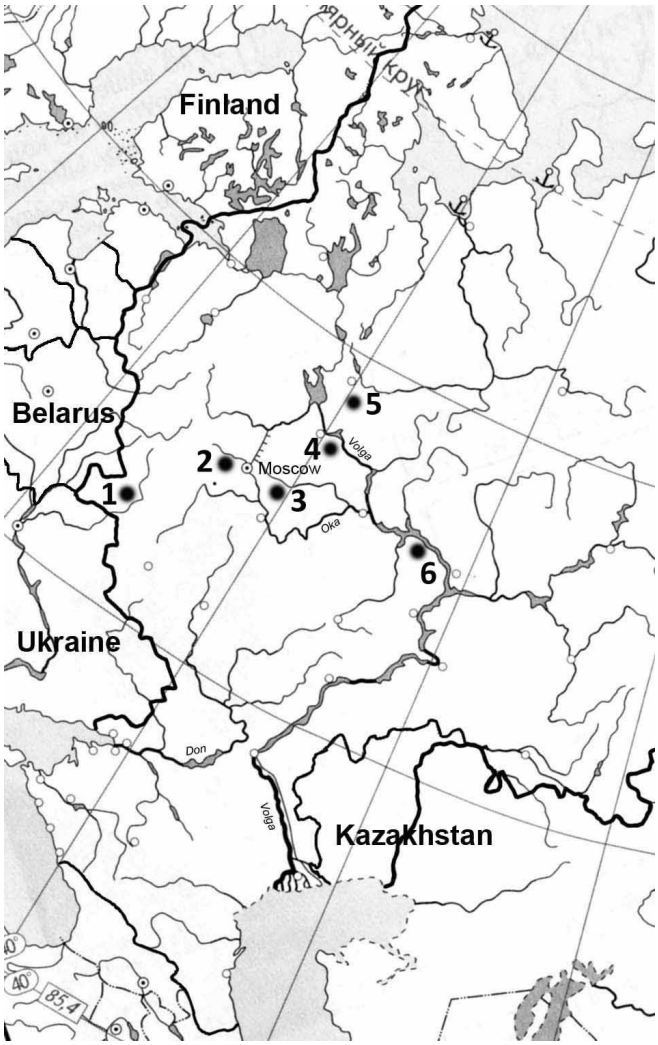

Fig. 1. Location of the sampling sites.

Infected potato seed tubers are the primary source of the inoculum (Burke, 1938; Santerre, 1972; Secor and Gudmestad, 1999). Once planted, the inoculum is transferred to the daughter tubers. Silver scurf incidence and severity increases with each new generation of crops (Geary and Johnson, 2006). Severity of the silver scurf in progeny tubers can be reduced by treating seed tubers with fungicides or elongation of periods between potatoes in crop rotation.

In 1968, thiabendazole was first found to be effective against a range of potato pathogens including *H. solani* and, since the mid-1970s, it has widely been used on potatoes (Hide *et al*., 1988). Applied as a part of postharvest or seed treatment, the fungicide has provided effective control of the silver scurf until the resistant isolates increased in frequency. Resistance of *H. solani* to thiabendazole was first reported in the UK in 1988 based on the strains collected from 1977 to 1986 in commercial fields of seed and ware potato (Hide *et al*., 1988). Thiabendazole resistance in *H. solani* was subsequently documented in the USA (Merida and Loria, 1990) and Canada (Kawchuk *et al*., 1994; Holley and Kawchuk, 1996; Platt, 1997). Thiabendazole insensitive strains were found in progeny tubers after one application of thiabendazole to seed infected with the sensitive strains (Hide and Hall, 1993). Loss of efficacy of thiabendazole, resulting from the increase in the frequency of resistant isolates, has led to the exploration of alternative fungicides for the control of the silver scurf.

Table 1. Origin of the tested *H. solani* strains.

Strain	Region	Site on the map	Potato cultivar	Year of isolation
Russian strains				
RB7	Bryansk reg.	1	Rosara	2013
RB11	Bryansk reg.	1	Vineta	2013
RMCh2	Moscow reg.	2	Nevskiy	2014
RMCh5	Moscow reg.	2	Nevskiy	2014
RMCh24	Moscow reg.	2	Nevskiy	2014
RM4d	Moscow reg.	3	Zhukovskiy ranniy	2013
RM32d	Moscow reg.	3	Zhukovskiy ranniy	2013
RM42	Moscow reg.	3	Zhukovskiy ranniy	2013
RKSt39	Kostroma reg.	4	Udacha	2013
RKSt68	Kostroma reg.	4	Udacha	2013
RKSu2/2	Kostroma reg.	5	Alwara	2014
RKSu7	Kostroma reg.	5	Delphine	2014
RKSu18	Kostroma reg.	5	Safia	2014
RKSu10	Kostroma reg.	5	Safia	2015
RCh1	Chuvashia rep.	6	Udacha	2014
RCh8	Chuvashia rep.	6	Udacha	2014
Strains from the imported seed potato tubers				
H16	Netherlands		Asterix	2013
H28	Netherlands		Asterix	2013
G3	Germany		Alwara	2013
G11	Germany		Delphine	2013
G12	Germany		Estrella	2013
G18	Germany		Saphia	2013
G20	Germany		Saphia	2013
G21	Germany		Saphia	2013

In this paper we have estimated the *in vitro* resistance of Russian and West-European *H. solani* strains to the fungicides used for tuber treatment: Score 250 SC (active ingredient difenoconazole 250 g/l), Quadris (azoxystrobin 250 g/l), Tecto 500SC (thiabendazole 500 g/l), Zeroxxe [colloidal silver particles (3 g/l) stabilized with an amphoteric surfactant].

MATERIALS AND METHODS

Source of isolates. Naturally infected samples of potato tubers from different regions of the Russian Federation and imported seed material from Germany and the Netherlands were used in the isolation trials of *H. solani*. The Russian strains were isolated from the seed material from Moscow, Vladimir, Bryansk, Kostroma areas and the Chuvashiya Republic (Fig. 1; Table 1).

Isolation of the *H. solani* strains. Tubers were washed with water, dried and cut into slices with a periderm layer, which were placed into Petri dishes and incubated at 21-23°C in the dark for 4-10 days. Using a binocular

Table 2. Primers for the amplification of the ITS regions and the β-tubulin gene of *H. solani*.

Name	Sequence	Melting temperature	Amplified region	Literature source
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG	58	Part of nuclear ribosome gene and ITS regions	White *et al*., 1990
ITS4	5'-TCCTCCGCTTATTGATATGC			
SS-f	5'-AGCATAGGCTGATGCTCGT	58	β -tubuline gene	McKay and Cooke, 1997
SS-r	5'-GACGATGAGTCCTGAGTAA			

microscope, *H. solani* conidia from the same conidiophore were removed with a sterile needle and placed in the center of 1.5% malt agar with an antibiotic solution (1000 U/ml benzylpenicillin sodium) on a Petri dish, then incubated at 25°C in the dark for 10-12 days. After that, the hyphal tips were transferred under a binocular microscope onto another Petri dish with clarified malt agar (1.5% of agar), sealed with Parafilm M and incubated for 15-20 days at 25°C. When the colony diameter reached 20-25 mm, the Petri dishes were transferred to a refrigerator and stored at 5°C until needed. From each tuber, only one strain was isolated.

DNA isolation. Mycelium was grown on a liquid pea medium (170 g of frozen green pea boiled for 10 min in 1 liter of distilled water, filtered through a cheesecloth and autoclaved during 30 min at 1 atm) and ground in liquid nitrogen. After grinding, 700 µl of CTAB buffer [1.4 M NaCl, 0.1 M Tris-HCl, 20 mM EDTA, and 2% hexadecyltrimethylammonium bromide (CTAB)] were added and each tube was agitated briefly. Tubes were incubated at 65°C for 1 h. After incubation, 500 µl of cold chloroform were added to the tubes that were centrifuged at 13,000 rpm for 10 min and the supernatants transferred to clean 1.5 ml microcentrifuge tubes. Isopropanol (400 µl) and 70 µl of 5 M potassium acetate (pH 4.6) were added to each supernatant, the tubes were shaken carefully and centrifuged at 13,000 rpm at room temperature for 10 min. Supernatants were discarded, 150 µl of 70% ethanol (v/v) were added and the tubes were centrifuged at 13,000 rpm at room temperature for 5 min. Supernatants were removed, pellets were air dried at room temperature for 20 min and resuspended in the 50 µl of sterile purified water (milliQ®).

PCR amplification. DNA amplifications were performed in a 25 µl total volume reactions containing 50 ng of the DNA template, 120 µM of each deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, dTTP), 0.2 µM of each primer (Evrogen Co, Russia) (Table 2), and 1.5 U of Taq polymerase (Promega, USA) in the reaction buffer supplied by the manufacturer. As the negative control, 1 µl of purified water (milliQ®) was used instead of the fungal DNA. The amplification was performed on a Biometra T1 cycler. DNA was denatured for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s, and the final extension at 72°C for 5 min. After amplification, PCR products were electrophoresed in the 1% agarose gel containing ethidium bromide (0.5 µg/ml) in 0.5 × Tris-borate EDTA (TBE) buffer at constant voltage (100 volts), for approximately 1 h, visualized and recorded with a UVP Image Store 7500 UV Transilluminator (UVP Inc., USA). PCR products were extracted from the gel, cleaned using the "Cytokine" kit (Cytokine Co., Russia), and used for the sequence analysis.

DNA sequencing. PCR amplicons were sequenced using the BigDye®Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the Applied Biosystems 3730 xl automated sequencer (Applied Biosystems, USA). Each fragment was sequenced in both directions using the same primers described above. Contigs sequences were used to identify the fungal isolates based on the sequences similarity in GenBank using the BLASTn program (version 2.0, NCBI United States National Institutes of Health, Bethesda, MD, USA).

Estimation of resistance to fungicides. Pure cultures of the fungal strains were sectioned into small agar inoculum blocks (5 mm in diameter) that were placed in the centre of Petri dishes (90 mm × 15 mm) containing 25 ml of malt agar with a fungicide at the rates of 0.1, 1, 10, 100 mg/l (accounted for the concentration of the active ingredient, AI). An additional concentration of 1000 mg/l (AI) was used in the case of thiabendazole and colloid silver. Malt agar without the fungicide was used as a control (Fig. 2). All fungicides were added to the cooling media before pouring. Three replicated inoculated dishes were incubated at 24 ± 1°C in the dark and the colony diameters were measured (in two directions per dish) after 18-25 days of growing, when the control colony had a diameter of approximately 50 mm. Mycelial growth for each isolate was converted to per cent inhibition compared with control. The fungicide concentration that inhibit linear growth of colony of 50% over control (EC_{50}), or of 90% over control (EC_{90}) was determined for each isolate by linear interpolation using the two concentration that bracketed 50% or 90% of inhibiting correspondingly.

RESULTS

Sequence of the nuclear ribosomal genes and internal transcribed spacers (ITS). No difference between the strains of the tested cultures was found with respect to the

Table 3. Resistance of *H. solani* strains to fungicides.

Strain	Thiabendazole		Difenoconazole		Azoxystrobin		Colloidal silver	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Russian strains								
RB7	5.4	9.1	0.06	11.5	>100	>100	10.0	93.6
RB11	6.6	41.5	0.06	8.9	0.08	>100	40.9	100.1
RMCh2	>1000	>1000	0.06	4.2	1.45	>100	40.0	98.7
RMCh5	738	>1000	0.09	24	0.08	>100	23.2	127.0
RMCh24	>1000	>1000	0.06	4.8	>100	>100	47.5	96.6
RM4d	0.5	0.9	0.08	9.0	0.09	>100	7.6	77.5
RM32d	6.1	9.2	0.06	3.7	5.5	>100	45.2	89.0
RM42	7.3	64.5	0.05	0.1	0.07	>100	7.0	86.4
RKSt39	5.9	9.2	0.06	1.0	>100	>100	52.6	125.3
RKSt68	5.7	9.1	0.06	0.7	0.8	>100	19.0	87.4
RKSu2/2	0.7	6.4	0.06	1.5	7.0	77.5	61.6	120.5
RKSu7	0.7	6.4	0.06	7.5	0.1	>100	76.7	178.0
RKSu10	>1000	>1000	0.07	1.0	1.2	>100	14.6	92.3
RKSu18	5.8	9.2	0.07	18	0.1	>100	7.1	86.2
RCh1	0.8	7.5	—*	—	—	—	—	—
RCh8	1.7	8.3	0.06	0.6	0.08	>100	8.0	90.2
Strains from the imported seed potato								
H16	>1000	>1000	0.07	6.1	0.09	>100	48.6	108.5
H28	818	>1000	0.06	3.6	>100	>100	62.2	145.0
G3	1.0	8.2	0.07	12.3	0.1	>100	43.5	95.5
G11	0.5	0.9	0.09	8.2	85.00	>100	69.4	112.2
G12	3.2	8.8	—	—	—	—	—	—
G18	0.5	0.9	0.06	0.4	0.1	96.1	26.5	115.0
G20	5.8	9.2	0.12	1.0	>100	>100	55.0	94.2
G21	5.3	9.1	0.06	5.5	>100	>100	72.7	120.4

*— - not tested.

analyzed DNA regions. Obtained ITS sequences for all 24 tested strains had a 100% identity with the sequences previously identified as *H. solani* [GenBank accession Nos. KC106739 (Al-Mughrabi *et al.*, 2013) and AF073904 (Olivier and Loria, 1998)]. The obtained molecular data confirmed the results of morphological identification based on the width and the length of conidia, the shape of conidiophores and the colony morphology.

Evaluation of resistance of the *H. solani* strains to fungicides. The difference in the fungicide efficiency against different strains of *H. solani* were revealed (Table 3). Difenoconazole demonstrated the highest level of fungistatic efficiency against *H. solani* ($EC_{50} \leq 0.12$ mg/l, $EC_{90} \leq 24$ mg/l). Another effective fungicide was colloidal silver with EC_{50} up to 76 mg/l, and EC_{90} up to 178 mg/l. No *H. solani* strains resistant to difenoconazole or colloidal silver were identified.

Azoxystrobin was effective against most *H. solani* strains ($EC_{50} \leq 7$ mg/l). However, among the Russian, Dutch and German strains there were several strains resistant to azoxystrobin. They were able to grow in the medium with a high concentration of the fungicide ($EC_{50} > 100$mg/l). The strain RMCh24 was resistant to both azoxystrobin and thiabendazole.

Difenoconazole, colloidal silver, and azoxystrobin appeared to have a strong fungistatic effect on *H. solani*.

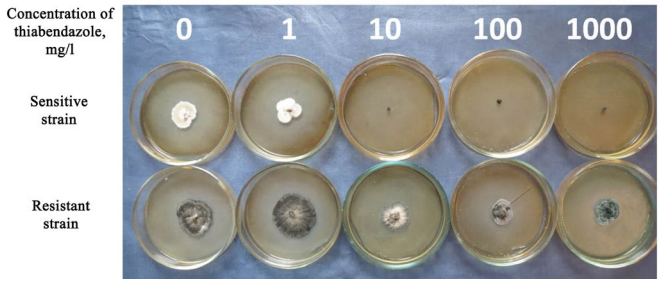

Fig. 2. Growth of sensitive and resistant strains on the agar media with different concentrations of thiabendazole (42nd day of cultivation).

After 25-30 days of incubation the mycelium started to grow very slowly on the fungicide-containing medium.

Thiabendazole inhibited the majority of the tested *H. solani* strains. Fig. 2 represents the sensitive and resistant strains on the agar media with different concentrations of this fungicide. The growth of the sensitive strains was restricted by evidently low concentrations of thiabendazole ($EC_{50} \leq 7.3$ mg/l). The mycelium did not expand into the fungicide medium from the inoculum block even after 40 days of incubation. The tested strains from the German tubers were all sensitive to thiabendazole. Among the Russian and Dutch samples there were strains 1000 times more resistant to thiabendazole than the sensitive ones (Table 3).

Identification of the thiabendazole resistance mutations. Thiabendazole is a benzimidazole fungicide that binds to the fungal β-tubulin protein and inhibits microtubule function. Resistance to benzimidazoles has been detected for many fungal species including *H. solani*. A mutation in the β-tubulin gene leads to the substitution within the β-tubulin molecule due to the point mutations in the codons 198 or 200 (Davidse and Flach, 1978; Koenraadt *et al.*, 1992). We have determined the structure of a β-tubulin gene fragment for a total of 17 *H. solani* strains differing in the level of the thiabendazole resistance. All analyzed β-tubulin sequences of sensitive strains were identical and matched the GenBank sequence Y10670 of the β-tubulin gene for the thiabendazole sensitive strains (McKay and Cooke, 1997). The only nucleotide mutation in the 198 codon was identified in the case of highly resistant strains RMCh24 and H16 isolated from the tubers of cv. Sante from the Moscow area and cv. Asterix imported from the Netherlands, respectively. The substitution of Glu (GAG) with Gln (CAG) during translation was the result of the SNP. The same SNP in the 198 codon was identified in the strain RKSu10 (sample from the Kostroma region, cv. Safia grown from German seed material). The strain RMCh2 was isolated from infected tubers together with the strain RMCh24 (both tubers from the same field in the Moscow region). It was resistant to thiabendazole; however, there was another SNP in the 200 codon resulting in a substitution of Phe (TTC) with Tyr (TAC) (Table 4). This mutation

Table 4. The structure of the β-tubulin gene of *H. solani* strains with different levels of resistance to thiabendazole. The codons with mutation are in bold.

Strain	Resistance to thiabendazole, EC_{50}, mg/l	Sequence of codons 197-200 of the β-tubulin gene
RB7	5.4	GACGAGACCTTC
RB11	6.6	GACGAGACCTTC
RMCh2*	>1000	GACGAGACC**TAC**
RMCh24**	>1000	GAC**CAG**ACCTTC
RM4d	0.5	GACGAGACCTTC
RKSt68	5.7	GACGAGACCTTC
RKSu2/2	0.7	GACGAGACCTTC
RKSu10**	>1000	GAC**CAG**ACCTTC
RCh1	0.8	GACGAGACCTTC
RCh8	1.7	GACGAGACCTTC
H16**	>1000	GAC**CAG**ACCTTC
G3	1.0	GACGAGACCTTC
G11	0.5	GACGAGACCTTC
G12	3.2	GACGAGACCTTC
G18	0.5	GACGAGACCTTC
G20	5.8	GACGAGACCTTC
G21	5.3	GACGAGACCTTC

*- strain RMCh2 with mutation in codon 200 resulting in the substitution of Phe (TTC) with Tyr (TAC).

** - strains RMCh24, RKSu10, H16 with mutation in 198 codon resulting in the substitution of Glu (GAG) with Gln (CAG).

is known to confer resistance to benzimidazole (Koenraadt *et al.*, 1992; McKay and Cooke, 1997).

DISCUSSION

It is difficult to control *H. solani* because the fungus survives and spreads both in the field and storage. However, the seed tuber treatment can reduce disease incidence. A number of preparations for seed treatment, including thiabendazole, imazalil, prochloraz, prochloraz manganese chloride, thiophanate-methyl with mancozeb, captan with mancozeb, fludioxonil and benomyl, appear to be effective in limiting disease incidence (Hide *et al.*, 1988, 1994a, 1994b; Denner *et al.*, 1997; Frazier *et al.*, 1998). In Russia, the fungicides like penflufen, thiabendazole, thiram, fludioxonil, difenoconazole, azoxystrobin, benomyl and pencycuron are used separately or in a combination for tuber preplant or postharvest treatment (Table 5).

In our research, difenoconazole appeared to be the most effective fungicide. None of the *H. solani* strains resistant to this fungicide have been detected among the tested samples. In the case of difenoconazole, we have revealed its effectiveness *in vitro* against *Colletotrichum coccodes*, *Alternaria solani*, and *Alternaria alternata* (Kutuzova *et al.*, 2015; Pobedinskaya *et al.*, 2012). It was found also that difenoconazole inhibits the oospore formation of *Phytophthora infestans* (Elansky *et al.*, 2016).

Zeroxxe, a colloidal silver-based fungicide, provided good protection of tubers against the *H. solani* strains. We have also shown that this fungicide is effective against *Phytophthora infestans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Alternaria solani*, *Colletotrichum coccodes*. Moreover, Zeroxxe has shown high antibacterial activity (Mita *et al.*, 2014; Khodykina *et al.*, 2014; Zherebin *et al.*, 2014).

H. solani strains differed greatly in resistance to azoxystrobin. Some strains grow well in agar media with azoxystrobin concentration above 100 mg/l (Table 3). Analyzed literature sources did not contain any evidence of *H. solani* being resistant to azoxystrobin, but the resistant strains of other plant pathogenic fungi and the mutation mechanisms are well known (Pasche *et al.*, 2004, 2005; Pobedinskaya *et al.*, 2012; FRAC, 2012). It is possible that owing to the start of azoxystrobin application for soil treatment before planting (Table 5) the resistant *H. solani* strains will soon prevail in Russian populations.

Thiabendazole turned out to be effective against the sensitive strains of *H. solani* only. Strains of *H. solani* resistant to thiabendazole have already been detected in the UK, the USA and Canada (Merida and Loria, 1990; Kawchuk *et al.*, 1994; Holley and Kawchuk, 1996; Platt, 1997; Hide and Hall, 1993). This study confirms the existence of the thiabendazole resistance of *H. solani* in Russia. There, thiabendazole is often used to protect potato tubers in storage. The effective application of thiabendazole is crucially dependent on the monitoring survey of the pathogen population including the imported seed material.

The challenge of screening in order to detect the fungicide resistance of the *H. solani* strains is the low growth rate of the fungus. The colony in the fungicide-free medium reaches the diagnostic size of 40-50 mm not earlier than 20-25 days of incubation and sometimes even later. The PCR-based assay is an appropriate method of studying the *H. solani* strains. This technique provides rapid identification of the *H.* solani strains either resistant or sensitive to thiabendazole directly from a tuber tissue within 1 day (Saunders and Errampalli, 2001). According to our data, the resistance of the Russian, European and American strains has the same genetic background and is conferred by the same mutations. Thus, the common approach to molecular diagnosis of the resistance to thiabendazole can be applied here in the form of the effective method of McKay and Cooke (1997) for detection of SNP in 198 codon and other PCR tests. Obviously, the PCR tests can be integrated into the strategy of the silver scurf disease management.

Spores forming the inocula initiating the disease transmission are an important factor to be considered during storage. *H. solani* is able to survive even in the clear storage. According to the data of Frazier *et al.* (1998), the *H. solani* spores were viable after nine months of storage in the soil between tubers or in the thermal insulation. The infected tubers from the fields are also the primary source of the inocula. Spores are being moved by the air from the ventilation system and pose a threat to healthy tubers (Rodriguez *et al.*, 1993). New infection may occur when warm and humid conditions favor the germination of the conidia (Rodriguez *et al.*, 1996). To limit the infection sources in

Table 5. Fungicides for tuber treatment registered in Russia.

Fungicide	Concentration of a fungicide in the treatment solution (g/l)	Amount of the treatment solution (l/t)	Mode of application
Azoxystrobin	2.1-9.4*	80-200 (l/ga)	Soil, before planting
Benomyl	28-83	2	Tuber, before planting
Pencycuron	10-15	10	Tuber, before planting
Thiram	80-100	<20	Tuber, before planting
Penflufen	1-2.3	10-20	Tuber, before planting
Difenoconazole	0.4-1.0	10-25	Tuber, before planting
Fludioxonil	0.5	10	Tuber, before planting, before or during storage
Colloid silver**	0.1-0.3	10-20	Tuber, before planting, before or during storage
Thiabendazole	4.8-5.6	10	Tuber, before planting, before or during storage
Benzoic acid	7.5-12	<10	Tuber, before planting or before storage

* According to the State Catalogue of Pesticides and Agricultural Chemical Substances allowed for use in the Russian Federation.
** Being registered in Russia.

the storage it is necessary to clean and sanitize the room before potato loading and treat the tubers with the chemicals before and during storage. Fungicides such as benzoic acid, fludioxonil and thiabendazole are registered for the post-harvest treatment of tubers in Russia (Table 5). The most popular method of the treatment in the storages of any size in Russia involves the use of pyrogenic pot "Vist". Thiabendazole is released during the process of burning, spreads through the ventilation system and protects the tubers.

Post-harvest fungicides spectrum in other countries is also quite limited. For example, in the EU only imazalil has been approved for post-harvest treatment of potato (EU Commission implementing regulation No. 540/2011). In the USA, Morocco and some other countries thiabendazole is a popular fungicide also. Besides imazalil and thiabendazole, fungicides like fludioxonil, azoxystrobin and difenoconazole are allowed to be used in the USA. Strains of fungi and oomycetes resistant to all of the aforementioned fungicides are found in different countries (FRAC, 2012). Resulting from the mass scale occurrence of strains resistant to conventional fungicides the search for new fungicides for potato post-harvest treatment is essential.

We have studied the new fungicide Zeroxxe which provided good protection of tubers against the *H. solani* strains. This fungicide is being registered in some countries of South-Eastern Asia and Latin America and it is also in the final stages of registration process in Russia, Kazakhstan and Uzbekistan. As a part of toxicological studies for the registration of Zeroxxe fungicide a variety of tests was performed by the Centro Toxicologico S.A.C – CETOX (Lima, Peru): (i) acute oral toxicity (OECD TG 423), acute dermal toxicity (OECD TG 402), acute inhalation toxicity (OECD TG 403), repeated dose (28 days) oral toxicity (OECD TG 407) and repeated dose (28 days) dermal toxicity (OECD TG 410) in *Rattus norvegicus*; (ii) acute eye irritation (OECD TG 405) and acute dermal irritation (OECD TG 404) in *Oryctolagus domesticus*; (iii) skin sensitization (OECD TG 406) in *Cavia porcellus* (Centro Toxicologico S.A.C., 2017). The fungicide was found to be non-toxic for mammals, thus it could be added to the national lists of chemicals for seed and ware potato tubers pre- and post-harvest treatment in potato growing countries.

Within our study, difenoconazole, which is approved for potato tuber treatment in the USA (EPA Reg. # 100-1386), has shown a high efficiency with respect to the *H. solani* strains studied. It may be recommended for application as a fungicide for seed potato tubers pre- and post-harvest treatment in other countries.

To improve the chemical protection of the stored tubers against *H. solani* and prevent the appearance of resistant pathogen strains it is reasonable to apply the rotation of active ingredients.

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REFERENCES

Al-Mughrabi K.I., Vikram A., Peters R.D., Howard R.J., Grant L., Barasubiye T., Lynch K., Poirier R., Drake K.A., Macdonald I.K., Lisowski S.L., Jayasuriya K.E., 2013. Efficacy of *Pseudomonas syringae* in the management of potato tuber diseases in storage. *Biological Control* **64**: 315-322.

Burke O.D., 1938. The silver scurf disease of potatoes. *Bulletin of Cornell University Agricultural Experimental Station* **692**: 1-30.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de toxicidad oral (DL_{50}) aguda – metodo de las clases de toxicidaden rata albina (*Rattus norvegicus*) de la muestra Zeroxxe (Report #T-OCT-17-1249) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de toxicidad dermica (DL_{50}) aguda en rata albina (*Rattus norvegicus*) de la muestra Zeroxxe (Report #DAR-17-0136) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de toxicidad inhalatoria (CL_{50}) aguda en rata albina (*Rattus norvegicus*) de la muestra Zeroxxe (Report #IA-17-1297) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de toxicidad oral enroedores dosis repetidas 28 dias de la muestra Zeroxxe (Report #ODR-17-0036) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de toxicidad dermica con dosis repetidas a 28 dias de la muestra Zeroxxe (Report #EBBK-17-0022) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de irritacion ocular en conejo albino (*Oryctolagus domesticus*) de la muestra Zeroxxe (Report #ID-17-1362) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba de irritacion dermica en conejo albino (*Oryctolagus domesticus*) de la muestra Zeroxxe (Report #IO-17-1347) Lima, Peru.

Centro Toxicologico S.A.C. – CETOX (2017) Prueba sensibilizacion cut an eaen cobayos (*Cavia porcellus*) de la muestra Zeroxxe (Report #SC-17-1569) Lima, Peru.

Davidse L.C., Flach W., 1978. Interaction of thiabendazole with fungal tubulin. *Biochimica et Biophysica Acta* **543**: 82-90.

Denner F.D.N., Millard C., Geldenhuys A., Wehner F.C., 1997. Treatment of seed potatoes with prochloraz for simultaneous control of silver scurf and black dot on progeny tubers. *Potato Research* **40**: 221-227.

Elansky S.N., Mita E.D., Skolotneva E.S., Pobedinskaya M.A., Kokaeva L.Y., 2016. Effect of difenoconazole on the formation of oospores by *Phytophthora infestans* (Mont) de Bary. *Journal of Plant Pathology* **98**: 123-127.

European Commission, 2011. EU Commission implementing regulation No. 540/2011: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:153:0001:0186:EN:PDF

FRAC - Fungicide Resistance Action Committee, 2012. List of plant pathogenic organisms resistant to disease control agents. http://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/group/list-of-resistant-plant-pathogens_2012-edition.pdf

Frazier M.J., Shetty K.K., Kleinkopf G.E., Nolte P., 1998. Management of silver scurf (*Helminthosporium solani*) with fungicide seed treatments and storage practices. *American Journal of Potato Research* **75**: 129-135.

Geary B., Johnson D.A., 2006. Relationship between silver scurf levels on seed and progeny tubers from successive generations of potato seed. *American Journal of Potato Research* **83**: 447-453.

Gore M.E., 2017. Fungal seedborne pathogens infecting potato seed tubers from Turkey, 2011-2014. *Journal of Plant Diseases and Protection* April 2017: 1-13.

Hide G.A., Hall S.M., 1993. Development of resistance to thiabendazole in *Helminthosporium solani* (silver scurf) as a result of potato seed tuber treatment. *Plant Pathology* **42**: 707-714.

Hide G.A., Hall S.M., Boorer K.J., 1988. Resistance to thiabendazole in isolates of *Helminthosporium solani*, the cause of silver scurf disease of potatoes. *Plant Pathology* **37**: 377-380.

Hide G.A., Hall S.M., Read P.J., 1994a. Control of skin spot and silver scurf on stored cv. King Edward potatoes by chemical and non-chemical methods. *Annals of Applied Biology* **125**: 87-96.

Hide G.A., Boorer K.J., Hall S.M., 1994b. Controlling potato tuber blemish diseases on cv. Estima with chemical and non-chemical methods. *Annals of Applied Biology* **124**: 253-265.

Holley J.D., Kawchuk L.M., 1996. Distribution of thiabendazole and thiophanate-methyl resistant strains of *Helminthosporium solani* and *Fusarium sambucinum* in Alberta potato storages. *Canadian Plant Disease Survey* **76**: 21-27.

Kawchuk L.M., Holley J.D., Lynch D.R., Clear R.M., 1994. Resistance to thiabendazole and thiophanate-methyl in Canadian isolates of *Fusarium sambucinum* and *Helminthosporium solani*. *American Potato Journal* **71**: 185-192.

Khodykina M.V., Polityko V.A., Kyrova E.I., Krutyakov Y.A., Zherebin P.M., Ignatov A.N., 2014. Antibacterial activity of antibiotics and a silver preparation Zeroxxe against plant pathogenic bacteria. *Zasita Kartofelya* **2**: 84-86 (Russian).

Koenraadt H., Somerville S.C., Jones A.L., 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology* **82**: 1348-1354.

Kutuzova I.A., Grigorovich I.A., Pobedinskaya M.A., Belov G.L., Elansky S.N., 2015. Resistance of Russian and European strains of *Colletotrichum coccodes* to selected fungicides *Zasita Kartofelya* **1**: 30-34 (in Russian).

McKay G.J., Cooke L.R., 1997. A PCR-based method to characterize and identify benzimidazole resistance in *Helminthosporium solani*. *FEMS Microbiology Letters* **152**: 371-378.

Merida C.L., Loria R., 1990. First report of resistance of *Helminthosporium solani* to thiabendazole in the United States. *Plant Disease* **74**: 614.

Mita E.D., Elansky S.N., Kokaeva L.Y., Pobedinskaya M.A., Ignatov A.N., Kuznetsova M.A., Kozlovsky B.E., Denisov A.N., Zherebin P.M., Krutyakov Y.A., 2014. New pesticide Zeroxxe – assessment of fungicidal and bactericidal properties in vitro. *Dostizheniya Nauki I Tekhniki APK* **28**: 16-19 (in Russian).

Olivier C., Loria R., 1998. Detection of *Helminthosporium solani* from soil and plant tissue with species-specific PCR primers. *FEMS Microbiology Letters* **168**: 235-241.

Pasche J.S., Piche L.M., Gudmestad N.C., 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Disease* **89**: 269-278.

Pasche J.S., Wharam C.M., Gudmestad N.C., 2004. Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. *Plant Disease* **88**: 181-187.

Platt H.W. 1997. Resistance to thiabendazole in *Fusariu*m species and *Helminthosporium solani* in potato tubers treated commercially in eastern Canada. *Phytoprotection* **78**: 1-10.

Pobedinskaya M.A., Plutalov P.N., Romanova S.S., Kokaeva L.Y., Nikolaev A.V., Alexandrova A.V., Elansky S.N., 2012. Resistance of potato and tomato early blight pathogens to fungicides. *Mikologiya I Fitopatologiya* **46**: 402-408 (in Russian).

Read P.J., Hide G.A., 1984. Effects of silver scurf (*Helminthosporium solani*) on seed potatoes. *Potato Research* **27**: 145-154.

Rodriguez D., Secor G.A., Gudmestad N.C., Francl L., 1993. Production and dispersal of *Helminthosporium solani* conidia in storage. *American Potato Journal* **70**: 836-837.

Rodriguez D.A., Secor G.A., Gudmestad N.C., Francl L.J., 1996. Sporulation of *Helminthosporium solani* and infection of potato tubers in seed and commercial storages. *Plant Disease* **80**: 1063-1070.

Santerre J., 1972. New studies on transmission of silver scurf in potatoes by contaminated seeds. *Canadian Journal of Plant Science* **52**: 625-632.

Saunders J.M., Errampalli D., 2001. Comparison of methods to detect resistance of *Helminthosporium solani* to a thiabendazole fungicide. *Phytoprotection* **82**: 103-112.

Secor G.A., Gudmestad N.C., 1999. Managing fungal diseases of potato. *Canadian Journal of Plant Pathology* **21**: 213-221.

White T.J., Bruns T., Lee S.J.W.T., Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Taylor J., Innis M.A., Gefland D.H., Sninsky J.J. (eds). PCR Protocols: a Guide to Methods and Applications, pp. 315-322. Academic Press, NY, USA.

Zherebin P.M., Ignatov A.N., Elansky S.N., Pobedinskaya M.A., Lisichkin G.V., Denisov A.N., Krutyakov Y.A., 2014. Zeroxxe – a silver-based preparation for the effective control of bacterial and fungal epidemic diseases of agricultural plants. *Zasita Kartofelya* **2**: 43-45 (in Russian).

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