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IDENTIFICATION OF POTATO CYST NEMATODES (*GLOBODERA ROSTOCHIENSIS, GLOBODERA PALLIDA*) SPREAD IN SAMTSKHE - JAVAKHETI AND SAMEGRELO – ZEMO SVANETI REGIONS OF GEORGIA

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ABSTRACT

The aim of the present work was detection and identification of potato cyst nematodes (PCN) in two geographically distinct regions of Georgia – Samtskhe-Javakheti and Samegrelo – Zemo Svaneti. For this, 11 villages in Samtskhe-Javakheti and 9 villages in Samegrelo-ZemoSvaneti were studied. Two forms of cystic nematodes were found in the soil samples; Their morphological and morphometric studies werecarried out and identified species were confirmed by the multiplex PCR; Analysis of PCR products confirmed that PCN in 7 samples from Zemo Svaneti and in 2 samples from Samtskhe-Javakheti belongs to *G.rostochiensi*, identification of another form of PCN, *Globodera*spp. remains the subject of farther research. Thus, PCN were found in two villages (Vale and Skhvilisi) of Samtskhe-Javakheti region, 5 villages (Ushxvanari, Lanchvali, Ifari, Tsvirimi, potato fields adjacent to the airport) of Samegrelo- ZemoSvaneti region.

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INTRODUCTION

Among the plant-pests that limit potato production and quality, the potato cyst nematodes (PCN) are the harmful around the world. Yield losses caused by PCN are estimated up to 30% (Bates *et al*., 2002; Hodda and Cook, 2009).Two species of PCN – *Globodera pallida* (Stone, 1973; Behrens, 1975) *and Globodera rostochiensis* (Wollenveber, 1923; Behrens, 1975)– are recognized as plant quarantine pests and are added to the EPPO A2 list (OEPP/EPPO, 2017) *G. rostochiensis* present in all EU countries. It is globally recognized as one of the most important factors of yield loss (Wollenweber, 1923). *G. pallida* is present in all EU Member States except Denmark, Estonia, Latvia, Lithuania and Slovakia (EFSA, 2012). Cyst nematodes live on the roots of host plants and can damage them to the extent of causing growth retardation, water stress, nutrient deficiency, early wither and ultimately yield loss (EFSA 2012).

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PCN are among the most difficult plant pests to control. Cysts protected by the durable wall can survive for over 30 years (Winslow and Willis, 1972). Control measures for cyst nematodes are: use of healthy planting material, crop rotation, chemicals, solarisation, bio-fumigation, weed removal. Currently, the most reliable control method against the cyst nematodes is breeding of resistant potato cultivars (Trudgill *et al*.,1987). Council Directive 2007/33 /EEC, which establishes the measures against populations of PCN in order to determine their distribution and prevent their spread (Council Directive 2007/33 /EC), regulates control of PCN (*G. pallida* and *G. rostochiensis*). There was no legislative regulation of these pests before 2016 in Georgia. The Government Resolution #302 from July 1, 2016, developed within the action plan of DCFTA (Deep and Comprehensive Free Trade Area) Agreement for legal acts approximation to EU *G. pallida* is present in all EU Member States except Denmark, Estonia, Latvia, Lithuania and Slovakia (EFSA, 2012). Cyst nematodes live on the roots of host plants and can damage them to the extent of causing growth retardation, water stress, nutrient

deficiency, early wither and ultimately yield loss (EFSA 2012). Concerns the approval of rules for the control of PCN and necessitates researches of these pests. The aim of the present work was to detectthe potato cyst nematodes (*G. rostoshiensi* and *G. pallida*) in two geographically distinct regions of Georgia - Samtskhe - Javakheti and Samegrelo - Zemo Svaneti; and to identify PCN on the species level based on morphological - morphometric and molecular methods.

MATERIALS AND METHODS

The field surveys were carried out for detection of nematode distribution areas in potato-growing zones of Georgia (Samtskhe-Javakheti, Svaneti). Soil and potato plant samples were collected accordingto EPPO protocols (OEPP/EPPO Bulletin, 2013). The coordinates of sample collecting sites (potato fields and farmers private storehouses) were adjusted via GPS. For study PCN (*G. pallida* and *G. rostochiensis*) samples were taken in May, August and September of 2018, in potato growing villages of Samtskhe-Javakheti (Vale, Sxvilisi, Ude, Arali, Aspindza, Mubareti, Uraveli, Tckhroma, Tcnisi, Agara and Atckuri) and Samegrelo - ZemoSvaneti (Ushkhvanari, latali, Lanchvali, Cholashi, Lakhiri, Ifari, Tcvirimi, Lalaidi and the fields adjacent to the airport). Soil samples were taken in each village, in a zig-zag pattern on the potato fields of the private sector, using the Metlitsky method (Metlitsky, 1985). Samples were taken also from infested potato plant roots; All samples were collected at 15-20 cm depth; Size of each sample was from 500 gr upto1000 gr.

Isolation of cysts from the soil: To extract nematode cysts from soil samples Fanwick can was used according to standart methods by EPPO (Fenwick, 1940; OEPP/EPPO Bulletin, 2013). Moist soil samples were dried at room temperature or in thermostat/draing oven at 25-30C. 250-300 gram soil sample was placed in the Fanwick can, through the flow of water nematodes flew over the spout into sieves. Sieved samples were dried and cysts were handpicked under stereoscope microscope (Leica M50) using brush or entomological needles; nematodes were collected on microscope glass slides or petri dishes for farther preparations.

Permanent and temporary slides: Permanent and temporary slides were prepared. Cysts were measured under stereoscopic microscope (Leica M50) by Bezooijen (Bezooijen, 2006). Cyst was transferred to water drop; vulva and anus were removed using fine lancets; larvae and eggs were removed using brush and slides were transferred on glass slide with glycerin drop in paraffin ring. Slide was covered with coverslip and placed on moderately hot plate to melt paraffin and prepare permanent slides. To prepare temporary slides larvae from cysts were placed in wated drop on glass plate; For morphological – morphometric study of permanent and temporary slideswere used biological microscopes (Leica DME and Olympus Bx51).

Identification of cyst nematodes: Identification of the nematod species were carried out by taking into consideration the morphological and morphometric characteristics of the cysts and larvae andusing the appropriate protocols (OEPP/EPPO Bulletin, 2017). Attention was paid to the shape and color of the cyst and the shape of the larvae stylet. For the cysts were measured: the body length, width; The number of cuticular ridges and the Granek`s ratio was determined; Inthe case of the second stage larvae was measured the length of the body, the length of the stylet, the width and shape of knob, the length of the hyaline region and tail. The morphological-morphometric study of nematodes was carried out with special protocols (OEPP/EPPO Bulletin, 2017). Data nematodes were processed in accordance with the statistical program.

Molecular study -DNA Extraction, amplification and separation of PCR products: DNA isolation was conducted using the Nematode DNA extraction& purification kits (Invitrogen) according to manufacturer's protocol. A multiplex PCR test (Bulman and Marshall, 1997) were used for molecular identification. PCR was performed by the universal ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3) and cyst nematodes' (*G. pallida, G. rostochiensis*) specific primers (PITSp4: 5′-ACAACAGCAATCGTCGAG-3'; PITSr3: 5′-AGCGCAGACATGCCGCAA-3). PCR was conducted in thermocycler (SimpliAmp™ Thermal Cycler _Applied Biosystems"). PCR mixture in a total volume of 25µl contained 25-50 ng DNA, and 0.25 µM ITS5, PITSp4R and PITSp4 primers. Positive controls contained *G. pallida and G. rostochiensis* from the Cheech collection of bank of Plant Pathogens DNA as matrices. Negative control contained PCR grade water instead of DNACycling conditions were: one cycle 95^0C for 5 min, 35 reaction cycles of 95^0C for 1 min, 55^0C for 30 s min, 72^0C for 1 min, followed by a final extension of 10 min at 72˚C and 4˚C hold. Analyses of obtained PCR fragments were conducted by horizontal electrophoresis on 1,5% agarose gel containing ethidium bromide (0.2µg/ml) in TAEbuffer (40mM Tris acetate, pH 8.0, 2mM EDTA-Na2) Sambrook and Russell, 1989). Visualization of fragments was done on transilluminator Benchtop UV**.** The sizes of the PCR products were determined by comparing the bands with a 100bp DNA mass ladder (Biolabs).

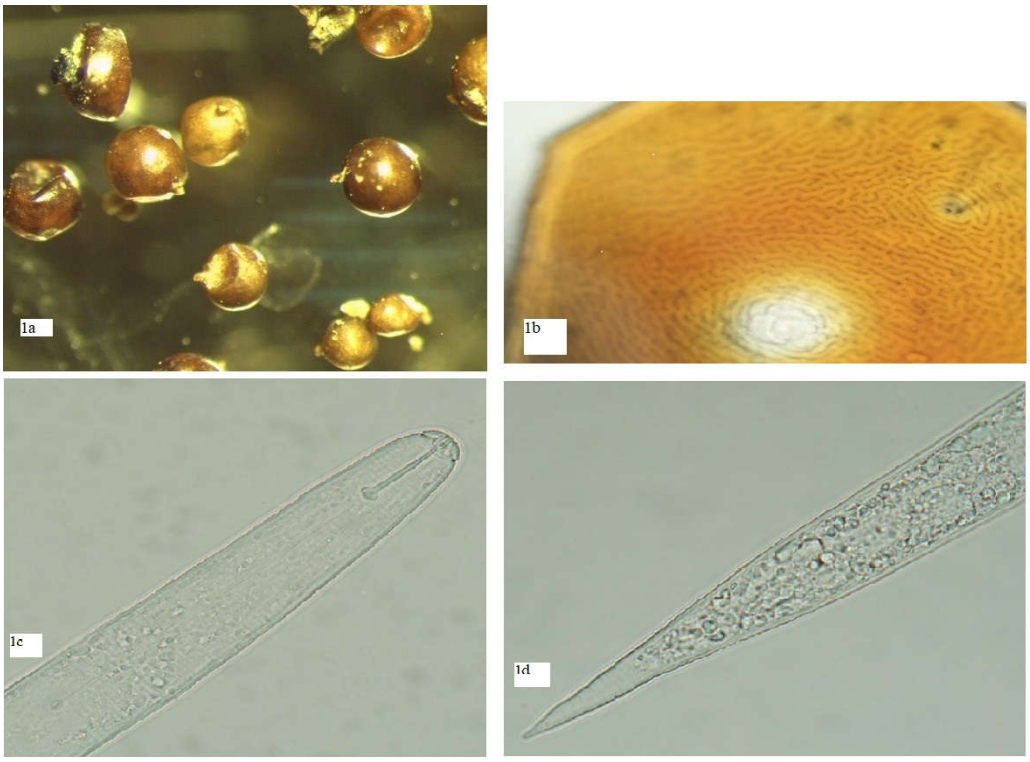
RESULTS AND DISCUSSION

To identify the species of potato cyst nematodes (PCN) in Samtskhe-Javakheti and Samegrelo-ZemoSvaneti were taken 135 samples, among them: 80 samples in Samtskhe-Javakheti region and 55 in Samegrelo-ZemoSvaneti region. Samples in Samtskhe-Javakheti were taken 1000 meters above the sea, and in Samegrelo-ZemoSvaneti were taken 1500 meters above the sea.

Cysts of Globiodera spp. were revealed in 8 samples obtained from Samtskhe-Javakheti, among them, 6 in samples from v. Skhvilisi (see GPS coordinates, Table 1) and 2 in samples from v. Vale (Picture 1 a). In Samegrelo-ZemoSvaneti**PCN** were revealed in 7 samples, among them 3 samples obtained from v. Ushkhvanari and in eachsamples obtained from v. Lanchvali, Ifari, Tcvirimi and fields adjacent to the airport. *G. rostoshiensi* larvae were revealed in one sample from Samtskhe-Javakheti (village Vale) and in 5 samples obtained in Samegrelo-ZemoSvaneti (Picture 1 c, d) v. Ushkhvanari, Lanchvali, Ifari, Tcvirimi and airport adjacent fields (Table 1). In the investigated samples from both regions, have not been reported male exemplars of larvae. Despite different geographic location, larvae and cysts of *G. rostoshiensi* were found in both regions. But cysts and larvae of *G. pallida* were not reported. Several measurements were taked for research: cyst body length, width, anus – fenestral edge distance; Granek`s ratio was calculated, determined the number of cuticularridges (Picture 1b). For the larvae of second stage were measured: body length, stylet length, width of of knob, length of hyaline region, length of tail (Table 2).

Table 1. Potato cyst and larvae nematode spread area in Samtskhe-Javakheti and Samegrelo-Zemo Svaneti regions

Site	Number of samples	Data of sampling	*G. rostochiensi*		Globodera sp.		Geographical position
			Cist	Larvae	Cist	Larvae	
Samtskhe-Javakheti region							
v. Vale	4	6.05.2018	+	-	-	-	41^{0}36'88" C; 42^{0}57'81" B
,,----------,,	6	24.09.2018	+	+	-	-	41^{0}60'20" C; 42^{0}85'37" B
v. Skhvilisi	5	6.05.2018	+	-	+	-	41^0 38'25" C; 42^{0}55'55" B
,,----------,,	4	,,------,,	+	-	-	-	41^{0}38'37" C; 42^{0}55'92" B
,,----------,,	5	,,------,,	+	-	-	-	41^{0}38'41" C; 42^{0}55'96" B
,,----------,,	5	,,------,,	+	-	-	-	41^{0}38'44" C; 42^{0}56'38" B
,,----------,,	4	,,------,,	+	-	-	-	41^{0}38'45" C; 42^{0}56'41" B
,,----------,,	7	24.09.2018	+	-	+	-	41^0 62'09" C; 42^{0}89'49" B
v. Ude	4	7.05.2018	-	-	-	-	41^{0}37'32" C; 42^{0}47'34" B
v. Arali	3	,,------,,	-	-	-	-	41^{0}38'29" C; 42^{0}50'03" B
v. Rustavi/Aspindza	4	,,------,,	-	-	-	-	41^{0}36'97" C; 43^{0}07'18" B
v. Mubareti	5	,,------,,	-	-	-	-	41^{0}41'64" C; 43^{0}04'73" B
v. Uraveli, Tckroma	4	,,------,,	-	-	-	-	41^{0}34'60" C; 43^{0}03'66" B
v. Tcnissa & Akhaltcikhe	5	,,------,,	-	-	-	-	41^{0}39'62" C; 43^{0}02'78" B
v. Tcnisi	6	8.05.2018	-	-	-	-	41^{0}40'42" C; 43^{0}03'86" B
v. Agara	4	,,------,,	-	-	+	-	41^{0}41'80" C; 43^{0}08'12" B
v. Atckuri	5	,,------,,	-	-	-	-	41^{0}43'15" C; 43^{0}08'85" B
Samegrelo-Zemo Svaneti region							
v. Ushkhvanari	5	2.08.2018	+	+	-	-	43^{0}02'85" C; 42^{0}36'70" B
,,----------,,	4	,,------,,	+	-	-	-	43^{0}02'89" C; 42^{0}36'62" B
v. Latali	6	,,------,,	-	-	-	-	43^{0}00'84" C; 42^{0}37'04" B
v. Lanchvali	5	,,------,,	+	+	-	-	43^{0}02'89" C; 42^{0}43'79" B
,,----------,,	5	,,------,,	+	-	-	-	43^{0}02'81" C; 42^{0}43'93" B
v. Cholashi	4	3.08.2018	-	-	-	-	43^{0}02'82" C; 42^{0}43'94" B
v. Laxiri	4	,,------,,	-	-	-	-	43^{0}03'23" C; 42^{0}48'68" B



Picture 1. 1a. Cysts of *G. rostochiensi*; 1b: the perianal region of *G. rostochiensi* cyst; 1c: G. *Rostochiensi* (J_2) head part of the larvae; 1d: tail part of the larvae

According to morphological and morphometric data, PCN found in Samtskhe-Javakheti and Samegrelo-Zemo Svaneti is similar to *G. rostoshiensi*, has round shape and light-brown colour (Picture 1 A). Simiarity was confirmed by morphometric measurements such as body length (440-649µm - v. Vale), body width (380-595µm - v. Lanchvali), cuticular ridges (17019 µm v. Lanchvali), Granek`s ratio (3.1 - v. Lanchvali), anus – fenestral edge distance (53-72µm v. Vale). Morphometric data concerning larvae also indicates similarity to *G. rostoshiensi*: body length (405-442 µm, v. Vale), length of stylet (20-23 µm, v. Vale, Ushkhvanari, Lanchvali, Ifari, Tcvirimi, and airport territory), length of hyaline region (19-28 µm, v. Tcvirimi), length of tail (44-51 µm v. Vale, 44-49 µm v. Ifari) (Table 2). After morphological and morphometric analysis, we used multiplex PCR for Globodera sp. species identification. Electrophoresis of the PCR product on an agarose gel shows 434 bp band, that confirms that PCN in all 7 samples from Svaneti and 2 samples from Samtskhe-Javakheti (v. Skhvilisi N1, Loc. 2 and v. Vale, N1, Loc. 8) belongs to *G. rostochiensi* (Picture 2).

Picture 2. Multiplex polymerase chain reaction (PCR) *Globodera* sp. (*rostochiensis, pallida* identification using primers PIp4, PIr3, and ITS. Lane1-negativecontrole (H_20); Lane 2, 10- positive control (DNA *G. rostochiensis*-434bp*);* lanes - 3, 4, 5, 6, 7, 8, 9 *G. rostochiensis* from Svaneti; lanes 11, 12 -*G. rostochiensis* from Samtskhe-Javakheti; lane13- 100 bp DNA ladder (Life Technologies); lane 14–positive control (DNA *G, pallida*-265 bp)

Table 2. Morphometric characteristics of Potato cyst nematodes revealed in Samtskhe-Javakheti and Samegrelo-Zemo Svaneti regions

Population	Samtskhe-Javakhetiregion				Samegrelo-ZemoSvaneti region				
	v.Vale *G. rostoch.*	v. SkhvilisiG. *rostoch.*	v. Skhvilisi Globodera sp.	v. Agara Globodera sp.	v. Ushkhvanari *G. rostoch.*	v. Lanchvali *G. rostoch.*	v. Iphari *G. rostoch.*	v. Tcvirimi *G. rostoch.*	near the airport *G. rostoch.*
Cysts paratipes	n	8 1035	10	1420	17	11	12		
Length	558±97 (440-689)	593±95 (512-754)	595±80 (517-728)	527±91 (437-682)	642±32 (590-688)	540±89 (452-666)	594±64 (495-693)	646±87 (499-690)	593±34 (475-694)
Width	485±79 (332-620)	509±65 (416-616)	515±53 (437-668)	442±75 (280-581)	525±42 (468-565)	463±58 (380-595)	493±68 (415-623)	603±51 (476-642)	524±62 (396-613)
Length/Width	1.2±0.07 (1.1-1.3)	1.1±0.06 (1.1-1.2)	1.1±0.02 (1.0-1.1)	1.1±0.08 (1.1-1.5)	1.2±0.07 -	1.1±0.08 (1.1-1.2)	1.1±0.08 (1.1-1.2)	1.1±0.05 (1.0-1.2)	1.1±0.04 (1.1-1.2)
Vulval areas									
Distance from anus to fenestral	65±7 (53-72)	72±8 (47-86)	72±5 (68-78)	69±6 (58-93)	60±6 (49-71)	58±4 (53-64)	97±9 (79-116)	73±5 (60-82)	74±4 (62-86)
Fenestra diam.	19±1 (18-23)	20±2 (16-25)	22±1 (21-23)	21±0.7 (20-22)	15 -	14±1 (12-15)	16±3 (12-20)	23±8 (15-37)	15 -
Granek`sratio	3.1±0.1 (2.9-3.3)	3.4±0.3 (3.1-4.0)	3.2±0.07 (3.1-3.3)	3.0±2 (1.7-4.6)	3.9±0.6 (3.2-4.7)	4.0±0.8 (3.6-4.3)	6.2±0.6 (5.8-6.6)	3.5±1.1 (2.2-5.0)	4.9±0.9 (4.1-5.7)
Cuticular ridges	16	≥10-17	16-17	11-22	17-19	17-18	17-25	13-17	16-22
Larvae cysts(J^2)									
n	14	-	-	-	12	20	15	6	3
Body length	421±11 (405-442)	- -	- -	- -	344±13 (330-359)	369±10 (360-379)	416±6 (401-420)	397±19 (367-415)	329±8 (316-346)
Stilet length	20	-	-	-	20-21	22-23	22-23	21-23	20-22
Knob width	3.5	-	-	-	3	4	3	4	3
Hialine length	22	-	-	-	24	18-22	19-22	19-28	20-22
Tail lengt	48±2 (44-51)	- -	- -	- -	41±2 (39-44)	42±1.5 (40-44)	45±2 (44-49)	47±2.5 (42-49)	45±2.8 (42-50)

As a conclusion, morphological, morphometric and molecular analysis of two forms of PCN found in investigated regions of Georgia shows that one form of PCN belongs to G. *rostoshiensi;* the second form is the different species of *Globodera sp*. And it is the subject of future research.

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