

RESEARCH REGARDING THE IDENTIFICATION OF *GLOBODERA* SPP. USING MORPHOLOGICAL CHARACTERS AND POLYMERASE CHAIN REACTION IN ROMANIA

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Abstract

*The control of potato cyst nematodes *Globodera rostochiensis* (Wollenweber) Behrens and *Globodera pallida* (Stone) is regulated by Directive 2007/ 33 EC, which is transposed in Romanian legislation by 139/2010 Ministerial Order. These species are included in the list of harmful organisms of potato which are monitored annually. Both species were detected in the soil samples from Brasov, Covasna, Harghita County in 2010-2011 period. The detection of potato cyst nematodes from soil was carried out with Schuiling centrifuge. The identification of potato cyst nematodes species was based on a combination of morphological, morphometric characters and molecular technique (Multiplex-PCR). It is presented our results regarding criteria, materials and methods which were used for identification of these dangerous pest allover the world and in EU states. In order to investigate species and distribution of the Potato Cyst Nematode (PCN), *Globodera* spp., present in Romania, soil samples were collected from different fields. Identification of *Globodera* species was based on some morphological criteria and confirmed by PCR. This is the first report of molecular identification of PCN populations which was done in Romania.*

INTRODUCTION

The potato cyst nematodes (PCN) *Globodera rostochiensis* (Wollenweber) Behrens and *Globodera pallida* (Stone) are quarantine nematodes on potato. The control of these nematodes is regulated by Directive 2007/33 EC, which is transposed in Romanian legislation by 139/2010 Ministerial Order. This Order establishes the measures to be taken against PCN in order to determine their distribution, to prevent their spread and to control them. Soil samples are taken from the areas where producers intend to cultivate potato and plants listed in Annex I of 139/2010 Ministerial Order, before planting, annually.

Studies on PCN in Romania have been performed by Man Simion (Rojankovschi and Deheleanu, 1986) since 1984, and Szabó demonstrated the presence of both

species (Szabó, 1994). The identification of PCN species were performed using only morphological methods. The aim of the present study was to analyse the cyst nematode species *Globodera* that occur in Romania using PCR analysis.

MATERIAL AND METHODS

Positive soil sample were collected from different fields prior to the planting of potato seed. The flotation method with application of Schuiling's centrifuge was used for extracting cysts from dried soil. The extraction was carried out in Regional Laboratory for Nematology of Brasov (samples from Braşov and Harghita county) and Central Phytosanitary Laboratory of Bucharest - Nematology Division (samples from Covasna county).

Identification of the two species of *Globodera* was based on a combination of morphological, morphometric characters and Multiplex-PCR.

G. rostochiensis and *G. pallida* are morphologically and morphometrically closely related (Stone, 1973a,b). We used a combination of cysts and stage juveniles characteristics. For cysts, the most important diagnostic differences are in the perineal area: number of cuticular ridges between vulva-anus and Granek's ratio and for second stage juvenile characteristics are length and stylet knob shape (EPPO Bulletin 39, 2009).

For molecular identification, we used Multiplex-PCR (Bulman&Marshall, 1997) with some changes indicated below. The method was set up together with our colleagues from Nematology Division of the National Plant Protection Laboratory, Le Rheu, France. DNA extract was obtained from crushed juveniles incubate for 1h at 60°C, 10 min at 95°C and 5 min at 10°C. Were used one universal primer ITS 5 and also specific primers PITSp4 for *G. pallida* and PITSr3 for *G. rostochiensis*. For amplification was used Taq DNA polymerase (Qbiogene).

DNA amplification was carried out in a 25 µl final volume of reaction mixture containing 1x Taq buffer with Mg Cl₂, 0.5 mM Mg Cl₂, 0.25 mM dNTPs, 0.64 µM for each primer, 0.6U Taq DNA Polymerase, 5µl DNA extract. PCR cycling parameters were: 2 min - 94°C, 35 cycles of 30s - 94°C, 30s - 60°C, 30s - 72°C, final elongation 7 min - 72°C. A negative control with no template DNA and positive controle whith DNA of *G. rostochiensis* and *G. pallida* were used.

DNA fragments were separated by horizontal electrophoresis on 1.5% agarose gel with ethidium bromide and visualized under UV light. The size of DNA fragments were estimated using the 100bp DNA Ladder.

PCR analyses were performed in Central Phytosanitary Laboratory from Bucharest.

RESULTS AND DISCUSSION

Our studies were developed during 2010-2011. Analysis of different field areas and the previous crop showed that the majority of cysts were found in fields with grain crops, mainly wheat. We identified *G. pallida* in Braşov County (Fagăraş - 3 ha) and Harghita County (Miercurea Ciuc - 12 ha, Lăzarea- 5 ha). *G. rostochiensis* was identified in Covasna county (Sânzieni - 8 ha, Târgu Secuiesc - 4.79 ha, Catalina - 15 ha) and Harghita County (Lăzarea).

The cysts of *G. pallida* were identified for the first morphologically and morphometrically (Figures 1, 2). After that we performed PCR to confirm the species.

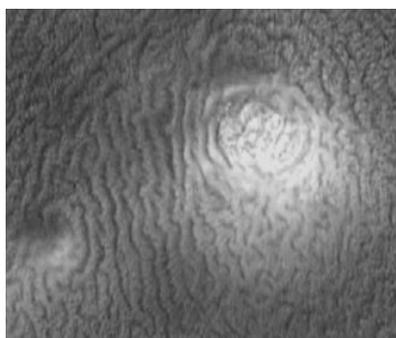


Fig. 1. *G. pallida* - perineal area

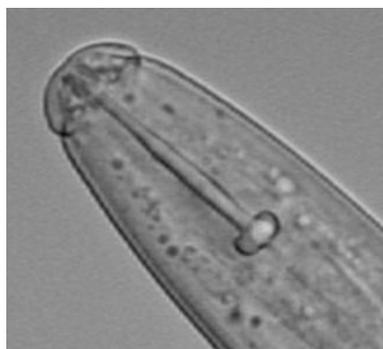


Fig. 2. *G. pallida* - anterior part of juvenile

For cysts of *G. rostochiensis* (Figures 3, 4), some of them were identified only morphologically and morphometrically when the measurements were very clear. We performed PCR when the cysts presented similar characteristics with *G. pallida*.

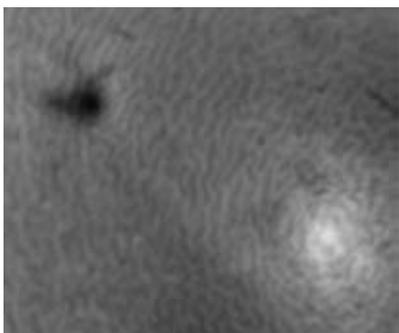


Fig. 3. *G. rostochiensis* - perineal area

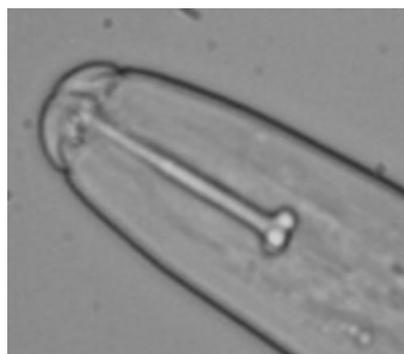


Fig. 4. *G. rostochiensis* - anterior part of juvenile

PCR analysis revealed that the lengths of amplified DNA bands were specific to *G. pallida*, by 265 bp (Figure 5) and *G. rostochiensis*, by 434bp (Figure 6).

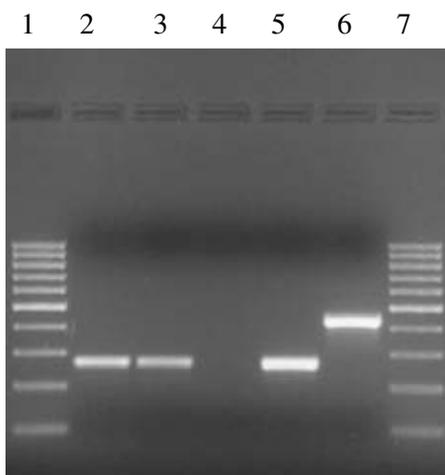


Fig. 5. PCR products of *G. pallida*

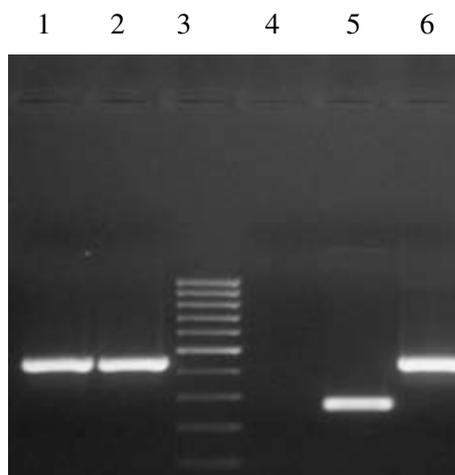


Fig. 6. PCR products of *G. rostochiensis*

Figure 5 shows PCR products of *G. pallida* of sample from Braşov County: 1, 7- ladder; 2,3- *G. pallida*; 4 - negative control; 5 - positive control *G. pallida*; 6 - positive control *G. rostochiensis*.

Figure 6 shows PCR products of *G. rostochiensis* of samples from Covasna county: 1,2 - *G. rostochiensis*; 3 - ladder; 4 - negative control; 5 - positive control *G. pallida*; 6 - positive control *G. rostochiensis*.

PCR products of *G. pallida* and *G. rostochiensis* are showed in figure 7: 1,2 - *G. pallida*; 3 - both species (*G. pallida* and *G. rostochiensis*); 4,5 - *G. rostochiensis*; 6 - ladder; 7 - negative control; 8 - positive control *G. rostochiensis*; 9 - positive control *G. pallida*.

CONCLUSIONS

1. *G. pallida* and *G. rostochiensis* were found in Romania in potato growing areas (Braşov, Covasna, Harghita Counties), using morphological analysis and a sensitive PCR method based on DNA analysis.
2. Based on the results obtained it can be concluded that on were detected mixed populations in Harghita County (Lăzarea) and also isolated populations of *Globodera rostochiensis* (Covasna) and *Globodera pallida* (Braşov and Harghita Counties).
3. All populations of nematodes were found in natural diapauses, the eggs following to hatch in a short time once the host culture was established.

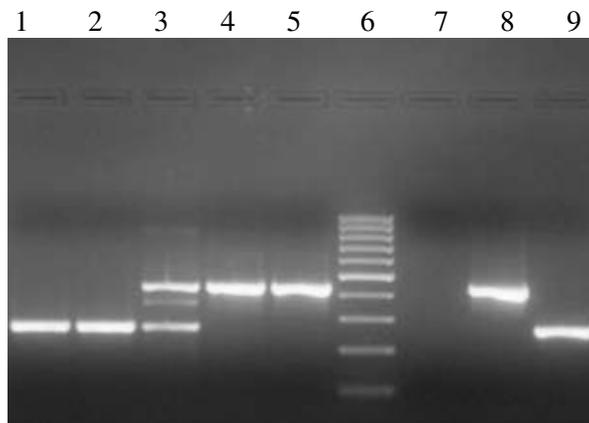


Fig. 7. PCR products of *G. pallida* and *G. rostochiensis*

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