FIRST MOLECULAR DETECTION OF CROATIAN POTATO CYST NEMATODE (PCN) POPULATIONS USING THE POLYMERASE CHAIN REACTION (PCR)

Dinka Grubišić[1,*], Ivana Pajač Živković[1], Tanja Gotlin Čuljak[1], Mirjana Brmež[2], Teuta Benković-Lačić[3] & Aleksandar Mešić[1]

[1*]Department of Agricultural Zoology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, 10000 Zagreb, Croatia, djelinic@agr.hr

[2] Faculty of Agriculture in Osijek, Department for plant protection, Petra Svačića 1d, 31 000 Osijek, Croatia

[3] University of Applied Sciences, Dr. Mile Budaka 1, 35000 Slavonski Brod, Croatia

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida* are economically important pests of the potato (*Solanum tuberosum* L. subsp. *tuberosum*) and are recognized as quarantine pests internationally. In Croatia, PCN were discovered for the first time in Međimurska county in 2001. Since then all identifications of PCN populations for monitoring purposes have been based on morphological characters. In order to introduce the use of the precise and reliable molecular method of PCN identification, the polymerase chain reaction (PCR) was conducted, for the first time in Croatia. Another aim of this investigation was to find out the composition of PCN populations in Croatia. For this purpose ten PCN populations from three Croatian counties (Međimurska, Varaždinska, Primorsko-goranska) were analysed. All populations in investigation were identified as *G. rostochiensis*.

Key words: Croatia, diagnostics, *Globodera rostochiensis*, *Globodera pallida*, PCR, potato cyst nematodes

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Krumpirove cistolike nematode (engl. PCN), *Globodera rostochiensis* i *Globodera pallida* ekonomski su štetnici krumpira (*Solanum tuberosum* L. subsp. *tuberosum*) koji se nalaze na međunarodnoj listi karantenskih štetnika. U Hrvatskoj su krumpirove cistolike nematode prvi put utvrđene 2001. godine u Međimurskoj županiji. Od tada sve identifikacije populacija ovih vrsta radi monitoringa temeljile su se na morfološkim karakteristikama. Metoda lančane reakcije polimerazom (eng. PCR) provedena je po prvi put u Hrvatskoj u svrhu uvođenja brze i pouzdane molekularne metode za identifikaciju krumpirovih cistolikih nematoda. Cilj ovog istraživanja bio je i utvrditi sastav populacija ovih nematoda u našoj zemlji. U tu svrhu analizirano je 10 populacija krumpirovih cistolikih nematoda uzorkovanih u tri hrvatske županije (Međimurskoj, Varaždinskoj i Primorsko-goranskoj). U svim analiziranim populacijama identificirana je vrsta *G. rostochiensis*.

Ključne riječi: Hrvatska, dijagnostika, *Globodera rostochiensis*, *Globodera pallida*, PCR, krumpirove cistolike nematode

Introduction

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber, 1923) Behrens and *Globodera pallida* (Stone, 1973) Behrens, 1975 have proved to be major economic and phyto-quarantine pest species of potato worldwide. These pests attack potato plants and cause significant tuber yield losses (Marks & Brodie, 1998). Because of the greater movement of potato tubers across frontiers and the freeing of markets, a greater emphasis on the monitoring and control of PCN is required. For the first time in Croatia, the golden potato cyst nematode, *G. rostochiensis,* was discovered in 2001 in the major potato-growing area, Međimurska county in the locality Belica (Ivezić et al., 2005; Grubišić et al., 2007; Grubišić et al., 2008) which is famous for its long tradition of potato growing, high yields (up to 40 t ha^{-1}) but also by the lack of rotation. After the first discovery of *G. rostochiensis*, intensive monitoring of the distribution of PCN in Croatia started. Since 2001, *G. rostochiensis* has been reported in four Croatian counties (Međimurska, Varaždinska, Zagrebačka, Primorsko-goranska) and *G. pallida* only in three soil samples from three localities in Međimurska and Varaždinska counties, where it occurred in mixed populations with *G. rostochiensis* (Grubišić et al., 2007). In the UK and other European countries, the increased distribution of *G. pallida* over *G. rostochiensis* in recent years is due not only to the availability of potato cultivars with full resistance only to *G. rostochiensis,* in contrast to the deficiency of varieties resistant to *G. pallida* worldwide, but also to the use of nematicides, which control *G. pallida* poorly (Whitehead et al., 1984). This could be the consequence of the relatively short persistence of nematicides in the soil and the prolonged period of emergence of *G. pallida* juveniles (Whitehead et al., 1994). Increasing restrictions on the application of nematicides and fumigants will necessitate the use of alternative methods of nematode control such as long crop rotations, catch crops and the use of resistant cultivars (Fleming et al., 1998). A very important step in the use of all these measures is the accurate identification of target nematode species in the field and an assessment of the actual numbers of nematodes. In Croatia, high densities of *G. rostochiensis* were found mainly in private fields, where the use of resistant cultivars but also lack of rotation occurs. Because of such practices in private fields, permanent control of PCN populations is necessary and it will facilitate the long-term management of these pests. Since 2001 all identifications of PCN population in Croatia have been based on morphological characters (Fleming & Powers, 1998; Turner, 1998). This method is slow and very laborious.

Over the past decades, molecular methods (PCR-based tests) have been developed and used successfully for the identification of PCN populations (Mulholland et al., 1996; Bulman & Marshall, 1997; Fullaondo et al., 1999; Fleming et al., 2000). The polymerase chain reaction (PCR) offers the prospect of a simple, rapid and reliable diagnostic tool for plant parasitic nematodes that enable identification and quantification of PCN populations from field samples (Burrows & Perry, 1988; Fleming et al., 1998; Stratford et al., 1992). Conventional (morphological) identification requires a minimum of 2 days to complete (Nakhla et al., 2010) while PCR results can be obtained in the same day (Ibrahim et al., 2001). An additional advantage of

the PCR-based method is that it can be used on very poor quality field samples which are very difficult to identify by conventional methods. DNA from single egg or juvenile is sufficient for successful identification, which indicates the great sensitivity of this method (Ibrahim et al., 2001). Ultimately, cyst samples that have been collected, processed and stored can be subjected to PCR diagnostic analysis after considerable periods of time without the requirement of expensive maintenance on host plants (Pylypenko et al., 2005).

The aim of the present work was to introduce the use of the precise and reliable PCR method of distinguishing of both PCN species and the more precise detection of the composition of PCN populations for monitoring purpose.

Materials and Methods

Soil sampling was conducted after the harvest of the potato crops in three Croatian counties: Međimurska (localities Belica, Kramarišće, Gardinovec, Hržišće), Varaždinska (locality Vidovec) and Primorsko-goranska (locality Kupjak). Soil sampling was conducted by the sampling procedure recommended by OEPP/EPPO Quarantine procedurefor *G. pallida* and *G. rostochiensis* (OEPP/EPPO, 1991) with a semihalfcylindrical sampling tool, from no deeper than 5 cm in the soil. Soil samples consisting of 100 4-5 ml soil cores were taken in a grid pattern throughout the plots. Samples of approximately 500 g were processed completely in the laboratory. The standard number of samples taken per hectare was four, whilst one sample was the minimum for areas of less than ¼ ha. Following this procedure, soil samples were taken as follows: five soil samples from five potato fields at the locality Belica, and one soil sample at the localities Kramarišće, Gardinovec, Hržišće, Vidovec and Kupjak. The soil samples were mixed thoroughly and were dried at room temperature (s'Jacob & van Bezooijen, 1984). Cysts were extracted using the Spears flotation method (Spears, 1968) from 100 ml sub-samples. *Globodera* sp. cysts were detected after morphological/morphometrical characters (Fleming & Powers, 1998; Turner, 1998). Cysts were collected and were kept dry at room temperature in plastic specimen tubes prepared for species identification using PCR.

Total genomic DNA was extracted from approximately 10 mg cysts of each nematode population using the GenEluteTM Mammalian Genomic DNA Kit (Sigma-Aldrich) and multiplex (MP) polymerase chain reaction (PCR) was conducted using the Qiagen multiplex PCR kit following the manufacturer's guidelines. PCR amplification of species-specific ribosomal 18S and ITS1 sequences from each nematode population was conducted according to the recommendations of OEPP/EPPO (2009) and MP-PCR assay and cycling conditions used are outlined by Bulman & Marshall (1997). The same method was used with excellent results for PCN population identification in England and Wales (Ibrahim et al., 2001), USA (Nakhla et al., 2010) and Ukraine (Pylypenko et al., 2005). According to this method the *G. rostochiensis* primer (5'-AGCGCAGACATGCCGCAA-3') (PITSr3) and the *G. pallida* primer (5'-ACAACAGCAATCGTCGAG-3') (PITSp4) binds to the ITS1 region. They

were used in combination with a universal primer (5′-GGAAGTAAAAGTCGTA-ACAAGG-3′) (ITS5) that binds to the 18S region. The PCR products were separated on 3% agarose gel buffered in 1X TAE, stained with GelRed dye (Olerup) and were visualised under UV light using Gel Doc™ XR imaging system (Bio-Rad).

Results and Discussion

All DNA extracts from ten Croatian samples produced amplification products of the same size (434 bp) as those reported for *G. rostochiensis*, while none of them produced amplification products reported for *G. pallida* (265 bp) (Figure 1).

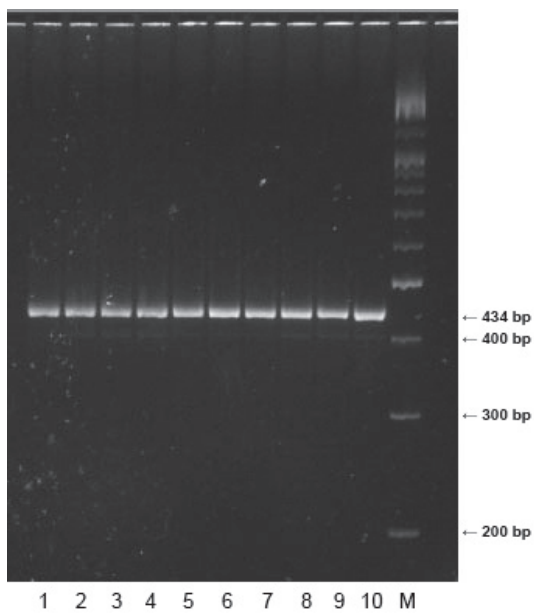

Figure 1. Molecular differentiation of Croatian PCN populations. Multiplex polymerase chain reaction using species-specific primers PITSr3, PITSp4 and the universal primer (ITS5): Lane 1 to Lane 10, *G. rostochiensis* (10 PCN populations); Lane M, Molecular weight markers (M) = O'GeneRuler DNA Ladder Mix, Ready-to-Use 100-10,000 bp (Thermo Fisher Scientific, Inc., Waltham, USA).

Although species *G. pallida* was discovered in mixed populations with *G. rostochiensis* in three soil samples collected from the three localities in Međimurska and Varaždinska counties in 2002 (Grubišić et al., 2007), the golden cyst nematode *G. rostochiensis* is still predominant or, according to the results from the use of the PCR identification method, was the only species present on the sampled potato fields. This could be the result of potato production being better organised in recent years,

involving longer crop rotation and the growing of resistant potato cultivars. An effective PCR based identification of PCN would greatly enhance the diagnostics of these crop pests in Croatia, as stated in many prior investigations (Burrows & Perry, 1988; Bulman & Marshall, 1997; Fleming et al., 1998; Stratford et al., 1992).

Conclusions

Unlike the conventional identification method, based on morphological/morphometrical characters, which is slow and very laborious, a PCR method for PCN identification is not affected by mistakes of subjective evaluation and facilitates the processing of a great number of samples in a relatively short time. The Bulman & Marshall (1997) molecular method of PCN identification, which was applied during this research for the first time in Croatia, will be of great help in the continuous monitoring of PCN populations conducted, in the main potato growing areas, since the first discovery of PCN in Croatia in 2001. Ten PCN populations collected from three potato growing areas from Međimurska, Varaždinska and Primorsko-goranska counties were analysed with the PCR method. All DNA extracts from ten Croatian samples yielded amplification products of the same size (434 bp) as those reported for *G. rostochiensis*, while none yielded the amplification products reported for *G. pallida* (265 bp). The results obtained indicate the slow emergence and spread of *G. pallida* in Croatia, which is of great significance for potato growers in those areas infested by PCN.

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