African Phytosanitary Journal http://africanphytosanitaryjournal.go.ke
ISSN 2617-1856 (Paper) DOI: 10.52855/IKEM3740
Volume 3, Issue 1, 2022

Development of a real-time PCR assay to detect and quantify the tropical root-knot nematode (*Meloidogyne arenaria*) in latently infected potato seed

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Abstract

Tropical root-knot nematodes (*M. incognita*, *M. arenaria* and *M. javanica*) are a serious problem to most crops, both cultivated and non-cultivated. In the recent past, accurate detection and discrimination of tropical *Meloidogyne* spp. has been achieved through the development of DNA-based diagnostic assays among others real-time polymerase chain reaction (qPCR) to complement results obtained using morphological and biochemical-based diagnostic methods. However, despite the developments, most of the current methods have not been optimized to quantify and characterize the tropical *Meloidogyne* spp. particularly in latent infections. Currently, this is a big problem in potato seed production where latently infected tubers often get distributed resulting in the distribution of root-knot nematodes to new areas. In this study, we sought to develop and validate a diagnostic method of quantifying root-knot nematode, *M. arenaria* in latently infected potato seed tubers. Study findings indicated that there is a high ($R^2 > 0.953$) and significant ($P < 0.05$) positive correlation between target DNA concentration and Ct values, and the assays can be used to quantify as low as 1.53/100th of DNA associated with individual juvenile nematodes. Using high resolution melting curve analysis, *Meloidogyne arenaria* samples produced specific melting peaks (79.32 ± 0.029°C, $P < 0.05$) clearly distinguishing themselves from other tropical *Meloidogyne* species (*M. incognita* 79.50 ± 0.022°C and *M. javanica* 79.96 ± 0.046°C). The development of the high-resolution melting curve (HRMC) analysis method for quantification and characterization of *M. arenaria* in this study will greatly improve on accurate screening of this pathogen during latent infections to curb potato losses often associated with distribution of infected seed.

Key words: Root-knot nematode, real-time PCR, high resolution melting curve analysis, South Africa, *Meloidogyne* spp. and potato seed

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Introduction

Among the major six *Meloidogyne* spp. (*M. chitwoodi*, *M. hapla*, *M. arenaria*, *M. incognita*, *M. javanica* and *M. fallax*) that parasitize crops worldwide, the tropical root-knot nematodes are associated with substantial vegetable losses in the tropics (Lamberti, 1997; Koenning *et al*., 1999; Trudgill and Blok, 2001). *Meloidogyne arenaria*, *M. javanica* and *M. incognita* were first reported in South Africa in 1951 and have since spread to all the sixteen major potato growing areas of South Africa (Kleyhans *et al*., 1996; Fourie *et al*., 2001). This distribution was also observed in the recent survey conducted on root-knot nematodes in potatoes (Onkendi and Moleleki, 2013). The tropical *Meloidogyne* spp. are closely related making most of the morphological and biochemical-based diagnostic methods labour intensive while trying to accurately distinguish the pathogens from one another (Landa *et al*., 2008). Furthermore, in the past, based on morphological and morphometric characters, some studies failed to distinguish *M. arenaria* and *M. hispanica* (Kleynhans, 1993).

In the past, accurate detection and delineation of tropical *Meloidogyne* spp. has been achieved through the development of DNA-based diagnostic assays (Zijlstra *et al*., 2000; Tigano *et al*., 2005). Among these methods is the real-time PCR assay which has greatly revolutionized molecular assays due to its accuracy, flexibility, reproducibility and robust nature (Gachon *et al*., 2004). The use of conventional PCR assays improved accuracy and sensitivity of diagnostics relative to morphological and biochemical approaches (Blok, 2005). However, laborious post PCR procedures such as electrophoresis, use of insensitive and carcinogenic intercalating dyes (such as ethidium bromide), end point determination of PCR product and irreproducibility of results demerits wide adoption of conventional PCR in nematode quantification and characterization compared to qPCR (Gachon *et al*., 2004; Cao *et al*., 2005).

Real-time PCR involves the use of highly sensitive and nucleotide sequence specific fluorescent dyes which hybridize to the target DNA sequence segment as dictated by the primers being used. Usually the amount of fluorescence from these fluorescent dyes is directly related to the amplification product being produced (Bohm *et al*., 1999). Unlike in conventional PCR where the final signal does not always reflect the initial template concentration, qPCR allows template quantification at the exponential phase, where the amplification efficiency is at the peak and the reaction is more reproducible since the template is the only limiting factor. The fluorescence emitted is directly related to a threshold number of cycles (Ct) and is usually determined from the linear part of the area within which amplification is taking place with the best

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efficiency (Gachon *et al*., 2004). Therefore, the total number of individual nematodes present in a given sample can be estimated through direct correlation with the number of target DNA copies in the sample (Madani *et al*., 2005). This is another specific benefit of using real-time PCR over conventional PCR.

Several quantitative real-time PCR assays have been developed for detection of specific nematode species from a mixture (Berry *et al*., 2008). Toyota *et al*. (2008) designed real-time PCR assays which could be used successfully to detect and quantify the cyst nematode *Globodera rostochiensis* and *M. incognita* from the soil. To accurately detect low levels of *M. minor* in the soil and plant materials De Weerdt *et al.* (2011) developed hydrolysis TaqMan® probes together with specific ITS-rDNA based primers that could detect a single second stage juvenile of *M. minor*. Tropical *Meloidogyne* spp. are regarded as one of the highly damaging root-knot nematodes worldwide and therefore an accurate method of quantification and characterization is a pre-requisite to better disease management particularly in potato seed industry. The aim of this study was therefore to develop a real-time polymerase chain reaction (qPCR) assay based on SYBR green I dye to detect and quantify the tropical *Meloidogyne* spp. in potato seed tubers in South Africa. *Meloidogyne arenaria* was used as the test species.

Materials and methods

Biological materials

Root-knot nematode infected potato tubers submitted to The Agricultural Research Corporation (ARC) from various parts of the country were used in this study (Table 1). Samples were processed to isolate second stage juveniles (J2s) and other developmental stages of *Meloidogyne* spp. using the centrifugal floatation method according to Bezooijen (2006) with modifications.

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Table 1: *Meloidogyne* species used in this study

Meloidogyne species	Code	Origin	Host
M. arenaria	A7	Mpumalanga[1]	Potato
M. arenaria	54	Limpopo[1]	Potato
M. arenaria	68	Free State[1]	Potato
M. chitwoodi	51	Free State[1]	Potato
M. arenaria	L32	French West Indies[2]	Unknown
M. incognita	L15	Thailand (Race 1)[2]	Unknown
M. javanica	L16	Crete[2]	Unknown

[1] Region in South Africa
[2] Dr. Vivian C Blok, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK

DNA extraction

Using Peter's 1 ml slide, different J2 numbers (1, 2, 4, 8, 16, 32 and 64) from isolated *M. arenaria* were counted ten times under a magnification of ×10 using the Olympus (SZX2-ILLK, Japan) dissecting microscope, aspirated from the sample into 0.5 ml eppendorf tubes and the nematodes crushed using a micro-pestle/blunt end of a pipette tip. The resulting solution was then incubated at -80 °C for 10 min, heated at 95 °C for 10 min, spun at 13500 rpm for 2 min and finally the supernatant collected as genomic DNA. Genomic DNA obtained from different groups of J2s was quantified using the Nanodrop spectrophotometer (Nanodrop Technologies, Inqaba Biotechnologies, South Africa).

Molecular identification of isolated nematodes

To precisely identify isolated nematodes, the IGS-rDNA region was amplified using primers 194 and 195 as adopted by Blok *et al*. (1997) and other species specific primers as outlined in Table 2. All PCR amplifications were performed in a final volume of 25µl mixture containing 30ng crude gDNA, 200µM dNTPs (Fermentas), 0.4µM primers, 0.5U Taq DNA polymerase (Fermentas) and 10× Taq DNA polymerase reaction buffer with 20mM $MgCl_2$ supplement (Fermentas). Thermal amplification profile included initial denaturation at; 94°C for 2min, followed by 45 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 90s with a final extension at 72°C for 10min in a Biometra Analytica Jena thermocycler (Göttingen, Germany). Species identification was done based on characteristic amplicon sizes.

Table 2: List of oligonucleotide primers used for real-time PCR studies

Primer code	5'-3' sequence	Reference
JMV1	GGATGGCGTGCTTTCAAC	Wishart *et al*., 2002
JMV2	TTTCCCCTTATGATGTTTACCC	Wishart *et al*., 2002
JMVhapla	AAAAATCCCCTCGAAAAATCCACC	Wishart *et al*., 2002
JMVtrp	GCKGGTAATTAAGCTGTCA	Wishart *et al*., 2002
194	TTAACTTGCCAGATCGG ACG	Blok *et al*., 1997
195	TCTAATGAGCCGTACGC	Blok *et al*., 1997
Far	TCGGCGATAGAGGTAAATGAC	Zijlstra *et al*., 2000
Rar	TCGGCGATAGACACTACAACT	Zijlstra *et al*., 2000
Fjav	GGTGCGCGATTGAACTGAGC	Zijlstra *et al*., 2000
Rjav	CAGGCCCTTCAGTGGAACTATAC	Zijlstra *et al*., 2000
MI-F	GTGAGGATTCAGCTCCCCAG	Meng *et al*., 2004
MI-R	ACGAGGAACATACTTCTCCGTCC	Meng *et al*., 2004

Quantification of target DNA from second stage juveniles using qPCR

To determine if real-time PCR assay is capable of quantifying target DNA from individual J2s of *M. arenaria* in a sample with plant debris, genomic DNA extracted from different J2 numbers (1, 2, 4, 8, 16, 32 and 64) was serially diluted to obtain six groups of DNA dilutions. Starting with 98.10 ng/µl of DNA from a single juvenile nematode, the DNA concentration was subjected to serial dilutions to reduce it to 25 ng/µl. Further dilutions were made to obtain 13 ng/µl, 6.0 ng/µl, 3 ng/µl, 1.5 ng/µl and 0.75 ng/µl for each group of J2 numbers. Six independent real-time PCR assays were then performed in a total volume of 10 µl using each of the DNA dilutions as a template, 5 µl of SYBR green I dye, 0.3 µM each of the JMV primers; JMV1, JMV2, JMVhapla and JMVtrp (Wishart *et al*., 2002). Amplification with the cocktail of JMV primers using the LightCycler 480 (Roche, Basel, Switzerland) was carried out using the following profiles; initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s.

High resolution melting curve analysis (HRMC)

To carry out HRMC analysis, 0.5 ng of each purified IGS PCR product was used together with the cocktail of JMV primers (JMV1, JMV2, JMVhapla and JMVtrp) on a LightCycler 480 (Roche, Basel, Switzerland). All populations identified as *M. javanica*, *M. arenaria*, *M. incognita* and *M. chitwoodi* were used in 10µl final reactions consisting of 0.5ng of DNA template, 5µl of SYBR green I dye, 0.3µM each of the JMV primers; JMV1, JMV2, JMVhapla and JMVtrp (Wishart *et al*., 2002). Amplification with

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the mixture of JMV primers using the LightCycler 480 (Roche, Basel, Switzerland) was carried out using the following profiles; initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 90s. After PCR phase, data for HRMC was measured during a temperature ramp from 65°C to 95°C for 10min with an acquisition of five units of data per °C.

Statistical analysis

Data obtained was statistically analyzed for variance (ANOVA) and mean comparison by student paired t- test using JMP v.5 software (SA Institute Inc., Cary, NC, USA). In this analysis, all the six independent experiments were analyzed together.

Results

Quantification of target DNA from second stage juveniles using real-time qPCR

In this study, for each number of J2s extracted from the potato tuber samples, sufficient DNA was obtained which could be quantified using the Nanodrop spectrophotometer. Starting with 25 ng/µl of DNA as the standard DNA amount from each group of juvenile numbers, the serially diluted DNA from all samples provided enough templates to run at least six independent real-time PCR assays (Table 3) using the JMV primers and the LightCycler 480 (Roche, Basel, Switzerland).

Table 3: Table showing means for One-way ANOVA for logarithm of DNA amount and the Ct values obtained from juveniles using the JMV primers ($P < 0.05$).

DNA conc. (ng/µl)	Log of DNA	Assays	Ct Mean	Std Error	Lower 95%	Upper 95%
0.75	-0.1249	6	32.26	0.37949	31.482	33.045
1.5	0.1761	6	32.33	0.26834	31.779	32.884
3.0	0.4771	6	31.63	0.32865	30.956	32.309
6.0	0.7782	6	31.18	0.26834	30.627	31.733
13.0	1.1139	6	30.82	0.26834	30.269	31.374
25.0	1.3979	6	29.69	0.26834	29.139	30.244

Real-time PCR was able to detect consistently 1.5 ng/µl diluted from the average 25.0 ng/µl of DNA originating from a single juvenile translating to 1.53/100th of the total amount of DNA detectable from a single juvenile. From the results obtained in Figure 1, there was a significant correlation in a positive way between different target DNA amounts from the juvenile nematode and the Ct values ($R^2 = 0.953$, $P < 0.05$, paired t-test).

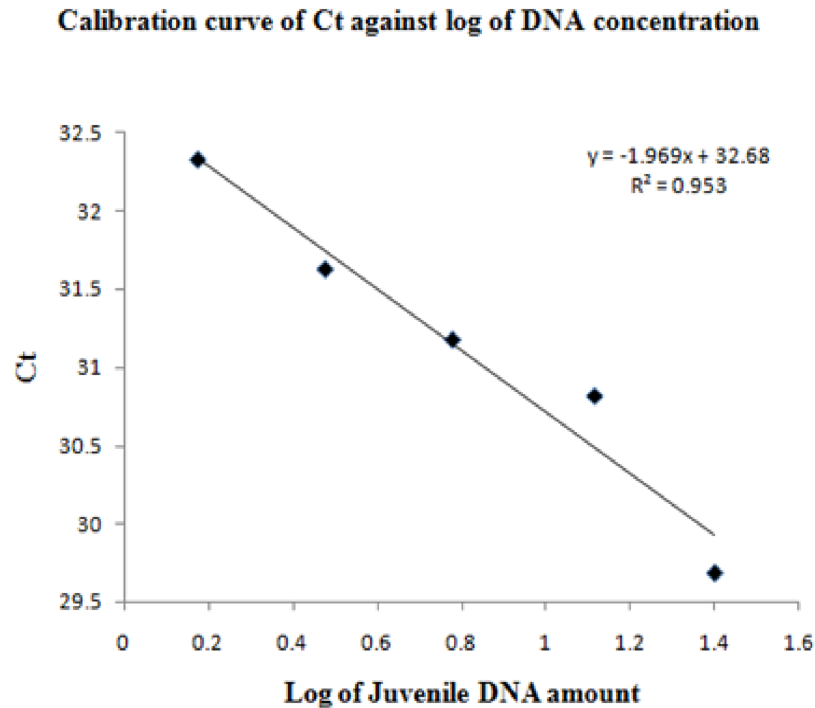

Fig 1: Curve illustrating a calibration for logarithm of DNA amount (1.5-25.0 ng/µl) from individual juvenile nematode against Ct values. Each point represents the mean of six independent real-time PCR assays with JMV primers using *M. arenaria* samples.

With low concentrations (0.75 ng/µl), the Ct means tended to be skewed and therefore they could not be plotted on a graph. Therefore this informed the exclusion of the Ct mean for 0.75 ng/µl DNA from the individual juvenile nematode. Overall, the calibration curve indicated that this quantification assay was able to detect as low as 1.53/100th of an individual juvenile nematode. The high ($R^2 > 0.953$) and significant ($P < 0.05$) positive correlation between target DNA concentration and Ct values using real-time PCR indicated that this assay can be used to detect and quantify target DNA from individual juvenile nematodes in infected potato tubers.

High resolution melting curve analysis

Having quantified target DNA and positively identified *M. arenaria* samples, we next developed HRMC analysis to characterize and distinguish *M. arenaria* from the other closely related tropical *Meloidogyne* species. The IGS PCR products for various *Meloidogyne* species in this study were purified and quantified using the Nanodrop spectrophotometer. A standardized DNA amount of 0.5 ng/µl from the IGS PCR products was then used consistently as the DNA template in real-time PCR. Using the JMV primers, HRMC was applied to evaluate the T_m values of various *Meloidogyne* species in

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each real-time PCR assay. Reproducibility of HRMC was also evaluated during these assays. From the six independent assays, the average T_m values for *M. arenaria* samples in this study (A7, C, 54, 68 and L32) ranged from 79.18 to 79.33. *Meloidogyne arenaria* samples produced specific melting peaks (79.32 ± 0.029°C, $P < 0.05$) clearly distinguishing themselves from other *Meloidogyne* species (*M. incognita*; 79.50 ± 0.022°C and *M. javanica*; 79.96 ± 0.046°C). *Meloidogyne* samples (L15, L16 and L32) obtained from TJHI, UK were included in the assay as positive controls with which we compared our study samples. Sample L15 recorded a mean melting temperature (T_m) value of 79.50 ± 0.022°C, L16; 79.96 ± 0.046°C and L32; 79.18 ± 0.074°C ($P < 0.05$) (Figure 2). The range of T_m values obtained in this study indicates that the assay has to be done at least twice for one to confidently confirm the identity of any given *Meloidogyne* species.

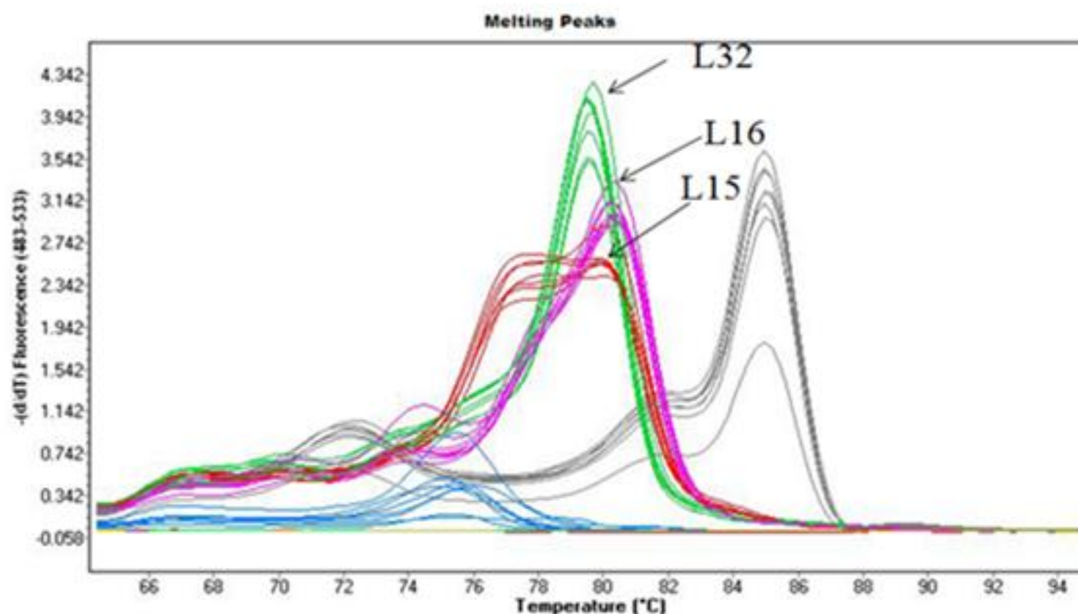

Figure 2: Melting peak profiles of *M. arenaria*, *M. incognita* and *M. javanica*. *Meloidogyne arenaria* sample SCRI L32 gave an early peak (79.18±0.074°C) compared to the other two mitotically parthenogenetic *Meloidogyne* species; *M. incognita* and *M. javanica* (L15 and L16) respectively. *Meloidogyne javanica* had the highest T_m value (79.96±0.046°C) compared to *M. incognita* (79.50±0.022°C) *M. arenaria* with 79.18±0.074°C ($p<0.05$).

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Discussion

Accurate quantification and characterization of various *Meloidogyne* spp. in infected potato tubers is increasingly becoming more important in deciding and implementing proper control programmes. To carry out accurate quantification and characterization, it entails adopting reliable and robust methods for *Meloidogyne* spp. isolation before they are subjected to other stages of the process. In this study, the centrifugal floatation method was chosen as the most appropriate method to isolate *Meloidogyne* spp. from infected samples. This was due to the fact that the method is quick and efficient in specifically isolating *Meloidogyne* spp. from a variety of samples including potato tissues (Coyne *et al*., 2009). During quantification assays in this study, the high ($R^2 > 0.953$) and significant ($P < 0.05$) positive correlation between target DNA concentration and Ct values in this study using real-time PCR indicated that this assay can be used to quantify as low as 1.53/100th of DNA associated with individual juvenile nematodes during quantifications. Subbotin *et al*. (2005) were able to detect as low as 1/100th of the fourth stage juvenile of the nematode *Ditylenchus dipsaci*. In addition, studies done by Berry *et al*. (2008) on three different nematode species (*Meloidogyne javanica*, *P. zeae* and *X. elongatum*) demonstrated a detection limit of 1/40th of the body of each nematode species. In this study, amplification assays of DNA extracted from individual J2s demonstrated that quantification of target DNA from individual J2s from potato tubers and vegetable samples can be done in a single step to yield reliable results. However, there is a detection limit with this assay.

We were able to differentiate *M. arenaria* from other *Meloidogyne* spp. at sequence level using IGS-rDNA based primers which are specific M*eloidogyne* spp. (Blok *et al*., 1997). This was the first step in laying a proper ground for characterizing *M. arenaria* using real-time PCR assays. Confirmation of the identities of these *Meloidogyne* samples using SCRI-UK *M. arenaria* positive control sample, species specific primers in SCAR PCR amplifications and sequence results gave us more confidence to utilize the DNA purified from the IGS PCR products in designing high resolution melting curve (HRMC) analysis for accurate characterization of *M. arenaria*. Real-time PCR assays with a cocktail of JMV primers was rapid and efficient in differentiating various *Meloidogyne* spp. and therefore it was easy to carry out HRMC analysis during these assays (Wishart *et al*., 2002; Holterman *et al*., 2012). The JMV primers are designed from IGS sequences within the rDNA of *Meloidogyne* genus and they give different band sizes for each *Meloidogyne* spp. (Wishart *et al*., 2002). The presence of *M. arenaria* results into an amplicon about 513bp in size while that of *M. chitwoodi* gives an amplicon of

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about 499bp in size (Holterman *et al*., 2012). The identity of *Meloidogyne* spp. was confirmed in our study by subsequent sequencing of the resultant PCR products.

High resolution melting curve analysis is a novel method which can be used to characterize various *Meloidogyne* spp. based on the melting temperatures (T_m) of their PCR products. With a multiplex PCR approach, HRMC is capable of distinguishing different *Meloidogyne* spp. within one assay (Bates *et al*., 2002). The melting temperature (T_m) is defined as the temperature at which a half of the double stranded DNA melts while a half of it is still intact (Bates *et al.*, 2002). On a particular gene target, different *Meloidogyne* spp. have variable nucleotide composition which results in the unique melting temperature of their PCR products. In this study we were able to detect and differentiate the closely related tropical *Meloidogyne* spp. which is challenging while using only the band sizes in IGS PCR. Furthermore, unlike in SCAR PCR where the primers can only target one *Meloidogyne* spp. in one assay, the JMV primers were useful (in a multiplex approach) in detecting and distinguishing various *Meloidogyne* spp. within one real-time PCR assay. Moreover, incorporation of HRMC technique in this study provided a reliable and quick method of distinguishing various *Meloidogyne* spp. in the study samples.

Analysis of all the six independent real-time PCR assays with *M. arenaria* samples and other *Meloidogyne* spp. allowed us to indicate with greater confidence that *M. arenaria* positive samples have a melting peak of 79.3183 ± 0.0295°C ($P < 0.05$). *Meloidogyne arenaria* was found in this study to have a lower (T_m) value compared to the other two mitotically parthenogenetic *Meloidogyne* spp. (*M. javanica* and *M. incognita*). During this study all *M. arenaria* samples gave an average melting peak which was different from peaks of other *Meloidogyne* spp. used. On the other hand, data analysis of all the individual results of other *Meloidogyne* spp. showed that they demonstrated a certain pattern in terms of their T_m values. This re-affirmed the concept of reproducibility and reliability of HRMC in diagnostics (Krypuy *et al*., 2006; Jas *et al*., 2012). The JMV primers were able to give us a single melting peak for study samples as expected (Holterman *et al*., 2012). Immense agreement between study sequence data, SCAR PCR results and HRMC analysis is a good indication that HRMC technique can be used in routine laboratory characterization of *M. arenaria* and other *Meloidogyne* spp. High resolution melting curve analysis is a fast and reliable method which can be multiplexed to detect several *Meloidogyne* spp. at the same time. This process has a high potential for reproducibility and it can be done in one single step to realize results rather than

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the two step approach adopted in this study.

Conclusions and recommendations

Determining potato tuber infection by Meloidogyne spp., typically involves making visual inspection for presence of symptoms such as galls. This seems to be an easy and faster way of identifying infected tubers particularly when infestation is high. But, for surveillance of quarantined and all other Meloidogyne spp., these methods rarely detect latently infected potato seed tubers. Currently this is a big problem in seed production where latently infected tubers often get distributed resulting in the distribution of Meloidogyne spp. to new areas. Accurate quantification and characterization of various Meloidogyne spp. from latently infected tubers is important to the seed industry in controlling the dissemination of Meloidogyne spp. from one area to another. The development of the high-resolution melting curve (HRMC) analysis method for quantification and characterization of M. arenaria in this study will greatly improve on accurate quantification and characterization of this pathogen to curb potato losses incurred in South Africa. Quantification and detection of target DNA as low as 1.53/100th of a single juvenile nematode in this study can be improved on to carry out future surveillance of Meloidogyne spp. in potato tubers or vegetable samples. High resolution melting curve (HRMC) analysis provides an alternative way of characterizing Meloidogyne spp. compared to conventional PCR. Furthermore, HRMC analysis can be used to obtain results in a rapid way with ordinary primers and fluorescence probes such as SYBR green I dye without using labeled probes which are usually very expensive. This technique can also be used in combination with other approaches such SCAR PCR to analyze bulky samples.

Acknowledgement

We thank Potato South Africa (PSA), National Research Foundation (NRF-Thutuka linked grant) and the University of Pretoria for financial support. Individual contributions both technical and material are also highly acknowledged by the authors.

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