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Technical and economic efficiency of methods for extracting genomic DNA from *Meloidogyne javanica*

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ABSTRACT

Plant parasitic nematodes reduce the production of agricultural crops. Species diagnosis is essential to predict losses, determine economic damage levels and develop integrated pest management programs. DNA extraction techniques need to be improved for precise and rapid molecular diagnosis of nematodes. The objective of the present study was to evaluate the efficiency of DNA extraction and amplification by PCR, cost and execution time by Chelex, Worm Lysis Buffer Method (WLB), Holterman Lysis Buffer Method (HLB) and FastDNA methods for nematodes of the *Meloidogyne* genus. The qualitative and quantitative efficiency of DNA extraction varied between methods. The band size of the amplified PCR product with WLB, Chelex and HLB methods was 590 bp. Extraction with the FastDNA is not recommended for DNA extraction from nematodes because it results in a low DNA concentration without bands in PCR amplification, besides presenting high cost. The efficiency of the WLB method to extracting DNA from *Meloidogyne javanica* was greater, ensuring a higher concentration and purity of the extracted material and guaranteeing lower costs and greater ease of PCR amplification.

1. Introduction

Plant parasitic nematodes reduce the production of agricultural crops such as coffee, cotton, oats, sorghum, tomato and wheat (Abad et al., 2008; De Brida et al., 2017, 2018). These microorganisms can survive on host plants for long periods or soil, making it difficult to eliminate them and causing losses of billions of dollars per year in agriculture (Nicol et al., 2011; Kyndt et al., 2013; Ravichandra, 2014).

Meloidogyne spp. induce the gall formation on plant roots, the primary and external parasitism symptom, and internally disorganize the cortex and vascular cylinder affecting the water and nutrients absorption and transport, which lead to secondary symptoms such as yellowing, wilting and reduced plant growth (Williamson and Gleason, 2003; Anwar and Javed, 2010). In addition, nematode-infected plants are more susceptible to other pathogens such as bacteria, fungi and viruses (Al-Hazmi and Al-Nadary, 2015; Ali et al., 2017).

The diagnosis of root-knot nematode species is essential to predict losses, establish economic damage levels and to develop integrated management programs for these microorganisms. Nematodes can be identified by morphological, biochemical and molecular techniques (Premachandra and Gowen, 2015; Seesao et al., 2017), but new tools have been developed for this process to reduce costs and execution times and increase accuracy (Oliveira et al., 2014).

Root-knot nematodes are identified based on the morphology of the female perineum and the male anterior region (Oliveira et al., 2017; Zhao et al., 2017). This method is inexpensive, although, susceptible to errors (Seesao et al., 2017) because morphological and morphometric differences are small and it requires taxonomic knowledge (Oliveira et al., 2009). Additionally, stages may be missing (Barea et al., 2004) and a sample including diverse species may hinder accurate identification (Floyd et al., 2002; Chitwood, 2003). The choice of a method depends on the question to be addressed. Among all nematode identification methods that exists; many can be used in fundamental studies such as morphological identification (Seesao et al., 2017). For this reason, several methods could be applied to different research fields.

Biochemical methods depend on variations in the isoenzyme esterase and malate dehydrogenase (Mdh) profiles. These techniques provide reliable diagnoses and are widely used for *Meloidogyne* spp., but have low importance to identify other pathogens (Kolombia et al., 2017). Additionally, it is only applicable on young adult females and

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presents limitations for interpretation of profile variants between and within species (Hyman, 1990; Blok and Powers, 2009).

Molecular techniques provide new perspectives for the identification of *Meloidogyne* species and for studies of intraspecific variability of root-knot nematodes. DNA-based analyses, such as polymerase chain reaction (PCR), represent a reliable tool to supplement or replace morphological and biochemical methods (Blok, 2005; Roeber et al., 2013). However, the cuticle of nematodes needs to be ruptured for DNA extraction (Mcmanus et al., 1985; Dawkins and Spencer, 1989; Seesao et al., 2014). This shows the importance of extraction techniques for accurate, rapid and low cost molecular DNA diagnostics.

The objective of the current study was to evaluate the efficiency of DNA extraction and amplification by PCR and the costs and execution time of four DNA extraction methods for plant parasitic nematodes of the genus *Meloidogyne*.

2. Material and methods

2.1. Obtainment and breeding of nematodes

Meloidogyne nematode samples were obtained from the "Departamento de Proteção Vegetal da Faculdade de Ciências Agronômicas da Universidade Estadual Paulista "Júlio de Mesquita Filho" - Campus of Botucatu (FCA/UNESP)" in Botucatu, São Paulo, Brazil.

Meloidogyne javanica was inoculated in tomato plants (*Lycopersicon esculentum* Mill. 'Rutgers') and cultivated in sterile substrate, composed of sand, organic material and soil (1:2:1), in plastic cups (2 L) in a greenhouse.

After 90 days of nematode inoculation in the soil, plant shoots were discarded and its roots with galls washed in tap water and 0.5% sodium hypochlorite (NaOCl) and centrifuged twice at 10,000*g* for 1 min, the first with water and the second with sucrose solution (456 g L^{-1}) when the nematodes fluctuate. The solution was screened with 250 μM (60 mesh) and 38 μM (500 mesh) sieves porosity and centrifuged to obtain the suspension with nematodes extracted (Coolen and d' Herde, 1972). The nematodes were counted on Peters slides under an optical microscope.

2.2. DNA extraction

Four methods for nematode DNA extraction to amplify this material using the PCR technique were performed on samples with 50 adult nematodes in 50 μl of 0.85% NaCl in triplicates.

2.2.1. Method 1- extraction with Chelex 100® resin (BioRad)

A total of 80 μl of 10% Chelex 100® resin diluted in sterile water with 8 μl of proteinase K (20 mg/ml) was added per tube with a solution containing 50 nematodes. The nematode samples were incubated in a heat block at 60 °C for 1 h and then at 95 °C for 15 min. Then, the samples were mixed using a vortexer, centrifuged 112 *g* in a microfuge and stored at −20 °C (Willard et al., 1998; Coombs et al., 1999).

2.2.2. Method 2- Holterman Lysis Buffer method (HLB)

A total of 50 μl of proteinase K (800 μg/ml), 1% (v/v) β-mercaptoethanol, NaCl (0.2 M), Tris HCl (0.2 M pH 8) and 25 μl Milli water-Q, was added per tube containing the nematode samples, for DNA extraction by the Holterman Lysis Buffer (HLB) (Holterman et al., 2006). Samples were incubated at 65 °C for 2 h, shaken for 5 min at 99 °C and stored at −20 °C.

2.2.3. Method 3- Worm Lysis Buffer method (WLB)

Extraction by the Worm Lysis Buffer (WLB) method (Williams et al., 1992) was adapted. Solution with 50 ul of KCl (50 mM), 10 ul of Tris (10 mM pH 8.2), 2.5 ul of MgCl 2 (2.5 mM), 4.5 ul of Tween 20 (0.45%), 3.3 μl of proteinase K (200 mg/ml) was used. Samples were stored at −70 °C for 15 min, incubated for 1 h at 60 °C, then 95 °C for 15 min to the DNA release process from the cells. Samples were stored at −20 °C.

2.2.4. Method 4- extraction with FastDNA Spin kit (MpBio)

The procedure for DNA extraction using the FastDNA Spin kit (MpBio) was performed according to manufacturer instructions. A solution consisting of 800 μl of CLV_VF and 200 μl of PPS was added, was homogenized for 40 s Fast Prep-24 Classic Instrument (MpBio) at a speed setting of 6.0 and centrifuged at 14,000 *g* for 5 min. The supernatant (2 ml) was transferred to the tube and 2 ml of Binding Matrix added. The solution was stirred vigorously. The tubes were incubated with gentle shaking for 5 min at room temperature. The suspension (800 μl) was transferred to Spin Filter tube and centrifuged at 14,000 *g* for 1 min and then the liquid from the collection tube was discarded and the spin procedure repeated by adding 500 μl SEW-SM, prepared with 100% ethanol and centrifuged at 14,000 *g* for 1 min. Subsequently, dry centrifugation was performed for 14,000 *g* for 2 min. The DNA, extracted from the column, was eluted by adding 40 μl DES, incubated in a water bath at 55 °C for 5 min and centrifuged at 14,000 *g* for 1 min. Samples were stored at −20 °C.

2.3. DNA amplification and quantification

The genomic DNA was amplified by the polymerase chain reaction (PCR) technique with the universal primers D2A (5′-CAAGTACCGTGA GGGAAAGTT G-3′) and D3B (5′-TCGGAAGGAACCAG CTACTA-3′) aiming to amplify the 28S rDNA (Al-Banna et al., 2004). Analysis was performed on INFINIGEN thermocycler (model TC-96CG) with 12.5 μl of Polymerase Mix Master Red (Ampliqon, Germany), 7.5 μl of Nuclease Free Water (Promega, USA), 1 μl of each primer (10 mM) and 3 μl of genomic DNA from each sample, totaling 25 μl of solution per tube, in each reaction. The cycles were preceded by initial denaturation at 94 °C for 7 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min (Mracek et al., 2006). The amplifications were done in biological replicates. Quantitative evaluation and purity of PCR amplification were done with 2 μl on an optical density spectrophotometer (NanoDrop® ND-1000 UV–Vis).

Simple agarose gel electrophoresis of the DNA preparation was performed to indicate if any of the samples were degraded. The samples of *M. javanica* were previously identified with specific primers (5′-GGTGCGCGATTGAACTGAGC -3′) and Rjav (5′- CAGGCCCTTCAGTGG AACTATAC -3′) (Zijlstra et al., 2000) and confirmed by Sanger's sequencing and compared to the database (GenBank, http://www.ncbi.nlm.nih.gov) for the identification of genetic similarities (accession number KP411880). Analysis was performed with an INFINIGEN thermocycler (model TC-96CG) with 12.5 μl of Polymerase Mix Master Red (Ampliqon, Germany), 7.5 μl of Nuclease Free Water (Promega, USA), 1 μl of each primer (10 mM) and 3 μl of genomic DNA from each sample, totaling 25 μl of solution per tube, in each reaction. The cycles were preceded by initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The expected fragment size was 670 bp (Zijlstra et al., 2000).

The DNA quality was evaluated with a solution (5 μl) of the DNA extracted with Bromophenol Blue dye and PCR product visualized in electrophoresis in 1% TAE buffer in 1% agarose gel, with Brilliant Green DNA intercalator Plus DNA (Neobio, Brazil) and low mass DNA (Invitrogen, Brazil) displayed on a UV light transilluminator (Major Science).

2.4. Cost and time estimates

The cost per sample of each DNA extraction method was estimated considering the prices of reagents and materials used, without including labor costs or wear of equipment. The total execution time for each

Table 1
Extraction methods, quantity of DNA extracted (ng/ul) (DNA), purity (230/260 and 260/280 absorbance readings), cost (US$) and execution time (minutes) of the genomic DNA extraction methods for *Meloidogyne javanica*.

Methods	DNA	Purity		Cost	Time
		230/260	260/280		
Chelex 100®	24.2 ± 0.93 c	1,27 ± 0,02 c	1.24 ± 0.02 c	8.00	85
HLB	32.4 ± 1.52 b	1,07 ± 0,04 d	1.11 ± 0.04 d	5.49	130
WLB	40.2 ± 0.92 a	1,87 ± 0,06 a	1.86 ± 0.02 a	2.81	95
FastDNA Spin Kit	8.7 ± 0.78 d	1,68 ± 0,07 b	1.53 ± 0.02 b	13.65	30

Means followed by the same letter per column did not differ by Tukey test ($P < 0.001$).

method per sample was estimated by the sum of the period (minutes) required to perform each procedure.

2.5. Statistical analysis

DNA purity and quantity data of three replications were submitted to variance analysis and the means compared using Tukey test ($p < 0.001$) using the SAS User v. 9.0 (SAS Institute, 2002).

3. Results

The quantity ($X^2 = 20.787$; df = 3; $p = 0.000$); and degree of purity of DNA of 230/260 ($X^2 = 0.27$; df = 3; $p = 0.000$) and 260/280 ($X^2 = 0.23$; df = 3; $p = 0.000$) were higher when extracted by the WLB method and lower with the FastDNA Spin Kit (Table 1). DNA extracted by the Chelex, HLB and FastDNA methods presented a lower purity degree.

The cost and time of DNA extraction varied between methods, with higher value for the FastDNA Spin Kit and lower for the WLB per sample. The execution time of the extraction procedures was shorter with the FastDNA Spin Kit and longer with the HLB (Table 1).

The qualitative efficiency to extracting DNA from nematodes with WLB, HLB and Chelex methods presented a band with a 590 bp size in 1% agarose gel, lower than the 1.000 bp Low Mass and molecular marker 1000 bp (Neobio, Brazil). The DNA extracted with the FastDNA Spin Kit did not present bands in any of the amplifications using the D2/D3 primers (Fig. 1). Single agarose gel electrophoresis of the DNA preparation indicated that the samples were not degraded. The sample extracted with WLB had the stronger band, however all samples have a band from around 2 kb (Fig. 2).

4. Discussion

Genomic DNA extraction from nematodes is a complex process because the nematode cuticle layer is robust (460 nm thick) and resistant to chemical, enzymatic and physical disturbances (Dawkins and Spencer, 1989; McManus et al., 1985). Isolation of high quality nucleic acids is essential for molecular analysis, with DNA extraction being the first step, which influences subsequent analyses. DNA extraction methods should be efficient, sensitive, rapid, simple and cost effective. The critical point of DNA extraction from nematodes is to ensure that the microorganism is well macerated, homogenized and its cells completely lysed (Park and Crowley, 2005; Ariefdjohan et al., 2010; Mikaeili et al., 2013).

The highest DNA yield with the WLB method proved promising since DNA extraction from nematodes is difficult due to the small size of these microorganisms and the complex structure of their cuticle (Seesao et al., 2014). On the other hand, the lower DNA yield extracted with the FastDNA kit without band visualization in the amplifications, agrees with reports for the extraction of coral DNA using this kit (Santos et al., 2012), limiting its use.

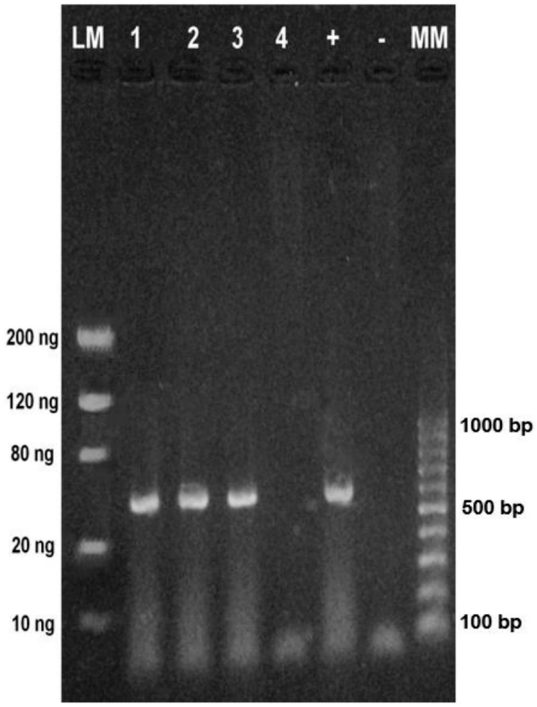

Fig. 1. Electrophoretic profile on 1% agarose gel generated on the basis of the amplification of the 28S rDNA region (590 bp) of *Meloidogyne javanica* using the D2A and D3B primers by four DNA extraction methods. LM: Low Mass (quantification in nanograms) (Invitrogen); 1: Chelex 100® (BioRad); 2: Holterman Lysis Buffer; 3: Worm Lysis Buffer and 4: FastDNA Spin Kit (MpBio); +: Positive Control; −: Negative Control; MM = 1000 bp molecular weight (Neobio).

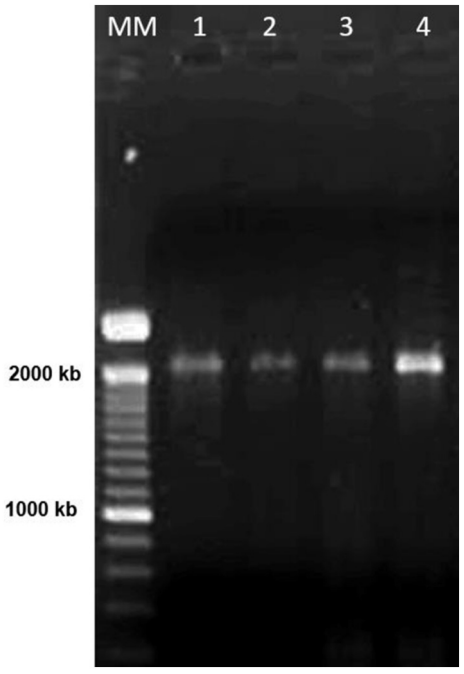

Fig. 2. Eletrophoresis of genomic DNA in 2% agarose gel. MM = 2 kb molecular weight (Neobio), 1: *Meloidogyne javanica* DNA extracted with protocol chelex 100® (biorad), 2: FastDNA Spin Kit (MpBio), 3: Holterman Lysis Buffer and 4: Worm Lysis Buffer.

The DNA purity extracted by the WLB method, was satisfactory with an absorbance ratio 230/260 and 260/280 > 1.80 ensuring the removal of most contaminants by solvents (Seesao et al., 2014). The

removal of substances that may interfere with the quality of the DNA extracted is important to avoid degradation of these molecules (Butler, 2011; Oliveira et al., 2014), although those with an adequate purity degree may remain adequate for amplification up to at least one year without the need for antioxidants (Schuurman et al., 2007). The purity degree between 1.6 and 2 indicates good quality and low protein concentration in the DNA solution (Lee et al., 2010). The deficiency of the HLB method may be due to the use of β-mercaptoethanol, which may interfere with the purity degree of the material extracted (Bielawski et al., 2001; Gerstein, 2001). The Chelex method gives lower stability of the material during storage but it does not ensure the removal of inhibitors, DNA contaminations by RNA, lipids, proteins, polyphenols and polysaccharides (Simonato et al., 2007; Butler, 2012). The complex structure of the nematode cuticle makes the purification of genomic DNA difficult (Seesao et al., 2014).

The WLB method was the least expensive as a casual method using small reagent amounts, while the cost was found to be highest for the FastDNA Kit. These kits are generally expensive and not always available in every country (Mikaeili et al., 2013). The HLB method had the longest duration due to the longer sample incubation period and, consequently, the greater demand for extraction. This method does not seem adequate when many samples need to be analyzed.

The absence of DNA band amplifications extracted by the FastDNA kit may be due to the low concentration of DNA extracted (Devran et al., 2017) with the same inadequacy for molecular diagnosis of *Meloidogyne* nematodes as reported for the electrophoresis extraction in denaturing gradient gel (DGGE) of micro-eukaryotes (Santos et al., 2012).

5. Conclusion

The precise and rapid identification of root-knot nematodes is important to define the crop or plant breeding (Devran and Sogut, 2009; Devran et al., 2017). Extraction with FastDNA is not recommended because this method only extracts low DNA concentrations, not allowing PCR amplification and presenting high costs. The Worm Lysis Buffer (WLB) method was the most efficient to extract DNA from *Meloidogyne* spp. with higher concentration and purity of DNA extracted with lower cost and better PCR amplification.

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Competing interests

The authors declare no competing interests.

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