Talanta 93 (2012) 182–185

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Rapid and highly sensitive detection of single nematode via direct MALDI Mass Spectrometry

Faheem Ahmad [a,b], Judy Gopal [a,b], Hui-Fen Wu [a,b,c,*]

[a] Department of Chemistry, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan
[b] Center for Nanoscience and Nanotechnology, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan
[c] Doctoral Degree Program in Marine Biotechnology, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan

ARTICLE INFO

*Article history:*
Received 1 November 2011
Received in revised form 3 February 2012
Accepted 5 February 2012
Available online 10 February 2012

*Keywords:*
Single nematode detection
MALDI-TOF MS
Protein
Sample preparation
Juvenile infective stage

ABSTRACT

For the first time, we demonstrate the feasibility for the use of MALDI-TOF MS for rapid, direct and sensitive detection of single adult root-knot nematode, *Meloidogyne incognita* and their second stage juvenile ($J_2$). We have proposed simple pretreatment protocols and have demonstrated that the crushed and washed nematodes yielded better spectra. We also report the differentiation between the harmless and harmful stages of the nematode based on mass spectrometric profiling. Peaks at $m/z$ 4350, 4692, 4933, 8725 were only present in the adult stages, while $m/z$ 3220, 3433, 3485, 3830, 6540, 7444, 7770 were unique to the $J_2$ infective stage. The only common peak to both the phases was at $m/z$ 3277. Thus, we show that MALDI-TOF MS can be used to differentiate between the infective and non-infective stage of the nematode and the detection sensitivity of MALDI-MS could be applied to a single nematode analysis.

© 2012 Elsevier B.V. All rights reserved.

## 1. Introduction

The root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid and White) Chitwood, is a sedentary endoparasite that retards growth and development of economic crops by attacking the root system, causing galling, stunting, and other adverse effects. *M. incognita* is distributed worldwide especially in tropical and sub-tropical regions, causing crop losses. The root-knot nematodes have evolved a sophisticated mode of parasitism in that they are able to alter gene expression in specific host cells and to modify them into specialized feeding cells. Infective second stage juveniles ($J_2$) migrate in the soil and are attracted to root tips where they penetrate and migrate towards a suitable site in the host root. The juveniles become sedentary and establish an intimate relationship with their host by induction and maintenance of specialized feeding cells on which they are dependent for their entire life cycle [1]. The simple life cycle has four juvenile stages in addition to egg-laying adult female. Unlike other stages of the life cycle, the $J_2$ is the only infective stage and enter to the root, usually near the root tip. In the zone of elongation where it then migrates toward the root tip. Once feeding begins, the $J_2$ losses its ability to move within the root. $J_2$ causes little physical damage to the roots during the penetration process. Most damage to the host plants results from physiological and biochemical changes caused by nematode feeding.

*M. incognita* is a major constraint for poor productivity of vegetables. Thus, a clear understanding of direct detection of the infective stage of *M. incognita* by MALDI-TOF MS would prove to be very useful for diagnosis of plant infection. MALDI-TOF Mass Spectrometry has been employed in microbiology to characterize specific peptides or proteins directly desorbed from intact bacteria, viruses, spores including nematodes [2–5]. The ability to detect peptide/protein ions over a broad $m/z$ range from microorganisms which are unique and the peptide/protein peaks which correspond to biomarker constituents can represent the identification of microorganisms. This is the basis of taxonomic identification of microbes by MALDI-TOF-MS with pathological relevance for the identification of many infectious diseases. To date, limited work has been devoted to detect $J_2$ of *M. incognita* [6–9] which requires extensive/tedious sample pretreatment techniques. For example, two-dimensional gel electrophoresis has been applied to protein variability in isolates of *Meloidogyne* species and several proteins were selected to discriminate *Meloidogyne* populations [10]. Although, the protein fingerprint of the nematode has been analyzed by MALDI-TOF MS, the mass profile of the infective phase of the nematode has never been studied. In this study, we proposed a rapid and simple approach using direct MALDI-TOF MS to detect a single (adult female) nematode and single second stage juvenile ($J_2$) form, which is the infective phase of the nematode lifecycle. We also project the differences in the protein profiles between these two phases in the lifecycle.

* Corresponding author at: Department of Chemistry, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan. Tel.: +886 7 5252000 3955; fax: +886 7 5253908.
*E-mail address:* hwu@faculty.nsysu.edu.tw (H.-F. Wu).

0039-9140/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2012.02.009

## 2. Materials and methods

### 2.1. Chemical and reagents

Chemicals were purchased from Sigma (St. Louis, MO, USA) including 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), tri-fluoro acetic acid (TFA; HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade). Deionized water (18.2 MΩ cm) was obtained from a Milli-Q Plus pure water system (Millipore, Bedford, MA, USA) and autoclaved at 121 °C before use. All microbiological procedures were carried out in a biosafety level 1 cabinet (NuAire, Plymouth, MN, USA).

### 2.2. Real sample collection of root-knot nematode, M. incognita

The roots of diseased eggplant (*Solanum melongena*) were obtained from vegetable fields near Punjipur village of Aligarh District, U.P., India. Root samples was placed in polythene bags, labeled, brought to the laboratory and examined for the presence of root galls. Identification of the species of *Meloidogyne* was performed by examining the perineal pattern [11]. Mature females were dissected from galls of eggplant roots and their Perineal patterns studied by mounting them on glass and examined under digital inverted fluorescent microscope (ESPA F-140) to study their characteristics.

### 2.3. Preparation and maintenance of pure culture

On the basis of the perineal pattern characteristics, the root-knot nematode infection was identified [11]. Single egg mass was inoculated onto eggplant. Sub culturing was performed by inoculating new eggplant seedlings with at least 30 egg masses. Egg masses were hand picked using sterilized forceps from heavily infected roots and washed in distilled water; placed in 15 mesh sieves (8 cm in diameter) containing crossed layers of tissue papers in Petri-dishes with water just deep enough to contact the egg masses and incubated at 28 + 2 °C to obtain freshly hatched second stage juveniles ($J_2$) of *M. incognita*.

### 2.4. MALDI-TOF MS analysis of single adult female of M. incognita

Adult females of *M. incognita* were dissected out from the root galls for MALDI-TOF MS analysis. A single adult female was transferred into an Eppendorf tube containing 1 mL of sterile distilled water. The Eppendorf tube containing the nematode was washed by gentle vortexing for 5 min. The single mature female was immediately transferred with the help of a needle to the well of the MALDI target plate. For the unwashed nematode sample, the single adult female was dissected out from root galls and directly transferred to the well of the MALDI plate. For crushed sample analysis, a single mature female was transferred to the well of MALDI plate and then it was crushed with a sterile thick needle. The well of the MALDI plate was immediately overlaid with 0.5 μL of matrix solution (50 mM, Sinapinic acid, SA). The matrix (SA) was freshly prepared in 3:1 (acetonitrile:water) containing 0.1% trifluoroacetic acid (TFA, Sigma). The sample/matrix mixtures were allowed to dry at room temperature in the air and then sent for MALDI-TOF MS analysis. Fig. 1 represents the steps in the procedure involved in the experimental process.

### 2.5. MALDI-TOF MS analysis of the infective single second stage juveniles ($J_2$) of M. incognita

Second-stage infective juvenile (J2) of *M. incognita* that had hatched from eggs were transferred into 1 mL of Eppendorf tubes

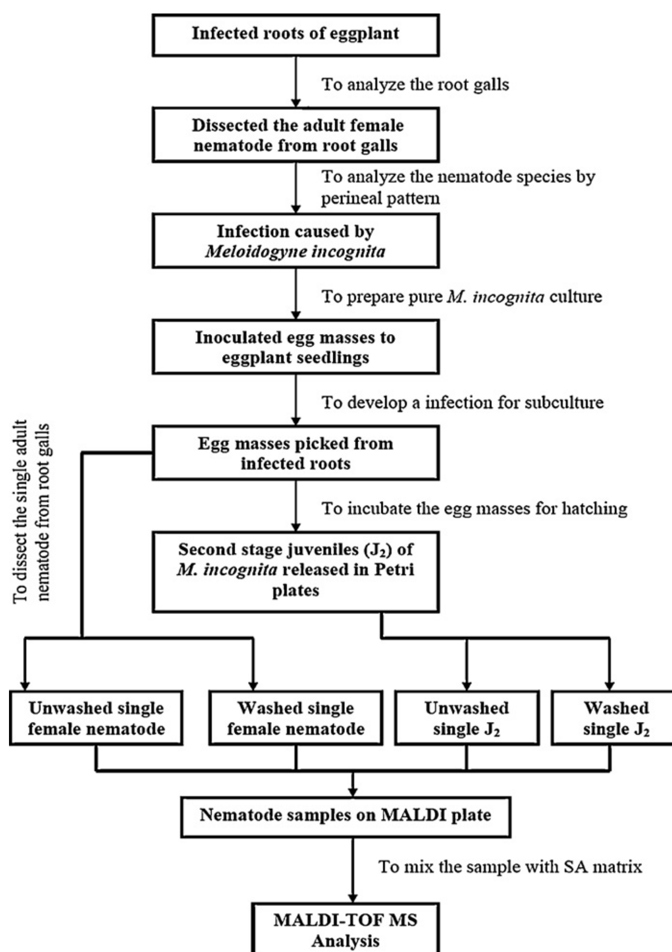


**Fig. 1.** Outline scheme showing methodology employed when using MALDI-TOF MS to detect single root-knot nematode, *Meloidogyne incognita*.

at room temperature and the samples were washed by gentle vortexing for 5 min. The samples were then centrifuged for 10 min at 4000 rpm, the supernatant was carefully removed, 1 mL of distilled water was added and then vortexed again for 5 min. The juvenile samples were again centrifuged gently as described in this section. The supernatant was removed and 1 mL of sterilized distilled water was added. The $J_2$ samples were transferred to a counting dish under the microscope. One single $J_2$ was picked from the counting dish using a sharp thin needle, and immediately transferred to the MALDI target plate and then crushed with a thick sterilized needle prior to analysis. The unwashed juveniles were picked from the counting dish containing unwashed hatched infective $J_2$. The picked $J_2$ was also transferred to a well of the MALDI plate and crushed before analysis. The wells of MALDI plate were immediately overlaid with 0.5 μL of matrix solutions (50 mM, SA) as described above (Fig. 1). The sample matrix mixtures were also allowed to dry at room temperature in the air and then sent for MALDI-TOF MS analysis.

### 2.6. Instrumentation

All mass spectra were generated in the positive ion mode using a MALDI-time-of-flight mass spectrometer (Microflex Bruker Daltonics, Bremen, Germany); a 337 nm nitrogen laser was used for irradiation of the analytes. The accelerating voltage was set at 20 kV. All mass spectra (150 laser shots for per spectrum) were generated in the linear mode for proteins. Other parameters were set the same as those described previously [12]. All experiments were at

least repeated three times in order to check the reproducibility of experiments.

## 3. Results and discussion

The research work was based on the MALDI-TOF MS protein profiling of isolated single *M. incognita* (adult) and their second stage infective juveniles ($J_2$). The diagnostic protein peaks were generated by using MALDI-TOF MS for the above selected root-knot nematode, *M. incognita*. Generated protein profiling and sample preparations were optimized for sensitivity, reproducibility and mass accuracy. The SA matrix was used to generate diagnostic protein peaks for washed and unwashed nematode samples to obtain good quality and reproducible spectra. The MALDI-TOF MS analysis of single mature female was carried out employing pretreatments which included washing (vortexing) and those which were unwashed (direct analysis). The protein profiles were obtained from both washed and unwashed samples separately. It is important to note that the washed nematode produced reproducible spectra with stronger mass peaks even for intact single cell nematode study. The unwashed (Fig. 2A) single (adult) female of *M. incognita* showed signals at *m*/*z* 4693.780, 4809.948, 4933.465, 5150.715 and 9233.905. While the washed (Fig. 2B) nematode samples which consisted of simple pretreatments described in the experimental sections showed more mass peaks. Distinct peaks at *m*/*z* 3167.033, 3277.515, 4692.048, 4809.029, 4933.536, 5150.239, 5440.814, 6058.901, 7185.454, 7541.795, 8019.547 and 9232.253 Da were observed. In this study, we reported for the first time protein mass peaks of single *Meloidogyne incognita* using MALDI-TOF MS. The single nematode spectra were generated with acceptable signal to noise ratios, peak resolution, baseline stability and detection sensitivity. Five protein peaks (*m*/*z* 4692, 4809, 4933, 5150, and 9232) were consistently found for *M. incognita* (Fig. 2A and B). These results confirm that the MALDI-TOF MS was successful in generating protein peaks that allow for the examination of even one intact single *M. incognita* using the simple pretreatment.

To further study a lysed single nematode, we also investigated the direct MALDI-TOF MS analysis of a crushed single (mature female) of *M. incognita*. The results (Fig. 3A for unwashed and Fig. 3B for washed nematode) revealed that the protein peaks were more intense as compared to those of unwashed (intact) nematode samples in Fig. 2. Comparing Fig. 3A with B, the washed (crushed adult female) nematode produced more intense protein peaks compared to that of the unwashed (crushed female) nematode. The unwashed (crushed/adult female) nematode produced protein profiles at *m*/*z* 3277.387, 4350.486, 4692.722, 4809.991, 4933.665, 5150.318, and 8725.127 (Fig. 3A). The washed crushed (adult female) nematode produced most number of protein peaks at *m*/*z* 3072.433, 3167.029, 3277.824, 4350.181, 4692.974, 4809.628, 4933.164, 5150.642, 8725.186, 9173.360, and 9232.685 (Fig. 3B). Reproducible protein peaks at *m*/*z* 3277, 4350, 4692, 4809, 4933, 5150, and 8725 were obtained from unwashed and washed nematode in all the experimental runs.

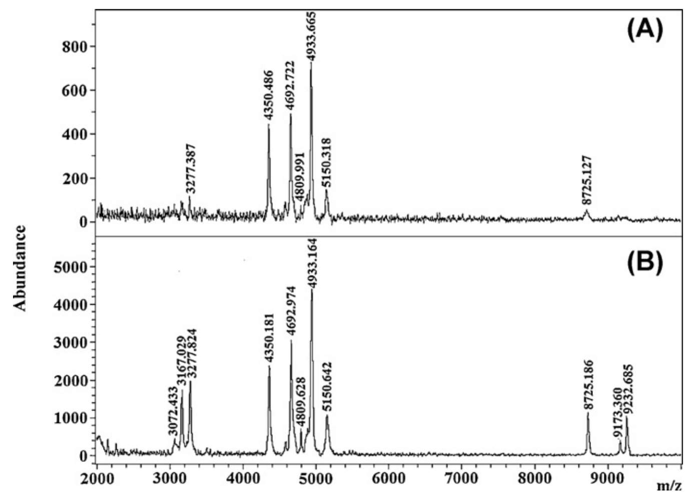


**Fig. 3.** Protein spectra of crushed single adult female of *Meloidogyne incognita* obtained from MALDI-TOF MS analysis. (A) Unwashed crushed single adult female of *M. incognita* and (B) washed crushed single adult female of *M. incognita*.

The second stage juveniles ($J_2$) are the infective stage of *M. incognita* and this is the stage that infects the economic plants. We have also made efforts for the first time to study the detection of a single $J_2$ infective stage using MALDI-TOF MS so that we can see if there is any differences in the protein profile of both stages in order to differentiate the harmless stage of the nematode from the infective stage. Again, the crushed washed juvenile generated more number of intense protein peaks than those of the crushed unwashed one as shown in Fig. 4. The protein peaks at *m*/*z* 3220.008, 3277.715, 3433.982, 3485.088, 3718.754, 3830.031, 4034.391, 4485.596, 4911.493, 4970.811, and 6540.662 were obtained from crushed unwashed single juvenile of *M. incognita* (Fig. 4A). The peaks at *m*/*z* 3220.703, 3277.892, 3433.843,

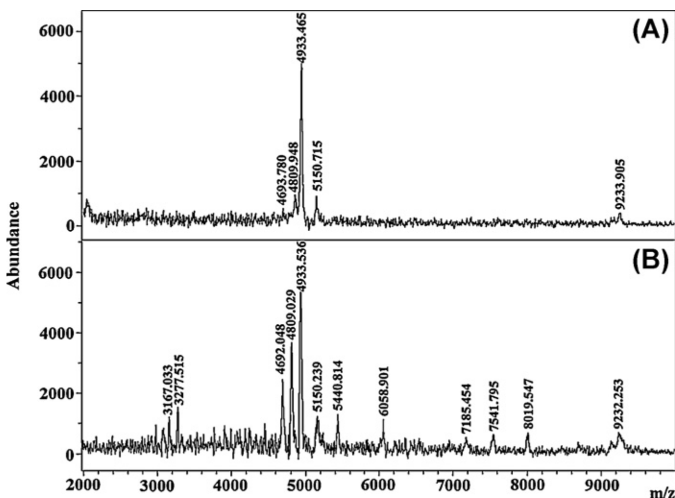


**Fig. 2.** Protein spectra of single adult female of *Meloidogyne incognita* obtained from MALDI-TOF MS analysis. (A) Unwashed single adult female of *M. incognita* and (B) washed single adult female of *M. incognita*.

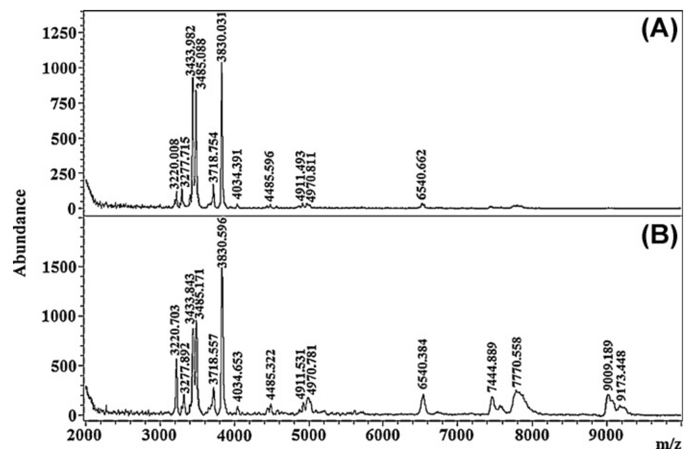


**Fig. 4.** Protein spectra of crushed single second stage juvenile ($J_2$) of *Meloidogyne incognita* obtained from MALDI-TOF MS analysis. (A) Unwashed single juvenile ($J_2$) of *M. incognita* and (B) washed single juvenile ($J_2$) of *M. incognita*.

3485.171, 3718.557, 3830.596, 4034.653, 4485.322, 4911.531, 4970.781, 6540.384, 7444.889, 7770.558, 9009.189 and 9173.448 were observed from the crushed washed single juvenile of *M. incognita* (Fig. 4B). As we can see, the protein profiles of the adult stage and the juvenile infective stage were distinctly different. Peaks at *m*/*z* 4350, 4692, 4933, 8725 were only present in the adult stages, while *m*/*z* 3220, 3433, 3485, 3830, 6540, 7444, 7770 were unique to the $J_2$ infective stage. The only common peak to both the phases was at *m*/*z* 3277. There are a few additional peaks other than the characteristic peaks, which were detected under the same instrument setting and the same target sample. But as we can observe the characteristic peaks were generally stable. Generally, different culture conditions, e.g., culture mode or temperature, do not affect the MALDI fingerprints a lot [13], but such appearances of additional peaks are normal. For example, it is reported that the secretion of a particular compound in the stationary phase may lead to additional mass peaks [14]. In addition, the lysed nematode used in the analysis may also produce additional cytoplasmic/secondary metabolite products, the unwashed nematode and the washed nematode would also differ. This is why irrespective of the additional peaks, we have regular characteristic peaks, on whose basis we do the detection and differentiation. Based on these results, it is evident that this study demonstrates that MALDI-TOF MS can be used for rapid and direct discrimination between the harmful infective juveniles and the harmless adult nematodes. Also, the MALDI-TOF MS technique was sensitive enough to detect a single nematode and juvenile, reducing the sample size requirement by this technique to just one nematode.

## 4. Conclusion

For the first time, we applied MALDI-TOF MS for rapid and direct analysis of a single nematode. We have demonstrated the ability of MALDI-MS to differentiate between the harmless adult stage and the juvenile infective stage of the nematode *M. incognita*. which is a popular plant pathogen. These studies indicate that the use of MALDI-TOF MS for rapid, direct and sensitive detection of a single nematode and also differentiation between the infective nematode from the non-infective stage is feasible.

## Acknowledgement

We thank National Science Council for financial support.

## References

[1] B.Y. Endo, Annu. Rev. Phytopathol. 13 (1975) 213–238.
[2] G.Z. Huanz, B.L. Gao, T. Maier, R. Allen, E.L. Davis, T.J. Baum, R.S. Hussey, Mol. Plant Microbe Interact. 16 (2003) 376–381.
[3] S. Jaubert, J.B. Laffaire, T.N. Ledger, P. Escoubas, E.Z. Amri, P. Abad, M.N. Rosso, Int. J. Parasitol. 34 (2004) 873–880.
[4] C. Neveu, S. Jaubert, P. Abad, P. Castagnone-Sereno, Mol. Plant Microbe Interact. 16 (2003) 1077–1084.
[5] B. Vanholme, J. De Meutter, T. Tytgat, M. Van Montagu, A. Coomans, G. Gheysen, Gene 332 (2004) 13–27.
[6] A. Navas, J.P. Albar, Proteomics 4 (2004) 299–302.
[7] C. Tastet, F. Val, M. Lesage, L. Renault, L. Marche, M. Bossis, D. Mugniery, Eur. J. Plant Pathol. 107 (2001) 821–832.
[8] H. Thiellement, M. Zivy, C. Plomion, J. Chromatogr. B 782 (2002) 137–149.
[9] M.R. Perera, V.A. Vanstone, M.G.K. Jones, Rapid Commun. Mass Spectrom. 19 (2005) 1454–1460.
[10] A. Navas, J.A. López, G. Espárrago, E. Camafeita, J.P. Albar, J. Proteome Res. 1 (2002) 421–427.
[11] J.D. Eisenback, H. Hirschmann, J.N. Sasser, A.C. Triantaphyllou. A guide to the four most common species of root-knot nematodes (*Meloidogyne* spp.) with a pictorial key. North Carolina State Uni. Graphics and USAID, Raleigh, 1981, pp. 48.
[12] K. Shrivas, K.K. Suresh, H.F. Wu, Proteomics 9 (2009) 2656–2667.
[13] N. Valentine, S. Wunschel, D. Wunschel, C. Petersen, K. Wahl, Appl. Environ. Microbiol. 71 (2005) 58–64.
[14] M. Vargha, Z. Takats, A. Konopka, C.H. Nakatsu, J. Microbiol. Methods 66 (2006) 399–409.