MOLECULAR DETECTION AND QUANTIFICATION OF *HETERODERA SCHACHTII* USING PCR AND ELISA

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ABSTRACT

Heterodera schachtii is a cyst-forming nematode that can cause severe production loss in sugar beet crop. The morphological identification and quantification of this parasite is a difficult and labour-intensive procedure.

In this paper we describe a fast method for detection and quantification of *H. schachtii*, by performing a PCR using specific primers developed by Amiri *et al.* (2002) followed by an immuno-assay (ELISA). PCR-products obtained by 2 primers, one carrying at its 5'-ends biotin and the other carrying fluorescein can be quantitatively analysed by this serological test. The biotin labelled primer allows the PCR-product to bind to a microtitre plate coated with strepavidin or avidin. After washing and removing primer residues, anti-fluorescein antibodies linked to horse-radish-peroxidase can bind to the PCR-products. Unbound antibodies are removed by washing. Identification and quantification can then be realised by adding a substrate that colours in the presence of the peroxidase. The optical density, measured by a photo-spectrometer and compared to the optical density of a standard sample that has a known amount of eggs and juveniles of *H. schachtii*, represents the quantity of this parasite in a given sample.

The PCR/ELISA procedure is a fast and sensitive method, which allows automation. Furthermore this method is relatively cheap in comparison with quantitative PCR.

ABRÉGÉ

Le nématode à kyste de la betterave *Heterodera schachtii* peut causer des dégâts importants en culture betteravière. L'identification et la quantification de ce ravageur sur base de la morphologie sont des opérations difficiles qui exigent beaucoup de temps.

Dans cette communication nous présentons une méthode d'identification et quantification rapide de *Heterodera Schachtii*, au moyen d'un test PCR utilisant des amorces, développées par Amiri *et al.* (2002), suivi d'un test immunologique (ELISA). Les produits PCR obtenus par 2 amorces portant à leur extrémité 5' de la biotine et de la fluoresceïne peuvent être quantifiés par ce test sérologique. L'amorce portant de la biotine permet au produit PCR de se fixer sur une

plaquette couverte de streptavidine ou d'avidine. Après lavage et élimination des résidus des amorces, les anti-corps anti-fluoresceine liés à de la peroxydase peuvent se lier aux produits PCR. Les anticorps non fixés sont éliminés par lavage. L'identification et la quantification peuvent alors être réalisées par addition d'un substrat qui se colore en présence de peroxydasse. La densité optique, mesurée par un photo-spectromètre et comparée à la densité optique d'un échantillon de référence dont on connaît le contenu en œufs et larves de *H. schachtii*, indique la quantité de ce parasite dans un échantillon donné.

Le procédé PCR/ELISA est une technique rapide et précise et qui peut être automatisée. De plus son coût est relativement faible par rapport au PCR quantitatif.

KURZFASSUNG

Heterodera schachtii ist ein zystenbildender Nematode, der in Zuckerrüben starke Schäden verursachen kann. Die morphologische Identifizierung und Quantifizierung des Schädlings ist schwierig und aufwendig.

In der vorliegenden Arbeit wird eine Methode zur Identifizierung und Quantifizierung von *H. schachtii* mit Hilfe einer PCR und nachfolgendem ELISA-Test beschrieben. Die PCR-Produkte enthalten zwei spezifische Primer, die an ihrem 5'-Ende entweder Biotin oder Fluorescein enthalten und quantitativ durch einen immunologischen Test bestimmt werden können. Der mit Biotin markierte Primer bindet die PCR-Produkte an eine mit Avindin oder Streptavidin beschichtete Mikrotiterplatte. Nach einen Waschschritt zur Beseitigung von Primerrückständen, können Anti-Fluoresceinantikörper in Verbindung mit Ölrettich-Peroxidase mit den PCR-Produkten binden. Nicht gebundene Antikörper werden durch einen weiteren Waschschritt entfernt. Identifikation und Quantifizierung wird durch Zugabe eines Substrates, dass sich durch die Peroxidase färbt, möglich. Die optische Dichte, gemessen mit einem Photospektrometer und verglichen mit der optischen Dichte eines Standards mit bekannter Menge Heterodera-Eier und Larven, repräsentiert die Schädlingsdichte in der zu bestimmenden Probe.

Die PCR/ELISA Analyse erlaubt die Automatisierung und die Ausrüstung ist im Vergleich zur quantitativen PCR (real-time PCR) kostengünstiger.

INTRODUCTION

Heterodera schachtii is a cyst forming nematode, which can cause severe production loss in sugar beet crop. The genus *Heterodera* can be subdivided into four groups of which only one holds species harmful to sugar beets, namely *H. schachtii*.

Infestation can only be suppressed through crop rotation and the use of nematode resistant varieties like Nemo and Paulina. Till date nematode resistant varieties yield a lighter crop in non- or low-infested fields when compared to classical non-resistant sugar beets. Therefore it is of great importance to identify and quantify the nematodes present in the soil. However,

identification, based on morphological characteristics, and quantification require great expertise and are labour-intensive.

Amiri *et al.* (2002) developed a molecular identification test using primers specific for the cyst forming nematode *H. schachtii*. This test offers a reliable qualitative method for detection and identification of the species *H. schachtii*.

In this paper we describe a fast method of quantification of *H. schachtii*, by performing a PCR using these specific primers followed by ELISA. The PCR/ ELISA procedure is sensitive method, which allows automation. Furthermore this method is relatively cheap in comparison with quantitative PCR.

METHODOLOGY

To obtain the DNA from the nematodes, first the cysts have to be extracted from the soil, using a Seinhorst-extractor and a binocular microscope. After the disruption of the cysts, DNA is extracted using the DNeasy Tissue Kit for animal tissues (Qiagen, Hilden, Germany).

The PCR is performed using the specific forward primer carrying at its 5'-ends biotin and the reverse primer carrying at its 5'-end fluorescein (Landgraf *et al.*, 1991). This results in specific PCR-fragments carrying at one end biotin and at the other end fluorescein (Figure 1).

To avoid the formation of primer dimers and non-specific fragments the primers, developed by Amiri *et al.* (2002), were adapted.

During the ELISA procedure the biotin label allows the PCR-products to bind to a microtitre plate coated with strepavidin or avidin. After washing off primer residues, anti-fluorescein antibodies linked to horse-radish-peroxidase (HRP) can bind to the PCR-products. Unbound antibodies are removed by washing.

Identification and quantification can now occur by adding a substrate that colours in the presence of the peroxidase. The optical density (OD), measured by a photo-spectrometer and compared to the OD of a standard dilution series that has a known amount of eggs and juveniles, represents the infestation level of the parasite in a given sample.

RESULTS AND DISCUSSION

Although the procedure has yet to be optimised first results showed to have great potential. When performing the procedure on a standard dilution series we got a correlation coefficient of 0.94 (Figure 2).

When using this new approach the analysis of 50 soil samples required approximately 60 hours of work. When compared to the classical, morphological method of identification and quantification, which can take up to 3 hours of work per sample, this means a gain of 90 hours. When the nematodes were identified by PCR and quantified through visual counting the procedure for 50 soil samples required approximately 100 hours of work.

Furthermore, due to the extension of the primers at the 5'-end, the formation of primer dimers was reduced and the amplification of non-specific PCR-products avoided.

CONCLUSION

The PCR/ELISA technique is a relative cheap, fast and sensitive method, which allows automation. Classical, morphological identification and visual counting which both require great expertise and are time-consuming, can thus be replaced by this fast molecular approach. This could not only result in an important gain of time but also in an increase of sensitivity and reliability of the obtained results.

Steps are being taken to eliminate the formation of primer dimers, which cause a large background signal during the procedure, and the appearance of false positive signals. For this purpose we are examining the effect of adding nucleotides at the 3'-end of the primers. Also the usage of larger primers and other enzymes are being tested.

Till now the most time-consuming step in this procedure is the extraction of the cysts out of the soil. This step takes up to 80% of the required time of the analyses. Furthermore the sensitivity and the reliability of this approach is mainly set by the accuracy at which the cysts are being picked out of the soil. Therefore we are currently investigating the possibility to extract the DNA directly out of the soil. This could reduce the required time for the test dramatically and simultaneously increase the reliability and the reproducibility of the method significantly.

REFERENCES

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2. LANDGRAF, A., RECKMANN, B. & PINGOUD, A.: Direct analysis of polymerase chain reaction products using enzyme-linked immuno-sorbent assay techniques. *Anal. Biochem.*, 198, 86-91, 1991

Figure 1: Principle of quantification by PCR/ELISA

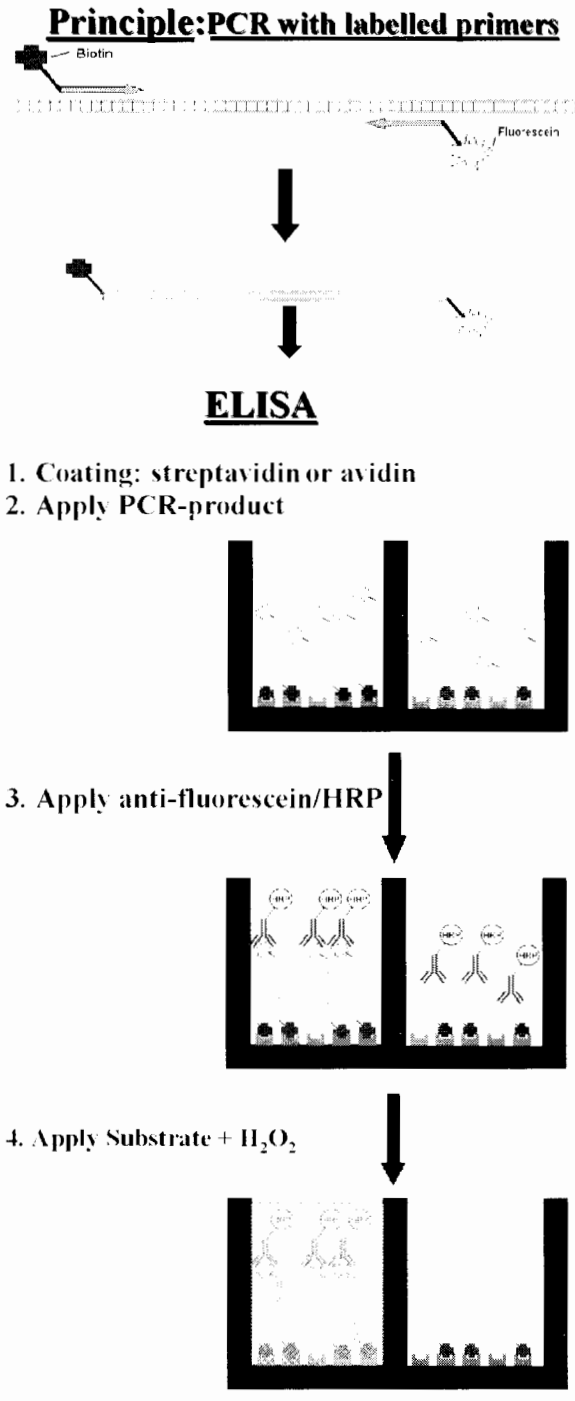

Figure 2: Optical Density of a standard dilution series