Detection of *Plasmodiophora brassicae* in soil by PCR method

A. Czubatka, J. Robak, A. Czajka, W. Szczechura, M. Staniaszek

Research Institute of Horticulture, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

anna.czubatka@inhort.pl

Introduction

Plasmodiophora brassicae Wor. fungus is the cause of clubroot - one of the most dangerous and most frequent diseases of brassicas. Resting spores of this pathogen are able to remain viable in soil even up to 8 years. The most effective way to protect cultivations against this disease is introduction resistant plants varieties and analysis of fields in the presence of this pathogen before planting brassicas.

Detection of *P. brassicae* relied on bioassays are long and require large amount of infected soil and space in greenhouse. Molecular methods use in the detection of this pathogen should be more sensitive and faster than traditional plant bioassays. Furthermore, application real-time PCR method could allow detect spores of *P. brassicae* and specify their amount in soil samples.

Development of reliable and sensitive method to detect this pathogen is necessary to protect cruciferous crops cultivation against clubroot.

The aim of this work was compare traditional bioassays and PCR methods as a tools for the detection of *P. brassicae* in soil.

Material and methods

Infected by *P. brassicae* roots of Chinese cabbage cv. Granaat were used to prepare resting spore suspension which was used to prepare soil samples. The clubs were macerated in water and filtrated through cheese loch. Autoclaved soil were inoculated with *P. brassicae* resting spores suspension to obtain studied concentrations: 10^3, 10^5, 10^6, 10^7 and 10^8 spores per 1 g^{-1}. Soil samples containing series of different concentrations of studied pathogen between 0 to 10^8 spores per 1 g^{-1} were analyzed using bioassay on Chinese cabbage cv. Granaat, nested PCR and real-time PCR.

Soil samples were distributed into plastic trays. Fifty seeds of Chinese cabbage were sown into the each tray. The plants were placed in the glasshouse and harvested 60 days after sowing and assessed for clubroot development. Genomic DNA for PCR reactions were isolated from studied concentrations of soil samples using NucleoSpin® Soil kit (Macherey - Nagel).

Used nested PCR primers were designed by Faggian (1999). PCR conditions used in accordance with Staniaszek (2001). A eppendorf Thermal cycler was used. PCR products were visualized by electrophoresis in 1,5% agarose gels and staining with bromide ethidium.

Real-time PCR reactions were performed using primers designed by Sundelin (2010) and KAPA SYBR® FAST qPCR Kits (Kapa Biosystems). The reaction was conducted in 480 LightCycler (Roche).

Results

In a biological test *P. brassicae* was detected in the soil when the resting spores concentration was 10^5 of spores per 1 g^{-1}.

In nested PCR where the first PCR contains an external pair of primers (PbITS1 – 5’ ACTTGCATCGATTACGT CCC 3’, PbITS2 – 5’ GGCATTCTCGAGGGTATCAA 3’), while the second contains two inner primers (PbITS6 – 5’ CAACGAGTCAGCTTGAATGC 3’, PbITS7 – 5’ TGTTT CGGCTAGGATGGTTC 3’), the specific DNA fragment of *P. brassicae* genome (507 bp in size) was amplified in soil in which the concentration of spores was also 10^5 spores per 1 g^{-1}.

Preliminary results with primers Pb4-1 (5’ TACCATACCCAGGGCGATT 3’) and PbITS6 (5’ CAACGAGTCAGCTTGAATGC 3’) indicate that real-time PCR detects 10^3 spores per 1 g^{-1}, which gives a chance to use it as a sensitive method for the detection and quantification of *P. brassicae* in soil.

Bioassay on Chinese cabbage cv. Granaat

Table 1. Incidence of clubroot *P. brassicae* observed as changes on the roots in susceptible test plants (Chinese cabbage cv. Granaat) following inoculation of soil with different concentration of spores suspension.

No of spores per 1g^{-1}	Disease index*				Diseased plants (%)
	0	1	2	3	
Control (autoclaved soil)	50	0	0	0	0
10^3	50	0	0	0	0
10^5	43	0	7	0	14
10^6	42	0	3	5	16
10^7	34	2	10	4	32
10^8	0	0	0	50	100

**Disease index on scale 0-3 where: 0 = no symptoms, 3 = severe clubbing of the entire roots system.*

Fig. 1. Symptoms of the disease on the Chinese cabbage cv. Granaat obtained in the bioassay. Red circles marked clubbs which size and number correspond with disease index (1-3)

Nested PCR

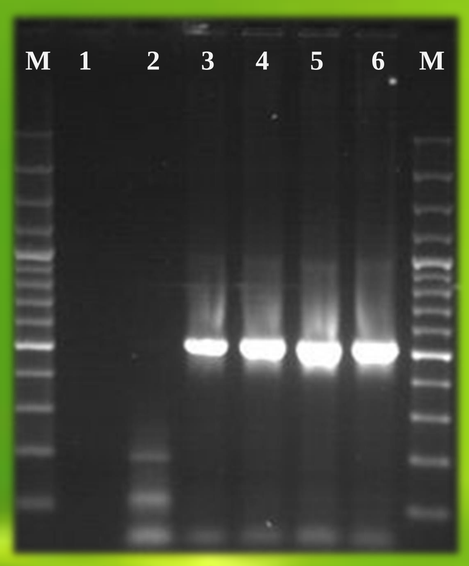

Fig.2. Ethidium bromide-stained 1,5% agarose gel of nested PCR products amplification (507 bp) from DNA extracted from *P. brassicae* – infested soil samples. Lines: M – Molecular weight marker: Gene Ruler™ 100bp (Fermentas); 1 – Control (autoclaved soil); 2 – 10^3 spores per 1 g^{-1}; 3 – 10^5 spores per 1 g^{-1}; 4 – 10^6 spores per 1 g^{-1}; 5 – 10^7 spores per 1 g^{-1}; 6 – 10^8 spores per 1 g^{-1}.

Real-time PCR

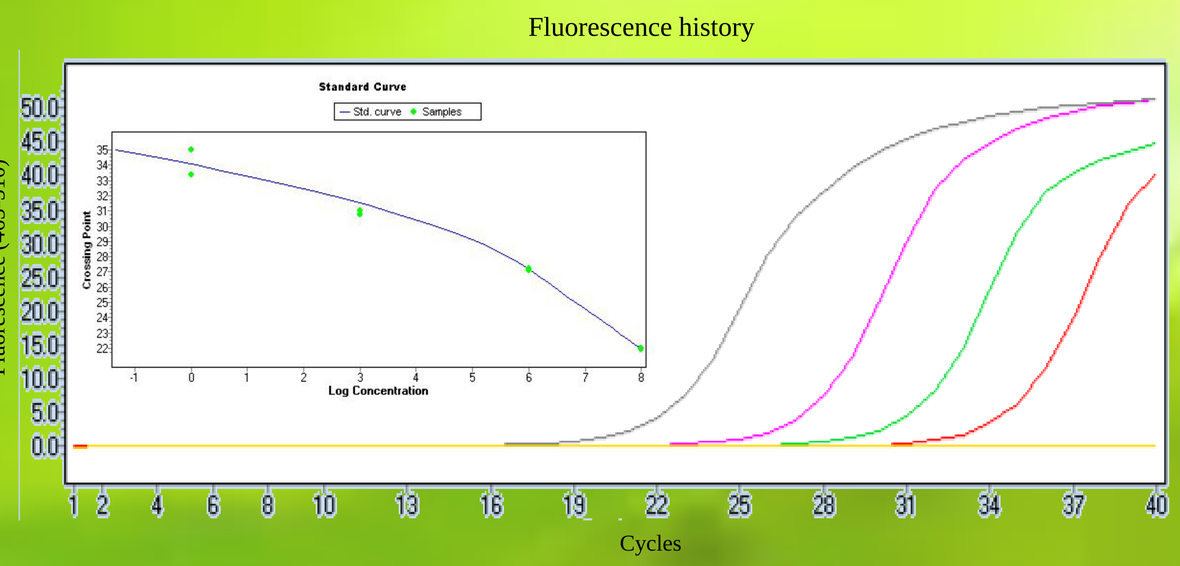

Fig.3. Amplification curves of the soil samples with different concentrations of *P. brassicae* spores. Obtained standard curve will be used for the quantitative determination of concentrations of *P. brassicae* spores in the unknown soil samples.
Lines: red curve – control autoclaved soil); green curve – 10^3 spores per 1 g^{-1}; pink curve – 10^6 spores per 1 g^{-1}; grey curve – 10^8 spores per 1 g^{-1}.

Conclusion

The highest sensitivity in the detection of *P. brassicae* from soil revealed real-time PCR method. The biggest advantage of this molecular method is the ability to quantify concentrations of *P. brassicae* spores in soil samples which can not be specified by nested PCR. However, this method still requires further work to become a routine diagnostic test in detection of *P. brassicae* in soil. Because of the difficulty of isolation pure DNA from soil, biological methods should be still use for the control of molecular tests.

References

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