THE SUITABILITY OF PCR-BASED TECHNIQUES FOR DETECTING *Verticillium dahliae* IN STRAWBERRY PLANTS AND SOIL

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A B S T R A C T

Three sets of primers specific for selected *Verticillium dahliae* genome fragments were tested for their suitability for detection of this pathogen in infected plants and soil. Using primers VMSP1/VMSP2 and VDS1/VDS2, detection of fungus DNA was possible only for single samples derived from leaves of inoculated plants. Primers possessing targeted sites internal to a sequence of product obtained in reaction with DV1/DV2 primers allowed generating specific products on the DNA templates isolated from all inoculated plants. Application of the same nested-PCR primers was partially successful in detecting pathogen in the soil. PCR product with size of 320 bp was obtained for 7 of 10 soil samples, for which the presence of fungus was confirmed earlier with biological method.

Key words: *V. dahliae*, detection, PCR-tests, inhibition

INTRODUCTION

Verticillium wilt is considered to be one of the most important diseases affecting roots system, which limits strawberry (*Fragaria* x *ananassa*) production in temperate climate (Pegg and Brady, 2002). The causal agent of this disease is *Verticillium dahliae,* described first by Klebahn in 1913 (cited after Smith, 1965). This soil-borne pathogen infects plants of many wild and cultivated species (Anonymous, 1997; Bhat and Subbarao, 1999). The significance of wilt disease for strawberry production drastically increased since the soil fumigation with methyl bromide has been banned in EU countries. Simultaneously, the last decade brought

about the changes in the profile of strawberry cultivation with focus on dessert cultivars, which are in majority very susceptible to this pathogen. Summarizing both facts, verticillium wilt disease is becoming one of the most serious problems on strawberry commercial plantations and effective prevention programmes should be applied to avoid the disease spread.

To make sure that the plantation will be established on pathogen-free soil, fast and sensitive tests for pathogen detection are certainly needed. Traditional biological assay is well known as very effective, but also time-consuming and laborious. Since early 80s, PCR-based molecular techniques have been introduced for pathogen detection making the test duration much shorter (Lievens and Thomma, 2005). For many pathogens molecular detection is fully successful (Johanson, 1994), whereas the methodology for other pathogenic organisms still needs validation and special developments taking into account biology of host-pathogen interactions (Heinrich et al., 2001). *V. dahliae* was successfully detected with PCR from fungus culture (Li et al., 1994, 1999) and partially in naturally infested soil (Volossiouk et al., 1995). However, the detection of this pathogen in both the plant and soil still brings about problems (Garcia-Pedrajas, 1999; Mascarello et al., 2001; Volossiouk, 1995) caused by biological properties of the pathogen, the character of infection as well as multi-dimensional environmental and biochemical interactions (Hu et al., 1993; Robb et al., 1994).

In this paper the results of preliminary study on application of PCR-based techniques for *V. dahliae* detection in strawberry plants and soil are presented.

MATERIAL AND METHODS

Plant material

Twenty five strawberry "frigo" plants from cultivars differing in susceptibility to *V. dahliae* ('Senga Sengana', 'Salut', 'Panon', 'Madeleine', 'Kent', 'Filon', 'Dukat', 'Camarosa'), and randomly selected plants from progeny of 'Dukat' x 'Camarosa' cross obtained from the collection of Department of Fruit Plant Breeding, were used for investigations.

Inoculum preparation

The following *V. dahliae* isolates were used for the study: t63 and t82 kindly supplied by dr Nadia Korolev from Volcani Center, Israel, and 424, 1320 purchased from Institute of Plant Protection, Poznan, Poland. The isolates were maintained as a pure cultures on Czapek-Dox Agar at 4°C (Stosz et al., 1996). Two weeks before inoculation they were passaged on the fresh medium and cultivated at 23°C to obtain appropriate mycelium density and conidia con-centration. Then the cultures of each isolate (mycelium with the medium) were removed from Petri-dishes, blended and suspended in 100 ml of sterile water, what resulted in inoculum concentration of approximately 3 x 10^5 conidia per ml. The conidia concentration was confirmed with use of Bürker cell counting chamber. The final suspension used for plant inoculation

consisted of mix of equal volume of each isolate (Bringhurst et al., 1961).

Inoculation

Plants were inoculated according to the method described by Olbricht et al. (2006). The roots of investtigated plants were washed under running water to remove soil remnants, trimmed to 2/3 of their initial length, and the plants were placed in the pathogen's culture suspension. After 24 hours the plants were potted into soil substrate (Hollas) and watered with inoculum suspension. Control plants were placed in and watered with sterile water. Plants were observed for the presence of symptoms typical for wilt disease starting two weeks after inoculation.

Soil samples

Soil samples were collected from naturally infested "provocation field" (0.5 ha) located in RIPF orchard. There were 10 randomly selected plots examined, for which the presence of the pathogen was conformed with biological test (Meszka B., unpublished data). Three soil samples of 10 g were taken from each plot with sterile polypropylene test tubes (Falcon) from the depth of 10 cm below the ground surface in proximity of plants' roots, then combined and mixed thoroughly. Finally, 1 g sample of mixed soil (one sample per each plot) were taken for the test.

DNA extraction

Total DNA was isolated: (A) from plants using method described by Doyle and Doyle (1990), (B) from soil with Garcia-Pedrajas and coworker method (1999), and (C) from fungal mycelium with simplified CTAB-based method (Doyle and Doyle, 1990).

(A) Strawberry leaves (2 g) with symptoms typical for wilt disease (inoculated plants) and without symptoms (control plants and asymptomatic inoculated plants) were collected 50 days after inoculation. Plant tissues were ground in liquid nitrogen and incubated for 30 minutes at 65°C in extraction buffer (2% CTAB; 100 mM Tris HCl pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% PVP; 0.2% β-mercaptoethanol). Polysaccharides were removed with 5 M NaCl. Nucleic acids were purified with chloroform/isoamyl alcohol (24:1) and phenol/chloroform-/isoamyl alcohol (25:24:1), and then precipitated with 700 µl isopropanol. DNA was pelleted by centrifugation (30 min, 12 000 rpm, 4°C) and dissolved in 50 µl of TE buffer.

(B) Soil samples were air dried and then ground to a fine powder using a pestle and mortar. 75 mg of obtained powder was thoroughly mixed with 200 µl of 3.2% skimmed milk solution. Afterwards soil residues were removed by centrifugation (10 min, 10 500 rpm) and supernatant was mixed with extraction buffer (0.3% SDS, 1.4 M NaCl, 50 mM NaAc pH 5.1). Nucleic acids were purified as described above, precipitated with 2.5 volumes of ethanol and dissolved in 25 µl of TE buffer.

(C) Mycelium from fungus culture was collected from the agar

surface with sterile spatula, ground in liquid nitrogen and incubated at 65°C in 800 µl of extraction buffer (2% CTAB; 100 mM Tris HCl pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% PVP; 0.2% β-mercaptoethanol). Nucleic acids were purified with chloroform/isoamyl alcohol (24:1) and then precipitated with 550 µl of isopropanol and 80 µl of sodium acetate. DNA was pelleted by short centrifugation in 12 000 rpm at 4°C and dissolved in 50 µl of TE buffer.

DNA concentration in each sample was measured spectrophotometrically at 260 nm. Additionally, sample quality and concentration of nucleic acids were estimated by electrophoresis in 1% agarose gel and comparison to λ DNA concentration standards (Invitrogen).

Polymerase chain reactions

PCR was performed in a volume of 20 µl. The reaction mixture contained 1 x PCR Buffer; 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Taq* DNA Polymerase (Invitrogen), 0.5 µM of each forward and reverse primer and 10 ng of DNA template. The same concentration of mixture compounds was used for direct PCR and for both rounds of nested PCR. For the second round of nested PCR 1 µl of reaction mixture obtained after first round was used as a template.

Two primer sets were used for direct PCR: VMSP1 (forward: 5'cataaaagactgcctacgccg) and VMSP2 (reverse: 5'aagggtactcaaacggtcag) (Li et al., 1994) and VDS1 (forward: 5'cacattcagttcaggagacg) and VDS2 (reverse: 5'ccgaaatactccagtagaag) (Li et al., 1999). For the first round of nested PCR primers DV1 (forward: 5'ctcataaccctttgtgaacc) and DV2 (reverse: 5'ccgaggtcaaccgttgccg) (Volossiouk et al., 1995) were used, whereas for the second round D1 (forward: 5'tccgatgcgagctgtaac) and D2 (reverse: 5'ccatcagtctctctgtttatac) primer set (Nazar et al., 1991) were applied.

All reactions were carried out with the following thermal profile: 60 s at 94°C, 60 s at 60°C, 120 s at 72°C (30 cycles) in a MJ Research thermocycler. PCR products were separated in 1.4% agarose gel, dyed with ethidium bromide, and visualised under UV light. Products' size was determined by comparison with 100 bp Ladder DNA Marker (Axygen).

RESULTS AND DISCUSSION

Products with expected sizes of 140 and 540 bp were obtained in the reactions with pairs of primers VMSP1/VMSP2 and VDS1/VDS2, respectively, on the template of DNA isolated from inoculated symptomatic ('Kent') and asymptomatic ('Panon') plants. There were no specific products observed for other inoculated ('Kent' and 'Senga Sengana' without symptoms) as well as for control plants ('Filon', 'Madeleine' and 'Salut') (Fig. 1 a, b). In reaction with primers D1/D2 no specific PCR products were obtained for all the plants tested.

Using primers D1/D2 for the second round of PCR on the templates obtained in direct PCR with primers DV1/DV2, product with expected

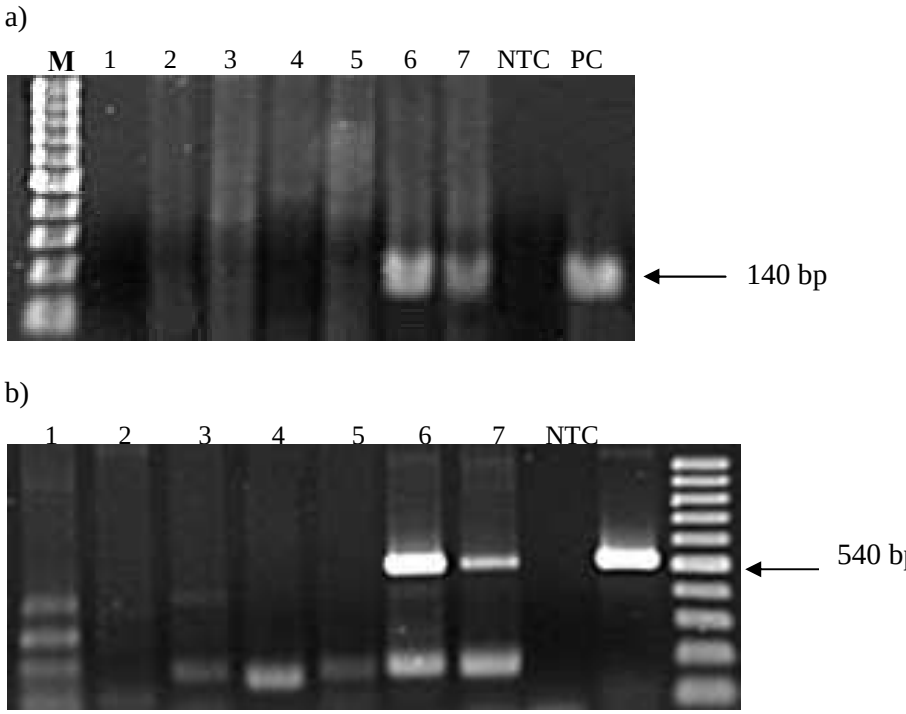

Figure 1. Electrophoretogram of direct PCR products on the template of total DNA isolated from strawberry plants of 6 cultivars inoculated and non-inoculated (control) with pathogen, in reaction with primers: (a) VMSP1/VMSP2, and (b)VDS1/VDS2. Non-inoculated (control) plants: 1 – 'Filon', 2 – 'Madeleine', 3 – 'Salut'; inoculated plants: 4 – 'Kent' without symptoms, 5 – 'Senga Sengana' without symptoms, 6 – 'Kent' with symptoms, 7 – 'Panon' without symptoms; NTC – negative control, no template; PC – positive control (DNA from *V. dahliae* mycelium); M – DNA ladder

size of 320 bp was generated. This product was amplified on the templates isolated from all inoculated strawberry plants (with and without symptoms). No specific PCR-product was observed in the reaction on the template of DNA isolated from control plants (Fig. 2).

The suitability of described PCR primers specific for three different *V. dahliae* genome fragments was fully confirmed in all reactions on the template of DNA isolated directly from fungal mycelium growing on Petri dishes. However, no specific PCR products were obtained on DNA derived from majority of inoculated plants, in which amplification of DNA fragments specific for *V. dahliae* was highly expected. This suggests the presence of some factors interfering with amplification of fungus DNA isolated from infested strawberries. Difficulties in PCR-based detection of fungus DNA in plant extract are often caused by the presence of plant components like polysaccharides (Demeke and

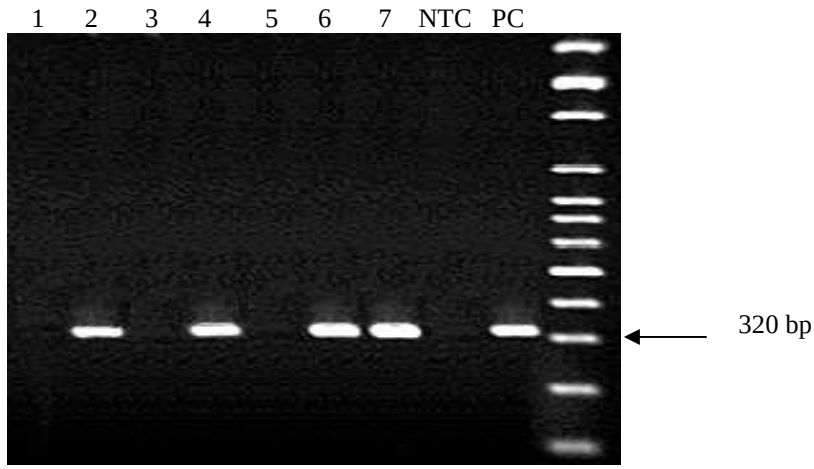

Figure 2. Electrophoretogram of nested PCR products (II amplification phase) with primers D1/D2 on the template of total DNA isolated from inoculated and non-inoculated (control) strawberry plants. 1 – 'Camarosa' control plant, 2 – 'Camarosa' inoculated plant without symptoms, 3 – 'Dukat' control plant, 4 – 'Dukat' inoculated plant without symptoms, 5 – 'Dukat' x 'Camarosa' control plant, 6 – 'Dukat' x 'Camarosa' inoculated plant without symptoms, 7 – 'Dukat' x 'Camarosa' inoculated plant with symptoms; NTC – negative control, no template; PC – positive control (DNA from *V. dahliae* mycelium); M – DNA ladder

Adams, 1992) and polyphenolic compounds (John, 1992), which are known to inhibit polymerase activity. In an attempt to overcome this problem, we have used Doyle and Doyle method described as one of the best techniques for preparation of pure DNA samples from strawberry plants containing high amount of polyphenolics (Korbin et al., 2000). However, the concentration of the inhibitors is strongly correlated with the stage of strawberry development and plant vigour and in some cases it is very difficult to avoid the residuals of these compounds in extracted DNA samples (Kuras and Korbin, in press). Taking into consideration that the pathogen's DNA makes up only a small portion of total DNA isolated from infected strawberry, even small amount of plant inhibitors can block enzymatic reaction being fundamental for PCR-based *V. dahliae* detection.

Results obtained in nested PCR confirmed this hypothesis. Application of nested PCR technique allowed to detect pathogen DNA in all inoculated plants, both symptomatic and asymptomatic, according to our expectation. In the second round of amplification the template was strongly diluted what decreased inhibitor's concentration. Simultaneously, dilution of fungus template was not the obstacle for amplification because nested PCR is known to detect nucleic acids at very low concentration (Nikaeen et al., 2005; Garg et al., 2007; Miller and Sterling, 2007).

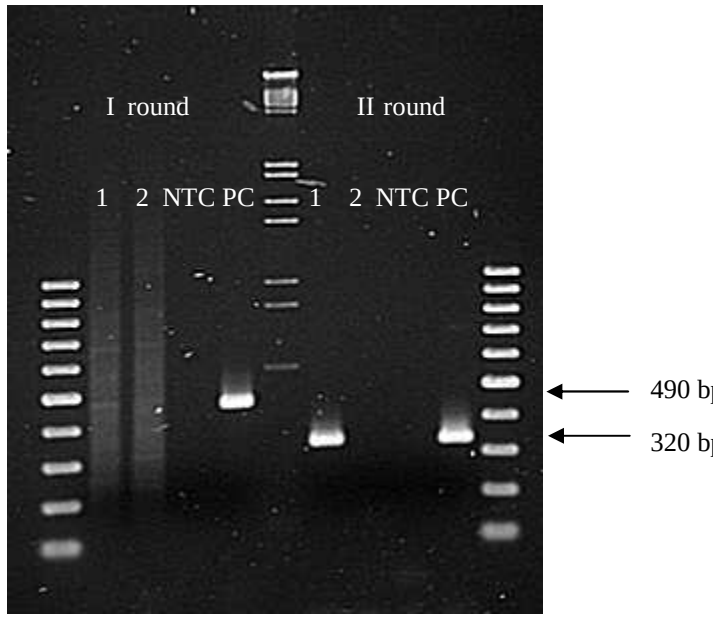

Figure 3. Electrophoretogram of nested PCR products obtained with primers DV1/DV2 (I phase) and D1/D2 (II phase) on the template of total DNA extracted from "provocation field" soil samples. 1, 2 – soil samples from "provocation field"; NTC – negative control, no template; PC – positive control (DNA isolated from fungus mycelium); M – DNA ladder

Similar results were obtained in detecting *V. dahliae* in the soil. Using primers DV1/ DV2 (direct PCR) no specific product was observed in reactions on the template of total DNA isolated from soil samples (data not shown). Specific products with expected size of 320 bp (Fig. 3) were generated in the second round of nested PCR with primers D1/D2 on the templates from almost all investigated soil samples. This result was obtained only for template isolated with Garcia-Pedrajas and co-worker method (1999). Using simple silica-based method for isolation of DNA from soil, neither after direct nor nested amplification specific PCR products were observed (data not shown), whereas the biological test carried out on "provocation field" revealed high pathogen concentration in the soil (Meszka B., unpublished data).

In three soil-samples pathogen was not detected in any PCR test. Unsuccessful results of amplification for individual samples is often correlated with very uneven concentration of a pathogen in naturally infested field (Xiao et al., 1997), thus it is highly probable that these investigated three soil samples did not contain *V. dahliae* or the concentration of the fungus was under threshold of technique detectability. Nevertheless,

it is also possible that the lack of specific PCR products is resulting from the presence of natural soil compounds (humic acid and its derivatives) known as inhibitors of *Taq* polymerase activity (Tsai and Olson, 1992). Application of skim milk into procedure has been suggested as a reasonable treatment aiming at amplification improvement, especially when dealing with DNA isolated from soil (Volossiouk et al., 1995; Garcia-Pedrajas et al., 1999). According to Garcia-Pedrajas and co-workers (1999), the efficiency of PCR is improved after using soil extract without dilution. In contrary to this result, Vollossiouk and co-workers (1995) observed that 50-fold dilution resulted in superior amplification.

To summarize, simple and very effective method for *Verticillium dahliae* detection has been successfully worked out. On the other hand, all remaining doubts should be a basic point for further development of the methodology of these PCR-based techniques for *V. dahliae* precise detection with considering all natural (field) circumstances and possible biochemical interactions.

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OCENA PRZYDATNOŚCI TECHNIK OPARTYCH NA PCR DO WYKRYWANIA *Verticillium dahliae* W ROŚLINACH TRUSKAWKI I W GLEBIE

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S T R E S Z C Z E N I E

W niniejszej pracy zostały przedstawione wyniki wstępnych badań nad przydatnością testów opartych na PCR do wykrywania *V. dahliae* w roślinach truskawki i w glebie. Zastosowanie starterów VMSP1/VMSP2 oraz VDS1/VDS2 specyficznych do dwóch różnych fragmentów genomu patogena pozwoliło na uzyskanie produktów PCR o długościach 140 i 540 pz dla pojedynczych próbek roślinnych. W reakcji z trzecią parą starterów D1/D2 nie uzyskano produktu dla żadnej z testowanych roślin. Wprowadzenie do badań testu opartego na zagnieżdżonym PCR umożliwiło potwierdzenie obecności patogena we wszystkich inokulowanych roślinach (produkt o długości 320 pz). Produkt o tej długości obserwowano także w reakcji przeprowadzonej na matrycy DNA pozyskanego z 7 spośród 10 testowanych próbek gleby pobranych z „pola śmierci". Badania nad adaptacją testów PCR są kontynuowane.

Słowa kluczowe: *V. dahliae*, wykrywanie, testy oparte na PCR, inhibicja