Pestic. Phytomed. (Belgrade), 24(3), 2009, 177-184
Pestic. fitomed. (Beograd), 24(3), 2009, 177-184

UDC: 632.4:634.711:579.64
Scientific paper ∗ Naučni rad

Methods for Detection of *Phytophthora fragariae* var. *rubi* on Raspberry

Mirjana Koprivica[1], Ivana Dulić-Marković[2], Radivoje Jevtić[3] and Dave E.L. Cooke[4]

[1]Ministry of Agriculture, Forestry and Water Management, Plant Protection Directorate, 11070 Belgrade, Omladinskih brigada 1, Serbia (mirjana.koprivica@minpolj.gov.rs)
[2]FAO SEEDEV
[3]Institute of Field and Vegetable Crops, 21000 Novi Sad, Maksima Gorkog 30, Serbia
[4] Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Received: July 17, 2009
Accepted: September 10, 2009

SUMMARY

Phytophthora fragariae var. *rubi* (Wilcox & Duncan), a causal agent of raspberry root rot, is a serious soil-borne pathogen listed by EPPO as an A2 quarantine pest. Root samples were collected from badly diseased raspberry plants showing a variety of characteristic and often dramatic symptoms during surveys carried out in western Serbia in 2002. Identification of the causal agent was performed in collaboration work with the Scottish Crop Research Institute (S.C.R.I.), Dundee, UK. Necrotic roots were plated on selective French bean agar (incorporating ampicilin, ryfamicin, bavistin and hymexasol). Detection of isolates was based on cultural and morphological features compared with referent cultures.

DNA was extracted directly from the sampled roots using extraction buffer (200 mM Tris- HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), purified by multi spin separation columns [Thistle Scientific (Axygen)] or in 24:1 mixture of chlorophorm- iso-amyl alcohol and amplified by nested PCR (ITS 4 and DC 6 for first round, DC 1 and DC 5 for second round). Diluted DNA extracts were also amplified by conventional PCR with modified "universal" *Phytophthora* primers (ITS 6, ITS 7 and ITS 8, Cooke et al., 2000) and digested with *Msp1*. Digestion patterns of the universal primers PCR product from infected roots matched those of Scottish strains.

P. fragariae var. *rubi* occured on 8 out of 14 sites. Our results indicate that nested PCR (ITS 4 and DC 6 for first round, DC 1 and DC 5 for second round) or digestion of the "universal" *Phytophthora* primers PCR product for detection of *P. fragariae* var. *rubi* are more sensitive and less time-consuming and therefore recommended for use.

Keywords: *Phytophthora fragariae* var. *rubi*; Raspberry root rot; PCR methods

INTRODUCTION

Raspberry root rot caused by species from the genus *Phytophtora* is known since 1937, but it was not considered an important disease of raspberries until mid-1980s. Several epiphytotics have been reported since then in central and eastern United States, Great Britain and Australia (Wilcox, 1997). It is considered that species of the genus *Phytophtora* are primary causes of raspberry decline in all production areas in the world (Figure 1).

Eight different *Phytophthora* species have been isolated from raspberry roots: *Phytophtora fragariae* var. *rubi*, *P. megasperma*, *P. erytroseptica, P. cactorum, P. citricola, P. cryptogea, P. drechsleri* and *P. cambivora*, but there is still a number of species isolated but not yet identified (Wilcox, 1989).

Diagnostics of *Phytophthora* species is often based on a combination of microscopic examination of raspberry roots, isolation of selective media and exposure of susceptible plants ("bait plants") to infection by material under examination (Hughes et al., 2000). The first two methods are unreliable and the third requires 5-6 weeks of experiment and different bait plants for detection of different *Phytophthora* species.

A range of quick, sensitive and very specific diagnostic methods based on polimerase chain reaction have been developed owing to an extensive DNA database of several thousands isolates of *Phytophthora*, which are almost all species described (Bonants et al., 1997). ITS sequences (Internal Transcribed Spacers) present parts of genes that code ribosomal DNA (rDNA) and are variable between species. Simple extraction of DNA, followed by nested PCR, is a quick and reliable method for identification of *Phytophthora* species (Duncan, personal communication). Generic primers ITS 4 and DC 6 in the 1st round of PCR amplify a part of ITS sequence of any *Phytophtora* including other *Oomycetes* in sample, while in the 2nd round primers DC 1 and DC 5 amplify a product specific only for *P. fragariae*. "Universal" *Phytophthora* primers amplify ITS sequences of almost all *Phytophthora* and none other genus. Primers ITS 6, ITS 7, and ITS 8 are versions of the "universal" ITS primers ITS 5, ITS 2 and ITS 3, respectively, of White et al. (1990), modified by D.E.L. Cooke to improve the amplification of rDNA from *Oomycetes*. Restriction digestion with frequent cutter enzyme enables detection down to species level based on the number and size of bands in gel when compared with known species.

This paper reports on detection of *P. fragariae* in raspberry samples showing root rot using isolation and molecular techniques.

MATERIAL AND METHODS

Visual examination and sampling

A survey for *P. fragariae* var. *rubi* was conducted early in the spring, while plants were still low and patches of wilting or individual wilted plants in plantation were easy to observe (Figure 1). A number of plants were taken during sampling, which was not standardized by EPPO in that moment and depended on the sampler's personal judgement.

Raspberry samples with root rot symptoms were collected from several locations in western Serbia during the survey and are presented in Table 1.

During visual inspection, plants were examined for a reduction in the number of smaller roots, change of their colour from white to dark, and appearance of reddish discoloration after removal of bark on older root parts (Figure 2).

Isolation

Selective French Bean Agar (FBA, French bean – 30 g, agar – 15 g, water up to 1 l) was used for isolation from roots. After autoclaving and cooling near melting temperature FBA was amended with fungicides (bavistin, 0.6% of final concentration, and hymexasol, 0.6% of final concentration) and antibiotics (rifamycin, 0.3% of final concentration, and piramycin, 0.1% of fi-

Figure 1. Wilting of raspberry plants in commercial plantations caused by *Phytophthora fragariae* var. *rubi*

Table 1. Root rot symptoms in raspberry samples

Sample Nº	Region	Location	Raspberry root rot (+ or -)
1	Ljubovija	Košica	-
2	Valjevo	Ostružanj 1	+
3	Valjevo	Ostružanj 1	+
4	Valjevo	Ostružanj 1	+
5	Valjevo	Ostružanj 2	+
6	Čačak	Krstac	-
7	Arilje	Pridvorica	-
8	Arilje	Cerova	-
9	Arilje	Gruda	-
10	Arilje	Vigošte	+
11	Arilje	Vigošte	+
12	Arilje	Vigošte	+
13	Arilje	Virovo	+
14	Čačak	Viča	-

Figure 2. Symptoms of raspberry root rot, small roots-reduction of number and change of colour from white to dark and apperance of reddish discoloration after removal of bark

nal concentration). Small raspberry roots, 1.5-2.0 cm in size, were sterilized in a 10% solution of NaOHCl, washed with sterile distilled water and, after drying on filter paper, put into media. Plates were incubated at 14°C for 3-4 weeks.

Extraction of DNA

Small raspberry roots were washed under tap water, dryed on filter paper and ground in liquid nitrogen. An amount of 0.03 g of ground sample was placed into 1.5 ml eppendorf tubes, and an equivalent volume of insoluble polyvinilpirrolidon (PolyclarSB100) and 1 ml of extraction buffer (200 mM Tris HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added to each. The tubes were placed onto a rotary shaker for 10 min, centrifuged at 10000 g for 5 min and 400 µl of supernatant was transferred to new 1.5 ml eppendorf tubes, then 400 µl of i-propanol was added and centrifuged again at 10000 g for 5 min. Supernatant was poured out and the pellet resuspended in 70% ethanol, and centrifuged at 10000 g for 5 min. The pellet was resuspended in 100 µl of sterile distilled water.

Mini spin columns (Thistle Scientific (Axygen) were filled up to 1 cm from top with polyvinilpirolidon (PVP), 250 µl of sterile distilled water was added and centrifuged at 4000 g for 5 min to allow collection of water at the bottom of tube. Columns were put in new eppendorf tubes with flat bottoms, 100 µl of extracted DNA was added and the tubes were centrifuged at 4000 g for 5 min. Pufiried DNA was stored at -20°C.

Beside this method of purification, another one was also amended for raspberry samples by adding 500 µl of sterile distilled water instead of 100 µl after the extraction of DNA. In tubes with 500 µl of extracted DNA, 400 µl of 24:1 mixture of chlorophorm: isoamyl alcohol was added and centrifuged at 10000 g for 5 min; 400 µl of the upper liquid phase was pippeted into new eppendorf tubes and 200 µl of 5M NaCl, 600 µl i-propanol was added, and the tubes were put at -20°C for 15

min to allow DNA to precipitate. The pellet was resuspended in 70% ethanol after centrifugation at 10000 g for 2 min and centrifuged again at 10000 g for 5 min. Purified DNA was resuspended in 100 µl of sterile distilled water and stored at -20°C before use.

The success of DNA extraction was checked by running on 1% agarose gel for 30 min.

PCR amplification

DNA was amplified by nested PCR using ITS 4 and DC 6 set of primers in the first round and DC 1 and DC 5 in the second. Besides this one, extracted DNA was amplified by a conventional PCR using "universal" *Phytophthora* primers.

Forward primer DC 6 (5′-GAGGGACTTTTGGGTAATCA- 3′, Bonnants et al., 1997) was designed based on sequences of 18S rDNA from a number of plants and fungi. When used with the reverse primer ITS 4 (5′-TCCTCCGCTTATTGATATGC - 3′, White et al., 1990), DC 6 gives 1310bp product specific for all *Oomycetes*.

Primers DC 1 (5′- ACTTAGTTGGGGGCCTGTCT- 3′, Bonnants et al., 1997) and DC 5 (5′ - CGCCGACTGGCCACACAG- 3′, Bonnants et al., 1997) were used in the second round to amplify 533 bp product only for *Phytophthora fragariae*.

Master mix for the first and second rounds of PCR was the same containing: 19 µl of sterile distilled water, 0.5 µl of each forward and reverse primer (10 µM), 5 µl of extracted DNA from sample (10 times diluted) and 1 Ready to Go TM PCR bead (Amersham pharmacia biotech). The first round of PCR was performed in a thermal cycler under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 60 s and final extension at 72°C for 10 min, while temperature was 66°C in the second round of PCR annealing.

Besides nested PCR, the samples were tested using conventional PCR with modified "universal" *Phytophthora* primers. ITS 6 (5'-GAAGGTGAAGTCGTAACAAGG-3', Cooke et al., 2000) is a forward primer which, together with two reverse primers, ITS 7 (5'-AGCGTTCTTCATCGATGTGC-3', Cooke et al., 2000) and ITS 8 (5'-GCACATCGATGAAGAACGCT-3', Cooke et al., 2000), gives the 820 bp product specific only for *Phytophthoras*. Master mix for conventional PCR with "universal" *Phytophthora* primers contained: 18 µl of sterile distilled water, 1 µl of ITS 6 primer (10 µM), 0.5 µl of each ITS 7 (10 µM) and ITS 8 (10 µM) primer, 5 µl of extracted DNA (10 times diluted) and 1 Ready to Go TM PCR bead. Amplification was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, elongation at 72°C for 60 s and final elongation at 72°C for 10 min.

The products of nested PCR were visualized after running on 1% agarose gel (60V) for 30 min. The gel was stained in 0.05% solution of ethidium bromide.

Digestion of products amplified with "universal" *Phytophthora* primers

Msp1 enzyme was used for digestion of products amplified with "universal" *Phytophthora* primers. Master mix for digestion was: 10 µl of PCR products, 2 µl of 10 x enzyme buffer, 1 µl of *Msp1* and 7 µl of sterile distilled water. Products were digested overnight at 37°C.

Products were visualized by running on 2.5% NuSieve agarose gel (30V) for 3 hours and 30 min. The gel was stained with 0.05% solution of ethidium bromide.

RESULTS

Isolation

Only 2 of 200 potential isolates were obtained after 4 weeks. Colony was observed after 14 days of incubation (Figure 3). It was white coloured, mostly immersed in media and partly aerial. Nonseptate mycelia were observed during microscopic examination (40x) which got some septae as the culture became older (Figure 4). Sympodial branching was not observed. Individual sporangia were not observed directly in the culture, but they were observed when mycelial discs were exposed to soil solution and +4°C temperature, and zoospores were observed directly. This was an indication that some *Phytophthora* was isolated.

DNA extraction

Extraction from samples originated from the localities Košice and Krstac (samples 1 and 6) was not performed because of an insufficient quantity of roots, but it was successful from the other samples (Figure 5).

Figure 3. *Phytophthora* spp. culture

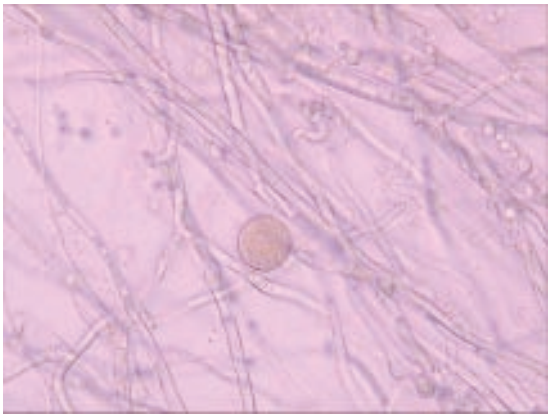

Figure 4. Sporangia of *Phytophthora* spp.

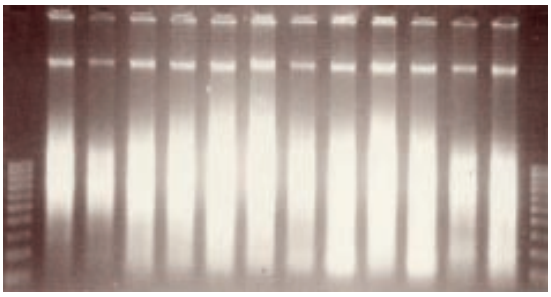

Figure 5. Extracted DNA from samples

DNA amplification

The product specific only for *Oomycetes,* obtained after amplification with ITS 4 and DC 6 primers, was found only in samples originating from the locations Ostružanj (samples 4 and 5) and Vigošte (sample 10) (Figure 6). Althougth DNA was successfully extracted from all except samples 1 and 6 (Figure 5), products from samples originating from Virovo, Košica, Pridvorica, Gruda and Viča were not observed.

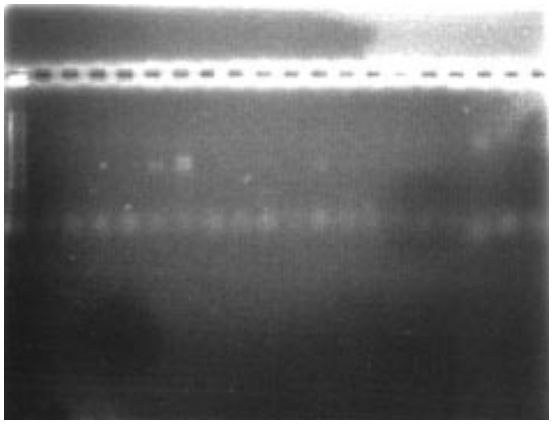

Figure 6. Products amplified with ITS 4 and DC 6 primers in the first round of PCR

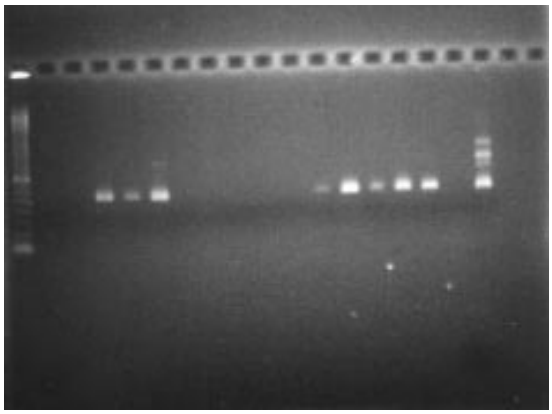

Figure 7. Products amplified with DC 1 and DC 5 primers in the second round of PCR

In the second round, when DC 1 and DC 5 primers were used, 533 bp product specific for *P. fragariae* was not only observed in samples from Ostružanj and Vigošte (product of 1310 bp observed in the first round) but also in samples from Pridvorica, Gruda and Viča (Figure 7).

After amplification with the "universal" *Phytophthora* primers, products were observed in samples from Ostružanj, Vigošte, Pridvorica, Gruda and Viča, but also in suspected samples (Ostružanj 2 and Gruda) in which the expected product was not detected by nested PCR (Figure 8). The "universal" *Phytophthora* primers provided better sensitivity and new bands were obverved (samples from Ostružanj 2 and Gruda) (Figure 8).

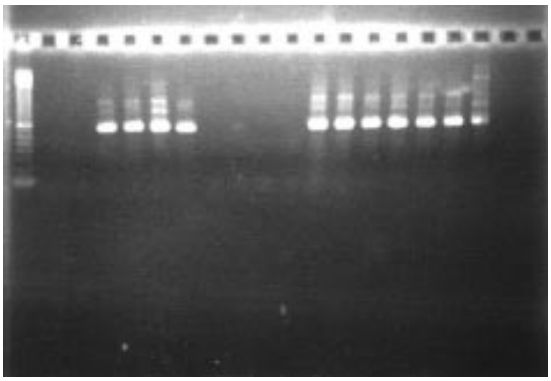

Figure 8. Products amplified with "universal" *Phytophthora* primers

Digestion of products amplified with "universal" *Phytophthora* primers

After digestion with *Msp1*, bands specific for *P. fragariae* were detected, which was confirmed by a corresponding number and size of bands, compared with the DNA extracted from a pure culture of *P. fragariae* (Figure 9). None of the other *Phytophthoras* was detected in tested samples (Table 2).

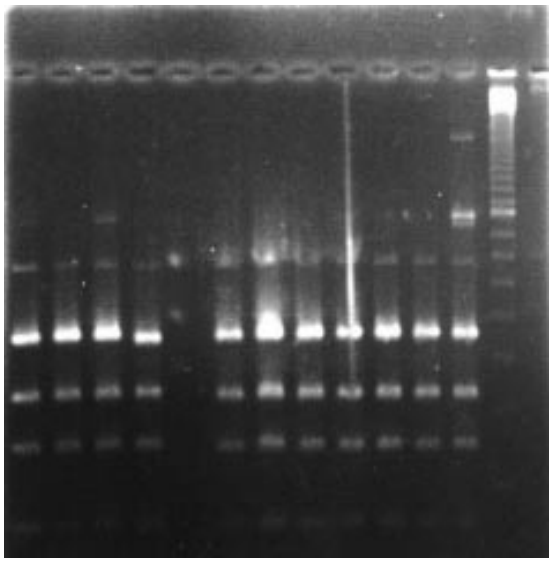

Figure 9. Digestion with Msp_1

DISCUSSION

Althought symptoms can be observed at almost any time of the year, the best time for visual inspection and sampling is springtime. In the summer, fungi are inactive and in the form of oospores because of higher temperatures. When the weather is cooler during sum-

Table 2. Samples with or without root rot symptoms from different locations and results of the first and second round of PCR, conventional PCR using "universal" *Phytophthora* primers and digestion with Msp_1

No.	Location	Root rot symptoms	First round PCR (ITS 4 & DC 6)	Second round PCR (DC 1 & DC 5)	Conventional PCR (ITS 5 & ITS 7 & ITS 8)	Digestion Msp_1
1	Košica	-	-	-	-	-
2	Ostružanj 1	+	-	+	+	*P. fragariae* var. *rubi*
3	Ostružanj 1	+	+	+	+	*P. fragariae* var. *rubi*
4	Ostružanj 1	+	+	+	+	*P. fragariae* var. *rubi*
5	Ostružanj 2	+	-	-	+	*P. fragariae* var. *rubi*
6	Krstac	-	-	-	-	-
7	Pridvorica	-	-	-	-	-
8	Cerova	-	-	-	-	-
9	Gruda	-	-	-	+	*P. fragariae* var. *rubi*
10	Vigošte	+	+	+	+	*P. fragariae* var. *rubi*
11	Vigošte	+	-	+	+	*P. fragariae* var. *rubi*
12	Vigošte	+	-	+	+	*P. fragariae* var. *rubi*
13	Virovo	+	-	+	+	*P. fragariae* var. *rubi*
14	Viča	-	-	+	+	*P. fragariae* var. *rubi*

mer fungi become active and can infect roots, but sampling needs to be very careful because advanced infection cannot be detected even by PCR test (Dave Cooke, personal communication). It is neccessary to take as many samples as possible, but the number has not yet been standardized.

Coinciding with the findings of Hughes et al. (2000), this examination showed that diagnostics of *Phytophthoras* based on a combination of microscope examination of small raspberry roots and isolation on selective media is unreliable and time consuming. This is very important for production of planting material because different *Phytophthora* can be detected that way, while only *Phytophthora fragariae* var. *rubi* is regulated for raspberries.

The most reliable PCR to be recommended is either nested PCR using DC 1 and DC 5 in the second round or conventional PCR using "universal" *Phytophthora* primers followed by digestion with restriction ensymes.

REFERENCES

Bonants, P., Hagenaar-de Weerdt, M., van Gent-Pelzer, M., Lacourt, I., Dooke, D.E.L. and Duncan, M.: Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. European Journal of Plant Pathology, 103: 345-355, 1997.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G. and Brasier, C.M.: A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology, 30: 17-32, 2000.

Hughes, K.J.D., Inman, A.J. and Cooke, D.E.L.: Comparative testing of nested PCR-based methods with bait-plant tests for detecting *Phytophthora fragariae* var. *fragariae* in infected strawberry roots from fruit crops in the UK. OEPP/EPPO Bulletin, 30: 533-538, 2000.

White, T.J., Bruns, T., Lee, S. and Taylor, J.: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Innis M.A., Gelfand D.H., Sninsky J.J. and White T.J., eds.), Academic Press, San Diego, U.S.A, 1990, pp. 315-322.

Wilcox, W.F.: Identity, virulence, and isolation frequency of seven *Phytophtora* spp. causing root rot of raspberry in New York. Phytopathology, 79: 93-101, 1989.

Wilcox, W.F.: *Phytophthora* Root Rot. Compendium of Raspberry and Blackberry Diseases and Insects. APS Press, St. Paul, Minnesota, 1997.

Metode za detekciju *Phytophthora fragariae* var. *rubi* na malini

REZIME

Phytophthora fragariae var. *rubi* (Wilcox & Duncan), prouzrokovač truleži korena maline, je veoma značajan zemljišni patogen A2 karantinskog statusa u EPPO. Uzorci korena su sakupljeni sa jako zaraženih biljaka maline koje su ispoljavale čitav niz karakterističnih i često dramatičnih simptoma tokom pregleda koji su vršeni u zapadnoj Srbiji 2002. godine. Identifikacija prouzrokovača je izvršena u saradnji sa Scottish Crop Research Institute (S.C.R.I.), Dundee, UK. Nekrotični korenčići su unošeni u selektivnu French bean podlogu, koja je sadržala ampicilin, rifamicin, bavistin i himeksazol. Detekcija dobijenih izolata je vršena posmatranjem kulture i morfoloških karakteristika i poređenjem sa autentičnim kulturama.

DNK je ekstrahovana direktno iz uzorkovanih korenova pomoću ekstrakcionog pufera (200 mM Tris-HCl pH 8,5, 250 mM NaCl, 25 mM EDTA, 0,5% SDS), prečišćena Multi spin separacionim kolonama (Thistle Scientific (Axygen) ili 24:1 mešavinom hloroforma i izoamil alkohola i amplifikovana nested PCR (ITS 4 i DC 6 za prvi krug, DC 1 i DC 5 za drugi krug). Rastvoreni DNK ekstrakti su takođe amplifikovani putem konvencionalnog PCR sa modifikovanim „univerzalnim" *Phytophthora* prajmerima (ITS 6, ITS 7 i ITS 8, Cooke i sar., 2000) i di-

gestirani sa *Msp1***.** Digestioni paterni proizvoda univerzalnih prajmera iz zaraženih korenova su se poklapali sa škotskim izolatima.

P. fragariae var. *rubi* se javila na 8 od 14 lokaliteta. Naši rezultati ukazuju da su nested PCR (ITS 4 i DC 6 za prvi krug, DC 1 i DC 5 za drugi krug) i digestija produkta univerzalnih *Phytophthora* prajmera u cilju detekcije *P. fragariae* var. *rubi* senzitivnije i zahtevaju manje vremena, pa ih stoga preporučujemo za korišćenje.

Ključne reči: *Phytophthora fragariae* var. *rubi*; trulež korena maline; PCR metode detekcije