

Rapid communication

A simple method for extracting DNA from rhododendron plants infected with *Phytophthora* spp. for use in PCR

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Abstract: Among the numerous protocols that describe the extraction of DNA, those relating to the isolation of DNA from infected plants, are rare. This study describes a rapid and reliable method of extracting a high quality and quantity of DNA from rhododendron leaves artificially infected with *Phytophthora cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citrophthora*, and *P. plurivora*. The use of the modified Doyle and Doyle protocol (1987) allowed us to obtain high quantity and quality DNA (18.26 µg from 100 mg of the fresh weight of infected leaves at the ratios of $A_{260/280}$ and $A_{260/230}$ – 1.83 and 1.72, respectively), suitable for conventional polymerase chain reaction (PCR) and real-time PCR amplifications.

Key words: conventional PCR, detection, infected rhododendron leaves, *Phytophthora* species, real-time PCR, total DNA extraction

Introduction

The detection of pathogenic organisms in environmental samples of plants, water or soil bases often on DNA markers obtained by the polymerase chain reaction (PCR) technique, which requires a high quality DNA. Weishing *et al.* (1994) showed that chemical heterogeneity of plant material may not allow the procurement of DNA in either sufficient quantity and quality, based on known isolation protocols. This is because even closely related species may require a specific modification. Additionally, commercially available kits for extracting DNA from plant tissue, despite being relatively expensive, do not always yield a satisfying quantity or quality of DNA that is especially needed for use in real-time PCR analyses. In current literature, there are numerous protocols of DNA isolation from the soil, and from different plants and plant tissues, also those producing large amounts of essential oils, containing high level of polysaccharides or other secondary metabolites (Porebski *et al.* 1997; Khanuja *et al.* 1999; Michiels *et al.* 2003; Bashalkhanov and Rajora 2008; Sahu *et al.* 2012). Protocols describing the isolation of DNA from diseased plants, however, are scarce. The specificity of these samples relies on the fact that they contain two kinds of biological material – microorganisms and plant tissues, which produce large amounts of secondary metabolites, mostly phenolics, in reaction to pathogen attack.

This study describes the DNA extraction protocol from plant tissue infected artificially with pathogens. The usefulness of this protocol was tested for the detection of the DNA of *Phytophthora cactorum* (Lebert et Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, *P. cinnamomi* Rands, *P. citroph-*

thora (R.R. Sm. & E.H. Sm.) Leonian, and *P. plurivora* T. Jung & T.I. Burgess, in rhododendron leaves. A conventional PCR amplification and SYBR Green real-time PCR assays were used. The above-mentioned species are multi-host pathogens which pose an increasing threat to trees, shrubs, and perennials (Orlikowski and Szkuta 2002, 2003; Orlikowski and Oszako 2005; Orlikowski *et al.* 2010).

Materials and Methods

Pathogen isolates, plant materials, and sampling

The isolates of five *Phytophthora* spp. were used in this study (Table 1). 'Nova Zembla' rhododendron leaves were wounded with the tip of a sterile scalpel. Inoculation was done by dripping of 30 µl of zoospore suspensions at a concentration of 1×10^4 ml⁻¹ into the wound. Deionized water was used as the control. Ten leaves per *Phytophthora* species were used. Inoculated leaves were placed on trays covered with sterile, moist blotting paper and plastic net and wrapped with polyethylene foil, and then incubated at 22°C at 12/12 h light/dark. The inoculated leaves were sprayed gently with sterile distilled water every day. After 14 days, the leaves were washed with deionized water, and leaf parts containing appr. 3/4 diseased and 1/4 healthy tissue were ground in a mortar with a pestle in liquid nitrogen. Then, according to the isolation protocol, 100 or 200 mg of infected tissue were sampled for DNA extraction. Infection was confirmed by placing 5 × 5 mm of necrotic tissues onto the PARP (Pimaricin + Ampicilin + Rifampicin + Pentachloronitro-

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Table 1. List of *Phytophthora* species used in this study

| <i>Phytophthora</i> species | Isolate number | Host | Year of isolation | GenBank accession number |
|-----------------------------|----------------|--|-------------------|--------------------------|
| <i>P. cactorum</i> | W1-RH06/CAC | water from the reservoir in a nursery ¹ | 2006 | KF682436 |
| <i>P. cambivora</i> | W2-RH06/CAM | water from the reservoir in a nursery ¹ | 2006 | KF682437 |
| <i>P. cinnamomi</i> | RH-03/CIN | rhododendron from nursery of ornamental plants | 2003 | KF682434 |
| <i>P. citrophthora</i> | RH-04/CTPH | rhododendron from nursery of ornamental plants | 2004 | KF682433 |
| <i>P. plurivora</i> | RH-06/PLU | rhododendron from nursery of ornamental plants | 2006 | KF682435 |

¹isolated from water using rhododendron leaf blades as baits for *Phytophthora* species recovery (Themann *et al.* 2002)

beneze Agar) *Phytophthora*-selective medium (Erwin and Ribeiro 1996). After 24 to 48 h, colonies growing from the diseased leaf fragments were transferred onto Potato Dextrose Agar (PDA; Merck) medium. Species identity was made on the basis of mycelium morphology and propagation organs.

Extraction protocols

Below, the four DNA extraction protocols are compared in terms of quantity, and quality:

- I. The extraction protocol described by Aljanabi and Martinez (1997) (AM) was applied with the modification that 100 mg of fresh weight of tissue was ground in liquid nitrogen with a mortar and pestle, instead of homogenization being done in a Polytron Tissue Homogenizer.
- II. Extraction employing a commercial DNA extraction kit – DNeasy Plant Mini Kit (Qiagen) (Q) using 100 mg of fresh weight of tissue, was made according to the manufacturer's protocol (www.qiagen.com).
- III. The protocol of Doyle and Doyle (1987) unmodified (DD) used 200 mg of fresh weight of tissue and CTAB (Cetyltrimethylammonium bromide) buffer. The amount of CTAB buffer was proportionally lower in comparison to the original, where 500 to 1,000 mg of fresh tissue was used.
- IV. The protocol of Doyle and Doyle (1987) (DDm) was made with the following modifications: (i) DNA was extracted from 200 mg of fresh weight of tissue instead of 500–1,000 mg; (ii) 500 µl of CTAB buffer was used; (iii) precipitation of nucleic acids was carried out at –20°C, not at room temperature; (iv) precipitation time was 1 h instead of several hours; (v) nucleic acids were washed with 70% ethyl alcohol instead of a wash buffer containing 76% ethyl alcohol and 10 mM ammonium acetate without centrifugation but only gently pipetted.

After extraction, the RNase A was added to a final concentration of 10 µg · ml⁻¹ and then incubated for 1 h at 37°C for protocols I, III, and IV, while for the Qiagen protocol RNase A was added during the isolation process.

Three replicates of each isolation were performed simultaneously for each species and method. The whole experiment was carried out twice.

Qualitative and quantitative analyses of extracted DNA

The quality and yield of DNA was checked by electrophoresis in 1% agarose gel stained with ethidium bromide, and by calculating the absorbance ratio $A_{260/280}$ and $A_{260/230}$ with the DNA yield measured in Biophotometer (Eppendorf) at a wave length of 260 nm.

Conventional and real-time PCR amplification

To check the suitability of the isolated DNA for amplification, a PCR using plant primers FMPI-2b/FMPI-3b was done according to Martin *et al.* (2004). For the detection of *Phytophthora* species, the PCR reactions were performed using the following primers: for *P. citrophthora* and *P. plurivora*, primers available in literature (Pc2B/Pc7 – Ippolito *et al.* 2004; P5/P6 – Böhm *et al.* 1999, respectively). For *P. cactorum*, *P. cambivora*, and *P. cinnamomi*, the primers were newly designed by the authors, based on the *Ypt1* gene sequence: Pcac47F: 5'-AGCTCCAGATTTCCACCAGA-3' and Pcac143R: 5'-TGGAGCTTGATGTCTTTCC-3'; Pcomb54F: 5'-AGTTTTGACCTCCAGGCTGA-3' and Pcomb187R: 5'-GCACTTTGAACCAGCGAAAT-3'; Pcin59F: 5'-CGTCGTTGTTGT TTCTGTGC-3' and Pcin191R: 5'-TTCAGTCAGCTCCACGAACA-3', respectively. The reaction mixture (25 µl) consisted of 2.5 µl of DNA template diluted to concentration of 10 ng · µl⁻¹, 0.5 U of *Taq* DNA Polymerase (Fermentas, Thermo Scientific), 1.5 mM MgCl₂, 50 µM of each DNA nucleotide, and 400 nM of each primer specific for *Phytophthora* species. The amplification parameters were as follows: 8 min of initial DNA denaturation at 95°C and 35 cycles of amplification (30 s of denaturation at 95°C, 60 s of annealing at 55°C (except for *P. cactorum* which was annealed at 52°C), 45 s of elongation at 72°C), and 10 min of final elongation at 72°C. The PCR reactions were performed in the GeneAmp PCR System 9700 (PE Applied Biosystems) and 15 µl of PCR products was analysed by electrophoresis in 2.5% agarose gels stained with ethidium bromide. Each reaction was repeated twice.

Real-time PCR amplifications were carried out in a total volume of 25 μl using the Stratagene Mx3005P QPCR System (Agilent Technologies). Each reaction mixture contained 2 μl of DNA template diluted to concentration of 10 $\text{ng} \cdot \mu\text{l}^{-1}$ obtained from the four DNA extraction protocols, 12.5 μl of 2 \times Brilliant[®]II SYBR[®]Green (Stratagene), and 160 nM of each of the above mentioned primers. Negative control reactions contained 2 μl of sterile distilled water and DNA from uninfected rhododendron leaves as the DNA template. The amplification parameters were 95°C for 10 min followed by 35 cycles at 95°C for 30 s, 55°C (except for *P. cactorum* which was annealed at 52°C) for 60 s, 72°C for 45 s. To determine the specificity of the amplification products a dissociation curve analysis in the temperature range of 55–95°C using Bioanalyzer 2100 (Agilent Technology) was obtained.

Standard curves based on threshold cycles (C_t) for 10-fold dilution series of fungal genomic DNA, extracted from pure cultures using the method Aljanabi and Martinez (1997) and modified by Wiejacha *et al.* (2002) (1×10^2 ,

1×10^1 , 1×10^0 , 1×10^{-1} , 1×10^{-2} $\text{ng} \cdot \mu\text{l}^{-1}$), were constructed for each *Phytophthora* species. A calculation of the C_t values was done using MxPro QPCR software program version 4.10 (Stratagene).

Statistical analysis

Statistical analyses were performed using the STATISTICA 10 package. Values of DNA concentration and quantities of DNA pathogen were analysed by one-way ANOVA and compared using the Tukey's test at $p = 0.05$. The amount of 200 versus 100 mg of the sample was corrected by dividing the results by 2. For statistical analyses the twice-reduced results were used.

Results

The average DNA concentrations obtained from artificially infected leaves varied from 0 with the AM to 18.26 μg with DDm protocol as measured in Biophotometer (Table 2).

Table 2. Quantification and purity of genomic DNA obtained by different extraction protocols

| Protocols | $\mu\text{g DNA}^1$ | $A_{260/280}^2$ | $A_{260/230}^3$ |
|---|---------------------|-------------------|-------------------|
| Aljanabi and Martinez 1997 | 0.0 \pm 0.0 a | 0.0 \pm 0.0 a | 0.0 \pm 0.0 a |
| DNeasy Plant Mini, Qiagen | 1.62 \pm 0.23 b | 1.65 \pm 0.07 c | 0.47 \pm 0.06 b |
| Doyle and Doyle 1987 | 9.21 \pm 2.26 c | 1.60 \pm 0.08 b | 1.40 \pm 0.11 c |
| Doyle and Doyle 1987 with modifications | 18.26 \pm 3.39 d | 1.83 \pm 0.05 d | 1.72 \pm 0.08 d |

¹calculated for 100 mg of plant tissue

²absorbance ratio $A_{260/280}$ was used for the evaluation of protein and phenol contaminants in the DNA

³absorbance ratio $A_{260/230}$ was used for the evaluation of carbohydrate contaminations in the DNA

The means in the columns followed by the different letters were significantly different ($p < 0.05$; one-way ANOVA, Tukey's test)

Standard deviation ($n = 30$ – three replicates \times two experiments \times five *Phytophthora* species)

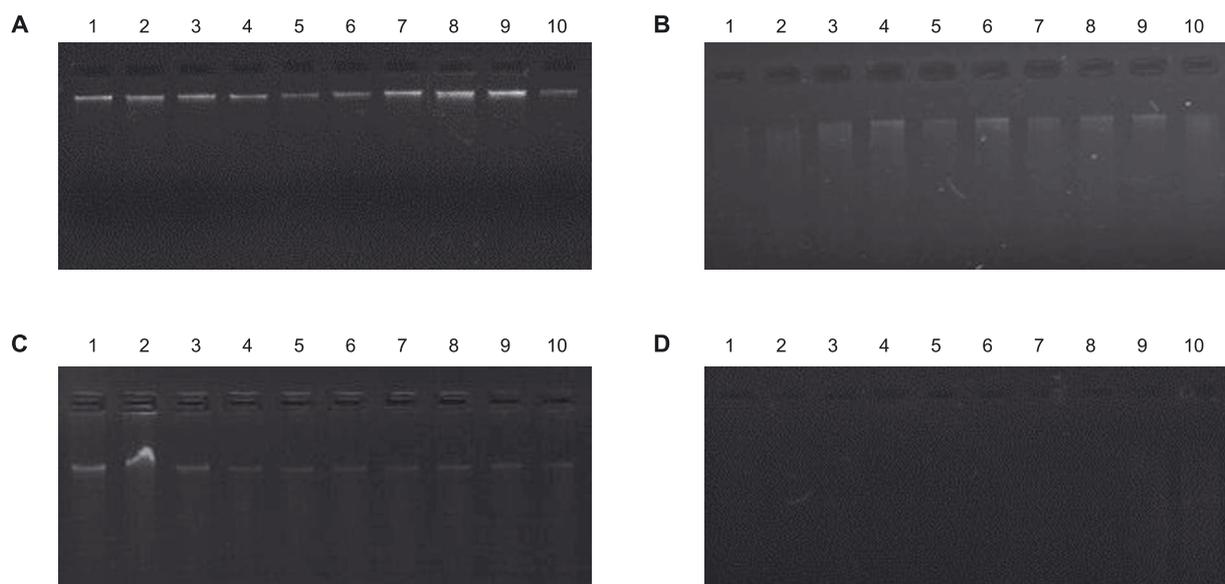


Fig. 1. Genomic DNA extracted from artificially infected rhododendron leaves, using: the Doyle and Doyle modified protocol (A), Doyle and Doyle protocol (B), DNeasy Plant Mini Kit (Qiagen) (C), Aljanabi and Martinez protocol (D). The electrophoresis on 1% (w/v) agarose gel; lanes 1, 2 – DNA from leaves infected with *P. cactorum*; 3, 4 – DNA from leaves infected with *P. cambivora*; 5, 6 – DNA from leaves infected with *P. cinnamomi*; 7, 8 – DNA from leaves infected with *P. citrophthora*; 9, 10 – DNA from leaves infected with *P. plurivora*

Table 3. Rate of positive pathogen DNA detection by real-time PCR with species-specific primers in samples from rhododendron leaves infected artificially with five *Phytophthora* species; in all combinations the number of analysed samples was 6

| Extraction protocols | <i>P. cactorum</i> ¹ | <i>P. cambivora</i> ¹ | <i>P. cinnamomi</i> ¹ | <i>P. citrophthora</i> ² | <i>P. plurivora</i> ³ |
|---|---------------------------------|----------------------------------|----------------------------------|-------------------------------------|----------------------------------|
| Aljanabi and Martinez 1997 | 0 | 0 | 0 | 0 | 0 |
| DNeasy Plant Mini, Qiagen | 5 | 4 | 4 | 4 | 4 |
| Doyle and Doyle 1987 | 4 | 4 | 4 | 3 | 4 |
| Doyle and Doyle 1987 with modifications | 6 | 6 | 6 | 6 | 6 |

¹primers designed by Trzewik and Nowak (unpublished), mentioned in Materials and Methods

²primers designed by Ippolito *et al.* 2004

³primers designed by Böhm *et al.* 1999

In addition, DDm protocol provided the best purity of DNA. Similar results were obtained on agarose gel, where DDm protocol also produced DNA of the highest quantity and quality (Fig. 1A). In this evaluation, DNA isolated by protocols Q and DD showed fragmentation (Figs. 1B, C), whilst DNA isolated by the AM protocol was not detected (Fig. 1D).

The PCR amplification with primers for plant DNA produced the expected amplicon (143 bp) from DNA obtained by the DDm, DD, and Q protocols, whilst no PCR product was obtained from putative DNA extract using the AM protocol (data not shown). The conventional PCR amplification with species-specific primers for *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citrophthora*, and *P. plurivora*, produced the expected amplicons of 97, 134, 133, 212, and 96 bp, respectively, but only when the DNA samples were derived using the DDm protocol. There was one exception in the sample isolated from *P. citrophthora* with the Q protocol (data not shown).

The amplification of DNA extracted from artificially infected rhododendron leaves using real-time PCR, generated the expected products for all five *Phytophthora* species but efficiency rate depended on extraction protocol (Table 3). No PCR product was obtained with a template of putative DNA derived from the AM protocol. Only DNA obtained using the DDm protocol consistently generated the expected products. A lower efficiency was obtained with DNA prepared by the DD and Q (Table 3). The use of standard curves based on known concentrations of DNA, enabled to quantify of pathogen DNA (data not shown).

The results showed that among the four methods used to extract DNA from rhododendron leaves artificially infected by five *Phytophthora* species, the modified Doyle and Doyle (1987) gave the best result. This method allows obtaining a PCR-ready DNA, which can be effective in detecting of *Phytophthora* DNA from infected rhododendron leaves using a conventional PCR amplification. In addition this method allows for the quantification of pathogen DNA using SYBR Green real-time PCR assays.

For obtaining a high quality and quantity of DNA suitable for use in PCR amplification we propose the following protocol.

Extraction protocol

1. Grind 200 mg of fresh weight infected rhododendron leaves in liquid nitrogen using a chilled mortar and pestle.

2. Scrape powder directly into a 1.5 ml tube and add 500 µl of CTAB isolation buffer (2% CTAB, 1.4 mM NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl).
3. Incubate sample at 60°C for 30 min with occasional gentle swirling.
4. Centrifuge at room temperature at 6,000 × g for 10 min, transfer the supernatant to a new tube and add a double volume of chloroform-isoamyl alcohol (24 : 1), mixing gently and thoroughly.
5. Centrifuge at 6,000 × g for 10 min at room temperature.
6. Remove aqueous phase, transfer it to a new tube, add 2/3 volumes of cold isopropanol, mix gently and keep at –20°C for 1 h to precipitate nucleic acids.
7. Centrifuge 8,000 × g at 4°C for 20 min and discard supernatant.
8. Add 70% ethanol to the pellet and mix gently by pipetting. Repeat this twice. Discard the supernatant and dry the pellet at room temperature.
9. Add 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) to dissolve the pellet.
10. Add RNase A to a final concentration of 10 µg · ml⁻¹ and incubate for 1 h at 37°C.
11. Store at –20°C for further use.

Discussion

The detection of pathogenic organisms in environmental samples is often based on markers generated by PCR using DNA extracted from environmental samples, e.g. from plants either with or without symptoms of disease. For detection, a reliable method for obtaining DNA of both sufficient quality and quantity is necessary. However, obtaining genomic DNA from diseased plant tissue is often difficult due to the presence of high amounts of secondary metabolites i.e. polyphenols. Secondary metabolites are produced by plants in response to infection and colonisation by a pathogen.

The DNA extraction method described by Aljanabi and Martinez (1997) was effective for tissues of wheat, barley, potato, beans, pear, and almond leaves as well as for the fresh tissue of fungi, insects, and shrimps. The authors showed that only 50–100 mg of young plant leaves or filtered and dried mycelium, was sufficient to obtain 500 to 800 ng of DNA from 1 mg of fresh tissue. Wiejacha *et al.* (2001), used this method successfully to isolate total DNA from the youngest leaves of lily obtained from *in*

in vitro cultures and from pure cultures of *Phytophthora* mycelium (Wiejacha *et al.* 2002). However, this method was useless in DNA isolation from infected rhododendron leaves, even with an additional purification with chloroform-isoamyl alcohol (24 : 1) (data not shown). Likewise, DNeasy Plant Mini Kit (Qiagen) cannot be recommended for this purpose, in spite of the fact that some authors have recommended the DNeasy Kit for the extraction of DNA from different plant specimens (Drábková *et al.* 2002; Hu and Vick 2003). The use of Qiagen kit for the extraction of DNA from diseased leaves, was unsatisfactory, because of the non-acceptable purity due to both proteins and carbohydrates contamination. The European and Mediterranean Plant Protection Organisation (EPPO) recommended DNeasy Plant Mini Kit (Qiagen) for the isolation of DNA from plant material to diagnose *Phytophthora ramorum*, but with the suggestion of an additional purification using the polyvinylpyrrolidone (PVPP) columns (Biorad) (OEPP/EPPO 2006). This procedure extends the time of isolation as well as increases the costs. Caprar *et al.* (2014) indicated that among three methods of DNA isolation from four species of *Rhododendron*, DNA extracted with DNeasy Plant Mini Kit (Qiagen) had a very low concentration when compared to the InnuSpeed Plant DNA Kit (Analytik Jena) and Isolate DNA Kits (Biolone). Moreover, Michiels *et al.* (2003) reported a low quality and quantity of DNA extracted from *Cichorium intybus* when using the DNeasy Plant Mini Kit.

The method described by Doyle and Doyle (1987) also did not show promising results in obtaining high quality DNA from infected rhododendron leaves. However, lowering the precipitation temperature to -20°C and shortening the precipitation time increased the yield and improved the purity of the obtained DNA. Our results confirmed the findings of Michiels *et al.* (2003), who stated that in the case of latex-containing Cichorioideae plants, the quantity and quality of isolated DNA depended on the temperature and duration of precipitation. Csaikl *et al.* (1998) reported that the extraction of DNA from *Rhododendron luteum* (silica dried and frozen) and *Quercus robur* (fresh material) using the Doyle and Doyle protocol, resulted in obtaining DNA of low quality and insufficient quantities. Csaikl *et al.* (1998) and Caprar *et al.* (2014) pointed out that extraction of DNA from plants such as rhododendron, oak, and conifers is difficult due to high amounts of different secondary metabolites. In this study, extraction of DNA was even more difficult possibly due to the additional phenolic compounds produced in the leaves as a reaction to pathogen attack and colonisation. The modifications of the original Doyle and Doyle protocol, especially those concerning the duration and temperature of precipitation, allowed us to obtain high quality and quantity DNA. Such DNA resulted in the high detection efficiency, both in conventional and real-time PCR amplifications, of the five *Phytophthora* species. In all the tested samples, pathogen DNA was detected. The rate of a positive DNA detection by real-time PCR was lower for DNA extracted using the DNeasy Plant Mini Kit (Qiagen) and the original Doyle and Doyle (1987) than when using the DDm method. The DNA extracted using the DNeasy Plant Mini Kit (Qiagen) was in a lower concentration and

had no optimal $A_{260/230}$ ratio compared with the DNA isolated when using the original Doyle and Doyle protocol. Yet, in the case of *P. cactorum* and *P. citrophthora*, the commercial kit gave a higher rate of positive DNA detection. Additionally, in conventional PCR amplifications, one sample from the extraction using the DNeasy Plant Mini Kit (Qiagen) gave a positive result in the amplification of the DNA of *P. citrophthora*. As has been proven here, the success of *Phytophthora* detection in rhododendron leaves depends on the DNA extraction procedure determining the quality, purity, and quantity of DNA. Thus, the extraction procedure affects the real-time PCR amplifications applied in diagnostics (Terry *et al.* 2002; Demeke and Jenkins 2010). It is important to note, that in conventional PCR also the crucial factor is the quality of the DNA template and the possible absence of reaction inhibitors such as polysaccharides and other secondary metabolites.

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