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PCR-detection of *Synchytrium endobioticum* (Schilb.) Perc.

PCR-Nachweis von *Synchytrium endobioticum* (Schilb.) Perc.

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Summary

The ITS-DNA region of *Synchytrium endobioticum* (Schilb.) Perc., the causal agent of potato wart disease, was used to generate specific PCR primers for molecular diagnosis of the disease. DNA was extracted from wart galls of all *S. endobioticum* pathotypes (1, 2, 6, 18) currently occurring and used for official testing purposes in Germany. Using the universal ITS primer # 4 and the *S. endobioticum*-specific primer Kbr1, a PCR fragment of 543 bp was obtained from all four fungal pathotypes. DNA for PCR-based diagnosis of the fungus could be extracted easily from summer sori, however, we did not succeed to extract amplifiable DNA from resting sori found in contaminated soils. To circumvent this problem, zoospores emerging from resting sori were used for DNA extraction and indirect detection. PCR also allowed to discriminate between weakly resistant and moderately susceptible responses of potato cultivars in addition to the routine visual inspection, since only alive summer sori of the weakly susceptible reacting potato cultivars released quantities of zoospores sufficient to be detected. No PCR signal was obtained from weakly or completely resistant potato cultivars.

Key words: *Synchytrium endobioticum;* ITS-DNA region; summer sori; resting sori; zoospores; resistant and susceptible potato cultivars

Zusammenfassung

Die ITS-DNA-Region von *Synchytrium endobioticum* (Schilb.) Perc., dem Erreger des Kartoffelkrebses, wurde verwendet, um spezifische Primer für PCR-basierte Diagnose zu entwickeln. DNA wurde aus Wucherungen aller vier *S. endobioticum* Pathotypen (1, 2, 6, 18) extrahiert, die zurzeit zur offiziellen Krebsprüfung bei Kartoffeln in Deutschland verwendet werden. Bei Verwendung des Universal-Primers ITS # 4 und des *S. endobioticum*-spezifischen Primers Kbr 1 wurde bei allen vier Krebspathotypen ein gleich großes Fragment von 543 bp mit der PCR amplifiziert. DNA zur PCR-Diagnose des Pilzes ließ sich problemlos aus Sommersori isolieren, allerdings konnte keine amplifizierbare DNA von Dauersori aus kontaminierten Böden extrahiert werden. Um dieses Problem zu lösen, wurden in einem indirekten Nachweis Zoosporen aus im Boden befindlichen Dauersori erfolgreich nachgewiesen. Die PCR eignete sich auch bei der visuellen Bonitierung zur Unterstützung einer Differenzierung von schwach resistenten und moderat anfälligen Reaktionen bei Kartoffelsorten, da nur von lebenden Sommersori bei den schwach anfällig reagierenden Kartoffelsorten genügend Zoosporen freigesetzt wurden, um nachgewiesen zu werden.

Stichwörter: *Synchytrium endobioticum;* ITS-DNA-Region; Sommersori; Dauersori; Zoosporen; resistente und anfällige Kartoffelsorten

1 Introduction

The genus *Synchytrium* is distributed world-wide, harbours obligately biotrophic plant pathogens, and is the most complex genus within the *Chitridiomycota*. *Synchytrium endobioticum* is the causal agent of potato wart disease which strictly depends on the living host (potato sprouts) for living and propagation and is thus an obligate biotroph. For the production and release of zoospores (biflagellated zygotes), free water is essential. Upon infection, potatoes develop galls. Successful infections, however, occur only at young sprouts of susceptible potato cultivars (Karling 1964).

After infection, *Synchytrium endobioticum* produces two different kinds of sori in the galls. Summer sori have a thin cell wall and form sporangia from where haploid zoospores are emerging and steady reinfection of the sprout tissue occurs. Resting sori (sometimes called "resting sporangia") are produced after zygotic infection (diploid stage) and form a thicker cell wall, enriched with a chitin layer. Resting sori are embedded deeper into the host tissue than the summer sori, and are designed for longer survival in the soil.

Importantly, resting sori can survive in the soil for more than 30 years, still being infective. When susceptible potatoes are grown in contaminated soils, an infection can occur during this period of time (Langerfeld 1984). Therefore, the potato wart disease is listed as a quarantine disease world-wide. Environmentally friendly and direct control methods of the potato wart are presently not available. However, growing resistant potato cultivars at wart-free growing areas helps to reduce the danger of spreading *S. endobioticum*.

In official evaluations of potato cultivars, problems usually arise when potato cultivars exhibiting weakly resistant or moderately susceptible defence reactions are used to be distinguished (Langerfeld and Stachewicz 1994). Meanwhile, new potato cultivars are available which have been bred for complete potato wart resistance.

Previously wart-contaminated fields can only be officially descheduled, when the contaminated soil has been surveyed microscopically for the lack of viable resting sori, in combination with a biotest by planting wart-susceptible potato cultivars (OEPP/EPPO 1999).

In order to detect either the resting sori in soils contaminated by *S. endobioticum* or to determine moderately susceptible reactions of the potato cultivars, a time-saving PCR detection procedure was developed by generating primers from the sequenced fungal ITS region. The non-coding internal transcribed spacers (ITS) regions of ribosomal (r) DNA are known to be highly variable. While the genes of this region code for the highly conserved ribosomal 18S, 5.8S and 28S sub-units, the ITS regions between these sub-units are less conserved and often show length heterogeneity (Klassen and Buchko 1990). The ITS regions lack a functional role (Nues et al. 1994), explaining why the high level of sequence variation within them can be tolerated. ITS regions have been sequenced as an attractive target for PCR-based diagnosis on the species level (White et al. 1990; Mazzola et al. 1996; Bunting et al. 1996; Lovic et al. 1995; Tisserat et al. 1994; Tooley et al. 1997).

We report here on the development of ITS-derived primers and a PCR application for detection of *S. endobioticum* in plant tissue. For a survey of plants and soils potentially contaminated with potato wart, zoospores released from sori have been found useful for PCR-based diagnosis of the pathogen.

2 Material and methods

2.1 Production of summer sori and resting sori in soils for experimental purpose

The procedure of generating sori in potato sprout tissue (galls) has been described by Langerfeld (1984). In emerging galls normally both summer and resting sori are produced simultaneously. Therefore, to enrich resting sori, old galls (1 month) were mixed with quartz sand and composted for a period of 6–12 months. The cell walls of the resting sori are more persistent than those of summer sori, and after several months, only resting sori have survived. The sori-enriched compost was used as soil sample containing resting sori.

2.2 Extraction of DNA from potato wart galls (summer and resting sori)

Young sprouts of susceptible potato tubers (cvs. 'Erstling' or 'Tomensa') were inoculated with zoospores from summer sori, generated from pre-infected and macerated tuber sprouts (galls). For all experiments, the most aggressive wart pathotype 18 was used. Since *S. endobioticum* is an obligate parasite, a pre-cultivation of this fungus on living sprouts was necessary. After inoculation of the young sprouts, the tubers were put into soil and incubated at 16 °C for 1 month, moistened occasionally to assure a steady high soil moisture. Freshly developed galls of infected sprouts were harvested and used for further work. DNA was extracted from galls using the CTAB method (Day and Shattock 1997).

2.3 Generation of *Synchytrium endobioticum*-specific primers

Primers with conserved rDNA sequences (White et al. 1990) were used to amplify the ITS region of ribosomal (r) DNA of *S. endobioticum*. ITS1 (5' TCCGTAGGTGAACCTGCGG 3', annealing at the 5' end of the 18S gene) and ITS 4 (5' TCCTCCGCTTATTGATATGC 3', annealing at the 3' end of the 28S gene) were used as forward and reverse primers allowing to amplify the two ITS1 and ITS2 regions. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One µl (20 ng/µl) of the PCR product was directly ligated into the plasmid vector pCR^R 2.1-TOPO (TOPO TA cloning kit, Invitrogen, Karlsruhe, Germany) and transformed into *E. coli* TOPO 10 cells according to the manufacturer's instructions. Screening of transformants was carried out on X-Gal agar plates. White colonies were transferred into 20 µl of a PCR reaction mix, containing 2.0 µl of 10 × PCR buffer (1.5 mmol/l $MgCl_2$, 200 µmol/l nucleotide mix, 1 pmol/l M13 universal, 1 pmol/l M13 reverse and 1 U *Taq* polymerase) (Amersham, München, Germany). The thermocycler was programmed as follows: After 5 min heating at 95 °C, DNA amplification was carried out in 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 55 °C and 90 s extension at 72 °C). Finally, a 10 min incubation at 72 °C and cooling to 4 °C were performed. The PCR products were either analyzed immediately or stored at –20 °C.

Transformants to be sequenced were inoculated into 10 ml of liquid LB_{amp}-medium. Bacteria were grown at 37 °C at 200 rpm overnight, harvested and transferred into 1.5-ml Eppendorf tubes. To isolate and purify bacterial plasmids, the pellets were processed with a QIA-prep spin miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid concentrations were adjusted to1 µg/µl. The purified plasmids could be directly sequenced or stored at –20 °C. Sequencing was performed using an automated DNA sequencer (ABI 310, Perkin Elmer, Weiterstadt, Germany). Suitable primers were derived from the sequences of the *S. endobioticum* ITS-region.

2.4 PCR conditions

PCR experiments using the universal primers ITS 1 and ITS 4 for proving as well as for the developed specific *S. endobioticum* primer Kbr1 for detection were as follows: Denaturation at 96 °C for 3 min, followed by 45 cycles at 96 °C for 45 s, annealing at 56 °C for 45 s, and elongation at 72 °C for 1 min. Finally, a 5 min incubation step at 72 °C was performed. The amplified fragments were analyzed by agarose gel electrophoresis on 1 % [w/v] gels.

2.5 Micro-organisms for testing the specificity of the primers

For testing the *S. endobioticum* specific primer pair, DNA of the following micro-organisms were used applying the PCR conditions given above:
Colletotrichum coccoides (Wallr.) Hughes
Fusarium oxysporum (Schlecht) Wo.
Plasmodiophora (Olpidium) brassicae Woron
Phoma exigua Desm.
Phytophthora infestans (Mont.) de Bary
Rhizoctonia solani Kühn
Spongospora subterranea (Wallr.) Lagerh.
Synchytrium endobioticum pathotypes 1, 2, 6, 18 (Schilb.) Perc.

Clavibacter michiganense ssp. *sepedonicus* (Smith) Davis
Erwinia carotovora atroseptica (Van Hall) Dye
Ralstonia solanacearum (Smith) Yabuuchi.

All fungi were obtained from the Federal Centre of Biology and Forestry strain collection.

2.6 Indirect detection of the existence of summer sori in plant tissue or resting sori in soils via zoospores

For indirect detection of active summer sori in plant galls, 5 g of plant tissue were used and submerged in distilled water for different time intervals to allow the release of the zoospores into the water. The supernatant was decanted at different time intervals (2 h, 4 h, 6 h and 24 h) after submerging in fresh water. All supernatants were directly adjusted to a final concentration of 70 % ethanol. To allow also resting sori to release their zoospores, a 6-month-old soil sample was used and the supernatant was decanted after 4 h incubation time as described above.

To collect the precipitated zoospores, the tubes were centrifuged at 10.000 × *g* for 10 min at 4 °C and the supernatant was decanted. After drying, the pellet was resuspended in CTAB buffer and the DNA was extracted according to Day and Shattock (1997).

3 Results

3.1 Specificity of the primers

A 15mer primer of the ITS1 region (Kbr1, 5' ATA CCC TTC CAA CAC 3') was chosen as forward primer, and the universal ITS 4 primer, derived from the conserved 26S rDNA region, served as the reverse primer. These primers allowed to amplify a DNA fragment of 543 bp, when *S. endobioticum* DNA was used. The specificity of the chosen primer pair for *S. endobioticum* was demonstrated with all four major pathotypes (1, 2, 6, 18; Fig. 1) occurring in Germany. Also with *S. endobioticum*-infected potato tissue as source of DNA, the PCR fragment of 543 bp was amplified. No PCR fragment was obtained with the other tested potato-pathogenic micro-organisms or with DNA from uninfected potatoes. Even the closely related Cytridiomycota *Olpidium brassicae* (host plant: cauliflower) and *Spongospora subterranea* (host plant: potato) did not show any cross-reactions, indicating the high specificity and applicability of the two primers Kbr 1 and ITS 4 under the PCR conditions chosen (Fig. 1). *Spongospora subterranea* was chosen because its life cycle is similar to that of *S. endobioticum* and, therefore, the symptoms can be confused in their early stages of infection. Other micro-organisms listed in the material section that exhibited no cross reactions are not shown in Figure 1.

3.2 Extraction of DNA from summer sori in plant galls and from resting sori in the soil

The CTAB method was used to extract DNA from plant tissue containing summer sori of *S. endobioticum.* DNA extraction from resting sori was not successful when applying the same extraction procedures as for the summer sori. Therefore, the extraction procedure has been modified using zoospores released from sori.

3.3 Extraction of DNA from released zoospores

Zoospores released from summer sori allowed DNA extraction by using the CTAB method. There were no PCR signals when extractions were made after incubating galls in water for 2 h. However, when the galls were incubated for a minimum of 4 h, extraction of DNA from zoospores was successful (Fig. 2). At all later sampling times, there were also zoospores detected via PCR. As a control, the unspecific primers ITS 1 and ITS 4 (generating a 650 bp DNA fragment) were used to prove that enough DNA was present for performing a PCR (data not shown).

To compare PCR-based diagnosis with zoospores formed on weakly resistant and partly susceptible potato cultivars, sprouts of potato lines previously visually determined to be moderately susceptible and weakly resistant were washed and incubated in water for 4 h to release the zoospores. Since the summer

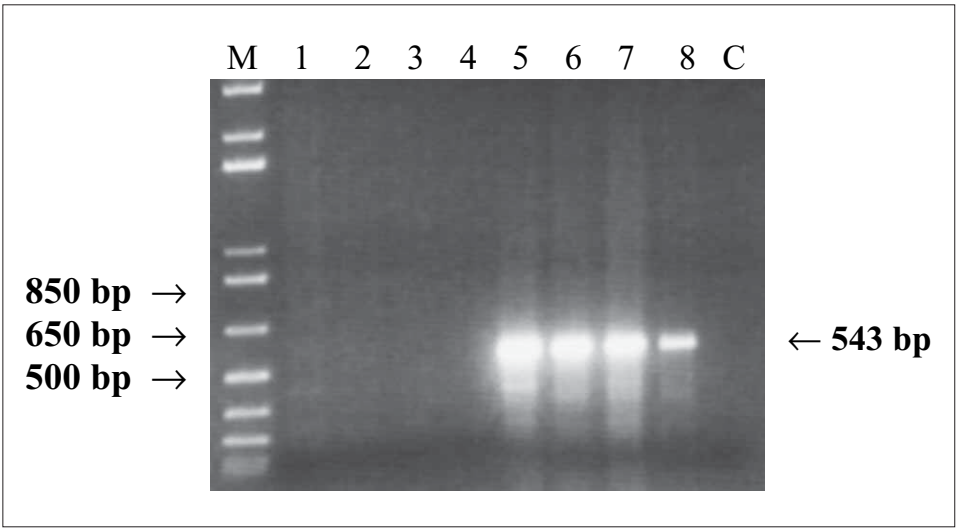

Fig. 1. Specificity of the primer combination Kbr1 and ITS 4. Only the tested pathotypes of *Synchytrium endobioticum* (pathotypes 1, 2, 6 and 18, lanes 5 to 8) yielded a PCR fragment. Lanes 1 to 2 represent the closely related fungi *Plasmodiophora brassicae* (1), *Spongospora subterranea* (2). Lane 3 shows *Phytophthora infestans.* Lane 4 represents healthy sprouts (potato cv. 'Erstling'). M is the DNA length standard and C the water control. The size of the amplified fragment is 543 bp.

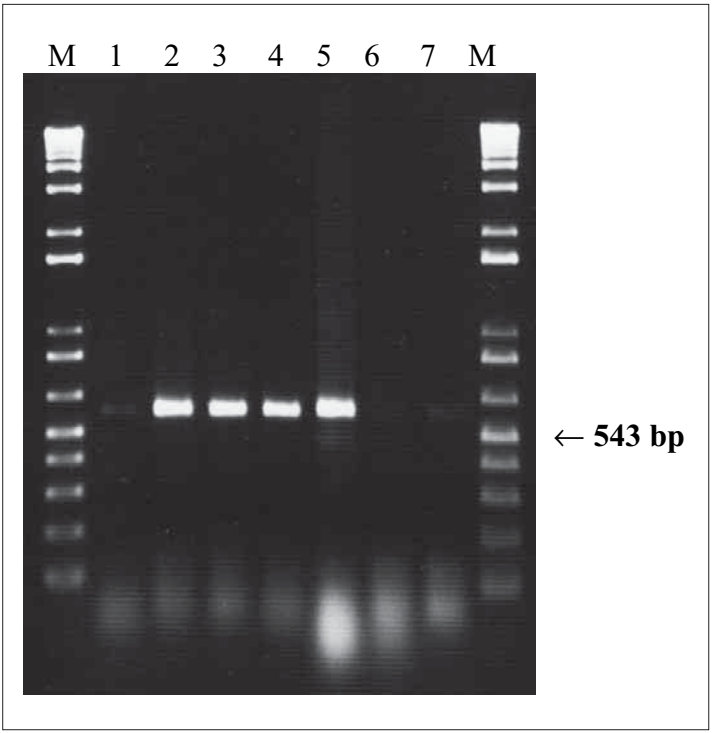

Fig. 2. Detection of zoospores released from wart galls (potato cv. 'Tomensa', *Synchytrium endobioticum* pathotype 4) into the water supernatant by the PCR and the primers Kbr1 and ITS 4 after different incubation periods. Lane 1 supernatant after 2 h, lane 2 after 4 h, lane 3 after 6 h, lane 4 after 24 h of incubation. Lane 5 represents the DNA extraction of galls (not sporulated sori), lane 6 represents healthy sprouts of the same potato cv. 'Tomensa' where the galls were obtained. Lane 7 is the water control and M represents the DNA length marker. The size of the amplified fragment is 543 bp.

sori release sufficient amounts of zoospores in highly susceptible cultivars at 4 h incubation, this point of time was also tested for moderately susceptible sprouts. After precipitating the complete water supernatant of each individual sprout in ethanol, DNA extraction was performed. Only in moderately susceptible cultivars, zoospores were released into the water, and ITS-DNA fragment could be amplified from the extracted zoospores by PCR. In weakly or strongly resistant cultivars, no PCR signal was obtained (Fig. 3). As a control, DNA from healthy sprouts was used. These results show that moderately susceptible and the weakly resistant cultivars can be distinguished applying the PCR protocol described (Fig. 3). An unspecific PCR fragment of ca. 700 bp did not interfere with the amplification of the 543 bp DNA fragment.

Using the supernatant of a soil sample incubated for 6 months, zoospores released from resting sori could be detected 11 days after moistening the soil. The extracted DNA allowed the amplification of the 543 bp DNA fragment (Fig. 4). This is in accordance with Hampson (1986), who reported a peaking of a release of zoospores about 11 days after submerging a 4-year-old compost containing resting sori. To demonstrate the ability of the released zoospores to penetrate the soil layers, small parts

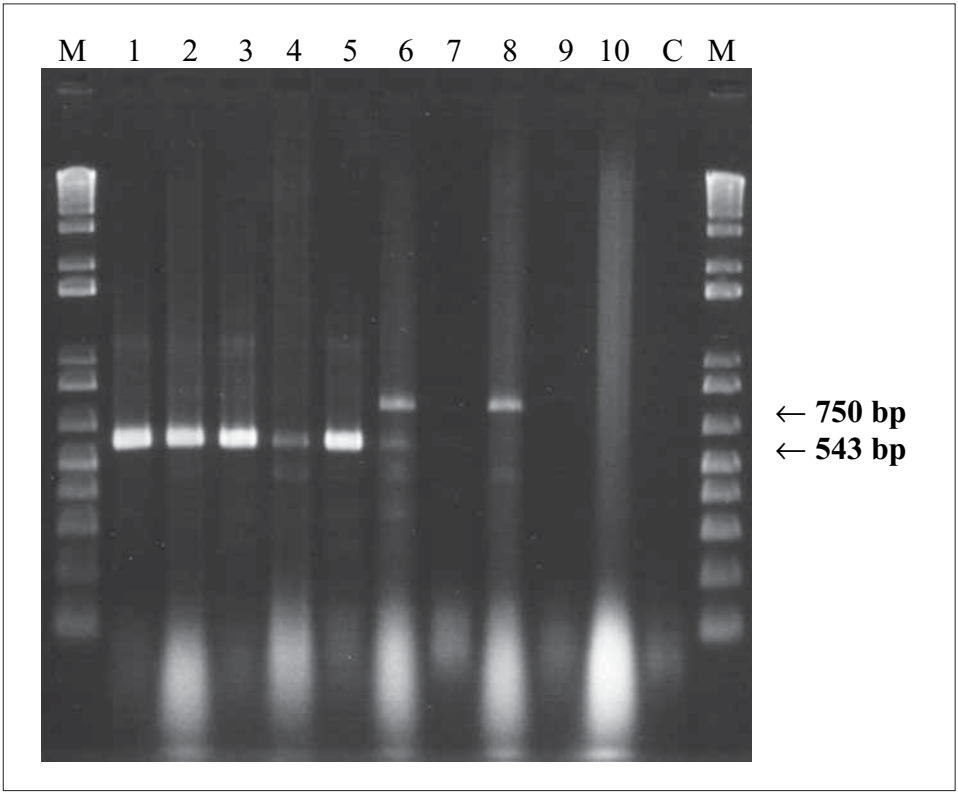

Fig. 3. PCR amplification of the 543 bp fragment from DNA extracted from moderately susceptible and weakly resistant reacting potato cultivars after inoculation with *Synchytrium endobioticum*. As a positive control, galls of a highly susceptible potato cultivar were used as a source of living zoospores (lanes 1 and 3). From a moderately susceptible potato cultivar, previously established by its symptoms, zoospores were released and detected with the specific primers Kbr1 and ITS 4 (lane 5). Lane 6 shows a faint PCR signal of 543 bp in size indicating the existence of intact summer sori. No PCR signal was obtained from the DNA extracted from a weakly (lane 7) or a highly resistant (lane 9) cultivar, indicating that no viable zoospores were present. Lanes 2, 4, 6, 8, and 10 represent the sprouts of the corresponding zoospore extractions remaining after zoospores were released. M = molecular weight standard (Invitrogen), lane 11 water control. The 750 bp fragment is unspecifically.

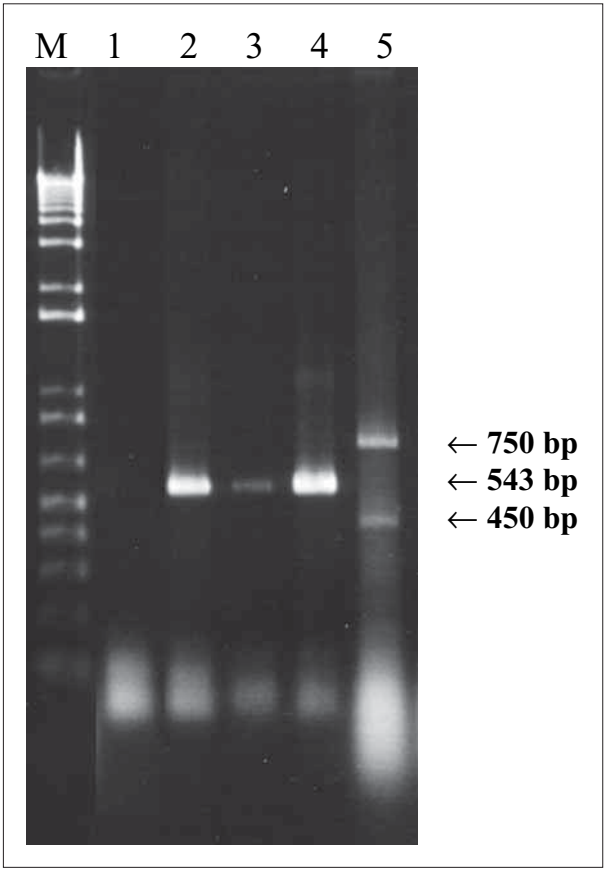

Fig. 4. Indirect detection of viable wart resting sori by PCR amplification of its released zoospores in artificially contaminated soil. Soil containing resting sori was maintained for 6 months by moistening and drying for several times during this period. Lane 1 represents the blank control and lane 2 the positive control of zoospores released from sommer sori. Lane 3 shows zoospores released from the contaminated soil sample after 11 days of moistening. Lane 4 is the positive zoospore control and lane 5 the supernatant of the contaminated soil sample after 24 h. Only in the 11-day-old supernatant, viable zoospores of resting sori with the fragment size of 543 bp were detected. Non-specific fragments of 750 bp and 450 bp (lane 5) did not interfere with the amplification of the *Synchytrium endobioticum*-specific 543 bp fragment.

of a wart gall were mixed with soil. Zoospores released from summer sori could be detected in the supernatant after 4 h of incubation, indicating that these spores were able to penetrate the soil.

4 Discussion

The PCR technique has been proven to be suitable for molecular diagnosis of potato-pathogenic fungi and for discriminating potato-pathogenic fungi and bacteria (Niepold and Rudolph 2001).

In addition to summer sori, newly formed galls contain an almost equal amount of resting sori, distinguishable only by their darker brownish colour visible under the microscope. As sufficient amounts of DNA, allowing the amplification of the *S. endobioticum* specific 543 bp ITS fragment, were not obtained from resting sori, we assume that the PCR-amplifiable DNA from infected plant material was from summer sori.

So far, in our hands, resting sori can only be detected indirectly based on the DNA extracted from released zoospores. Since the *S. endobioticum* zoospores are only of ca. 4 µm, they cannot be visualized easily under the microscope. In addition, the period of germination of the resting sori cannot be predicted, since resting sori release zoospores over an extended time interval. However, our PCR results show that the *S. endobioticum*-specific DNA fragment can be reliably detected using zoospores emerging from resting sori. After passing the dormancy and ripening period (the zoospores are released only in the infective stage), germination of the resting sori is dependent on soil temperature and moisture

(see review by Langerfeld 1984). In the experiment with the 6-month-old soil sample, germination might have been favoured by dry storage over several weeks, prior to re-moistening. Indirect detection of resting sori in soil via released zoospores might open the possibility to detect resting sori in naturally contaminated soils.

So far, no PCR detection procedures are available for monitoring hatched zoospores in potato fields, which had been contaminated with *S. endobioticum*. Due to the current quarantine regulations (OEPP/EPPO 1999), fields contaminated with potato wart are omitted to plant and grow wart-susceptible or resistant potato cultivars. Restrictions can be abolished after 20 years provided it can be proved that there are less than five living resting sori per g of soil. So far, the differentiation between alive and dead resting sori in soils can be facilitated by using lactophenol blue staining (Stachewicz 2004). Since germination of resting sori occurs over long periods of time (Langerfeld 1984), establishment of time-saving PCR-based detection methods would be beneficial. A first step in this direction has been made in this study by PCR-based detection of zoospores released from soil directly contaminated with resting sori. The detection of resting sori in soils is of special importance since in Germany a significant number of fields exists which had been contaminated with *S. endobioticum* years ago. A screening of these locations using a modified DNA extraction or detection procedure of released zoospores via PCR would be helpful to survey these locations.

In addition, it would be helpful to differentiate the major four wart pathotypes in Germany via PCR, since there are problems in obtaining the susceptible potato cv. 'Saphir', differentiating exclusively *S. endobioticum* pathotype 2. Especially this cultivar is no longer registered in the official German Breeding List. Having specific primers available for differentiating these four wart pathotypes would not only save time when identifying the pathotype on a newly infected field (a classical test would take longer than one vegetation period), but the presently high number of test potato cultivars required could be reduced. In order to obtain pure *S. endobioticum* DNA, it would be appropriate to work with zoospores. However, using the method described in this paper, i. e., extraction of DNA from precipitated zoospores could generate pure fungal DNA for the purpose of DNA-based discrimination between races.

Using zoospores released from weakly resistant and moderately susceptible potato cultivars turned out to be helpful in discriminating these two defence reactions. Since infectious zoospores are released only from moderately susceptible potatoes, which allow slow growth of potato warts, PCR reaction allows to detect the wart fungus under these conditions. Resistant cultivars, however, do not release zoospores and no ITS-DNA can be amplified. This approach could support the visual differentiation of moderately susceptible and weakly resistant reactions and might be applied for difficult evaluation cases in routine official testings performed on a yearly basis.

Using the ITS sequences has become a common procedure to generate specific primers for the detection of many plant pathogenic fungi. Especially the DNA region between the conserved 5.8S and 28S rDNA genes is known to be highly variable and can be used for generating specific primers, which even allow to distinguish closely related fungi. Applying this strategy, we were able to specifically detect *S. endobioticum* and to discriminate it from other potato pathogens. The primers used may be suitable for routine applications.

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