RPA-PCR couple: an approach to expedite plant diagnostics and overcome PCR inhibitors

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ABSTRACT

DNA extraction can be lengthy and sometimes ends up with amplification inhibitors. We present the potential of recombinase polymerase amplification (RPA) to replace plant DNA extraction. In our rapid 'RPA-PCR couple' concept, RPA is tuned to slower reaction kinetics to promote amplification of long targets. RPA primers amplify target and some flanking regions directly from simple plant macerates. Then PCR primers exponentially amplify the target directly from the RPA reaction. We present the coupling of RPA with conventional, TaqMan and SYBR Green PCR assays. We applied the concept to strawberry *Phytophthora* pathogens and the *Phytophthora* identification marker *atp9-nad9*. We found RPA-PCR couple specific, sensitive and reliable. The approach may also benefit other difficult samples such as food, feces and ancient samples.

METHOD SUMMARY

Crudely macerate plant tissue in water for a few minutes and transfer supernatant in a recombinase polymerase amplification (RPA) reaction. Incubate RPA reaction for 20 min at 39°C and thereafter directly utilize RPA reaction to exponentially amplify target through standard PCR.

GRAPHICAL ABSTRACT

KEYWORDS:

atp9-nad9 • PCR inhibitors • *Phytophthora* • *Phytophthora cactorum* • *Phytophthora fragariae* • plant DNA extraction • recombinase polymerase amplification

A standard method for diagnosis of plant diseases is PCR. Traditionally, PCR requires prior extraction of DNA from infected tissue. Plant DNA extraction not only is a time-consuming process but also sometimes co-extracts PCR inhibitors. To identify the presence of inhibitors in DNA samples, amplification of internal controls are often measured to ensure amplification efficiency is not affected. Although the internal controls spot false negatives, they also can increase cost and reduce amplification of the target amplicon when it is present in low concentrations. To combat reductions in amplification efficiency due to inhibitors, DNA extracts can be further purified, diluted or have the DNA extraction repeated. Although DNA dilution can reduce concentration of inhibitors, it also impacts the sensitivity of the assay by reducing the number of target DNA copies. For these reasons, DNA extraction from infected plant material may be a bottleneck in diagnosis of diseases [1].

An alternative DNA amplification method – recombinase polymerase amplification (RPA) – has been developed that addresses many of these limitations of DNA extraction [2]. RPA is an isothermal amplification where primers of 30–35 nucleotides length bind with recombinase enzyme, and these nucleoprotein complexes scan DNA to pair up with a homologous region. This results in D-loop structure formation, where recombinase dissociates from primers and polymerase extend the primers. Bidirectional extension of primers by polymerase with strand displacement activity leads to exponential amplification of the target nucleic acid. Commercially available formulations for RPA assays can be obtained from TwistDx Inc. (M Maidenhead, UK) the patent holder of the technology, and Agdia (Soisy-sur-Seine,

France). TwistDx Inc., in addition to supplying reagents for RPA technology, aims at supporting the deployment of RPA technology in various fields including life sciences, diagnostics, microfluidics/lab-on-chip applications, agriculture and defense.

RPA is reported to be tolerant of PCR inhibitors [3] and can amplify DNA directly from crudely macerated plant tissue. Crude macerates have been prepared with different buffers. Ready-to-use buffers from Agdia such as GEB [4], GEB 2 [5,6], GEB 4 [7] and AMP1 [8,9] have been utilized. Similarly, NaOH [10,11], standard ELISA grinding buffer [5] and TE buffer [12] have also been used for maceration. Besides maceration, extraction buffers have been employed to release nucleic acid from leaf disks [13,14]. Moreover, squeezed sap has been directly utilized for RPA [15].

In addition to tolerances to inhibitors, RPA is rapid and sensitive, and it requires simple instrumentation. For these reasons, RPA has gained attention from plant pathologists, but users may encounter some challenges when designing amplification primers with the necessary specificity. Although many researchers have reported satisfactory specificity of their RPA assays, some have also clued about the nonspecificity issue [16–18]. RPA assays have also been reported tolerating five mismatches [19], seven mismatches [20] and even nine mismatches [21] with target sites. The study reporting tolerance up to seven mismatches originally investigated the effect of different mismatches on RPA specificity and recommended selection of a target marker with at least 36% sequence difference with the closely related species while designing RPA assays [20]. RPA primers, in contrast to PCR, do not depend on melting temperatures to anneal with target sites. Amplification at low temperature ranging from 25 to 42°C may enable RPA primers to make dimers and even bind to matching nontarget sites, thereby generating nonspecific by-products that can be visible on an agarose gel. Moreover, a nonspecific by-product longer than 300 base pairs (bp) cannot be removed through a routine PCR purification kit, and in such a scenario, gel extraction of the target amplicon would be necessary. In an RPA reaction, the nonspecific products also compete with the target amplification for the limited energy available, and therefore targets are kept short, ideally of 100–200 bp. Longer amplicons require lengthier extension time, and thereby target amplification may be outcompeted by the background or nonspecific amplification. For longer amplicons, TwistDx manuals recommend slower reaction kinetics through lowering the incubation temperature and concentration of magnesium acetate and primers. In our experience, lowering the concentration of magnesium acetate and primers is beneficial for longer amplicons, but detection of a low amount of longer targeted amplicons (~500 bp) is still not possible. It should be noted that background amplification is less problematic for the real-time RPA chemistries designed for inclusion of a probe-based detection system, such as the TwistAmp exo kit.

Although both PCR and RPA have their disadvantages, a unique combination of them can result in improved detection capabilities. Coupling slower reaction kinetics RPA with PCR can exclude the need for time-consuming and problematic DNA extractions. The method is also expected to overcome the RPA shortcomings of nonspecificity (background amplification), short amplicon size (100–200 bp optimal) and poor resolution for quantification. In the coupled amplification, RPA slowly multiplies the target and some flanking region in the first-round amplification directly from plant macerate. Then in the second round of amplification, PCR exponentially amplifies the target region directly from the RPA reaction. Slower RPA reaction kinetics promote generation of longer amplicons. Similarly, the slower rate of RPA amplification generates less background noise and target amplicons that can be directly utilized for PCR (without dilution). The coupled nested PCR are run with normal chemistry.

In this proof-of-concept study, we will validate RPA-PCR couple as a simple, rapid, sensitive and specific approach to directly amplify target DNA from plant crude macerates. The study will target *Phytophthora* intergenic mitochondrial marker *atp9-nad9*, and the concept will be applied to two *Phytophthora* species causing devastating damage to strawberry. The *atp9-nad9* marker is a gene order that is highly conserved in the genus *Phytophthora* but different in the related genus *Pythium*, Eumycotan fungi and plants [5,22]. The pathogens will include *Phytophthora fragariae*, a causative agent of red stele of strawberry roots, and *Phytophthora cactorum*, an agent of strawberry crown rot. Our first round of RPA assay will be *Phytophthora* genus-specific, and it will amplify the intergenic spacer between the *atp9* and *nad9* genes at slower reaction kinetics. Then the RPA reaction will be coupled either with species-specific real-time PCR assays or genus-specific conventional PCR aimed at Sanger sequencing. The species-specific assays include SYBR Green and TaqMan assays for *P. fragariae* and *P. cactorum*, and their specificity originates from the intergenic spacer between *atp9* and *nad9*.

Materials & methods

RPA primers screening

Supplemental file 2 of Miles *et al.* [5] provided 274 partial *atp9-nad9* sequences of approximately 100 *Phytophthora* species. The sequences were aligned by Geneious 8.1.9 (Geneious, Auckland, New Zealand) to identify the *atp9* gene, intergenic spacer and *nad9* gene sequences. Ten forward RPA primers were picked from the *atp9* region and ten reverse primers from the *nad9* region. Primers were screened to find the optimal RPA primer pair generating the maximum amount of target amplicons. DNA extracted from *P. fragariae* hyphae was utilized as template, and the DNA volume was kept at 1 µl per reaction. DNA extraction from *Phytophthora* hyphae was described previously [23]. The concentration of the hyphal DNA was 100 pg/µl. All DNA concentrations in this study were quantified through Qubit 2.0 Fluorometer, utilizing the Qubit dsDNA HS Assay kit (Thermo Scientific, USA).

In primers screening, RPA reactions were run at normal reaction kinetics utilizing the TwistAmp Liquid Basic kit (TwistDx Inc.) and following manufacturer recommendations. RPA incubation was done at 39°C for 25 min with no agitation and using a PTC-0200 DNA Engine Cycler (BIO-RAD, USA) with no lid heating. Following incubation, reactions were purified with a GeneJET PCR Purification kit

Table 1. Primers and probe sequences and role.

Primer/probe	Sequence	Assay and specificity
ATP9F-M9	CCTTCTTTACAACAAGAATTAATGAGAAC	RPA forward primer, genus specific (Miles <i>et al.</i> [5] forward primer Atp9-F with 6 bp truncated from the 3' end)
Phy_Gen_R20	CGAATACTTAATAAATTATAAATAATTTCA	RPA reverse primer, genus specific
Phy_Gen_SeqF4	ACAACAAGAATTAATGAGAACTGC	Forward primer in all PCRs, genus specific (the first 16 bp are from the 3' end of PhyG_ATP9_2FTail of Bilodeau <i>et al.</i> [22])
Phy_Frag_PCRR4	TTTTTGTGTGAAAAGAGCTA	Reverse PCR primer in <i>Phytophthora fragariae</i> SYBR Green PCR assay, species specific
Phy_Cac_PCRR2	AATAGTGTTAATAATTATATAACATG	Reverse PCR primer in <i>Phytophthora cactorum</i> SYBR Green PCR assay, species specific (designed from the <i>P. cactorum</i> TaqMan probe described in Bilodeau <i>et al.</i> [22])
Phy_Gen_SeqR8	GGTAAATTTGTAATAAATATTGACT	Reverse PCR primer in conventional and TaqMan PCRs, genus specific
PfraVf_nad9sp_TaqMan2	/56-FAM/ATC TCG TAA /ZEN/TAG ATA TAT ATG TAT ATT TAA TAC GT/3IABkFQ/	Probe in <i>P. fragariae</i> TaqMan PCR assay, species specific [22]
Pcact_nad9sp_probe2	/56-FAM/TTA CAT GTT /ZEN/ATA TAA TTA TTA ACA CTA TTT ATA AAA /3IABkFQ/	Probe in <i>P. cactorum</i> TaqMan PCR assay, species specific [22]

RPA: Recombinase polymerase amplification.

Figure 1. Location of primers in recombinase polymerase amplification, SYBR Green PCR, TaqMan PCR and conventional PCR assay.

(Thermo Scientific) and results were examined by the end point determination method of gel electrophoresis. The forward RPA primer 'ATP9F-M9' and reverse RPA primer 'Phy_Gen_R20' (Table 1) were found to be the optimum RPA primer pair (data not shown).

Real-time SYBR Green PCR assays

All PCR reactions in this study were performed with 500-nM primer concentration and an amplification volume of 25 μ l. PCR primers were designed through Geneious 8.1.9, where default GC% and temperature of melting (T_m) were often lowered. All quantitative PCR amplifications were run with the Mx3000P QPCR System (Agilent, Waldbronn, Germany). All primers and probes were ordered from Integrated DNA Technologies Inc. (Leuven, Belgium). Primers and probe sequences are provided in Table 1, with their locations in the *atp9-nad9* region presented in Figure 1.

In species-specific SYBR Green PCR assays, reverse primers were designed from the intergenic spacer between the *atp9* and *nad9* genes, and forward primers from the genus conserved region of *atp9*. In the *P. fragariae* assay, the forward primer 'Phy_Gen_SeqF4' and reverse primer 'Phy_Frag_PCRR4' were utilized. The amplification program included 10-min predenaturation at 95°C, and 40 amplification cycles, where each cycle has 10-s denaturation at 95°C, 20-s annealing at 58°C and extension of 20 s at 67°C. The *P. cactorum* assay was done with the forward primer 'Phy_Gen_SeqF4' and reverse primer 'Phy_Cac_PCRR2.' Amplification conditions were similar to *P. fragariae* with the exception of a 55°C annealing temperature and extension of 30 s at 60°C. All SYBR Green assays were completed with LightCycler® 480 SYBR Green I Master (Roche, Rotkreuz, Switzerland). Melt curve analysis was always included in SYBR Green amplifications.

Real-time TaqMan assays

Bilodeau *et al.* [22] designed and validated species-specific TaqMan assays for several *Phytophthora* species including those used in this experimentation. In these assays, probes were placed in the intergenic spacer between the *atp9* and *nad9* genes, whereas forward and reverse primers were designed from the flanking genus conserved regions. The probes for *P. fragariae* 'PfraVf_nad9sp_TaqMan2' and for *P. cactorum* 'Pcact_nad9sp_probe2' were ordered as ZEN double-quenched probes to improve signal to background discrimination. The forward primer 'PhyG_ATP9_2FTail' and reverse primer 'PhyG-R6_Tail' were replaced with forward primer 'Phy_Gen.SeqF4' and reverse primer 'Phy_Gen.SeqR8', as the latter improved sensitivities. In the species-specific TaqMan PCR assays, probe concentration was kept at 100 nM, and Luminaris Probe qPCR Master Mix (Thermo Scientific) was used. The PCR program for the *P. fragariae* assay started with a 2-min incubation at 50°C to let Uracil-DNA glycosylase (UDG) remove possible carryover contamination. Then a 10-min incubation at 95°C to activate Hot Start Taq DNA polymerase followed by 45 amplification cycles. Each cycle consisted of a denaturation step of 15 s at 95°C and an annealing/ extension step of 90 s at 57°C. The amplification program for the *P. cactorum* assay was the same but with an annealing/extension step at 55°C.

Conventional PCR for sequencing

Conventional PCR amplification of the *Phytophthora* genus-specific amplicon was done to generate a template for Sanger sequencing. The amplification utilized the previously mentioned 'Phy_Gen.SeqF4' and 'Phy_Gen.SeqR8' primers. LightCycler® 480 SYBR Green I Master was utilized as the master mix due to its exceptional capability to amplify even after 50 cycles. The fluorescence signals were not considered; rather, gel electrophoresis was utilized to observe amplification of the target approximately 300 bp bands. The amplification program started with a 10-min preincubation at 95°C and followed 50 amplification cycles of 10 s at 95°C, 20 s at 55°C and 30 s at 65°C. PCR reactions with visible target bands were purified with GeneJET PCR Purification and sent for Sanger sequencing to GATC Biotech (Konstanz, Germany).

Coupling RPA with PCR

For the RPA-PCR couple method, the TwistAmp Liquid Basic kit was used to prepare an RPA reaction mix for amplifying a target of approximately 500 bp under conditions of slower reaction kinetics. A premaster mix was prepared in a 1.5-ml tube. The premaster mix 1X recipe included 25 µl of 2× reaction buffer, 2 µl of 10× Basic E-Mix, 1 µl of 40 mM dNTPs (total), 1.5 µl of forward primer 'ATP9F-M9' (10 µM), 1.5 µl of reverse primer 'Phy_Gen.R20' (10 µM) and 15.21 µl of water. The premaster mix was vortexed and spun briefly, and the 20× Core Reaction Mix was added to the lid and the tube was inverted ten-times to prepare the final master mix. For 1X recipe of the master mix, 20× Core Reaction Mix volume was kept at 1 µl. The 20× Core Reaction Mix was also warmed to room temperature and pipette mixed before use. Then 47.21 µl of the master mix was added to individual amplification tubes followed by 1 µl of macerate/template and 1.79 µl of 280 mM MgOAc that were added to the inside of the lid strip. Reactions were started by inverting the tubes to mix and the amplification mixture concentrated at the bottom of the tube prior to immediately incubating the tubes at 39°C for 20 min, with an agitation step after 4 min. Following 20-min incubation, reaction enzymes were denatured by heating at 85°C for 1 min to terminate amplification and cooling to 4°C. The RPA incubation was done in a PTC-0200 DNA Engine Cycler with no lid heating. Once the RPA reaction was completed, a 1-µl reaction was directly utilized for PCR or frozen at -20°C for future use. In RPA-PCR couple, the number of PCR amplification cycles were reduced. The coupled species-specific SYBR Green and TaqMan amplifications were done with 30 thermal cycles, whereas conventional PCR genus-specific amplification was done with 40 cycles.

For estimation of sensitivity of the RPA-PCR couple method, slower reaction kinetics RPA reactions were done with 1 µl of *P. fragariae* or *P. cactorum* hyphal DNA dilutions at concentrations ranging from 100 pg/µl to 0.1 fg/µl and coupled with related PCR assays. The RPA reactions with *P. fragariae* DNA dilutions were spiked with macerate of healthy rootlet tips, whereas reactions with *P. cactorum* DNA were spiked with macerate of healthy crown tissue. Macerate preparation was described previously [23] and added in amounts that would be used for assaying plant samples (1 µl).

Specificity

In laboratory evaluations of specificity, we first analyzed all PCR assays individually for possible cross reactivity with DNA from nontarget taxa. Then we analyzed RPA-PCR couple specificity by adding DNA from each taxa individually for RPA amplification and coupling those RPA reactions with all formats of PCR assays. DNA extracts concentrations ranged between 50 and 100 pg/µl, and 1 µl of each DNA extract was added in individual reaction tubes. We utilized DNA extracts of *P. fragariae*, *P. cactorum*, *Phytophthora taxon raspberry*, *Phytophthora megasperma*, *Phytophthora rosacearum*, *Phytophthora ramorum*, *Phytophthora plurivora*, *Phytophthora pini*, *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Pythium sylvaticum*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Mucor hiemalis*, *Fusarium avenaceum* and *Fusarium proliferatum*. All five fungal species, *P. sylvaticum*, *P. fragariae* and *P. cactorum* were isolated from strawberry plants. More details of the taxa included in laboratory evaluations of specificity are provided in Supplementary Table 1 in the Supplementary data. In addition to laboratory evaluation of specificity, we customized NCBI Primer-BLAST to analyze specificity of the species-level SYBR Green PCR assays. For this purpose, we utilized the partial *atp9-nad9* sequences of approximately 100 *Phytophthora* species provided in supplemental file 2 of Miles *et al.* [5] and complete *atp9* gene sequences from 28 *Phytophthora* species received from the US Department of Agriculture's Agricultural Research Service (CA, USA).

Field validation

For field validation, 22 fine rootlet samples and 22 crown samples were collected from the plants investigated for red stele and crown rot disease in the summer of 2018. The plants samples were from 25 different problematic fields of the Northern Savo region of Finland. Each rootlet sample was collected from three plants from a field (pooling of finest root tips). In contrast, crown samples were not pooled; rather, each crown sample was collected from individual plants. The crown samples always included rot if it was observed. Tissue samples weighing from 25 to 100 mg were collected in a round bottom 2-ml Eppendorf tube and macerated in autoclaved distilled water with a plastic pestle. Rootlet macerates were prepared as 1:10 (w/v) tissue weight by volume of water, whereas crowns macerates were 1:20 (w/v). To facilitate maceration, initially only 100 μ l of water was added, and following maceration, more water was supplemented to achieve the final desired dilution. Once macerates were ready, they were vortexed and left for particulate settlement for approximately 1 min. The maceration process is explicitly explained in Munawar *et al.* [23]. From tissue macerates, 1 μ l of supernatant was transferred to RPA amplifications done with slower reaction kinetics. Once the RPA amplification was completed, RPA reactions were coupled with the four species-specific real-time PCR assays and one conventional genus-specific PCR assay. The target bands from conventional PCR were also Sanger sequenced to confirm identification of the taxa present.

To validate RPA-PCR couple results and compare them with standard diagnostic procedure of DNA extraction-PCR, the macerates prepared were frozen at -80°C. Later, the samples were freeze dried, further ground with plastic pestles and DNA extracted as reported by Till *et al.* [24], directly starting from the step fifth of lysis buffer addition. In DNA extraction, we utilized 100 μ l of liquid silica stock per tube, guanidine thiocyanate as DNA binding buffer and LB2 as lysis buffer. Following DNA extraction, DNA volumes of 0.25, 0.5 and 1 μ l from each sample were tested with the four species-specific and one conventional PCR amplification. The products from conventional PCR were also Sanger sequenced. All DNA samples that showed no amplification were tested for the presence of PCR inhibitors or DNA extraction failure through a separate control PCR amplification targeting plant DNA. The control PCR was a SYBR Green PCR assay targeting plant sequences that included the FMP12b and FMP13b primers of Tooley *et al.* [25], master mix LightCycler® 480 SYBR Green I Master and 0.25- μ l DNA samples. The amplification program started with 10-min incubation at 95°C followed by 35 amplification cycles of 95°C for 10 s, 55°C for 20 s and 72°C for 20 s.

Results & discussion

Optimum PCR assays

Both species-specific SYBR Green assays detected as low as 1 fg of target genomic DNA (eight replicates) with linearity down to 10 fg. The melt curves produced double peaks as predicted by uMELT [26]. Amplification efficiency of the *P. fragariae* assay was 98.76% and the *P. cactorum* assay was 94.50%. Like the SYBR Green assays, both species-specific TaqMan assays amplified as low as 1 fg of target genomic DNA (eight replicates) with linearity down to 10 fg. Amplification efficiency of the *P. fragariae* assay was 90.10% and the *P. cactorum* assay was 82.91%. The conventional PCR genus-specific assay produced visible target band of approximately 300 bp for as low as 1 fg of *Phytophthora* hyphal DNA (two replicates).

Primer-BLAST predicted cross reactivity of the *P. fragariae* SYBR Green assay with only *Phytophthora alni*, *P. cambivora* and *Phytophthora rubi*. Similarly, Primer-BLAST foretold cross reactivity of the *P. cactorum* SYBR Green PCR assay with *Phytophthora pseudotsugae*, *Phytophthora hedraiaandra* and *Phytophthora idaei*. In laboratory analysis of specificity, the species-specific TaqMan and SYBR Green assays showed no cross reactivity for the taxa tested with the exception of the SYBR Green PCR assay for *P. fragariae*, which cross reacted with *P. cambivora* as predicted by Primer-BLAST. Similarly, the conventional genus-specific PCR amplified all *Phytophthora* species and showed no cross reactivity with any of the non-*Phytophthora* species DNA.

Optimizing PCR assays designed in this project from the low GC *atp9-nad9* marker was challenging, especially in the case of *P. cactorum*. In the *P. cactorum* SYBR Green assay, the extension temperature was lowered to 60°C and an additional 10 s were provided to assist completion of the extension step. Moreover, for the TaqMan PCR assays, the amplification curves shapes were more linear, missing exponential and plateau phases (shown later in Figures 3, 4B & D). This probably happened due to low T_m or GC content of the TaqMan probes. In the *P. fragariae* TaqMan assay, 'PfraVf_nad9sp_TaqMan2' has a GC content of 20.0% and T_m of 56.1°C, whereas in the *P. cactorum* TaqMan assay, 'Pcact_nad9sp_probe2' has a GC content of only 11.1% and T_m of 53.8°C. Ideally, the TaqMan probes used in this study should be redesigned with addition of minor groove binder to raise T_m to an adequate level. Otherwise, the TaqMan probe should be further elongated to raise T_m. Regarding the *Phytophthora* genus-specific conventional PCR, the extension temperature was lowered to 65°C. An extension temperature of 68°C worked for *P. fragariae* but failed for *P. cactorum*.

Optimum RPA-PCR couple

In sensitivity evaluation of RPA-PCR couple, slower reaction kinetics RPA reaction were done separately for 1/10 serial dilutions of *P. fragariae* and *P. cactorum* hyphal DNA. The RPA reactions were also spiked with healthy rootlets and crown macerate, respectively. Following amplification, aliquots of the RPA reactions were transferred to real-time TaqMan and SYBR Green PCR assays. RPA-PCR couple consistently amplified target *Phytophthora* hyphal DNA down to 10-fg starting concentration (six replicates). Figure 2 represents standard curves when slower kinetics RPA reactions were done with 1/10 serial dilution of *P. fragariae* DNA and healthy rootlets macerates, and the RPA reactions were coupled with *P. fragariae* specific SYBR Green PCR assay. Similarly, Figure 3 represents a similar setup of RPA-PCR coupled where RPA reactions were used with dilutions of *P. cactorum* DNA and spiked with healthy crown macerate, and later

Figure 2. Standard curve of recombinase polymerase amplification-PCR couple when the template was *Phytophthora fragariae* DNA and the coupled PCR is a *P. fragariae* specific SYBR Green PCR assay. The curves represent 1:10 serial dilutions of DNA from 100 pg to 10 fg used in the RPA amplification.

RPA: Recombinase polymerase amplification.

Figure 3. Standard curve of recombinase polymerase amplification-PCR couple when template was *Phytophthora cactorum* DNA and the coupled PCR is *P. cactorum* specific TaqMan PCR assay. The curves represent 1:10 serial dilutions of DNA from 100 pg to 10 fg used in the RPA amplification.

RPA: Recombinase polymerase amplification.

coupled with *P. cactorum* specific TaqMan PCR assay. The odd shape of the curves in Figure 3 is reflective of the TaqMan assay rather than the RPA assay. Overall, the RPA-PCR couple standard curves were a bit less steep and unevenly spaced compared with relevant PCR assays. The slight decrease in RPA-PCR couple curves steepness possibly indicates inhibition of PCR by RPA reagents. Moreover, as RPA is run first in RPA-PCR couple, the standard curves are more representative of the RPA and therefore unevenly spaced.

In laboratory specificity analysis of RPA-PCR couple, RPA amplification was done with DNA from nontarget taxa and later coupled with the four real-time PCR and one conventional PCR assays. We observed that only the RPA reaction with *P. cactorum* DNA was detected with the coupled *P. cactorum* specific real-time TaqMan and SYBR Green PCR assays. Similarly, only the RPA reaction with *P. fragariae* was detected with the coupled *P. fragariae* specific real-time TaqMan PCR assay, but the coupled *P. fragariae* real-time SYBR Green also detected *P. cambivora* besides *P. fragariae* as expected. Coupling the RPA reactions with genus-specific conventional PCR amplified all

Figure 4. Results of the field validation for a plant crown infected with *Phytophthora cactorum*. (A & B) Amplification curves for DNA extraction followed by *Phytophthora cactorum* specific SYBR Green and TaqMan PCR assay, respectively. (C & D) Amplification curves for RPA followed by *P. cactorum* specific SYBR Green and TaqMan PCR assay, respectively. RPA: Recombinase polymerase amplification.

of the *Phytophthora* species with no amplification observed with other taxa. Sequencing the *Phytophthora* amplicons confirmed their species identification.

In laboratory specificity analysis of RPA-PCR couple, DNA of the different taxa was diluted to a range between 50 and 100 pg/μl. Addition of DNA more than 100 pg in an RPA reaction tends to give false amplification in the coupled PCR assays, especially SYBR Green assays, but any false-positive amplifications in SYBR Green PCR assay can be identified through melt curve analysis. Moreover, such high DNA concentrations are not generally present in plant crude macerates.

In RPA-PCR couple optimization, we first screened RPA primers at normal RPA reaction kinetics, then optimized PCR assays and finally tested the coupling of slower kinetics RPA with PCR (RPA-PCR couple). We screened RPA primers at normal reaction kinetics and with a high concentration of template to have better results to compare through gel electrophoresis. We suggest that one can also adopt a reverse approach for optimizing RPA-PCR couple, meaning that first optimizing a PCR assay and later utilizing the PCR assay to compare the slower reaction kinetics RPA reactions added with a different combination of RPA primers. Screening RPA primers through the coupled real-time PCR will exclude the need for RPA reaction clean-up/gel electrophoresis and will provide better data to compare.

Field validation

For field validation, we compared the RPA-PCR couple method with the method of DNA extraction followed by PCR. We observed some difference in the results of the two methods for the field samples, but all five versions of PCR generated consistent results within each method. The five PCR assays were *P. fragariae* specific TaqMan PCR, *P. fragariae* specific SYBR Green PCR, *P. cactorum* specific TaqMan PCR, *P. cactorum* specific SYBR Green PCR and *Phytophthora* genus-specific conventional PCR (Sanger sequencing).

In the process of validation and comparison, we first did RPA-PCR couple from macerates and later extracted DNA from the macerates to perform PCR assays. All DNA extracts that gave no amplification with the five target PCR assays were further tested with a control PCR to exclude the possibility of inhibitors or extraction failure. For all of those samples, the control PCR generated a cycle threshold (Ct) value between 22 and 28, except one crown DNA that had a Ct approximately 33. The crown sample with the high Ct was excluded from the validation process. Regarding PCR from DNA extraction, all crown DNA samples amplified with 0.5 and 1 μl volume, whereas rootlet DNA samples that had more inhibitors and volumes of 0.25 and 0.5 μl were mostly successful.

In the comparison of RPA-PCR couple with the method of DNA extraction followed by PCR, the latter produced more positives results. Through RPA-PCR couple, among the 22 rootlet samples investigated, nine were found to be infected with *P. fragariae*, four with *P. cactorum* and one coinfecting with both *P. fragariae* and *P. cactorum*. The co-infection was detectable through species-specific PCR assays, but Sanger sequencing was not successful for that sample. Similarly, analysis of 21 crown macerates with RPA-PCR couple

revealed that eight were infected with *P. cactorum*, whereas 13 crown macerates gave no amplification. Regarding results of PCR from DNA extractions, all positive samples from RPA-PCR couple were also found to be positive. PCR assays from DNA extractions detected three additional weak or late positive samples: one rootlet sample infected with *P. cactorum* and two crown samples contaminated with *P. fragariae*. The Sanger sequencing of the coinfecting rootlet samples did not fail but rather generated good reads from the *P. fragariae atp9-nad9* marker (possibly due to bias of PCR). All of the Sanger sequences of *P. fragariae* were identical to JF771842 (GenBank accession number), and all sequences of *P. cactorum* were identical to MH094138 (GenBank accession number). In Figure 4, we present results for the method DNA extraction followed by PCR versus RPA-PCR couple for a field plant crown infected with *P. cactorum*.

Conceptualization of RPA-PCR couple

In prior conceptualization of RPA-PCR couple, we were only aiming at a direct and DNA-extraction-free amplification of the *Phytophthora atp9-nad9* marker from infected plant tissue through RPA to proceed with Sanger sequencing, but we faced problems. To succeed with Sanger sequencing, we needed to gel cut the target band, and often the concentration of the target amplicon was not enough. Therefore, we switched to coupling the RPA with PCR. The concept of coupling RPA with PCR was earlier presented by Miles *et al.* [5], where standard RPA reaction (normal reaction kinetics) was coupled with PCR to sequence and validate some RPA results. We observed that coupling of RPA reactions with PCR for our long target of approximately 500 bp length was not successful for the low target concentrations. Moreover, excessive amplification in RPA reaction, either target or nonspecific, required dilution of the RPA reaction before PCR. Following TwistDx recommendations for long amplicons, we observed that lowering magnesium acetate and primers favored amplification of the long target to some extent, but the issue was resolved through the liquid RPA basic kit (TwistAmp Liquid Basic), which provides flexibility to adjust RPA reaction components. We slowed RPA reaction kinetics by lowering concentrations of primers, MgOAc, ATPs (10X Basic E-Mix), building blocks (dNTPs) and related enzymes (20X Core Reaction Mix), then coupled the slower reaction kinetics with PCR (RPA-PCR couple).

The TwistAmp Liquid Basic kit is provided with components of 2× reaction buffer, 10× Basic E-Mix, 20× Core Reaction Mix and 280 mM MgOAc. The 2× reaction buffer is composed of high salt buffer, Tris, potassium acetate, PEG and dH₂O. Similarly, the 10× Basic E-Mix contains phosphocreatine, ATP, creatine kinase, high salt buffer and dH₂O. The 20X Core Reaction Mix is composed of GP32, UvsX, UvsY and polymerase (personal communication with TwistDx Inc.).

Advantages of RPA-PCR couple

RPA has the ability to amplify target nucleic acid directly from simple plant macerates and is a good choice for real-time detection (TwistAmp exo kit) of the pathogen or target region provided that the target marker is short and adequately unique. However, standard RPA cannot efficiently amplify long targets such as 500 bp with adequate sensitivity, and long amplicons can be desirable in different applications such as Sanger sequencing. Similarly, we observed that some nonspecific by-products generated in RPA were not removed through routine PCR purification kits, and purification of amplicons required gel extraction. RPA assays have also been reported tolerating up to nine mismatches. In contrast to RPA, the traditional method of plant DNA extraction followed by PCR sometimes encounters the problem of amplification inhibitors. The inhibitors enter the DNA sample during the lengthy process of DNA extraction, and therefore we proposed excluding the DNA extraction step and replacing it with RPA. An RPA run at slower reaction kinetics can amplify a long target in limited quantity. The limited or low number of amplicons is not a problem, as a coupled PCR can exponentially amplify those limited amplicons. We observed that slowing the RPA reaction not only favored amplification of long targets but also excluded the need for diluting the RPA reaction prior to doing PCR and provided a better resolution for quantification through real-time quantitative PCR assays compared with standard RPA assays (TwistAmp exo kit). Moreover, through RPA-PCR couple, we experienced quicker amplification of our target (compared with traditional methods) and simple purification of amplicons (compared with standard RPA) through a routine PCR purification kit. In short, in RPA-PCR couple, the RPA takes the role of initial replication of the target from crude macerate, whereas PCR takes responsibility for more specific and exponential amplification of the target.

Although RPA-PCR couple requires additional steps for preparation of RPA reaction mix, TwistDx Inc. also provides a custom freeze-drying service for RPA reaction pellets with variation in the concentration of components. Moreover, PCR in RPA-PCR couple also runs for fewer cycles (lesser time). The RPA-PCR couple procedure for a batch of 10–20 samples takes 5–7 h, whereas results for the traditional method of plant DNA extraction followed by real-time PCR are usually available the next day (8–11 h in total). It is notable that we utilized the cost-effective but lengthy DNA extraction method of Till *et al.* [24]; however, commercial kits such as the DNeasy Plant Mini kit (Qiagen, Germany) have relatively shorter procedures. Regarding the cost of RPA-PCR couple, it is comparable with the traditional method. For instance, the current price of a TwistAmp Liquid Basic (~100 reactions) is EUR200, whereas the DNeasy Plant Mini kit (50 columns) is priced at EUR207. In Table 2, we compare the performance of RPA-PCR couple with that of the traditional method of DNA extraction followed by PCR.

Applications of RPA-PCR couple

We conceived the idea of RPA-PCR couple for plant diagnostics, but it may prove beneficial for other difficult samples encountering the PCR inhibitors problem when DNA is extracted, such as food or fecal samples [27]. It is notable that woody plant tissues have a high content of phenolic compounds and polysaccharides that are co-extracted along with nucleic acid extraction and result in PCR

Table 2. Comparison of the performances of recombinase polymerase amplification-PCR couple and the method of DNA extraction followed by PCR.

Method	Sensitivity	Specificity	Time duration	Cost
DNA extraction followed by PCR	PCR consistently detected down to 1 fg <i>Phytophthora</i> genomic DNA	Satisfactory	5–7 h	Comparable
RPA-PCR couple	RPA-PCR couple consistently detected down to 10 fg <i>Phytophthora</i> genomic DNA	Equally satisfactory	8–11 h	Comparable

RPA: Recombinase polymerase amplification.

inhibition [28], but RPA has shown success in amplifying targets from woody plant macerates. The RPA assay of Miles *et al.* [5] was able to amplify the target from *Umbellularia californica* crude macerate. Similarly, the RPA assay of Zhang *et al.* [7] also amplified the target from some *Prunus* plants. Therefore, we expect the RPA-PCR couple concept to work with different recalcitrant sample macerates. We have presented the method for amplification of pathogen DNA from plant macerates, but it is equally applicable to amplify plant or macerate DNA. Moreover, RPA-PCR couple should also be able to amplify RNA targets by adding suitable reverse transcriptase in an RPA reaction.

Applying the RPA-PCR couple method to identification markers and doing Sanger sequencing afterward can expedite species identification. In the current study, utilization of the unique intergenic *atp9-nad9* marker provided a rapid *Phytophthora* identification assay with the potential to identify almost all *Phytophthora* species [5,22]. The assay is about crudely macerating *Phytophthora*-infected plant tissue in water and transferring the macerate supernatant into the RPA reaction added with forward primer 'ATP9F-M9' and reverse primer 'Phy_Gen_R20.' The RPA reaction is transferred to conventional PCR assay ('Phy_Gen_SeqF4' and 'Phy_Gen_SeqR8' primers), and the PCR product is cleaned and Sanger sequenced. It is notable that we have only tested the assay with ten *Phytophthora* species due to limited resources. Moreover, the assay will not be applicable to *Phytophthora bisheria* or *Phytophthora frigida* due to the possible unusual gene order of *atp9* and *nad9* genes [22].

RPA-PCR couple should be equally applicable to long and short targets, and it may even work with a target longer than 500 bp. In RPA-PCR couple optimization, we first screened RPA primers and found the optimum primer pair amplifying a target of approximately 500 bp length. Next, we tried to design a conventional PCR with a subtarget length of approximately 450 bp, but we ended up with a subtarget length of approximately 300 bp due to difficulty in primer designing from the GC-poor *atp-nad9* marker.

Preventing contamination

Although RPA generates a limited product in RPA-PCR couple, opening RPA tubes to transfer reactions into PCR poses risk of contamination. The RPA strip opening and reaction transfer should be carefully accomplished in some suitable hood of the post-PCR laboratory. Similarly, filter tips or positive displacement tips should be utilized to avoid contamination. Good molecular laboratory practice of physically separating pre- and postamplification laboratories, preparing macerates and reactions reagents in the preamplification laboratory, and including no template control (NTC) guarantee quality of results. The RPA reaction should also be added with an NTC (RPA NTC), which can be further coupled with PCR (coupled NTC). PCR should also include its own NTC. In addition, it is important to mark RPA lid strips to avoid direction inversion of the lid strip. In RPA-PCR couple, either RPA or PCR can be provided with the UDG enzyme and the dTTP partially or completely replaced with dUTP. Importantly, the UDG system cannot be applied to both RPA and PCR in the couple, as the product of RPA is being utilized by the coupled PCR as the initial template.

Future perspective

Isothermal nucleic acid amplification technologies including RPA often suffer from background amplification problems, and their utilization for initial and limited amplification of target nucleic acid to replace the DNA extraction step provides new insight. In this proof-of-concept study, we demonstrated RPA-PCR couple as a time-saving procedure to amplify target DNA from simple macerates, excluding the need for laborious plant DNA extraction. Although we utilized the *Phytophthora* identification marker *atp-nad9* and strawberry *Phytophthora* pathogens to prove the functioning of our RPA-PCR couple concept, the concept is quite general. We expect that this method can also amplify nucleic acid targets from other recalcitrant samples. To apply the concept to some defined targets and available PCR assays, one would only need to design RPA primers from flanking regions of their targets and screen them to find an optimal pair.

Supplementary data

Supplementary Table 1 provides a list of the taxa utilized in laboratory evaluations of specificity and their isolate numbers, sources and origins of recovery. To view the supplementary data please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0065

Author contributions

MA Munawar completed most of the laboratory work and wrote the manuscript, whereas all other coauthors contributed to improving the manuscript. F Martin also contributed by conceptualizing and improving experiment designs.

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Protocols.io link

<https://www.protocols.io/view/protocol-for-rpa-pcr-couple-bifrkbm6>

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