NOTE
Soilborne pathogens / Agents pathogènes telluriques

Improved extraction of *Rhizoctonia* and *Pythium* DNA from wheat roots and soil samples using pressure cycling technology

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Abstract: Soilborne pathogens are important biotic factors in yield reduction in the dryland cereal production region of the Pacific Northwest. *Rhizoctonia solani* AG-8, *Rhizoctonia oryzae*, and *Pythium* spp. are causal agents of root rot, bare patch, and damping-off of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). Although these pathogens can be rapidly and specifically quantified using quantitative real-time PCR, the extraction of *Rhizoctonia* DNA from agricultural samples is often inconsistent, especially at low pathogen population densities. Using a novel extraction system that uses pressure cycling technology (PCT), we improved the extraction of *R. solani* AG-8 DNA up to 16-fold and of *P. abappressorium* DNA up to 2-fold from three types of agricultural soils compared with a bead beating extraction method. PCT also yielded quantifiable amounts of *R. solani* AG-8 and *R. oryzae* DNA from lyophilized wheat roots that were otherwise recalcitrant to homogenization. Furthermore, the extractions were so consistent that pathogen quantification generally could be derived from two rather than three or four replicated extracts. Because PCT is performed in a closed system and minimizes sample shearing and heating, it confers a substantial advantage over conventional extraction systems. Here, we report for the first time the application of PCT in a laboratory setting for the improved extraction and quantification of three types of soilborne pathogens in soil samples. The effectiveness of PCT for three soils suggests that it will be beneficial for other hard-to-extract pathogen samples.

Key words: Barocycler™, necrotrophic pathogen, PULSE tube™, real-time PCR, root disease, soilborne pathogens.

Résumé : Les agents pathogènes telluriques sont d'importants facteurs biotiques de réduction des rendements dans la région sèche de production des céréales du Pacifique Nord-Ouest. Le *Rhizoctonia solani* AG-8, le *Rhizoctonia oryzae* et les *Pythium* spp. sont les agents responsables du piétin, du rhizoctone noir et de la fonte des semis du blé (*Triticum aestivum*) et de l'orge (*Hordeum vulgare*). Même si ces agents pathogènes peuvent être quantifiés rapidement et spécifiquement par la PCR en temps réel, l'extraction de l'ADN de *Rhizoctonia* à partir d'échantillons agricoles n'est pas toujours fiable, en particulier lorsque les densités de population de l'agent pathogène sont faibles. À l'aide d'un nouveau système d'extraction qui utilise la technologie dite PCT (*Pressure Cycling Technology*), nous avons amélioré jusqu'à 16 fois l'extraction de l'ADN de *R. solani* AG-8 et jusqu'à 2 fois celle de l'ADN de *P. abappressorium* à partir de trois types de sols agricoles par rapport à une méthode d'extraction avec broyage à billes. La PCT permet aussi d'obtenir des quantités mesurables d'ADN de *R. solani* AG-8 et de *R. oryzae* à partir de racines de blé lyophilisées, habituellement réfractaires à l'homogénéisation. De plus, la répétabilité est telle que la quantification des agents pathogènes peut se faire avec deux répétitions des extractions plutôt qu'avec trois ou quatre. Puisque la PCT est réalisée dans un système fermé et qu'elle minimise le cisaillement et le réchauffement des échantillons, elle possède des avantages considérables sur les méthodes d'extractions conventionnelles. Nous rapportons ici, pour la première fois, l'utilisation en laboratoire de la PCT pour l'extraction améliorée et la quantification de trois types d'agents pathogènes telluriques dans des échantillons de sol. L'efficacité de la PCT avec trois sols démontre qu'elle pourrait être efficace avec d'autres échantillons d'agents pathogènes difficiles à extraire.

Mots-clés : Barocycler[MC], agent pathogène nécrotrophe, PULSE tube[MC], PCR en temps réel, maladie racinaire, agent pathogène tellurique.

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Introduction

Root rot and damping-off are among the chronic, yield-limiting root diseases of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) in the Pacific Northwest (Cook 1992; Smiley et al. 1996; Cook et al. 2002). The causal pathogens, *Rhizoctonia* and *Pythium* spp., are refractory to chemical treatments and rotation (Chamswarng and Cook 1985; Ogoshi et al. 1990), and improved disease management practices are needed to better control the diseases. Acute rhizoctonia root rot, resulting in bare patches, occurs in low rainfall zones of the Pacific Northwest, whereas pythium root rot and damping-off are particularly problematic in wetter, clay soils. Development of improved management practices will require the accurate diagnosis and quantification of both *Rhizoctonia* and *Pythium*.

Current protocols for the diagnosis of root pathogens have drawbacks. Often, root pathogens cannot be distinguished on the basis of symptoms, especially at the seedling stage or when more than one pathogen is present. Culture-based quantification methods for enumerating pathogen levels are often labour intensive and impractical, especially at low pathogen population densities. Real-time quantitative PCR (Q-PCR) provides a rapid, specific, and sensitive alternative to culture-based quantification methods. We have developed Q-PCR assays for *Rhizoctonia solani* Kühn anastomosis group 8 (AG-8), *Rhizoctonia oryzae* Ryker & Gooch (unpublished data), and *Pythium abappressorium* Paulitz & Mazzola (Schroeder et al. 2006), three pathogens that are among the most pathogenic to wheat and barley (Mazzola et al. 1996; Paulitz et al. 2003a, 2003b).

Extraction of pathogen DNA from agricultural samples remains problematic despite advances in pathogen quantification. Hyphae of *R. solani* and *R. oryzae* occur as extensive networks in the soil and can survive as thick, melanized monilioid hyphae in roots (Butler and Bracker 1970). *Rhizoctonia oryzae* also forms abundant microsclerotia in plant tissues. Oospores of *P. abappressorium* can persist in root tissue and in soil. In addition, pathogen cells contained within lignified plant tissues, including mature roots, are protected from lysis by their host. Therefore, structural features of the pathogen and host present barriers to the efficient and reproducible extraction of pathogen DNA from agricultural samples.

Pressure cycling technology (PCT), developed by Pressure BioSciences, Inc. (West Bridgewater, Mass.),[2] subjects samples to rapid cycles of alternating high and ambient pressure. This procedure efficiently lyses cells and minimizes heating, shearing, and formation of cellular debris that result from bead beating methods. Sample mixing and contamination that can occur with the mortar and pestle or other conventional open-system homogenization methods also are greatly reduced (Schumacher 2006). Pressure cycling can be performed in a wide range of buffers or media, rendering it adaptable to the extraction of DNA, RNA, protein, and low molecular mass metabolites. This technology has applications in medicine and the medical industry (Bradley et al. 2000; Dusing et al. 2001), proteomics (Smejkal et al. 2006), and forensics (P. Froelich, unpublished data), and is amenable to automation (Schumacher et al. 2002; Smejkal et al. 2006).

We developed real-time PCR assays to quantify *Rhizoctonia* and *Pythium* spp. and noted that our assays were yielding about 10-fold less pathogen in agricultural samples than pathogen levels obtained by a private service laboratory. To test whether pathogen DNA extraction could be improved, we generated wheat root and soil extracts using either a standard bead beating protocol or PCT. We made comparisons based on two types of soilborne pathogens (an oomycete, *Pythium abappressorium*, and a true fungus, *Rhizoctonia* spp.), three soils, and three lines of wheat cultivar 'Bobwhite'. Here, we report for the first time, the application of PCT in a laboratory setting for the improved extraction and quantification of a soilborne pathogen in soil samples.

Materials and methods

Fungal isolates, soil, and root materials

Fungal pathogens used in this study were *Rhizoctonia solani* AG-8 isolates C1 (Smith et al. 2003), 070304, and 120203; *R. oryzae* isolates 0801387 and 120232 (Paulitz et al. 2003b); *Pythium abappressorium* isolate 020162 (Schroeder et al. 2006); and *Fusarium oxysporum* f. sp. *spinaciae* (Sherb) Snyder & Hansen isolate Fus001. Cultures were maintained on potato dextrose agar (PDA; Difco, Sparks, Nev.) slants at 4 °C and transferred to PDA dishes every 2 weeks or as needed.

Rhizoctonia solani AG-8 and *R. oryzae* were cultured on autoclaved oats (*Avena sativa* L.), ground, and sieved to obtain inoculum particle sizes of 250–1000 mm (Paulitz and Schroeder 2005) for soil-spiking experiments. Pathogen population densities were enumerated from 100 mg portions of homogenized material, suspended in 5 mL water in triplicate, and diluted 10-fold. Aliquots of 0.2 mL were spread onto water agar supplemented with 100 mg/mL chloramphenicol and 1 mg/mL a.i. benomyl (Benlate®, DuPont, Wilmington, Del.) and incubated at 24 °C in darkness for 3 days. Mean population densities were calculated on the basis of triplicate platings of both diluted and undiluted suspensions. Inocula were mixed with pasteurized nonagriculutral soil from a site near Quincy, Washington (Raaijmakers et al. 1997), and inoculated soils were stored at –20 °C prior to DNA extraction.

Approximately 500 g of agricultural soils were collected in June 2006 at four field sites in Ritzville, Washington, as part of an ongoing study to monitor *Rhizoctonia* in fields that sustained rhizoctonia root rot and bare patch. Soil samples also were collected at spring or winter wheat variety test sites (http://variety.wsu.edu/) located at Walla Walla and Connell, Washington, to establish baseline soil levels of *Rhizoctonia*. Each variety test site sample consisted of three soil cores (2.54 cm wide by 15.24 cm deep) containing a total of about 20 g of soil. All soils were stored at –20 °C until extraction.

Hexaploid wheat genetic lines designated that TaAB, TaC (P. Okubara and A. Blechl, unpublished data), and 'Bobwhite' were sown individually in 15 cm cones (Steuwe and Sons, Corvallis, Ore.) containing 70 g of soil infested with 80 propagules/g of *R. solani* AG-8 (for 'Bobwhite') or 100

[2] References to a company and (or) product by the USDA are only for the purposes of information and do not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

propagules/g each of *R. solani* plus *R. oryzae* (for TaAB, TaC) using inocula generated as previously described. Pasteurized nonagricultural soil from a site near Quincy, Washington (Raaijmakers et al. 1997), was used in all experiments. Plants were grown in a greenhouse at 15 °C ± 1 °C with 12 h of daily supplemental lighting (66–90 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Plants were kept under plastic until hypocotyl emergence and watered with 10 mL of distilled water or Miracle-Gro® (0.94 g/L; Scotts, Port Washington, N.Y.) as needed. Water was withheld for 1 or 2 days prior to harvest so that the soil was loose enough to be removed from the roots without root breakage. Roots were washed extensively to remove soil, frozen at –80 °C, lyophilized for 80–84 h, and stored at ambient temperature prior to DNA extraction.

DNA extractions

DNA was extracted from soil samples using bead beating or PCT sample preparation system (Pressure BioSciences, Inc, West Bridgewater, Mass.) and from lyophilized wheat root samples using PCT only. Twenty milligrams of lyophilized root tissue (about ten 1.5 cm sections) was transferred to disposable FT500 PULSE tubes™ (Pressure BioSciences, Inc) at the cap end. Extraction medium consisted of the UltraClean soil lysis solution, including garnet grit, 120 µL of S1 (sodium dodecyl sulfate solution), 400 µL of inhibitor removal solution (IRS), and 600 µL of bead solution (guanidine thiocyanate solution), which are provided in the UltraClean soil DNA kit (MO BIO Laboratories, Solana Beach, Calif.). Each root extract also contained 200 mg acid-washed Amberlite XAD-4 (Sigma Chemical Co., St. Louis, Mo.). All components were added to the cap end of the PULSE tube. Pressure cycling was conducted in a bench model Barocycler™ NEP 3229 (Pressure BioSciences, Inc) using 15 cycles of 35 000 psi (235 MPa) for 20 s alternated with ambient pressure for 10 s. After pressure cycling, samples were transferred to Eppendorf tubes and incubated at 4 °C for 5 min followed by centrifugation at 10 000 *g* for 1 min. Clarified supernatants were incubated with 400 µL S2 (acetate solution) and 1.8 mL S3 (guanidine HCl – isopropanol solution) buffers, passed through spin filter columns, and washed with 300 µL S4 (ethanol solution) as recommended for the UltraClean soil DNA kit. Total pathogen DNA was eluted with 60 µL S5 (Tris buffer solution) into an Eppendorf tube. "No PCT" controls consisted of the same amount of sample and buffer processed in parallel but without pressure cycling. Triplicate extracts were prepared from each sample.

Ritzville soil samples were extracted as described for root tissue except that each PULSE tube contained 0.2 g of soil, and the Amberlite was omitted. For variety test site samples, 0.5 g soil was combined with 60 µL of S1, 200 µL of IRS, and 300 µL of bead solution and processed with S2, S3, and S4 buffers as described for root extracts. Total soil DNA was eluted with 60 µL S5 into an Eppendorf tube containing 5 mg (50 µL of a 10% (*w*/*v*) aqueous suspension) of insoluble polyvinylpyrrolidone (PVP; Sigma Chemical Co., St. Louis, Mo.) to remove low molecular mass fluorescent compounds. The PVP was centrifuged at 13 000 *g* for 3 min to remove water before the pathogen extract was added. Extractions were done in triplicate for each sample.

Bead-beating extraction was performed in triplicate using the UltraClean soil DNA kit as described in Schroeder et al. (2006). Essentially, a 0.5 g aliquot of soil was added to a tube containing garnet grit and homogenization buffer supplemented with 60 µL of S1 and 200 µL of IRS. The mixture was homogenized using the FastPrep FP120 homogenizer (QBiogene, Carlsbad, Calif.) at a setting of 5.0 for 45 s. Extracts were chilled on ice for 5 min before and after the bead beating step. Extracts were processed with S2, S3, and S4 buffers as described for root extracts. Pathogen DNA was eluted in 45 µL S5 and treated with insoluble polyvinylpyrrolidone prior to real-time PCR.

Total DNA (genomic and mitochondrial) from cultured *R. solani* AG-8 isolate 070304, *R. oryzae* isolate 0801344, and *P. abappressorium* isolate 020162 were extracted for use as positive controls in Q-PCR assays. Mycelia were grown in potato dextrose broth for 5–7 days. Mycelia were washed three times with sterile distilled water under gentle vacuum on a Buchner funnel. Mycelial DNA was obtained using the FastDNA kit and FastPrep FP120 homogenizer at a setting of 5.0 for 45 s; solutions were used according to the manufacturer's alternate protocol for maximum yield. DNA was quantified using the fluorescent DNA quantitation kit (Bio-Rad, Hercules, Calif.) and Safire fluorometric plate reader (TECAN, Research Triangle Park, N.C.).

Size analysis of PCT-extracted DNA

Total DNA from cultures of *R. solani* AG-8 isolates C1 and 120203, *R. oryzae* isolate 120232, *Fusarium oxysporum* f.sp. *spinaciae* Fus001, frozen 'Bobwhite' wheat roots infected with *R. solani* AG-8 C1, oat inoculum of *R. solani* C1 used to infect the roots, and natural soils collected from Ritzville, Washington, were subjected to PCT and extracted as described in the previous section. Approximately 200 ng of each type of sample was used for the extractions. DNA (100–250 ng) was partitioned on 0.7% agarose in 0.5 × TBE (45 mmol/L Tris- HCl, 45 mmol/L borate, 1 mmol/L EDTA, pH 8.0) at 65 V for 4 h. The molecular size standard was the 1 kb DNA ladder (Invitrogen, Carlsbad, Calif.). DNA was visualized by staining in ethidium bromide.

Genomic DNA from lyophilized 'Bobwhite' wheat roots was amplified using "universal primers" 5′-CGAATCGGT AGACGCTACG-3′ and 5′-GGGGATAGAGGGACTTGAAC-3′ specific for chloroplastic noncoding DNA and PCR cycling profiles described in Taberlet et al. (1991). The 653 bp amplicons were partitioned for 30 min on a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, Calif.).

Real-time PCR of pathogen DNA

The Q-PCR was conducted using the FastStart DNA Master SYBR green I fluorescence chemistry and a LightCycler® thermocycler (Roche Applied Science, Indianapolis, Ind.). The Q-PCR assays targeted the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA genes (P. Okubara, unpublished data; Schroeder et al. 2006). Primers for *R. solani* AG-8 were *Rs8F* 5′ GGGGGAATTTATTCATTTATTGGAC and *Rs2.1R* 5′-GGTGTGAAGCTGCAAAAG-3′; for *R. oryzae* were *RoGr3F* 5′-CTGTTGAAACCGGTTTACTATG-3′ DNA and *RoGr3R* 5′-CTTCCAAGTCCAAATACAACAATC-3′; and for *P. abappressorium* were ABA1bF 5′-GTTGTTGTGCGT CTGCGGATTTG-3′ and ABA1R 5′-TGCATAAACGAATAT

ACCAACCGC-3′ (Schroeder et al. 2006). *Rhizoctonia* PCR reactions consisted of 0.5 µL of DNA extract, 5 pmol of each primer, and 4 mmol/L $MgCl_2$ in 10 µL. *Pythium* PCR assays were similar except that 2 µL of DNA extract and a final volume of 20 µL were used. Amplification was performed at 95 °C for 10 min and up to 50 cycles of 95 °C for 5 s denaturation, 10 s annealing, and 72 °C for 10 s extension. Annealing temperatures were 58 °C, 60 °C, and 70 °C for the *R. solani* AG-8, *R. oryzae*, and *P. abappressorium* assays, respectively. Melting profiles of the amplicons were generated by increasing the reaction temperature from 70 °C to 96 °C in 0.1 °C increments. Fluorescence was monitored after each annealing step and every 0.4–0.5 °C during melting. Genomic DNA from *R. solani* AG-8 070304, *R. oryzae* 0801344 and *P. abappressorium* 020162 served as positive controls; negative controls contained nanopure water instead of DNA. Duplicate Q-PCR reactions were run for each extract.

DNA was quantified using standard curves for *R. solani* AG-8, *R. oryzae* group III (unpublished data), and *P. abappressorium* (Schroeder et al. 2006). Standard curve equations were C_t = –3.749 log(pg DNA) + 24.987 for *P. abappressorium*; C_t = –3.805 log(pg DNA) + 28.388 for *R. solani* AG-8 in soil; C_t = –3.938 log(pg DNA) + 33.474 for *R. solani* AG-8 in root extracts; and C_t = –3.591 log(pg DNA) + 35.172 for *R. oryzae* group III in root extracts, where C_t is the mean cycle threshold value of two Q-PCR reactions.

Statistical analyses were performed using the linear general analysis of variance and least significant difference functions of Statistix version 8.1 (Analytical Software, Tallahassee, Fla.).

Results and discussion

The efficiency of extraction of pathogen DNA from an agricultural sample has consequences for the reliability of Q-PCR quantification and, ultimately, pathogen diagnosis. At C_t values >40, amplicons often displayed abnormal melting profiles, and a reliable diagnosis of the pathogen could not be made. Therefore, we considered the threshold for pathogen detection to be C_t ~ 40. In contrast, the threshold for quantification was C_t ~ 32 (unpublished data; Schroeder et al. 2006). At this value or below, the C_t values among replicate PCR reactions were very similar, and the mean C_t value could be converted to picograms or femtograms DNA with confidence using the appropriate standard curve. The SDs of the means of triplicate extractions ranged from 0.03 to 0.48 for PCT samples compared with 0.53 to 2.13 for bead beating samples (Table 1). Our studies showed that pressure cycling significantly ($P < 0.05$) enhanced DNA extraction to quantifiable levels in wheat root and soil samples.

PCT enables *Rhizoctonia* DNA recovery from infected wheat roots

Rhizoctonia is known to form thick, monilioid hyphae in roots, whereas *R. oryzae* produces microsclerotia in host tissues. In previous studies, these barriers to *Rhizoctonia* DNA extraction were partially overcome using lyophilization and additional homogenization. For example, extraction of DNA from mycelia of cultured *Rhizoctonia* requires four to six cycles of homogenization using the FastPrep FP120 plus lyophilization of the mycelia prior to extraction; these procedures enhance DNA yields about twofold (unpublished data).

Table 1. Variability of real-time PCR quantification of pathogen DNA in soil samples extracted using pressure cycling technology (PCT) or bead beating (BB) extraction.

	R. solani AG-8		*P. abappressorium*	
Sample	PCT	BB	PCT	BB
T4 R3a	28.58±0.48	28.98±1.20	30.95±0.27	28.81±0.06
T5 R2d(c)	29.81±0.38	32.98±1.66	26.30±0.72	25.92±1.00
T9–06 R3b	28.64±0.03	29.99±0.53	26.82±0.27	27.74±0.42
T9–06 R3c	28.95±0.32	28.60±2.13	26.75±0.09	25.35±0.43

Note: Values are means ± SDs of cycle threshold values of triplicate extractions from four soil samples, excepting the *P. abappressorium* assay of T4 R3a (two replicates). Each extraction was assayed in duplicate Q-PCR reactions.

In this study, we sought to quantify the amount of *R. oryzae* and (or) *R. solani* AG-8 associated with lyophilized roots of wheat cultivars 'Bobwhite', TaAB and TaC after 14 days of growth in the presence of the pathogens. Washed roots were lyophilized for about 3 days and appeared to be dry but became rubbery during storage. The roots were totally recalcitrant to pulverization in liquid nitrogen using a mortar and pestle and to bead beating and, subsequently, could not be extracted using our standard protocol.

Using PCT, quantifiable amounts of *R. solani* AG-8 DNA were recovered from all infected 'Bobwhite' root samples that were tested (Table 2). DNA yield was proportional to the amount of 'Bobwhite' root tissue used. Furthermore, both *R. solani* AG-8 and *R. oryzae* DNA could be quantified in roots of wheat lines TaAB and TaC after infection with a combination of the pathogens. In contrast, pathogen DNA were below the threshold for quantification ($C_t > 40$) in the "no PCT" samples. It is possible that the roots harbored monilioid hyphae, which would be more refractory to conventional extraction. Although PCT extractions were done on 10 and 20 mg root tissue in this study, up to 500 mg of root tissue can be extracted. Because PCT is carried out in closed disposable tubes, sample mixing (cross-contamination) that might occur with the mortar and pestle is eliminated. Also, extensive washing of mortars and pestles is avoided using PCT.

PCT improves extraction of *Rhizoctonia* DNA from soil samples

We quantified *R. solani* AG-8 in three Washington agricultural soils known to sustain rhizoctonia root rot and bare patch to test the impact of PCT on soil extracts. In Ritzville soil samples, PCT resulted in DNA yields of about 260–550 $pg \cdot g^{-1}$ soil compared with about 16–150 $pg \cdot g^{-1}$ for the bead beating method (a 4- to 16-fold increase) (Table 3).

The Ritzville soil extracts also were assayed for the presence of *Pythium abappressorium*, a root pathogen that is prevalent in cereal production sites throughout eastern Washington (Paulitz et al. 2003a; Schroeder et al. 2006). *Pythium abappressorium* was quantified in all of the samples (Table 3). Extraction of *Pythium* DNA from cultured mycelia and soils is more facile compared with *Rhizoctonia* because *Pythium* has thin-walled hyphae. This was evident in the similar yields of *Pythium* DNA obtained using both

Table 2. Detection and quantification of *Rhizoctonia solani* AG-8 and *R. oryzae* DNA in lyophilized wheat roots of cultivar 'Bobwhite' and 'Bobwhite'-derived lines TaAB and TaC with and without pressure cycling.

		R. solani		*R. oryzae*	
Plant[a]	Treatment[b]	pg[c]	ng/g[d]	pg[c]	ng/g[d]
'Bobwhite'					
10 mg	PCT	33±10	401±122	—	—
20 mg	PCT	63±21	381±127	—	—
20 mg	No PCT	1.0±0.2	5.9±0.1	—	—
TaAB					
10 mg	PCT	78±10	942±117	24±5.0	285±60
20 mg	PCT	281±60	1689±359	22±3.4	132±20
20 mg	No PCT	0.3±0.2	1.8±1.4	2.8±1.8	17±11
TaC					
20 mg	PCT	8.9±1.5	53±8.9	4.1±0.8	24±4.7
20 mg	No PCT	0.9±0.0	5.4±0.04	0.8±0.2	5.0±1.3

[a]'Bobwhite' wheat was grown in soil infested with 80 propagules/g *R. solani* AG-8; the remaining wheat lines were grown in soil infested with 100 propagules/g *R. solani* plus 100 propagules/g *R. oryzae*. Extractions were performed using 10 or 20 mg of root tissue.

[b]Pressure cycling was performed using 15 cycles of 35 000 psi (235 MPa) for 20 s followed by ambient pressure for 10 s for 15 cycles.

[c]Means and and SEs of pathogen DNA in 0.5 µL of extract (triplicate samples) determined from a standard curve of *Rhizoctonia* genomic DNA were added to wheat root extracts (P. Okubara, unpublished data). Standard curve equations were $C_t = -3.938 \log(\text{pg DNA}) + 33.474$ for *R. solani* AG-8, and $C_t = -3.591 \log(\text{pg DNA}) + 35.172$ for *R. oryzae* group III, where C_t is the mean cycle threshold value of two Q-PCR reactions.

[d]Means and SEs of triplicate extractions standardized to gram root tissue.

the PCT and bead beating extraction methods in three of the samples. However, PCT conferred about a twofold increase over bead beating in samples T5 R2d(c) and T9-06 R3b (Table 3). The reason for the modest enhancement of *Pythium* DNA in these samples is not known; it is possible that the samples harbored more oospores or possessed soil qualities, such as high organic content, that affected the extraction and (or) PCR quantification of pathogen DNA. Analysis of more soils that harbor *Rhizoctonia* and *Pythium* will contribute to our understanding of the benefits of PCT.

We sought to determine whether PCT improved the quantification of *R. solani* AG-8 DNA from other soil types. *Rhizoctonia solani* AG-8 was found at Walla Walla and Connell in a previous survey of Washington soils (P. Okubara, K. Schroeder, and T. Paulitz, unpublished data). A comparison of PCT and bead beating extractions of these samples showed that the former yielded quantifiable levels of pathogen DNA, whereas the latter did not yield levels that were even detectable (Table 4). PCT also improved *Rhizoctonia* DNA yields in pasteurized nonagricultural soil from Quincy, Washington, that harbored no detectable endogenous *Rhizoctonia*. Pathogen DNA was increased 1.8- and 3.4-fold in Quincy soil spiked with 10 and 10 000 propagules/g of *R. solani* AG-8 isolate C1, respectively, following PCT (Table 4). These findings indicate that PCT is effective for multiple soil types and pathogen populations.

Pathogen DNA yields from soil extracts are more consistent after PCT

PCT conferred a high degree of reproducibility in C_t values among triplicate extracts in our real-time PCR assays (Table 1). In the *R. solani* AG-8 assays, SDs of C_t values obtained from three independent extracts averaged 0.39 for the PCT extracts compared with 1.24 for bead-beater extracts. A similar but less marked effect of PCT on standard deviations in the *P. abappressorium* assays (means of 0.34 and 0.48 for PCT and bead beating, respectively) was observed. Higher assay reproducibility also was obtained for lyophilized roots extracted using PCT (data not shown). It is hypothetically possible to obtain reliable quantification from a single extract, although duplicate extracts are recommended. Nevertheless, two rather than three to four extractions represent a resource savings, especially for comprehensive studies involving a large number of samples.

We have not estimated the effect of heat on DNA yield but expect that it poses an unwanted variable. Heating of samples after two to six 45 s bead-beating treatments was somewhat reduced by chilling on ice immediately before and after homogenization; heating could have been further reduced by chilling between each homogenization, but this was considered to be too time consuming. The shearing that resulted from bead beating also was a limitation; two cycles of bead beating improved PCR quantification of *Rhizoctonia* DNA, but more than six cycles had a negative effect (unpublished data). Samples processed using PCT were not warm to the touch compared with beat beater extracts, and the mean size of DNA extracted using PCT was about 40 kb (C. Li, unpublished data). Reproducibility likely resulted from two advantages conferred by PCT: minimized heating and shearing of nucleic acids during cell disruption.

Quality of PCT-extracted DNA

Genomic DNA of about 7 to >12 kb in size were obtained from natural soils, frozen wheat roots, oat inoculum,

Table 3. Comparison of pressure cycling technology (PCT) and bead beating (BB) extraction of *Rhizoctonia solani* AG-8 and *Pythium abappressorium* DNA from agricultural soils from Ritzville, Washington.

		R. solani AG-8		*P. abappressorium*	
Sample[a]	Treatment[b]	DNA (pg/g soil)[c]	PCT/BB ratio	DNA (pg/g soil)	PCT/BB ratio
T4 R3a	PCT	551±94	3.6	2.3±0.24	1.0
	BB	152±68		2.3±0.07	
T5 R2d(c)	PCT	258±36	16	33±10	1.8
	BB	16±10		18±6.7	
T9-06 R3b	PCT	515±4.8	7.3	9.6±0.98	2.0
	BB	71±14		4.8±0.73	
T9-06 R3c	PCT	432±45	5.6	35±1.2	1.5
	BB	76±13		23±3.4	

Note: Soil type was classified as Ritzville silt loam (USDA Natural Resources Conservation Services website).

[a]Soil samples designated T* were collected from Ritzville, Washington, in September 2006.

[b]Pressure cycling was performed using 15 cycles of 35 000 psi (235 MPa) for 20 s followed by ambient pressure for 10 s for 15 cycles. Bead beating extraction was done using the UltraClean soil DNA kit and FastPrep FP120 homogenizer.

[c]Means and SEs of triplicate extractions of 0.2 g soil (PCT) or 0.5 g soil (BB), determined from standard curves of fungal genomic DNA added to soil extracts. Standard curve equations were $C_t = -3.805 \log(\text{pg DNA}) + 28.388$ for *R. solani* AG-8 and $C_t = -3.749 \log(\text{pg DNA}) + 24.987$ for *P. abappressorium*, where C_t is the mean cycle threshold value of two Q-PCR reactions.

Table 4. Comparison of pressure cycling technology (PCT) and bead beating (BB) extraction of *Rhizoctonia solani* AG-8 DNA from additional agricultural soils.

			DNA (pg/g soil)[a]		
Sample[b]	Location	Soil type[c]	PCT[d]	BB[e]	PCT/BB ratio
VT2–3SW	Walla Walla	Walla Walla silt loam	14±8.6	Not detected	—
VT2-4SW	Walla Walla	Walla Walla silt loam	5.0±1.6	Not detected	—
VT3-1WW	Walla Walla	Walla Walla silt loam	7.7±0.08	Not detected	—
VT3-5WW	Walla Walla	Walla Walla silt loam	4.3±0.0	Not detected	—
VT16-3SW	Connell	Ritzville silt loam	18±13	Not detected	—
QV-10ppg	Quincy	Very fine sandy loam	70±0.8	38±27	1.8
QV-10000ppg	Quincy	Very fine sandy loam	71 285 ± 14 876	20 677 ± 10 738	3.4

[a]Values are means ± SEs of triplicate extractions of 0.5 g soil (PCT and BB) determined from a standard curve of *Rhizoctonia* genomic DNA added to soil extracts. The standard curve equation was $C_t = -3.805 \log(\text{pg DNA}) + 28.388$, where C_t is the mean cycle threshold value of two Q-PCR reactions.

[b]Soil samples were collected from spring wheat (SW) or winter wheat (WW) variety test sites in Walla Walla and Connell, Washington, in June 2006. QV, nonagricultural soil from Quincy, Washington, spiked with 10 or 10 000 propagules/g of *R. solani* isolate AG-8 C1.

[c]Quincy soil type was described in Raaijmakers et al. (1997); the others were described on the USDA Natural Resources Conservation Services website.

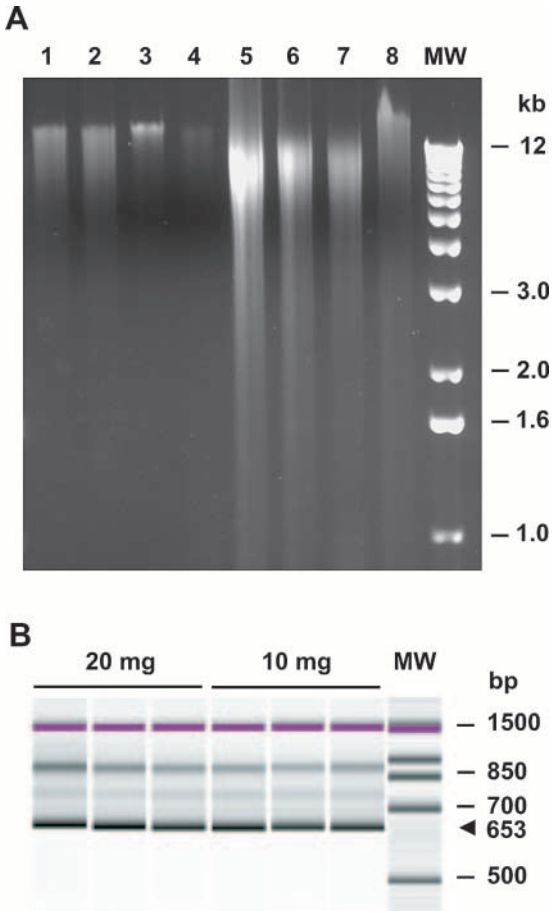
[d]Pressure cycling was performed using 15 cycles of 35 000 psi (235 MPa) for 20 s followed by ambient pressure for 10 s for 15 cycles.

[e]Bead beating extraction was done using the UltraClean soil DNA kit and FastPrep FP120 homogenizer.

and mycelia of *Rhizoctonia* and *Fusarium* after PCT treatment (Fig. 1A). A majority of the DNA exceeded 12 kb based on copartitioning with a 1 kb DNA ladder. PCT has also been used to extract DNA from fresh sprouts, frozen leaves and roots, and dried leaves of corn and seed tissue from rice, maize, tomato, wheat, apple, tobacco (Schumacher 2006), and onion (unpublished data). Fragments of up to 40 kb in size are recovered from these tissues. PCT-extracted plant DNA routinely yielded a chloroplast-specific amplicon of 653 bp; an example from lyophilized wheat root samples is shown in Fig. 1B. Furthermore, a 2.48 kb intergenic spacer fragment was recently amplified from PCT-extracted *F. oxysporum* f.sp. *spinaciae* Fus001 mycelial DNA (L. Harrison and L. du Toit, unpublished data). These findings indicate that PCT yields genomic DNA fragments suitable for PCR applications.

The additional manipulation that PCT adds to the diagnoses of *Rhizoctonia* spp. is offset by significant improvements in pathogen DNA extraction efficiency and real-time PCR quantification. In combination with protocols for the extraction of PCR-quality pathogen DNA from soil and root samples, PCT provides the means to more accurately monitor the incidence, distribution, and quantity of the predominate types of *Rhizoctonia* and *Pythium* associated with cereal cropping systems in the Pacific Northwest and worldwide. It will improve the assessment of disease management strategies for these pathogens.

Fig. 1. Molecular sizes of genomic DNA obtained using PCT (A) and an example of PCR amplicons derived from wheat root DNA following PCT (B). (A) Lanes are: total DNA was extracted from two different Ritzville soil samples (lanes 1 and 2), *Rhizoctonia solani* C1 oat inoculum (lane 3), frozen wheat roots infected with *R. solani* C1 (lane 4), mycelia of *R. solani* C1 (lane 5), *R. solani* 120203 (lane 6), *R. oryzae* 120232 (lane 7), and *Fusarium oxysporum* f. sp. *spinaciae* (lane 8); MW, 1 kb DNA ladder (Invitrogen, Carlsbad, Calif.). (B) Endpoint PCR analysis of chloroplast DNA amplicons derived from root DNA of 'Bobwhite' wheat. Ten and 20 mg lyophilized root segments were used for PCT. Arrowhead indicates the 653 bp amplicon.



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