DNA-based Protocols for Detection and Quantification of Soilborne Pathogens Affecting Almond

Project No.: **09-PATH2-Browne/Kluepfel**

Project Leaders:
Greg Browne
USDA-ARS, Crops Pathology and Genetics Research Unit,
Department of Plant Pathology
UC Davis
One Shields Ave.
Davis, CA 95616
(530) 754-9351
E-mail: gtbrowne@ucdavis.edu

Dan Kluepfel
USDA-ARS, Crops Pathology and Genetics Research Unit,
Department of Plant Pathology
UC Davis
One Shields Ave.
Davis, CA 95616
(530) 752.1137
E-mail: dakluepfel@ucdavis.edu

Project Cooperators and Personnel:
Ravi Bhat, Department of Plant Pathology, UC Davis
Leigh Schmidt, USDA-ARS, CPGRU, Davis

Objectives:
1. Develop an affordable macro array for soilborne pathogens of almond.
2. Develop and optimize specific quantitative PCR (qPCR) primers and hydrolysis probes for fungi positively and negatively associated with almond replant disease.
3. Validate qPCR system for quantification of target fungi in soil and root samples from almond orchards.

Interpretive Summary:

In December 2009 we reported on evaluations of a prototype macro array for simultaneous detection of multiple almond pathogens (*Development of DNA arrays for diagnosis and prediction of almond diseases*, Interim report to the Almond Board of California, Browne and Kluepfel, 2009). A prototype macroarray was constructed using genomic (i.e., total) DNA purified from *Cylindrocarpon* sp. (two isolates), *Trichoderma* sp., *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora cactorum*, *P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. drechsleri, P. gonapodyides, P. megasperma, P. persica, Pythium helicoides*, and *Pythium* sp. The array was optimized and tested to evaluate its specificity and sensitivity. It was determined that at the

optimal hybridization of temperature 65 ^{0} C, the probing could detect as little as 5 to 10 ng of pathogen DNA in a sample. This level of sensitivity may be sufficient for root and soil diagnostics. However, the specificity of the array was not sufficient; it distinguished at a genus level at best. For example, members of the genus *Phytophthora* were distinguished from *Pythium* and the fungal genera (*Cylindrocarpon*, *Verticillium*, *Trichoderma*, and *Colletotrichum*), and from peach, but the array did not distinguish among the fungal genera. It was concluded that this macroarray format would not fulfill its intended purpose. It was judged that a micro array format would be very costly to pursue and considered unlikely that it would afford the needed specificity and quantification capability.

As an alternative approach, we focused on the quantitative polymerase chain reaction (qPCR) for detection of target almond pathogens. It is known that qPCR can afford rapid, specific, and sensitive detection and quantification of pathogens (Gachon et al., 2004). However, unlike array-based detection systems where multiple microbes can be detected and quantified simultaneously, qPCR allows detection and quantification of one or a few target organisms at a time. The specificity of qPCR reactions is achieved by use of sequence-specific PCR primers (and in some cases, by use of sequence-specific reporter "probes" (e.g., TaqMan hydrolysis probes or molecular beacons). The quantification capability of qPCR is achieved by measuring the intensity of fluorescence from a reporter probe or a non-specific fluorescing dye (e.g., SYBR green) that binds with the double-stranded DNA product (Bilodeau et al., 2007, Lievens et al., 2006). Each qPCR reaction requires testing, optimization, and validation before it is suitable for diagnostic applications.

We designed and tested many qPCR primer pairs for *Trichoderma harzianum* (an organism associated with healthy trees in pre-plant fumigated plots in our replant trials) and *Cylindrocarpon* sp. and *Pythium helicoides* (organisms that were associated with RD-affected trees in non-fumigated plots). The testing revealed qPCR primer pairs that were specific to the target fungi, and experiments are in progress to optimize qPCR protocols using hydrolysis probes.

Materials and Methods:

Testing a macro array for soilborne almond pathogens. A modified CTAB method was used to extract DNA from 18 test isolates, including *Cylindrocarpon* sp. (two isolates), *Trichoderma* sp., *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora cactorum*, *P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. drechsleri, P. gonapodyides, P. megasperma, Pythium helicoides*, a *Pythium* sp. and Nemaguard and Lovell peach rootstocks. The purity of the extracted genomic DNA was good, with an average ratio of 1.90 for A_{260}/A_{280} and 1.55 for A_{260}/A_{230}.

In each of three experiments, multiple sets of positively charged Hybond-N^{+} nylon membranes (115 mm x 75 mm) were spotted with known concentrations of probe DNA (i.e., DNA fixed to the membranes in spots and used to probe samples for their target organisms). The probe DNAs were spotted on nylon membranes in amounts ranging

from 10 pg to 500 ng DNA. The specificity and sensitivity of the probes were tested using samples of DNA from the target organisms.

Development of qPCR protocols for soilborne almond pathogens. DNA was purified from target and non-target fungi and oomycetes in our laboratory collection. Details of isolates and field samples are listed in **Table 1**. Briefly, 52 isolates of fungi, 9 isolates of oomycetes, four samples of *Prunus* sp. grown either in tissue culture or green house environment, and 20 peach and Marianna 2624 root samples from two locations in California were included for DNA extraction. In addition, DNA was extracted from 12 soil samples from an almond replant trial near Firebaugh, CA. Several modifications of CTAB method and some published protocols were used to obtain good quality and quantity of DNA from soil. Mycelia were obtained from the fungi and oomycetes listed in **Table 1** by growing them for 4 to 7 days in potato dextrose broth and V8 juice broth, respectively. Leaves of peach rootstocks Nemaguard and Lovell and roots of field samples, frozen at -80^0 C for storage, were used for DNA extraction. Total nucleic acids were extracted from all samples using a modified CTAB method (Bhat and Browne, 2009). Extracted DNA was assessed for quality and quantity by measuring the absorbance at 230, 260, and 280 nm in a NanoDrop machine. DNA from all 85 samples was diluted to concentrations that ranged from 0.65 to 50 ng/µl. Separate 10-fold serial dilutions, 100 ng/µL to 10 fg/µL, were prepared for the DNA from *T. harzianum* (also known by its sexual stage name of *Hypocrea lixii* abbreviated below as "Hyplix")*, Cylindrocarpon* sp. (sexual stage name *Neonectria radicicola* abbreviated below as "Neorad") and *Pythium helicoides* (abbreviated below as "Pythel") for testing sensitivity in the qPCR protocols described below. All DNA samples were stored at -20^0 C.

The purified DNA samples described above were used for sequencing of the internal transcribed spacer (ITS) regions comprising ITS1-5.8S-ITS2 of the rRNA gene in our companion ABC project "*Development of improved strategies for management of replant problems*", Browne et al., 2010". These sequences were processed, blasted in NCBI web site and used in this project to design primers specific for *T. harzianum* (Hyplix), *Cylindrocarpon* sp*.* (Neorad)*, P. helicoides* (Pythel), and *Phaeonectriella lignicola* (Phalig). PRISE software (Fu et al., 2008) and other online resources were used for primer design. Twenty three primers listed in **Table 2** were thoroughly tested for specificity in conventional PCR (cPCR) before proceeding to quantitative PCR (qPCR). All primers were purchased from Operon Technologies.

Sixteen DNA samples (a subset of organisms listed in **Table 1**) and 8 serial dilutions of the appropriate target organism were used for the initial cPCR tests. Generally, each cPCR mix in 25 µl included 2.5 µl of 10X reaction buffer (100 mM Tris-HCl pH 8.0, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs mix, 2.0 µl of 2.5 µM forward primer, 2.0 µl of 2.5 µM reverse primer, 5.0 µl of 10% polyvinylpyrrolidone 40 (PVP40), 0.3 µl (1.5 units) of *Taq* polymerase (Gene Choice), 8.2 µl of ultra-pure distilled water (GIBCO, Invitrogen Corporation, Grand Island, NY), and 1.0 µl of working DNA suspension. In some experiments, 2.5 µl of SYBR Green (10X) and 0.3 µl of ROX dye (1:500 diluted) were included in the PCR mix. The forward

and reverse PCR primers are listed in **Table 3**. Amplifications were conducted in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) thermal cycler using the following parameters: initial denaturing at 94°C for 5 min, then 40 cycles of 94°C for 30 sec, 60°C to 72°C gradient for 30 sec, and 72°C for sec with a final extension at 72°C for 10 min, followed by a final step of cooling to 4°C. Ten microliters of PCR amplicons were separated by 1.4% agarose gel electrophoresis in 0.5X TAE buffer, and DNA bands were visualized by ethidium bromide (0.5 µg ml^{-1}) staining and UV illumination.

Primers performing satisfactorily in cPCR were then tested for qPCR using a Mx3000P PCR machine (Stratagene; Agilent Technologies, Santa Clara, CA). All qPCR reactions were performed in 25 µl, either in 8-tube strips or 96-well plates. Master mixes were prepared for the appropriate number of reactions, and they contained fluorescent dyes 1X SYBR Green (Molecular Probes, OR) and ROX (Applied Biosystems, CA) along with the chemical ingredients used for cPCR. ROX was used as a passive dye for normalization whereas SYBR Green was used as a binding dye to indicate the amplification of double stranded DNA. Three primer pairs, Hyplix240For and ITS4Long for *T. harzianum*, Neorad85For and Neorad440Rev for *Cylindrocarpon* sp., and Pythel494For and Pythel738Rev for *P. helicoides* were tested extensively at annealing temperature from 60 to 72°C and primer concentrations from 25 to 200 nM, In addition, *Taq* polymerase from GeneChoice was compared with HotStar *Taq* polymerase from Qiagen for qPCR efficiency. Thermal cycling conditions for qPCR were 94°C for 5 min, 40 cycles of 94°C for 30 sec, annealing temperature (varied from 60 to 72°C) for 30 sec, and 72°C for 30 sec. Fluorescence was read after the end of each annealing temperature step. After the completion of 40 cycles, fluorescence was collected at every 0.2°C increment from 60 to 94°C to obtain the dissociation curve. Data from Mx3000P software were exported as threshold (Ct) values and analyzed in Excel spread sheets.

Results and Discussion:

Testing of a macro array. In a first macroarray experiment, in which probe DNAs were fixed on the array at 200 ng per spot and 50 ng of test DNA was present in each sample, the probes distinguished at a genus level between samples of *Phytophthora* and peach DNA. However, the probes did not distinguish among sample DNA from different species of *Phytophthora* or between sample DNA from Nemaguard and Lovell peach. Probe DNAs for the *Phytophthora* spp. and for the peach rootstocks did not cross hybridize with sample DNA from *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* and vice versa. However, probe DNAs for *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* cross reacted (i.e., hybridized indiscriminately) among sample DNA preparations of these same species. The specificity of probes was similar at hybridization temperatures 60^0, 65^0 and 70^0 C. Decreasing the exposure time of X-ray films used to capture fluorescence from 30 to 5 min reduced fluorescence intensities but did not affect probe specificity. None of the labeled whole genomic DNA preparations hybridized with water control spots on the membrane.

In a second macroarray experiment, in which probe DNAs were fixed on the array at 50 ng, 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg, 25 pg, or 10 pg per spot, sample DNA concentration was 50 ng, and hybridization temperatures were 60^0, 65^0 and 70^0 C, specificity and sensitivity were not improved over the levels obtained in experiment 1 (**Table 3**). That is, regardless of probe spot DNA amount and hybridization temperature, the array distinguished between DNAs from *Phytophthora*, peach, and the fungi in the experimental samples, but it did not distinguish among DNAs from different species of *Phytophthora*, different genera of the fungi, or the two selections of peach (**Table 3**). The intensity of hybridization was proportional to the amount of DNA per spot and the exposure time. Based on results obtained in experiments 1 and 2, it was concluded that the optimum amount of DNA per spot for 5-min exposure to X-ray films was 200 ng.

In the third experiment, in which probe DNA amount was 500 ng per spot and sample DNA amount was varied (i.e., 50, 40, 30, 20, 10, 5, or 1 ng), and sample DNAs were from *P. cactorum*, *P. citricola*, or *Pythium helicoides*, sensitivity and specificity were similar to the levels obtained in the first and second experiments . It was determined that the macroarray could detect as little as 10 ng and 5 ng of target DNA in a sample for film exposure periods of 5 min and 30 min, respectively (**Figures 1A and 1B**).

Overall, it was concluded from the three experiments that the macroarray format would not fulfill its intended purpose. The macroarray exhibited adequate sensitivity, but it had inadequate specificity. As described below, we pursued qPCR as an alternative approach.

Development of qPCR protocols. Of the PCR primer pairs selected with software algorithms for specific detection of *T. harzianum*, *Cylindrocarpon* sp., *P. helicoides*, and *P. lignicola*, some functioned well in cPCR runs (**Table 4**). The other primer pairs amplified multiple DNA fragments or were non-specific and hence unsuitable for specific detection.

When the suitable primer pairs for *T. harzianum*, *Cylindrocarpon* sp., *P. helicoides* were tested using qPCR and the appropriate DNA sample dilution series, primer specificity was good in each case, but quantification of the target DNA generally suffered from background amplification of primer dimmers, which resulted in non-specific binding and fluorescence of SYBR green. The optimum annealing temperature for all three primer pairs was 68°C, and optimum primer concentrations ranged from 50 to 100 nM. R^2 values of >0.98 and qPCR efficiencies of >90% were obtained when dilution series of the target organisms were prepared (Fig. 2). The binding of SYBR Green to primer dimers often reduced the detection limits and sensitivity of the assay by projecting false Ct values. The suitable primer pair for *P. lignicola*, Phalig268For and Phalig361Rev, did not amplify from any of the 85 DNA templates, which included field samples, at annealing temperature range of 60 to 72°C. Because we did not have a positive control DNA template from cultured isolates of *P. lignicola* (we detected it only using culture-independent methods, see “Development of improved strategies for management of replant problems”, 2010 Comprehensive Report to ABC, Browne et al., 2010) we could

not properly test the qPCR primers and protocol developed for it. We are attempting to secure samples of a positive control for *P. lignicola*.

Further cPCR and qPCR tests were completed using dilutions of 20 DNA samples from roots of healthy and RD-affected almond trees from Firebaugh and Chico replant trials. We used the suitable qPCR primers described above in attempts to detect *T. harzianum*, *Cylindrocarpon* sp., *P. helicoides*, and *P. lignicola* in the root samples. Positive and negative control samples were used, with the exception that positive controls not available for *P. lignicola*. Both with cPCR and qPCR, we did not detect *T. harzianum*, *P. helicoides*, or *P. lignicola* in the test samples. However, we did detect *Cylindrocarpon* sp. among these samples using both cPCR (Fig. 3) and qPCR. Because of primer dimmers and resulting background fluorescence in the latter qPCR reactions, it was hard to accurately quantify the amount of target *Cylindrocarpon* sp. DNA. We are attempting to eliminate the background fluorescence problem by using sequence-specific hydrolysis probes as fluorescence indicators instead of using the non-specific SYBR green indicator.

When we tested the qPCR primers for *Cylindrocarpon* sp. using DNA extracted from soil from the RD-affected and healthy plots in the Firebaugh replant trial, no amplification resulted, although a positive control reaction using general ITS304F and ITS4 primers for fungi yielded a DNA product, suggesting the PCR inhibition was not preventing function of the qPCR primers. Further testing is needed to determine whether *Cylindrocarpon* sp. can be detected from soil.

It is likely that incorporation of specific hydrolysis probes will improve sensitivity of our qPCR detection protocols for *T. harzianum*, *Cylindrocarpon* sp., *P. helicoides*, and *P. lignicola*. We are proceeding with the work required to test this hypothesis. Given that the expected improvement results, we can then further optimize and validate the qPCR detection protocols for application with root and soil samples.

Literature Cited:

Bhat, R. G., and Browne, G. T. 2009. Specific detection of *Phytophthora cactorum* in diseased strawberry plants using nested polymerase chain reaction. Plant Pathology 59:121-129.

Bilodeau, G. J., Levesque, C. A., de Cock, A. W. A. M., Duchaine, C., Briere, S., Uribe, P., Martin, F. N., and Hamelin, R. C. 2007. Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and molecular beacons. Phytopathology 97:632-642.

Browne, G.T., Connell, J.H., and Schneider, S.M. 2006. Almond replant disease and its management with alternative pre-plant soil fumigation treatments and rootstocks. Plant Disease 90:869-876.

Fu, Q., Ruegger, P., Bent, E., Chrobak, M., and Borneman, J. 2008. PRISE (PRImer SElector) software for designing sequence-selective PCR primers. Journal of Microbiological Methods. 72:263-267.

Gachon, C., Mingam, A., and Charrier, B. 2004. Real-time PCR: What relevance to plant studies? Journal of Experimental Botany 55:1445-1454.

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma, B. P. H. J. 2006. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Science 171:155-165.

Table 1. Isolates of fungi, oomycetes and *Prunus* sp. used in designing and testing primers for conventional PCR (cPCR) and quantitative PCR (qPCR)

Organism	Isolate number (=DNA No.)	No. of isolates
Alternaria sp.	5374 WA bl-B1 (10)	1
Aspergillus sp	5376 WA ri-b2 (27), 5227 a2-ri (W40)	2
Bionectria ochroleucci	5379 WA bl-ei (19)	1
Chaetomium globosum	5373 WA bl-cz (91), 5371 WA bl-h (W17)	2
Colletotrichum acutatum	Ca270 (W270)	1
Corynascus sepedonium	5371 WA bl-ez (112)	1
Cylindrocarpon sp. (=*Neonectria radicicola*)	5848 bl-o1 (1), 5370 WA bl-g (W13), 5847 bl-f2 (7), 5851 bl-m (5), 5853 bl-l (6), 5853 ri-d2 (3), 5858 ri-f (4), 5861 bl-g (8), 5861 bl-h (2), 5376 WA bl-b (34)	10
Emericellopsis minima	5377 WA ri-c1 (113)	1
Fusarium incarnatum	5380 WA ri-d1 (21), 5372 WA bl-a (36)	2
Fusarium oxysporum	5378 WA bl-j (43)	1
Fusarium solani	5374 WA ri-e (61)	1
Fusarium sp.	5229 b-ri (W42b), 5229 m-bl (W15), 5231 i-ri (W43a), 5231 w-bl (W16), 5233 o-bl (W17), 5235 a-ri (W29), 5235 m-bl (W18), 5237 n-bl (W19)	8
Fusarium tricinctum	5370 WA bl-a (14)	1
Macrophomina phaseolina	5373 WA ri-L (8)	1
Microascus sp.	5229 n ble (W53)	1
Mortierella sp.	5372 PARP ri-C (15)	1
Mucor circinelloides	5374 WA ri-b (33)	1
Nectria haematococca	5369 WA bl-I (50)	1
Penicillium sp.	5369 WA bl-e (9)	1
Petriella sp.	5230 m-ble (W55)	1
Phomopsis sp.	5380 WA ri-l (57)	1
Phytophthora cactorum	1750 (W101)	1
Prunus sp.	Lovell, Mariana root_tc, Nemaguard root_tc, Nemaguard-gh	4
Psathyella candelleana	5372 WA bl-f (38)	1
Pythium cryptoirregulare	5374 WA ri-h (37)	1
Pythium helicoides	6038A (W6038A), 6038C (W6038C)	2
Pythium irregulare	5374 PARP ri-a (25), 5229 L1-ri (W52c), 5376 PARP ri-a (W29)	3
Pythium violae	5378 PARP bl-c (76)	1
Rhizopus oryzae	5374 WA ri-a2 (90)	1
Rhizoctonia sp.	5231 u rin (W50)	1
Trichoderma harzianum (=*Hypocrea lixii*)	5373 WA bl-d (4), 5229 a1-ri (W58), 5234 j-ri (W36), 5236 d2-ri (W59), 5373 WA bl-d (4), 5374 WA ri-I (W28), 5375 WA bl-l (W2), 5379 WA ri-d (W3), 5238 h-rin (W60)	9
Verticillium dahliae	T-9 (W271)	1
Prunus sp. (cv. Mariana)	Chico samples, 3975 to 3982 (W3975 to W3982)	8
Prunus sp. (cv. Nemaguard)	Firebaugh June07 samples, 5227 to 5238 (W5227 to W5238)	12

Table 2. Primers tested for optimizing reaction conditions of conventional PCR (cPCR) and quantitative PCR (qPCR) for detection of *Hypocrea lixii*, *Neonectria radicicola*, *Phaeonectriella lignicola* and *Pythium helicoides*

Target organism	Primer name	Primer sequence (5' to 3')
Trichoderma harzianum (=*Hypocrea lixii*)	Hyplix116for	CAGTATTCTGGCGGGCATGCCTGT
	Hyplix121For	TTGTATACCCCCTCGCGGGTTTTTT
	Hyplix160For	TCTCGGCGCCTCTCGTAGGCGT
	Hyplix240for	CGCAGCCTCTCCTGCGCAGTA
	Hyplix260rev	TACTGCGCAGGAGAGGCTGCG
	Hyplix55For	TCTCTGCCCCGGGTGCGT
	Hyplix67For	TGCGTCGCAGCCCCGGACC
T. harzianum and Cylindrocarpon sp. (=*Hypocrea lixii* and *Neonectria radicicola,* respectively*)*	ITS4Long	TCCTCCGCTTATTGATATGCTTAAG
Cylindrocarpon sp. *(=Neonectria radicicola)*	Neorad106For	AGAGGACCCCAAACCCTGATT
	Neorad173for	TTGGTGTTGGGGATCGGCGAGCCT
	Neorad366Rev	AGGCTCGCCGATCCCCAACA
	Neorad370Rev	AGGCTCGCCGATCCCCAACACCAA
	Neorad375Rev	TAGATTTAGGGGACGGCGGG
	Neorad418Rev	CAGTGCGAGGTGTGCTA
	Neorad433Rev	GTGCTACTACGCAGAGGAAGCTACA
	Neorad440Rev	GCCGCGCTGTTTCCCAGT
	Neorad63For	GTTGCCTCGGCGGT
	Neorad65For	CCTATTTGTTGCCTCGGCGGTG
	Neorad85For	GTGCCTGTTCCGACAGCCCG
Phaeonectriella lignicola	Phalig268For	CTGCGTAGTGGTTA
	Phalig361Rev	AAGTTCAGCGGGTAA
Pythium helicoides	Pythel494For	GTGCGTTGGGTGCCGGTGG
	Pythel738Rev	TGACACACTCCACGCACGA

Table 3. Specificity and sensitivity of a prototype nylon membrane-based macro array, experiment 2

Genomic probe DNA on Hybond+ Nylon membrane	Annealing temp. (^{0} C)	Minimum amount of genomic DNA (ng) in probe spot required to hybridize with 50 ng of target sample DNA[b]											
		P. cactorum		*P. citricola*		*P. cinnamomi*		*P. megasperma*		Nemaguard		Lovell	
		5 min[a]	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min
P. cactorum	60	25	25	-	25	-	50	-	-	-	-	-	-
	65	25	10	-	-	-	-	-	-	-	-	-	-
	70	25	25	-	25	-	-	-	-	25	25	25	25
P. citricola	60	25	25	25	10	50	25	-	-	-	-	-	-
	65	50	25	25	5	-	25	-	50	-	-	-	-
	70	5	2	5	2	25	5	-	25	50	50	50	50
P. cinnamomi	60	-	25	-	10	50	5	-	-	-	-	-	-
	65	25	10	-	25	25	5	-	-	-	-	-	-
	70	25	10	10	5	5	2	-	25	-	-	-	-
P. megasperma	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	50	25	-	50	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
C. acutatum	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Trichoderma sp.	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Nemaguard	60	-	-	-	-	-	-	-	-	-	25	-	-
	65	-	-	-	-	-	-	-	-	-	25	-	25
	70	-	-	-	-	-	-	-	-	25	2	25	5
Lovell	60	-	-	-	-	-	-	-	-	25	25	-	25
	65	-	-	-	-	-	-	-	-	25	0.01	25	10
	70	-	-	-	-	-	-	-	-	-	25	-	25

[a] Exposure time of X-ray films to antibody-bound membranes that were covered with CDP-*Star* substrate solution.
[b] "-" indicates that no hybridization was detected under any of the conditions tested.

Table 4. Suitability and annealing temperatures of primer pairs for use in conventional PCR (cPCR) and quantitative PCR (qPCR) to amplify ITS regions of the rRNA gene from genomic DNA of target organisms

Target organism	Primer pair	Forward primer name	Reverse primer name	Expected band size (bp)	Remarks based on cPCR[b]	Optimum annealing temp (^{0} C)
Trichoderma harzianum (=*Hypocrea lixii*)	HyplixP01[a]	Hyplix240for	ITS4Long	200	Good	65 to 70
	HyplixP02	Hyplix116for	Hyplix260rev	144	Not Good	
	HyplixP03	Hyplix55For	Hyplix260rev	400	Not Good	
	HyplixP04	Hyplix67For	Hyplix260rev	387	Good	60 to 72
	HyplixP05	Hyplix121For	Hyplix260rev	320	Good	60 to 72
	HyplixP06	Hyplix160For	Hyplix260rev	280	Good	60 to 72
Cylindrocarpon sp. (=*Neonectria radicicola*)	NeoradP01	Neorad173for	ITS4Long	200	Not Good	
	NeoradP02	Neorad85For	Neorad433Rev	348	Not Good	
	NeoradP03	Neorad85For	Neorad366Rev	281	Not Good	
	NeoradP04	Neorad85For	Neorad370Rev	285	Not Good	
	NeoradP05	Neorad65For	Neorad375Rev	330	Not Good	
	NeoradP06	Neorad106For	Neorad440Rev	352	Not Good	
	NeoradP07	Neorad63For	Neorad418Rev	371	Not Good	
	NeoradP08	Neorad106For	Neorad375Rev	289	Not Good	
	NeoradP09	Neorad65For	Neorad440Rev	393	Not Good	
	NeoradP10	Neorad106For	Neorad418Rev	338	OK	60 to 72
	NeoradP11	Neorad65For	Neorad418Rev	379	Not Good	
	NeoradP12	Neorad85For	Neorad375Rev	311	Not Good	
	NeoradP13[a]	Neorad85For	Neorad440Rev	374	Good	60 to 72
	NeoradP14	Neorad173for	Neorad418Rev	98	OK	66 to 72
	NeoradP15	Neorad85For	Neorad418Rev	360	Good	60 to 72
	NeoradP16	Neorad173for	Neorad440Rev	112	Good	66 to 72
	NeoradP17	Neorad173for	Neorad375Rev	49	Not Good	
Phaeonectriella lignicola	PhaligP01	Phalig268For	Phalig361Rev	110	?	?
Pythium helicoides	PythelP01[a]	Pythel494For	Pythel738Rev	244	Good	66 to 72

[a] Primer pairs used for optimization of annealing temperature and primer concentrations in cPCR and qPCR. These primer pairs were specific for the intended target microorganisms when used in cPCR system. Because of binding of SYBR Green dye to primer dimers formed after 30 thermal cycling, sensitivity of the qPCR assay was reduced substantially.

[b] Good = A specific and robust DNA fragment was amplified only from the intended target microorganism, Not Good = Non-specific multiple DNA fragments were amplified, OK = A specific and faint DNA fragment was amplified at certain annealing temperature, ? = No DNA fragment was amplified.

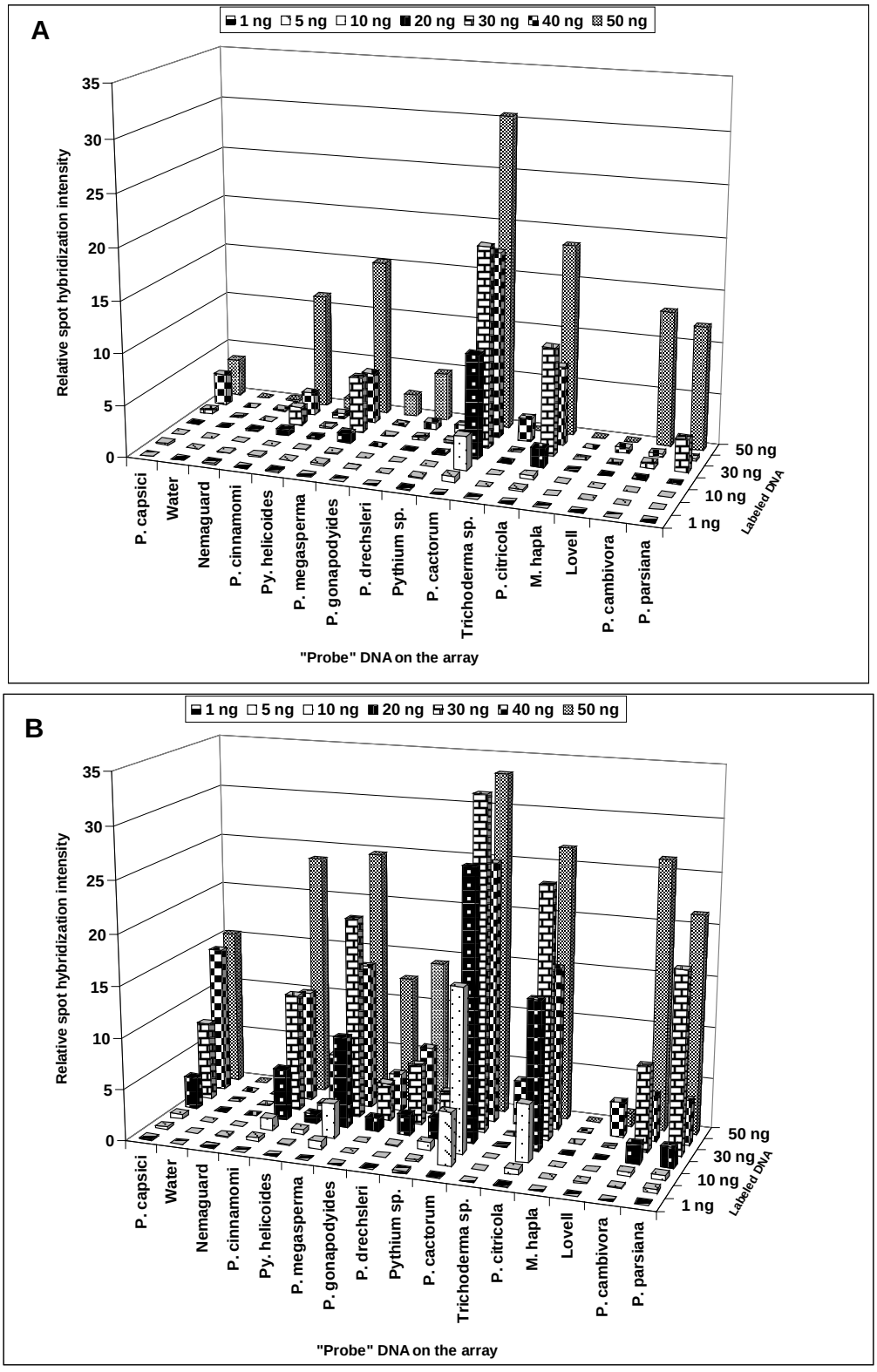

Figure 1. Relative spot intensity values of whole genomic macroarrays hybridized with different amounts of labeled *Phytophthora cactorum* (sample) DNA after exposing hybridized-arrays to films for 5 min (**A**) and 30 min (**B**), experiment 3.

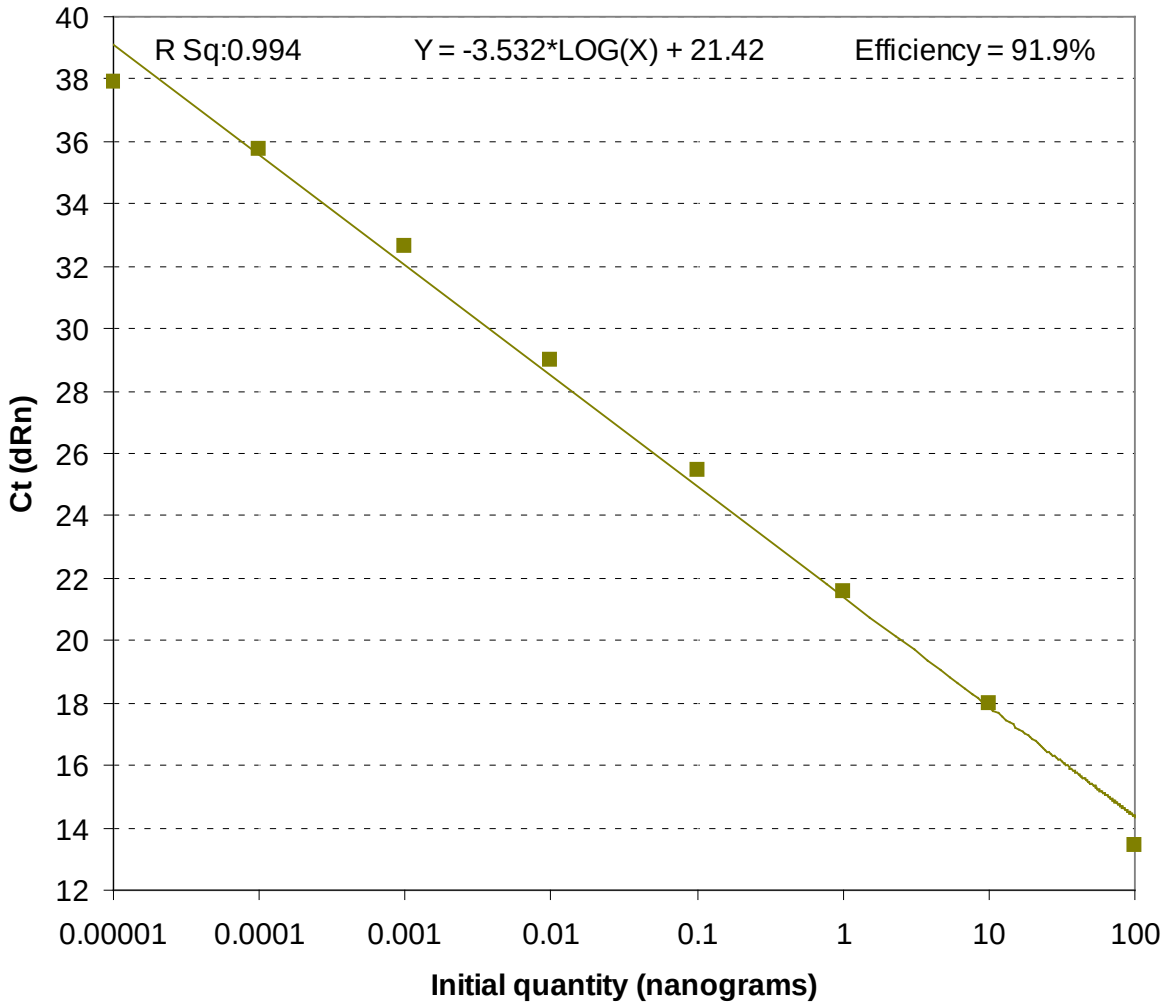

Figure 2. Standard curve for 1:10 serial dilutions of *Cylindrocarpon* sp. (=*Neonectria radicicola*) in a qPCR analysis using Neorad85For and Neorad440Rev primers and SYBR Green dye.

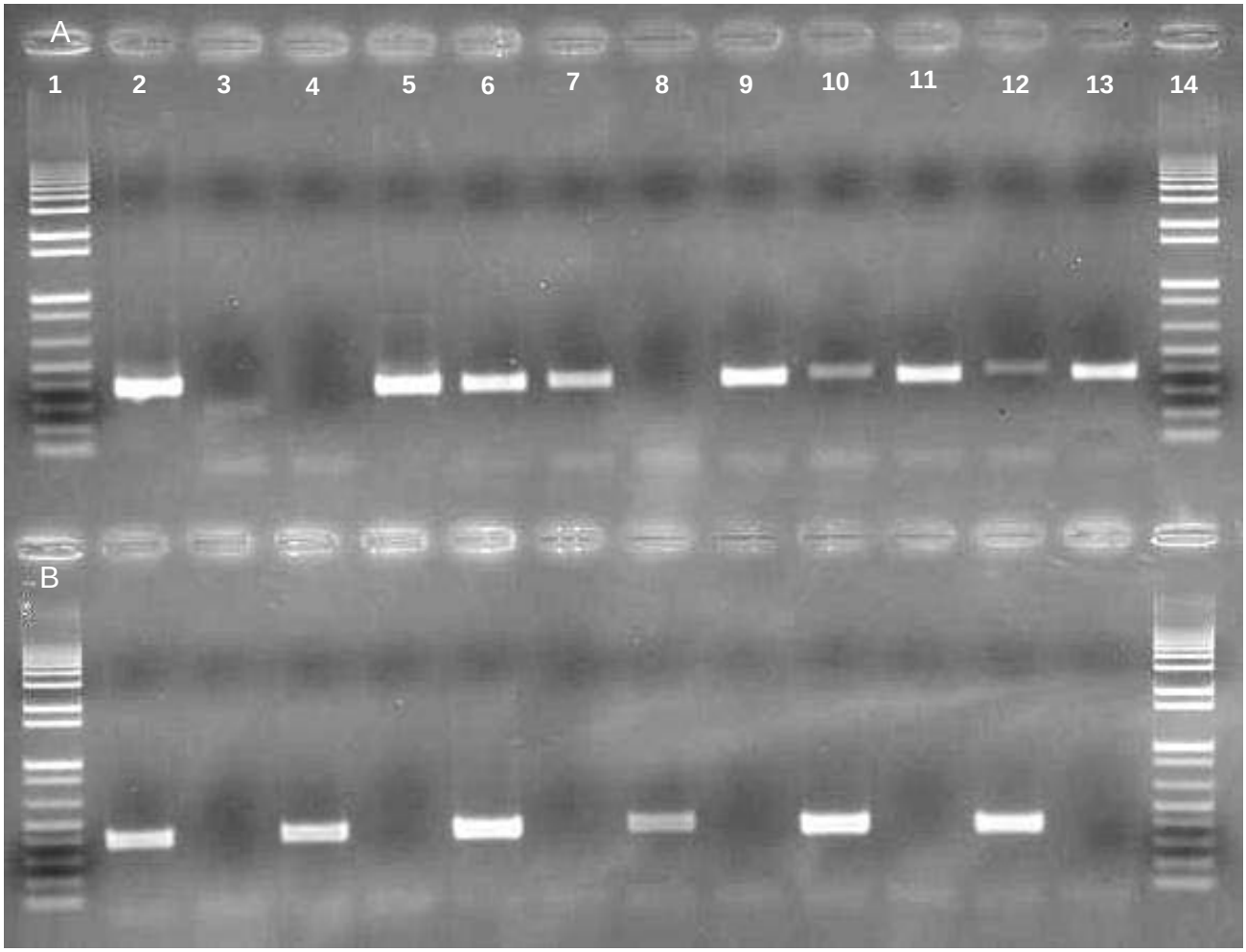

Figure 3. DNA banding patterns of conventional PCR for specific detection of *Cylindrocarpon* sp. (=*Neonectria radicicola*), using primers Neorad85For and Neorad440Rev. Lanes 1 and 14 in panels A and B, 1-kb plus DNA ladder. In the upper panel A: lane 2, DNA from *N. radicicola*; lane 3, DNA from *Trichoderma harzianum* (=*Hypocrea lixii*); lane 4, DNA from *Pythium helicoides*; lane 5, DNA from *Cylindrocarpon* sp.; lanes 6, 8, 10, and 12, DNA from roots of Marianna rootstock from chloropicrin-treated field plot in Chico; lanes 7, 9, 11, and 13, DNA from roots of Marianna rootstock from untreated field plot (control) in Chico. In the lower panel B: lanes 2, 4, 6, 8, 10, and 12, DNA from roots of Nemaguard rootstock from untreated field plot (control) in Firebaugh; lanes 3, 5, 7, 9, 11, and 13, DNA from roots of Nemaguard rootstock from chloropicrin-treated field plot in Firebaugh.