Range of Phytoplasma Concentrations in Various Plant Hosts as Determined by Competitive Polymerase Chain Reaction

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ABSTRACT

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For competitive polymerase chain reaction (PCR), an internal standard DNA template was developed that consisted of a highly conserved, internally deleted 16S rDNA fragment of an aster yellows phytoplasma. The internal standard was calibrated using a quantified culture of *Acholeplasma laidlawii*. Serial dilutions of the internal standard and fixed amounts of target templates from infected plants were coamplified with the same primers, and the products obtained were quantified using an enzyme-linked immunosorbent assay procedure. Analysis of the data revealed that the phytoplasma concentration in the plants examined differed by a factor of about 4×10^6 . Phytoplasma concentrations of 2.2×10^8 to

1.5×10^9 cells per g of tissue were identified in periwinkles infected with various phytoplasmas. High to moderate concentrations were detected in *Malus domestica* (apple) genotypes infected with the apple proliferation phytoplasma, *Alnus glutinosa* (alder) genotypes infected with the alder yellows phytoplasma, and most aster yellows-infected *Populus* (poplar) genotypes examined. Very low phytoplasma concentrations, ranging from 370 to 34,000 cells per g of tissue, were identified in proliferation-diseased apple trees on resistant rootstocks 4551 and 4608, yellows-diseased *Quercus robur* (oak) trees, and *Carpinus betulus* (hornbeam) trees. Such low concentrations, which corresponded to about 4 to 340 cells in the reaction mixture, could only be detected and quantified by nested PCR.

Additional keyword: tobacco.

Phytoplasmas are nonculturable prokaryotes of the class Mollicutes (trivial name mycoplasmas) that cause diseases of several hundred plant species (29,42). There is evidence that phytoplasma concentration can vary greatly in infected plants. Low numbers are often observed in woody hosts. For example, electron microscopical examinations show that American elms (*Ulmus americana*) infected with the elm yellows agent have a very low phytoplasma titer (7) and that flavescentia dorée-infected grapevines have a phytoplasma concentration that is often below the detection level of microscopical methods (10). Using 4'-6-diamidino-2-phenylindole (DAPI) fluorescence microscopy, a wide range of phytoplasma concentrations, ranging from not detectable to fairly high numbers, were identified in *Malus* (apple) genotypes that were experimentally inoculated with the apple proliferation (AP) phytoplasma (20). In poplars infected with the aster yellows (AY) agents, phytoplasmas were detectable by DAPI fluorescence microscopy in *Populus alba*, rarely in *P. tremula*, and not in *P. nigra* cv. Italica. However, lower phytoplasma concentrations were detected by polymerase chain reaction (PCR) using the nested version (5). There are also examples from eucalyptus and hazel in which phytoplasma numbers were too low to be detected by one-round PCR and in which nested PCR yielded positive results (27,28). High phytoplasma concentrations are usually observed in periwinkle (*Catharanthus roseus*). However, no firm data is available on the concentration range of phytoplasmas in plant tissue, although phytoplasma concentrations may be an important factor in pathogenicity.

The method of choice to quantify nonculturable disease agents is competitive PCR. With this method, the target DNA is coamplified with internal standard (IS) DNA, which competes for primers.

Quantification is achieved by comparing the relative yield of the PCR products amplified from the target and IS. The relative amounts of each remain constant during the amplification process (3,13). As the IS must be distinguishable from the wild-type target, the competitor is either characterized by the presence or absence of a unique restriction site or differs from the target in length because of either an insertion or deletion of the intervening sequence. Competitive PCR has many applications including quantifying mRNA (3,13) and infecting viruses (32), phytopathogenic, walled bacteria (18), and fungi (30). For quantitation of extremely low amounts of target templates, competitive nested PCR is also being used (15,16).

Phytoplasma quantification by competitive PCR is uncommon. Pressacco and Firrao (34) estimated the amount of phytoplasma DNA in infected periwinkles, and Liu et al. (23) determined the concentrations of an AY phytoplasma in the aster leafhopper (*Macrostelus fascifrons*). In both of these studies, the relative amounts of target DNA were estimated by comparison with a cloned bacterial DNA fragment as the competitor. The objective of our work was to quantify phytoplasma numbers in various plants hosts for which considerable differences in phytoplasma concentration could be expected. Quantification was performed by comparing the PCR products obtained from the target template with that of the competitor, which was calibrated using a *Acholeplasma laidlawii* liquid culture.

MATERIALS AND METHODS

Sources of phytoplasmas and DNA extraction. The phytoplasmas examined included strains maintained in the greenhouse and field-collected samples. Greenhouse-kept phytoplasmas were maintained in periwinkle and included strains COL, SAY, and AV2192 of the AY phytoplasma group and strain AT of the AP phytoplasma. All strains were sampled 4 months after graft inoculation from fully symptomatic plants. Strain AT was also main-

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tained in *Nicotiana tabacum* (tobacco) cv. Samsun and was sampled at different times after inoculation. Field samples were collected from the following: (i) five 18-year-old 'Golden Delicious' apple trees on rootstocks M 11 (*Malus domestica*), 4608 (*M. sieboldii* × *M. purpurea*), and 4551 (*M. sieboldii* × *M. domestica*) graft-inoculated with the AP phytoplasma 15 years prior to sampling or naturally infected and grown on the same plot; (ii) four 3-year-old black alder (*Alnus glutinosa*) trees graft-inoculated with the AY agent 2 years prior to sampling; (iii) two 80-year-old oak (*Quercus robur*) trees naturally infected with AP-group phytoplasmas (the Munich area of Germany); (iv) one 20-year-old hornbeam (*Carpinus betulus*) tree naturally infected with an AP-group phytoplasma (southwestern Germany); (v) one 20-year-old tree each of *P. tremula* (aspens) and *P. nigra* cv. Italica (Lombardy poplar) naturally infected with AY-type phytoplasmas (southwestern Germany); (vi) two 10-year-old *P. alba* trees naturally infected with AY-type phytoplasmas (France); and (vii) two 4-year-old *P. nigra* cv. Italica trees and one 4-year-old *P. tremula* tree, all graft-inoculated with AY-type phytoplasmas 2 years prior to sampling. Berges et al. (5) provide more information on the poplar phytoplasmas and Seemüller et al. (42) provide more information on the taxonomic positions of the phytoplasmas used. As healthy controls, one apple tree and one periwinkle plant, both kept under insect-proof conditions, were included in the study.

Samples were collected from several parts of each plant. From periwinkle and tobacco, DNA was isolated from leaf midribs. From apple trees, either root phloem or leaf midribs were taken. From other trees, both stem phloem and midribs were sampled and mixed prior to extraction. Phloem was prepared as previously described (2). DNA was isolated from 1.0 g of plant tissue using a phytoplasma-enrichment procedure (1). Ethanol-precipitated DNA from each sample was dissolved in 100 µl of water.

Generation of IS. Conserved rDNA of strain SAY was amplified by PCR using primers P1/P7, which anneal to the 5' end of the 16S rRNA gene and the 5' region of the 23S rRNA gene (Fig. 1), respectively, and amplify a 1,800-base pair (bp) DNA fragment (40). The amplification product was purified by phenol-chloroform extraction, and the DNA was digested with restriction endonuclease *KpnI* (MBI Fermentas, Vilnius, Lithuania). The digestion products were resolved by agarose gel electrophoresis, which revealed the presence of the expected three fragments of 425, 475, and 900 bp (5'→3') (Fig. 1). The two distal fragments were excised from the gel, extracted using the JETsorb kit (GenoMed, Bad Oeynhausen, Germany), and ligated with T4 DNA ligase (Stratagene, La Jolla, CA) according to the protocol of the supplier. For cloning, the ligated distal fragments were reamplified using primer pair P1/tRNA (44), yielding a DNA fragment of about 1,200 bp (Fig. 1).

Cloning was carried out using standard procedures (38). The internally deleted fragment was ligated into the *EcoRV* restriction site of plasmid vector pBluescript II SK (Stratagene). Ligation efficiency was increased by preincubating the digested vector with *Taq* polymerase, polymerase buffer, and 2 mM dTTP to create 3' thymidine overhangs that are complementary to the 3' adenine overhangs generated by the polymerase during PCR amplification (26). After incubation, the ligation mixture was used to transform competent cells of *Escherichia coli* strain XL-1 Blue MRF' (Stratagene). Recombinant plasmids were prepared as described by

Sambrook et al. (38). The insert was excised with *PvuII* and its identity examined by *KpnI* digestion (39). One of the clones (AY1) containing the desired 1,200-bp insert was purified and quantified by spectrophotometry for use as an IS template in competitive PCR.

Calibration of IS. The close phytoplasma relative *A. laidlawii* (strain PG-8) was chosen according to the approach described by Hu et al. (18) to quantify the walled bacterium *Clavibacter michiganensis* subsp. *sepedonicus* in plant tissue. *A. laidlawii* was grown at 32°C for 3 days in liquid M medium (12). Ten-fold serial dilutions with five replicates of each dilution were made in M medium, and 200 µl of each dilution was plated on standard petri dishes containing agar-solidified M medium. After 3 days of incubation at 32°C, colonies were counted under the microscope. Immediately after plating, the cells from each dilution were harvested by centrifugation and genomic DNA extracted according to Doyle and Doyle (11). DNA from 1 µl of the dilutions was dissolved in 100 µl of Tris-EDTA and quantified by coelectrophoresis with dilutions of defined amounts of calf thymus DNA and with the DNA preparation after it was treated with RNase and precipitated with ethanol by spectrophotometry. The relationship between numbers of colony-forming units (CFU) and the amount of DNA obtained in the two approaches was determined by dividing the DNA quantities by the colony counts. Cell numbers were estimated by considering that the mass of genomic DNA of a single cell of *A. laidlawii*, whose chromosome size is about 1,580 kilobases (17), is approximately 1.6 fg.

To calibrate the PCR product to CFU from the *A. laidlawii* culture, competitive PCR was performed with 1 µl of a 10⁻⁵ dilution of recombinant plasmid AY1 (containing 40 pg of DNA) of internally deleted IS and 1 µl of template DNA extracted from 10-fold serial dilutions of the *A. laidlawii* culture. For amplification of the 405-bp, internally deleted IS, primer pair fU5/rU3 (5'-CGG CAA TGG AGG AAA CT-3'/5'-TTC AGC TAC TCT TTG TAA CA-3') (24) was used (Fig. 1). The corresponding fragment of *A. laidlawii*, which was about 880 bp, was amplified with primers fU5Al/rU3Al (5'-CGG CAA TGG GGG AAA CC-3'/5'-TAT CGC TTC TCT TTG TTC CA-3'), which differ from fU5/rU3 in two and six positions, respectively. Amplification was conducted as described (24), except that the amounts of polymerase, polymerase buffer, and dNTPs were duplicated. PCR was repeated four times for estimation of an average value. In preliminary work and for rapid examination, the PCR products were compared and quantified by electrophoresis in an ethidium bromide-stained agarose gel. The competition equivalence point was determined when both products showed similar fluorescence intensity, considering that fluorescence is affected by the different sizes of the two fragments (880 versus 405 bp).

For more precise quantification, an enzyme-linked immunosorbent assay (ELISA) procedure based on protocols described by Lüneberg et al. (25) and Berg et al. (4) was used. As described above, the IS template and dilutions of the *A. laidlawii* DNA template were amplified with primer pairs fU5/rU3 and fU5Al/rU3Al, respectively. Primers fU5 and fU5Al were labeled with biotin to mediate immobilization of the amplification products on streptavidin-coated microtiter plates (Labsystems, Helsinki, Finland). After denaturation by alkali treatment, digoxigenin-labeled oligonucleotide probes U1comp (5'-TTT GCT CCC CAC GCT TTC GT-3') and U2comp (5'-CTG ATA ACC TCC ACT AT-3') were hybridized to the single-stranded immobilized PCR fragments. Since the target sequence of probe U1comp was deleted in the IS, it could hybridize only to the PCR product obtained from the *A. laidlawii* template. In contrast, due to six mismatches with the *A. laidlawii* sequence, probe U2comp hybridized only to the IS. Therefore, two sets of samples were prepared in triplicate, one of which was hybridized with probe U1comp and the other with probe U2comp. Hybrids were detected by ELISA using an alkaline phosphatase-conjugated antibody (Roche Diagnostics, Mannheim, Germany) and 4-nitrophenyl phosphate as substrate (Roche Diagnostics). Optical density was measured with a Titertek Multiscan MCC/340

Fig. 1. *KpnI* restriction map of the rDNA fragment polymerase chain reaction amplified with primer pair P1/P7. Positions of the primers used in the work are also shown.

ELISA reader (Flow Laboratories, Bioggio, Switzerland) at 405 nm. The cut-off value was determined by adding three standard deviations to the mean of the negative control.

Values obtained by competitive amplification of the 10^{-5} dilution of the IS template and the serially diluted *A. laidlawii* template were related by determining the competition equivalent point at which the ELISA readings from the IS and the *A. laidlawii* competitor are equal. This was done by plotting the $\log_{10}$ of the PCR product ratio (amount of IS product/*A. laidlawii* product) for each DNA concentration against the $\log_{10}$ of the values obtained from the DNA dilutions of the *A. laidlawii* competitor as a function of the $\log_{10}$ of the number of copies of competing template added (31). Graphically, this transformation yielded a linear plot (Fig. 2C). Interpolation on the plot for a Y value of 0 ($\log_{10} 1 = 0$) gives the number of copies corresponding to the equivalence point and, hence, to CFU from the *A. laidlawii* culture.

Phytoplasma concentrations in plants. To determine phytoplasma concentrations in infected plant tissue, 1 μ l of sample DNA and a 10-fold serial dilution of the calibrated IS DNA template were amplified by competitive PCR using primer pair fU5/rU3, which amplifies a fragment of about 880 bp from the target template. Primer fU5 was biotin-labeled when the PCR products were quantified by ELISA. For phytoplasma detection in trees, in which the phytoplasma concentrations were too low to be detected by one-round PCR, nested PCR with primer pair P1/tRNA in the first round and primer pair fU5/rU3 in the second round were employed. For nested amplification, in which the PCR parameters were the same as in the first round, 1:100 dilutions of the products of the first round were used as a template. As described above, two sets of samples were prepared. One of them was hybridized with probe U1comp and the other with probe U2comp. As probe U2comp hybridized to both the IS and target, the readings obtained with probe U1comp were subtracted from the U2comp readings in order to determine the values for the competitor alone. The competition equivalence point was determined as described above for the IS and *A. laidlawii* templates, using the IS as the competitor and sample DNA as the target template. Identities of plant-associated phytoplasmas were determined by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified DNA using *AluI* and *RsaI* restriction endonucleases that allow classification of major phylogenetic groups (39).

Detection thresholds of PCR procedures. To determine the detection thresholds of the procedures used, samples from four different plant species were selected for which the phytoplasma concentrations were previously determined by competitive PCR. From these samples, 10-fold serial dilutions were made that were amplified by direct or nested PCR. Amplification products were detected by agarose gel electrophoresis or ELISA as described above.

RESULTS

Calibration of IS. Colony counts from 10-fold serial dilutions of a 3-day-old liquid culture of *A. laidlawii* resulted in an average value of 1.17×10^9 CFU/ml. At this titer, a mean DNA concentration of 150 μ g/ml was determined by both comparative electrophoresis and spectrophotometry, resulting in a calculated mass of 128 fg of DNA per CFU. Considering a DNA mass of 1.6 fg for a single *A. laidlawii* chromosome, a CFU/cell ratio of 1:80 was calculated. This value is about in the middle of the range found in previous work in which 1 CFU was shown to be equivalent to 10 to 1,000 mycoplasma cells (36). From the CFU/ml value of the *A. laidlawii* culture and the CFU/cell ratio, an achleoplasma concentration of 9.4×10^{10} cells per ml was calculated.

To calibrate the IS, competitive amplification of a 10^{-5} dilution of the IS template together with a 10-fold serial dilution of the *A. laidlawii* competitor was performed. With decreasing amounts of *A. laidlawii* DNA, the amount of the 880-bp PCR product from *A.*

laidlawii decreased, whereas the amount of the 405-bp product from the IS increased (Fig. 2A and B). Plotting $\log_{10}$ ratios of the values for the IS and *A. laidlawii* DNA dilutions against $\log_{10}$ values obtained from the *A. laidlawii* dilutions resulted in a linear regression line (Fig. 2C). Interpolation of the plot for a Y value of 0 gave an equivalence point at $\log_{10}$ of 2.68, which corresponded to a dilution of 4.79×10^2 for the *A. laidlawii* culture. This dilution corresponded to a concentration of 1.95×10^8 cells per ml of

Fig. 2. Calibration of the internal standard (IS) by competitive polymerase chain reaction (PCR) amplification of 40 pg of the IS template and a 10-fold serial dilution of the *Acholeplasma laidlawii* culture. **A**, Electrophoretically resolved, ethidium bromide-stained PCR products from the IS template (lower band) and decreasing amounts of the competitive *A. laidlawii* template (upper band). Additional bands are due to heteroduplex formation. M = size marker, A.I. = product from the undiluted *A. laidlawii* template, and IS = product from the IS alone. At a 10^{-2} to 10^{-3} dilution of the competitor, the amount of products is similar. **B**, Quantitation of the amplification products shown in **A** by enzyme-linked immunosorbent assay (ELISA). C.r. healthy = template from healthy periwinkle. **C**, Determination of equivalence point ($\log_{10}$ of ratio IS/competitor = 0) by plotting the ELISA data shown in **B**. At a $10^{-2.68}$ dilution of the competitor, the amounts of products from the IS template and the calibrated *A. laidlawii* template are identical.

the *A. laidlawii* culture. This value differs from the concentration of the IS. Forty picograms of IS DNA, which is contained in the 10^{-5} dilution, corresponded to nearly 10^7 copies of the recombinant plasmid. Since phytoplasmas and *A. laidlawii* both possess two rRNA operons, this number corresponds to about 5×10^6 mol-

licute cells, which is equivalent to about 5×10^8 cells per ml. This value is about 2.4 times greater than the corresponding cell number of the *A. laidlawii* culture.

Phytoplasma concentrations in plants. For quantitation of phytoplasma concentrations in infected plants, the IS value based

Fig. 3. Quantitation of phytoplasma concentrations in various diseased plants by plotting the ratios of polymerase chain reaction products from the internal standard/phytoplasma template against the products from 10-fold serial dilutions of the internal standard as the competitor. **A**, Periwinkle-maintained strains COL, SAY, AT, and AV2192; **B**, samples from apple proliferation-infected 'Golden Delicious' apple trees on various rootstocks; **C**, samples from infected poplars (*Populus alba* 3 is not shown); **D**, samples from alder yellows-infected alder trees; **E**, samples from oak and hornbeam; **F**, samples from strain AT-infected tobacco, collected 3 (1), 6 (2), and 10 (3) weeks postinoculation. Table 1 provides equivalence points, phytoplasma concentrations, and more information about samples. The order of the regression lines (from left to right) corresponds to the order of the data listed in the boxed area (from top to bottom).

its calibration on *A. laidlawii* culture. It was carried out by competitive amplification of 1 µl of target template with a 10-fold serial dilution of the IS competitor. ELISA readings with the DNA template from healthy plants and other controls were usually below 0.034. The cut-off value for positive samples was defined as 0.066. Correlation coefficients of the regression lines (R^2) obtained by plotting the ratios of the values obtained from the target template and IS dilutions against the IS dilutions were mostly near 0.9 or above, indicating a high level of accuracy (Fig. 3).

In periwinkle infected with strains AT, SAY, AV2192, and COL, phytoplasma numbers differed significantly. When $\log_{10}$ ratios of values obtained from the target template and IS dilutions were plotted against the $\log_{10}$ of the IS dilutions, $\log_{10}$ equivalence points between 4.12 and 4.94 were determined (Fig. 3A). These values correspond to between 1.5×10^9 and 2.2×10^8 phytoplasma cells per 1.0 g of infected periwinkle tissue, considering that a 10^{-5} dilution of the IS corresponds to 1.95×10^8 *A. laidlawii* cells per ml and that the total volume of DNA extract per gram of tissue was 100 µl (Table 1).

Examination of samples from infected trees yielded an extremely wide range of phytoplasma concentrations. The phytoplasma concentration in the root phloem of an AP-diseased 'Golden Delicious' apple tree on rootstock M 11 was 1.0×10^8 cells per g of tissue, a value near that of periwinkle infected with strain AV2192. In the 'Golden Delicious' scion cultivar of a second apple tree on rootstock M 11, which showed witches' broom symptoms like the above tree but from which leaf midribs were examined, the phytoplasma concentration was markedly lower. Low to extremely low phytoplasma numbers, ranging from 1.7×10^4 to 6.5×10^2 cells per g of tissue, were detected in leaf midribs from three diseased trees of the same cultivar grown on experimental rootstocks 4551 and 4608 (Table 1; Fig. 3B). These trees showed no or only mild yellowing symptoms.

A wide range of phytoplasma concentrations were also identified in *Populus* spp. infected with AY-type phytoplasmas showing yellowing and decline symptoms (Table 1; Fig. 3C). Values ranged from 8.3×10^7 to 3.4×10^4 cells per g of tissue for *P. nigra* cv. Italica, whereas numbers detected in *P. alba* and *P. tremula* were between these concentrations. In *Alnus glutinosa* trees experimentally inoculated with the alder yellows phytoplasma and exhibiting mild yellowing symptoms, phytoplasma concentrations were relatively uniform and in the upper range of values found for trees (Table 1; Fig. 3D). Very low concentrations were identified in yellowish and declining trees of *Q. robur* (oak) and *Carpinus betulus* (hornbeam) (Table 1; Fig. 3E). Both oak and hornbeam were infected with AP-group phytoplasmas as determined by RFLP analysis of the PCR products obtained with primer pair fU5/rU3 (data not shown). A more detailed differentiation of the infecting agents was not possible based on the method used.

Development of phytoplasmal infection in tobacco. Three weeks after graft inoculation with the AP agent, phytoplasma concentrations in tobacco were very low, just reaching 8.9×10^2 cells per g of tissue. After this point, phytoplasma numbers increased rapidly and by the end of the sixth week a titer of 3.5×10^6 cells per g of tissue was determined. Further growth was slow, and at 10 weeks postinoculation (p.i.) the phytoplasma concentration reached 5.1×10^6 cells per g of tissue (Table 1, Fig. 3F). A similar time course has been described for other plants inoculated with AY phytoplasmas (43).

Sensitivity of PCR procedures used. By competitive one-round PCR, the lowest concentration detected was for the AY phytoplasma in *P. tremula* 28, estimated to be 4.9×10^3 cells in the reaction mixture (4.9×10^5 cells per g of tissue). For all lower concentrations, starting with AY phytoplasma in *P. nigra* cv. Italica 72, nested PCR was required for detection. The sample from *P. nigra* cv. Italica 72 was estimated to contain 340 cells in the reaction mixture. The three lowest phytoplasma concentrations detected by nested PCR ranged from about four to nine cells (Table 1).

Tests to determine detection thresholds were done by amplifying 10-fold serial dilutions of target templates from strain AT in periwinkle, strain AT in tobacco (10 weeks p.i.), AP phytoplasma in rootstock M 11, and AY phytoplasma in *P. tremula* 28, selected from the samples listed in Table 1. Results largely confirm the values obtained by competitive PCR (Table 1). The end point for detection of amplification products depended on the method used (Table 2). When products from one-round PCR were visualized by agarose gel electrophoresis, 490 to 1,000 phytoplasma cells could be detected in the reaction mixture, which contained 1 µl of sample DNA. Detection sensitivity of ELISA was similar or 10 times better than agarose gel electrophoresis. Nested PCR was 10 times more sensitive than one-round PCR followed by ELISA detection,

TABLE 1. Phytoplasma concentrations in various plant hosts as determined by competitive polymerase chain reaction^y

Phytoplasma ^z	Host plant	Log ₁₀ of equivalence point	Cells/g tissue
AY strain COL	<i>Catharanthus roseus</i> , experimentally inoculated	4.12	1.5×10^9
AY strain SAY	<i>C. roseus</i> , experimentally inoculated	4.20	1.2×10^9
AP strain AT	<i>C. roseus</i> , experimentally inoculated	4.45	6.9×10^8
AY strain AV2192	<i>C. roseus</i> , experimentally inoculated	4.94	2.2×10^8
AP phytoplasma	Apple 1, M 11 rootstock, naturally infected	5.27	1.0×10^8
AY phytoplasma	<i>Populus nigra</i> cv. Italica 12, naturally infected	5.37	8.3×10^7
ALY phytoplasma	<i>Alnus glutinosa</i> 1f/98, experimentally inoculated	5.44	7.1×10^7
ALY phytoplasma	<i>A. glutinosa</i> 4j/98, experimentally inoculated	5.71	3.8×10^7
AY phytoplasma	<i>P. alba</i> 3, naturally infected	6.08	1.6×10^7
ALY phytoplasma	<i>A. glutinosa</i> 4j/97, experimentally inoculated	6.18	1.3×10^7
AP strain AT	<i>Nicotiana tabacum</i> cv. Samsun, 10 weeks postinoculation	6.58	5.1×10^6
ALY phytoplasma	<i>A. glutinosa</i> 1f/97, experimentally inoculated	6.58	5.1×10^6
AY phytoplasma	<i>P. tremula</i> 19, naturally infected	6.71	3.8×10^6
AP strain AT	<i>N. tabacum</i> cv. Samsun, 6 weeks postinoculation	6.75	3.5×10^6
AP phytoplasma	Apple 2, 'Golden Delicious' on rootstock M 11, naturally infected	6.86	2.7×10^6
AY phytoplasma	<i>P. alba</i> 2, naturally infected	7.29	1.0×10^6
AY phytoplasma	<i>P. tremula</i> 28, experimentally inoculated	7.60	4.9×10^5
AY phytoplasma	<i>P. nigra</i> cv. Italica 72, experimentally inoculated	8.76	3.4×10^4
AP phytoplasma	Apple 3, 'Golden Delicious' on rootstock 4551, experimentally inoculated	9.07	1.7×10^4
AP-group phytoplasma	<i>Quercus robur</i> Schöng., naturally infected	9.35	8.7×10^3
AP phytoplasma	Apple 4, 'Golden Delicious' on rootstock 4608, experimentally inoculated	9.82	3.0×10^3
AP-group phytoplasma	<i>Q. robur</i> No. 2, naturally infected	10.07	1.7×10^3
AP phytoplasma	<i>N. tabacum</i> cv. Samsun, 3 weeks postinoculation	10.34	8.9×10^2
AP phytoplasma	Apple 5, 'Golden Delicious' on rootstock 4551, experimentally inoculated	10.48	6.5×10^2
AP-group phytoplasma	<i>Carpinus betulus</i> , naturally infected	10.72	3.7×10^2

^y Nested polymerase chain reaction was required for quantification of the last eight samples listed in the table.

^z AY = aster yellows; AP = apple proliferation; and ALY = alder yellows.

irrespective if the amplification products were detected by ELISA or agarose gel electrophoresis. Nested PCR allowed the detection of 5 to 69 cells in the reaction mixture. An example of comparing direct and nested PCR and detection of the amplification products by agarose gel electrophoresis and ELISA is shown in Figure 4. The detection threshold for target templates from the *A. laidlawii* culture and the internal control were in the same range as the phytoplasma samples (Table 2).

DISCUSSION

In this work, a sensitive method is described to quantify phyto- plasmas in infected plants by competitive PCR. With this proce- dure, a fixed amount of sample DNA was coamplified with a dilu- tion series of calibrated IS DNA as the competitor. This competitor consisted of a highly conserved 16S rDNA fragment. Thus, both competitor and sample target could be amplified with primer pair fU5/rU3, which is known to amplify the target sequence from templates of all phyto- plasmas (24,41). The IS was characterized by an internal deletion that enabled distinction from the wild-type target. For estimation of phytoplasma numbers in infected tissue, the competitor was calibrated using a *A. laidlawii* culture of known cell concentration. CFU and cell number values obtained from this culture were in agreement with values reported by Razin (35,36). Since phyto- plasmas and *A. laidlawii* both have two sets of rRNA operons (37), *A. laidlawii* culture cell numbers could, via IS, be directly related to phytoplasma concentrations in infected plant tissue.

Calculation of phytoplasma concentrations on the basis of the copy number of the competitor resulted in values that were about 2.4 times higher than those based on IS parameters obtained by calibration to the *A. laidlawii* culture. The difference between these two values is insignificant compared with the variance in phytoplasma numbers observed between infected plants of the same species or between different stages of disease of the same plant. The principle findings, identification of the range of phytoplasma concentrations in various plants, are not altered. The reason for the differences in the calibration of the IS is unclear. It is possible that it was more efficiently amplified than rDNA of *A. laidlawii*.

Amplification of sample DNA with primer pair fU5/rU3 yielded a fragment that was more than twice the size of the IS product. However, as the elongation time was adjusted for amplification of the larger target fragment, there is no indication of a negative effect on the amplification efficiency of the two fragments, as shown in other work with differently sized targets (13,14,18).

TABLE 2. Detection thresholds of one-round and nested polymerase chain reactions (PCR) in phytoplasma detection, depending on the amplicon detection method

Template ^w	Detection thresholds (cells in reaction mixture)		
	One-round PCR		Nested PCR
	Agarose gel	ELISA ^x	Agarose gel/ELISA
Strain AT in periwinkle ^y	690	690	69
AP phytoplasma/ Rootstock M 11 ^y	1,000	100	10
Strain AT/ Tobacco (10 weeks p.i.) ^y	510	51	5
AY phytoplasma/ <i>Populus tremula</i> 28 ^y	490	490	49
<i>Acholeplasma laidlawii</i>	940	n.d. ^z	9
Internal standard	1,950	n.d.	20

^w AP = apple proliferation and AY = aster yellows.
^x ELISA = enzyme-linked immunosorbent assay.
^y Table 1 provides phytoplasma concentrations in an undiluted sample. p.i. = postinoculation.
^z Not done.

Also, the differences in fragment size did not affect the quantification of the PCR products, because they were measured by ELISA following a hybridization procedure that was not affected by fragment length. In contrast, quantitation of the products by electrophoresis in an ethidium bromide-stained gel depends on fragment size because of the intercalating dye and was thus only used for quick comparisons and preliminary estimations.

Previous work by electron and fluorescence microscopy, serology, nucleic acid hybridization, and standard PCR indicated that there must be a wide range of phytoplasma numbers in infected plant tissue. Our work showed that phytoplasma concentrations in the plants examined differed by a factor of 4.0×10^6 . The differing phytoplasma concentrations determined in this work by quantitative PCR agree with previous observations based on other methods. Highest concentrations were detected in periwinkles infected with various phyto- plasmas. This plant is generally considered to be a high-titer host and is, for this reason, preferentially used as phytoplasma source for molecular and other studies. Relatively high to moderately high concentrations were also identified in apple trees on *M. domestica* rootstocks, in alder, and in most poplars examined. These findings also confirm observations from previous work (5,20,21). While phytoplasma concentrations in the above plants could be quantified using one-round PCR, nested PCR was required for phytoplasma quantification in apple trees on the apomictic rootstock selections 4608 and 4551, oak, hornbeam, and one *P. nigra* cv. Italica tree. These results also agree with previous work that showed that in some *Populus* genotypes phyto- plasmas can be detected only by nested PCR (5) and that very low phytoplasma concentrations occur in apple rootstock 4551 (19,20), which may affect phytoplasma concentrations in the scion. There is also a report of very low phytoplasma numbers in oak (2). Although only one sample per

Fig. 4. Detection thresholds of apple proliferation phyto- plasmas (strain AT) in infected periwinkle by one-round and nested polymerase chain reactions (PCR). Amplification products from 10-fold serial dilutions of the target template were detected by either electrophoresis in an ethidium bromide-stained agarose gel (A, one-round PCR; B, nested PCR) or C, enzyme-linked immuno- sorbent assay. The starting concentration of the target template is shown in Table 1, and the threshold levels are shown in Table 2. M = size marker. Arrow indicates the cut-off value for samples rated as positive as described in text.

plant was examined, the fact that our data confirmed the expected differences in phytoplasma concentrations between various host plants indicates that the values obtained are not due to variability among samples. This conclusion is also supported by the finding that some groups of plants such as periwinkle, alder, and oak showed the same range on concentrations. In other cases, such as apple and tobacco, the differences can be explained by rootstock and time of inoculation, respectively.

In our work, nested PCR increased sensitivity considerably. Whereas the detection threshold of one-round PCR was approximately 500 cells, nested PCR extended the range of detection and quantification to about five cells. A progressive extension of the results obtained with nested PCR over one-round PCR gives an indication of the reliability of this method. The accuracy of our phytoplasma quantification study is also evidenced by the fact that the detection thresholds of both standard and nested PCR were largely similar, whether determined by competitive PCR or by PCR amplification of a dilution series of the calibrated target templates. Nested PCR was usually 10 to 100 times more sensitive than one-round PCR followed by agarose gel electrophoresis of the amplification products. ELISA detection increased sensitivity of one-round PCR but not of nested PCR. A higher sensitivity of PCR-ELISA than of electrophoretic detection of the amplification products following one-round PCR was also reported by Poggi Pollini et al. (33).

Pressacco and Firrao (34) estimated that the detection threshold of competitive one-round PCR is 0.3 to 3.0 ng of target DNA. Considering a chromosome size of about 1 Mb, these amounts correspond to 300 to 3000 phytoplasma cells, a range similar to that identified in our work. However, Liu et al. (23) were able to quantify with a similar method 1 to 10^7 copies of phytoplasma templates extracted from the leafhopper *Macrostes fascifrons*. Also, 1 to 250 genomes of human-pathogenic mycoplasmas could be detected by one-round PCR (4,9,45). These results may indicate that amplification of phytoplasmal DNA extracted from plants is less efficient than that of mycoplasma DNA extracted from in vitro cultures or insect and mammalian tissues, perhaps because of the presence of endogenous inhibitory substances in plant or other tissues. However, by the use of nested PCR, the sensitivity for samples derived from plants was similar to that extracted from pure cultures or animal tissues.

Although phytoplasma-host interactions are poorly understood, there is an indication that phytoplasma numbers in infected plant tissue may be an important pathological factor. It is possible that different mechanisms of pathogenicity exist in high-titer hosts and low-titer hosts. It is also conceivable that plants differing in host suitability, and thus phytoplasma titer, may respond differently to infection. For example, AP phytoplasma-infected trees on *M. domestica* and *M. silvestris* roots show relatively high pathogen titers and form witches'-brooms as a specific symptom, but sieve tubes are little affected and the trees usually do not decline. In contrast, in genotypes of *M. tschonoskii*, *M. sargentii*, and *M. kansuensis*, phytoplasma titers are extremely low and only non-specific yellowing symptoms develop. Also, severe sieve tube necrosis is observed, and grafted trees often decline (19,20). A similar situation exists in elm yellows-infected species of the genus *Ulmus*. *U. americana* and *U. rubra* are low-titer hosts that develop nonspecific symptoms and decline, whereas *U. minor* (= *U. carpinifolia*) and *U. laevis* are relatively high-titer hosts that form specific witches'-broom symptoms and are less prone to decline (7,8). Another question is whether extremely low phytoplasma populations as quantified in this work by competitive nested PCR in oak and hornbeam trees showing nonspecific yellowing symptoms are disease causing or just contamination. There are reports on the detection of low phytoplasma numbers in European hackberry and fruit trees showing nonspecific, mild, or no symptoms in which the pathological significance of the colonizing mollicutes appears to be unclear (6,22).

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