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Difficulties with conventional phytoplasma diagnostic using PCR/RFLP analyses

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Abstract

Polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis is often used for phytoplasma identification and classification. Although these techniques are very sensitive and specific, in some cases, nonspecific reactions, false positives and negatives results, as well as unusual or illegible profiles after RFLP analyses, amplification of plant host´s DNA or other difficulties occurred. Experiences with suitability of positive and negative controls integration in PCR, evaluation of critical samples and other difficulties in phytoplasma PCR/RFLP identification are reported.

Key words: DNA extraction, PCR, primers, RFLP, positive and negative controls, critical samples.

Introduction

The '*Candidatus* Phytoplasma' taxon comprises prokaryotic wall-less pathogens of the class *Mollicutes* that inhabit plant phloem and insects. Polymerase chain reaction (PCR) with primers from sequencing of randomly cloned phytoplasma DNA, from 16S rRNA, from ribosomal protein gene sequences, from SecY and Tuf genes, and from membrane associated protein genes opened paths for phytoplasma finer identification and classification. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way enabling the construction of phylogenetic trees of many micro-organisms especially in the *Mollicutes* taxon (Bertaccini, 2007). However, sequence similarity of phytoplasma with hosts plants or other micro-organisms genes, their low concentration and uneven distribution as well as presence of phenolic substances and other inhibitors, especially when the extraction is performed from woody plants can make their detection difficult. Experiences with phytoplasma detection and identification using PCR/RFLP analyses to show several difficulties and their resolution is demonstrated and discussed.

Materials and methods

The nucleic acid extraction was performed from the following phytoplasma reference strains in *Catharanthus roseus* kindly provided by A. Bertaccini: peanut witches´ broom, PnWB (16SrII-A), peach X-disease, CX (16SrIII-A), German stone fruit yellows 1, GSFY/1 (16SrX-B), German stone fruit yellows 2, GSFY/2 (16SrX-B), Moliére disease, MOL (16SrXII-A). Phytoplasma strains: aster yellows, AY (16SrI-B, host: *Callistephus chinensis*), clover phyllody, CPh (16SrI-C, host: *C. roseus*), clover yellow edge, CYE (16SrIII-B, host: *Trifolium pratense*), apple proliferation, AP (16SrX-A, host: *Malus x domestica* ´Matčino´), pear decline, PD (16SrX-C, host: *Pyrus communis*) previously identified in our lab, and tissues from 18 healthy *C. roseus* plants were also used for DNA extraction. A phenol/chloroform method, a CTAB method and commercially available kits were tested. PCR assay was carried out with different primer pairs combination. To amplify region that includes the 16S rRNA gene, the spacer region, and the start of 23S rRNA gene of the phytoplasma genome, the primer pairs P1/P7 and P1A/P7A were used in direct PCR. PCR products were diluted with sterile distilled water (1: 29) prior to amplification by nested PCR using P1A/P7A, F1/B6, R16(I)F1/R, fU2/P7, fU5/rU3, 16R758F/16R1232R, F1/R0, Pc399 /Pc1694, R16F2n/R2 and F1/B6, R162n/R2 primer pairs, respectively. Double nested PCR was carried out by several ways with subsequent primer pairs combinations: P1/P7–P1A/P7A–R16F2n/R2, P1/P7–F1/B6–R16F2n/R2, P1/P7-R16F2/R2-R16(I)F1/R1, P1/P7–F1/B6–16R758F/16R1232R, and P1/P7–F1/B6–fU5 /rU3. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al.*, 1992) in a final reaction mixture volume of 25 µl. The DNA was amplified by 35 cycles in a MJ Research thermocycler (Watertown, MA, USA). To reduce handling errors, in some cases PCR reactions were repeated up to 6 times. Approximately 200 ng of DNA of each positive PCR product from positive controls and DNA originating from 5 asymptomatic *C. roseus* plants, which revealed often positive signals in PCR, were separately digested from R16F2n/R2 amplicons. Digestions were carried out with 2.5 U of *Mse*I, *Alu*I, *Hha*I and *Rsa*I restriction enzymes. Restriction patterns obtained were compared with positive controls and with those described in the literature (Lee *et al.*, 1998) after electrophoresis through an 8% polyacrylamide gel in 1x TBE buffer followed by staining with ethidium bromide and visualization under an UV transiluminator.

Results

DNA extracted by phenol/chloroform or CTAB techniques diluted in distilled water reacted positively also

after 5 years of storage in refrigerator. DNA extracted by used-friendly and quick commercial kits showed lower concentration and also low bands intensity under UV transilluminator after one month of storage in the same conditions as above or in conditions recommended by manufacturer. Some of these positives samples did not work in PCR or produced bands of nonspecific length after one year of storage.

Primers P1/P7–R16F2n/R2 and P1A/P7A-R16F2n/R2 amplified specifically DNA of all positive controls in nested PCR. Highly specific and sensitive was also the double nested PCR using P1/P7-F1/B6-R16F2n/R2 primer combination; no product was obtained by amplification of DNA from all 18 healthy *C. roseus* plants as well as from water. RFLP profiles with*Mse*I, *Alu*I, *Hha*I and *Rsa*I were in agreement with literature (Lee *et al.*, 1998). However the DNA from 5 asymptomatic out of 18 *C. roseus* examined, give amplification with some other primers combination. False positives were obtained sporadically using primer pairs combination P1/P7-fU5/P7, P1/P7-fU5/rU3 and exceptionally P1/P7-P1A/P7A. DNA amplicons from 5 healthy *C. roseus* plants, which gave positive reactions up to 9 primer combinations, were choose for RFLP. RFLP with all four endonucleases employed showed R16F2n/R2 patterns different from those characteristic for phytoplasmas. After repeated digestion, a very weak profile corresponding to ribosomal subgroup 16SrI-B was observed in one sample, with *Mse*I. The sequencing of this amplicon (1,500 bp) confirmed no phytoplasmas (data not shown).

Discussion

Though PCR/RFLP analyses are routine techniques for phytoplasma detection and identification, their still meet some difficulties, at least with some primers: several primer pairs and their combination are recommended (Heinrich *et al.*, 2001). Moreover, in some papers, nonspecific PCR amplifications are mentioned. For example, Siddique *et al.* (2001) described after PCR amplification with P1/P6 primers besides the band of expected size, additional bands of different sizes. The same was observed with primer pairs Pc399/Pc1694, P1/U3 and M1/P7 in our analyses. According to Heinrich *et al.* (2001), some primers can induce dimers, bands of nonspecific sizes. In these cases, false positives can be expected. In our hands, nested PCR with primer combination P1/P7–16R758F/16R1232R amplified products not only from all positive controls and asymptomatic *C. roseus* plants, but also with water used as template or when only master mix and primers were used for PCR amplifications. Similar reactions were observed using P1/P7-Pc399/Pc1694 and P1/P7-fU5/rU3 primer pairs in nested PCR. In the contrary, the same DNA samples amplified for example with P1/P7-P1A/P7A, P1A/P7A-R16F2n/R2 or P1/P7-P1A/P7A-R16F2n/R2, P1/P7-F1/B6-R16F2n/R2 never reacted with DNA from healthy *C. roseus* plants or with water controls.

According to our knowledge, it seems that in the case of phytoplasma positive samples, the primers preferentially amplified phytoplasma sequence of expected sizes, exceptionally, also additional bands could be observed. In the case of DNA isolated from healthy plants, some primers can react probably with sequences of plant genome or dimers and false positives could be observed. That is one of the reasons for including DNA originating from corresponding healthy plants and also water controls in PCR assays. In some cases, no visible products were obtained not only from healthy controls, but also from phytoplasma positive samples. This could be caused by inhibitor presence. In this case, higher dilution of DNA is advised (Heinrich *et al.*, 2001). The PCR alone is not sufficient enough for phytoplasma detection. Subsequent confirmation of phytoplasma presence and its identification must be accomplished at least by RFLP analyses using at least two or more endonucleases. In the case of critical samples, different primer pair combination, RFLP with more enzymes and also sequencing should be used for elucidation of phytoplasma presence.

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