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General phytoplasma detection by a q-PCR method using mycoplasma primers

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ABSTRACT

Phytoplasmas and mycoplasmas are bacteria belonging to the class *Mollicutes*. In this study, a fine tuning of quantitative polymerase chain reaction (qPCR) with a universal mycoplasma primer pair (GPO3F/MGSO) targeting the *16S rRNA* gene was carried out on phytoplasmas. The dissociation curves of DNAs from *Catharanthus roseus* phytoplasma-infected micropropagated shoots and from phytoplasma field-infected plant samples showed a single peak at 82.5 °C (±0.5) specifically detecting phytoplasmas belonging to several ribosomal groups. Assay specificity was determined with DNA of selected bacteria: '*Candidatus* Liberibacter solanacearum', *Xylella fastidiosa*, *Ralstonia solanacearum* and *Clavibacter michiganensis*. No amplification curves were observed with any of these tested bacteria except '*Ca.* L. solanacearum' that was amplified with a melting temperature at 85 °C. Absolute quantification of phytoplasma titer was calculated using standard curves prepared from serial dilutions of plasmids containing the cloned fragment GPO3F/MGSO from European stone fruit yellows phytoplasma. Phytoplasma copy number ranged from 10^6 to 10^3 according with the sample. The sensitivity evaluated comparing plasmid serial dilutions resulted 10^{-6} for conventional PCR and 10^{-7} for qPCR. The latter method resulted therefore able to detect very low concentrations of phytoplasma in plant material.

1. Introduction

Phytoplasmas and mycoplasmas are bacteria belonging to the class *Mollicutes* (trivial name mollicutes) because they lack a rigid cell wall. They are the prokaryotes with the smallest genome size having also a low G + C content, both capable of self-replication, but phytoplasmas has relatively limited metabolic capacities [1]. Both bacteria have economic and clinical importance, and despite their phylogenetic relatedness while phytoplasmas are plant pathogens, mycoplasmas are vertebrate pathogens [2]. Phytoplasmas are associated with devastating diseases in many agricultural important crops; they are transmitted by insects, grafting, dodder (*Cuscuta* spp.) or seeds [1]. They are classified using the sequencing of the *16S rRNA* gene as '*Candidatus* Phytoplasma' species [3] and by its RFLP analyses in ribosomal groups and subgroups [4]. Since the pure axenic culture of phytoplasmas is still difficult, the development of fast and reliable molecular methods increasing their detection sensitivity is still of uttermost importance. The nested-PCR method, using primers based on conserved genes, is the most used and sensitive methodology for phytoplasma detection, but it may encounter contamination problems, and reduced sensitivity due to the presence of inhibitors in both plant materials and cultures [4]. A quantitative PCR (qPCR) approach help to overcome the above mentioned problems because the pipetting steps are reduced, gel electrophoresis visualisation is not needed, moreover the testing is considerably faster than the conventional PCR. Several group-specific qPCR assays have been developed to detect phytoplasmas belonging to a specific ribosomal group [5,6], while only a few qPCR assays, based on both *16S rRNA* [7] and *23S rRNA* genes [8], were developed for a general phytoplasma detection. A general qPCR assay, able to verify phytoplasma presence in materials in which these pathogens are at low concentrations, such as dormant cuttings and seedlings, is of great practical relevance. A SYBR Green-based qPCR assay using primers described by Botes et al. [9] able to detect mycoplasmas, acholeplasmas, mesoplasmas, hemoplasmas, spiroplasmas and ureaplasmas in a highly sensitive

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and specific manner is under development (Ramírez et al. personal communication). In this study, a qPCR with these primers was tested to verify their ability in detecting phytoplasmas belonging to several ribosomal groups.

2. Materials and methods

2.1. DNA samples

Catharanthus roseus healthy and phytoplasma-infected micropropagated shoots were used as source of DNA [10]. The following phytoplasma strains were used: '*Candidatus* Phytoplasma asteris' (primula virescence, PRIVA, 16SrI-B), '*Ca.* P. australasia' (tomato big bud, TBB, 16SrII-D), faba bean phyllody (FBPSA, 16SrII-C), '*Ca.* P. pruni' (peach X-disease, CX, 16SrIII-A), '*Ca.* P. ulmi' (elm yellows, EY, 16SrV-A), lucerne virescence (LUM, 16SrVI), '*Ca.* P. faxini' (ash yellows, ASHY, 16SrVII-A), *Pichris echioides* yellows (PEY, 16SrIX-C), '*Ca.* P. prunorum' (European stone fruit yellows, ESFY, 16SrX-B), '*Ca.* P. mali' (apple proliferation, AP-15, 16SrX-A), '*Ca.* P. pyri' (pear decline, PD, 16SrX-C), leafhopper-borne phytoplasma (BVK, 16SrXI-C), '*Ca.* P. solani' ("stolbur", STOL, 16SrXII-A), Suriname virescence (SuV, 16SrXV) (Table 1). Field-collected samples showing symptoms referable to phytoplasma presence and asymptomatic were also tested from peach (*Prunus persica*), plum (*P. domestica*) and apricot (*P. armeniaca*). Moreover, tomato (*Solanum lycopersicum*) and corn (*Zea mays*) seedlings deriving from seeds produced by phytoplasma-infected and healthy mother-plants were analysed (Table 2). Total DNA was extracted grinding with liquid nitrogen one g of tissue from each sample with pestles in porcelain mortars. The DNA was then extracted with a phenol/chloroform method [11] and resuspended in 1× TE buffer, quantified by spectrophotometer at 260 nm and diluted until 20 ng/μl.

2.2. Phytoplasma detection by qualitative analyses

The phytoplasma presence was verified by PCR using 16Sr DNA universal primers. In particular, for the micropropagated shoots, P1 (5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′) as forward [12] and P7 (5′-CGT CCT TCA TCG GCT CTT-3′) as reverse [13], generic primers for phytoplasma detection were used in direct PCR. For the field-collected samples, primer pair R16F2n (5′-GAA ACG ACT GCT AAG ACT GG-3′)/R16R2 (5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′) [14] was used in direct PCR. Instead, for seedlings a nested PCR with R16F2n/R16R2 followed by 16R758f (=M1) (5′-GTC TTT ACT GAC GCT GAG GC-3′)/16R1232r (= M2) (5′-CTT CAG CTA CCC TTT GTA AC-3′) primers [15] was used on amplicons diluted 1:30.

Further direct PCR using GPO3F as forward (5′-TGG GGA GCA AAC AGG ATT AGA TAC C-3′) and MGSO as reverse (5′-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3′) [9], primers originally designed for mycoplasmas was used for all samples.

A 25 μl reaction was prepared by mixing 2.5 μl PCR 10× Buffer,

Table 2
Results of direct PCR on field-collected samples (with primer pair R16F2n/R2) and of nested-PCR on seedlings (with primer pair M1/M2 on R16F2n/R2 amplicons); and results of qPCR (with primer GPO3F/MGSO).

Sample	PCR	qPCR	
	RFLP results	Tm (°C)	Ct values (°) (±SE)
Seedlings			
Healthy corn	–	–	–
Corn 1.1	16SrI + 16SrXII-A	–	–
Corn 1.2	16SrXII-A	82.0	28.02 (±0.41)
Corn 4.1v	16SrXII-A	–	–
Corn 4.1n	16SrI	–	–
Corn 3.1v	16SrI + 16SrXII-A	83.0	30.79 (±0.15)
Corn 4.4v	16SrI	82.0	25.86 (±0.39)
Corn 4.3v	16SrI + 16SrXII-A	–	–
Corn 4.5	16SrI	–	–
Healthy tomato	–	–	–
Tomato 9	16SrI	–	–
Tomato 11	16SrI	82.5	27.44 (±0.24)
Tomato 23	16SrI	82.5	30.01 (±0.26)
Field-collected samples			
Asymptomatic apricot	–	–	–
Apricot 1A7	16SrX-B	–	–
Apricot 1C2	16SrX-B	82.5	24.55 (±0.39)
Asymptomatic peach	–	–	–
Peach Verona 5	16SrX-B	82.5	26.24 (±0.37)
Peach Verona 8	16SrX-B	–	–
Asymptomatic plum	–	–	–
Plum Rome 4	16SrX-B	82.5	24.69 (±0.37)
Plum Rome 7	16SrX-B	–	–

–, negative.

Table 1
Results of direct PCR on phytoplasma DNAs with primer pair P1/P7 and on other bacteria; and results of qPCR with primers GPO3F/MGSO.

Phytoplasma strain and acronym	Direct PCR	qPCR	
	RFLP results	Tm (°C)	Ct values (°) (±SE)
Healthy periwinkle	–	–	–
Primula virescence - PRIVA	16SrI-B	83.0	26.22 (±0.34)
Tomato big bud – TBB	16SrII-D	82.5	23.84 (±0.23)
Faba bean phyllody – FBPSA	16SrII-C	82.5	27.26 (±0.40)
Peach X-disease -CX	16SrIII-A	–	–
Elm yellows – EY	16SrV-A	82.5	24.72 (±0.38)
Lucerne virescence - LUM	16SrVI	82.5	20.91 (±0.13)
Ash yellows – ASHY	16SrVII-A	82.5	26.07 (±0.29)
Pichris echioides yellows - PEY	16SrIX-C	–	–
European stone fruit yellows - ESFY	16SrX-B	82.5	24.52 (±0.18)
Apple proliferation - AP-15	16SrX-A	82.5	26.68 (±0.21)
Pear decline – PD	16SrX-C	82.5	24.83 (±0.01)
Leafhopper-borne – BVK	16SrXI-C	83.0	23.37 (±0.08)
"Stolbur" – STOL	16SrXII-A	82.5	22.22 (±0.01)
Suriname virescence - SuV	16SrXV	83.0	25.35 (±0.43)
Other bacteria			
Ralstonia solanacearum	Positive*	–	–
Clavibacter michiganensis subsp. *sepedonicus*	Positive*	–	–
'*Ca.* Liberibacter solanacearum'	Positive*	85.0	27.52 (±0.01)
Xylella fastidiosa subsp. *pauca strain* CoDIRO-SC	Positive*	–	–

*, RFLP not applicable; –, negative.