Improved Molecular Methods for Detection of *European Stone Fruit Yellows* (ESFY) Phytoplasmas from In Vitro Shoots of Fruit Trees

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Abstract

***Prunus* species are prone to infections by viruses and phytoplasmas, against which no effective cure exists for already infected plants in the field. Phytoplasmas infecting fruit trees can cause severe symptoms and are considered as quarantine organisms in Europe and North America. However, detection often is hampered by their irregular distribution in host plants. In the frame of phytosanitary measures a sensitive, specific and quick detection system would be highly desirable for routine detection, mainly to avoid the use of infected planting material. The development of improved strategies for the production of elite plants of phytoplasma-free stone fruit cultivars to be used for the production of certified elite propagation material was achieved. In vitro thermotherapy and meristem culture to eliminate pathogens from stone fruit plants were applied and protocols were validated for survival rates of shoots and plants and on their effectiveness for pathogen elimination. To gain time and to confirm the elimination of these pathogens from planting material by the applied in vitro treatments, improved detection methods were tested on micropropagated material soon after the treatment. The use of diverse general primers in bi-nested PCR allows to detect phytoplasmas belonging to different groups. Broad spectrum PCR are advisable for fruit tree material in micropropagation when the sanitary status of the mother plants is not known, while specific PCR primers could be employed to detect the presence of a known phytoplasma.**

INTRODUCTION

Prunus species are prone to infections by a range of pathogens, e.g. viruses and phytoplasmas, against which no effective cure exists for already infected plants in the field. They cause considerable economic losses and are therefore of major concern worldwide. The development of improved strategies for the production of elite plants of pathogen-free stone fruit cultivars includes on one hand the application of rapid, reliable, user-friendly, sensitive and cost effective methods for the detection and elimination of the major stone fruit tree viruses and phytoplasmas to be used for the production of certified elite propagation material.

An increasing presence of phytoplasma associated diseases such as leptonecrosis on Japanese plum (*Prunus salicina*) and chlorotic leaf roll on apricot (*Prunus armeniaca*) has been observed in commercial orchards in several European regions in the last twenty-five years (Giunchedi et al., 1978; Desvignes and Cornaggia, 1982; Dosba et al., 1991; Bertaccini et al., 1993; Laimer et al., 2001). The name *European Stone Fruit Yellows* (ESFY) was proposed for disorders related to these prokaryotes. The most important fruit tree phytoplasmas currently present in Europe, namely Apple Proliferation (AP), *European Stone Fruit Yellows* (ESFY) and Pear Decline (PD), are closely related (Jarausch et al., 1994; 2000; Lorenz et al., 1995; Lee et al., 1995; Kison et al., 1997).

In vitro thermotherapy and meristem culture to eliminate pathogens from stone

fruit plants were applied and protocols were validated for survival rates of shoots and plants and on their effectiveness for pathogen elimination (Laimer, 2003, Balla et al., 2002). To validate the last step PCR methods that provide the most sensitive, specific and quick phytoplasma detection system were compared. A comparison of DNA isolation procedures aimed at reducing costs and time required to achieve DNA of a suitable purity for PCR detection was carried out together with comparison of selected ribosomal primers in nested PCR procedures.

MATERIALS AND METHODS

Phytoplasma detection tests were carried out on micropropagated shoots deriving from phytoplasma infected plants of *Prunus domestica*, *P. cerasus*, *P. mahaleb*, *P. armeniaca* as well as on shoots derived from thermotherapy and in vitro micropropagation. Extractions methods involving the use of chloroform/phenol (method 1) on fresh or frozen material (Prince et al., 1993) and a silica gel (method 2) (Veronesi et al., 2001) were compared on 0.6 and 0.3 g of micropropagated tissue respectively. PCR experiments were carried out on the nucleic acid samples diluted in TE buffer to give a final concentration of 20 ng per µl for method 1 and on 12 µl of template for method 2, in total 25 µl reaction mixtures containing 200µM of each dNTP, 1.25U Taq polymerase (Polymed, Florence, Italy) and 0.4µM of primers under the conditions described by Schaff et al. (1992). Nested and bi-nested PCR reactions were performed under the same conditions and using as template the products of the previous amplification diluted 1: 30 with sterile water. Positive control samples were DNAs extracted from periwinkle plants infected by phytoplasma strains from the micropropagated collection of DiSTA (University of Bologna). PCR products were subjected to electrophoresis in a 1% agarose gel and visualised by staining with ethidium bromide and UV illumination.

Different PCR protocols were compared by using broad spectrum primer pairs such as P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995); R16F2/R2 (Lee et al., 1995); U5/U3 (Lorenz et al., 1995), 16R738f/16R1232r (=M1/M2) (Gibb et al., 1995); Pa2f/r (Heinrich et al., 2001) and group 16SrX specific primers such as R16(X)F1/R1 (Lee et al., 1995) and f01/r01 (Lorenz et al., 1995). Phytoplasma identity was always confirmed by RFLP analyses: 3 to 15 µl of PCR products (according with band intensity) were digested using the enzymes *Mse*I, *Ssp*I and *Rsa*I at 37°C for at least 16 hours following the instructions of the manufacturer (Fermentas, Lithuania). The restriction patterns were then compared with those of control strains after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide.

RESULTS

While tests performed on chloroform/phenol-extracted material yield many negative results, the silica gel extraction allows the detection of the pathogen in some of the samples even in direct PCR (Tables 1 and 2). Minor differences were observed, when different PCR protocols were compared: by using primers specifically designed on an Austrian isolate of ESFY from apricot (PA2f/r) (Heinrich et al., 2001) it was possible to verify ESFY presence in direct PCR reactions. The use of different general primers in nested and/or bi-nested PCR allows detecting the presence of phytoplasmas, further identified by RFLP analyses using selected enzymes. However in some cases aspecific products were also observed (Tables 1 and 2). No amplification was obtained from the reaction mixture devoid of nucleic acid template. The PCR results were consistently confirmed in many of the different tests performed. ESFY phytoplasmas were identified in *P. mahaleb* samples confirming data reported in in vivo material from Hungary (Varga et al., 2000). The nested PCRs revealed also the presence of elm yellows related phytoplasmas in different species. Their role in possible disease expression appears not clear, especially considering the low titer in the tissues tested. Similar problem were encountered in some samples, where aster yellows type phytoplasma-specific products were identified.

DISCUSSION

From a large amount of tests carried out we can conclude that broad spectrum PCRs are advisable for fruit trees material in micropropagation, when the sanitary status of the mother plants is not known, while specific PCR primers should be employed to detect the presence of a known phytoplasma. It appears that for general phytoplasma detection the most sensitive system is the use of at least one nested PCR in the following sequence of amplification primers P1/P7+R16F2/R2+M1/M2. Further research will verify if shorter procedure could obtain similar results. The detection methods tested were sensitive and accurate to confirm the elimination of these pathogens from planting material by the applied treatment during the propagation in vitro. One of the most important reason that make difficulties in phytoplasma detection is the fact, that *Prunus* phytoplasmas are irregularly distributed in micropropagated plantlets, as it was observed from trees in the field. After initial difficulties in interpreting the obtained results, this factor was taken into account at the sampling step, and detection results became more reliable. Further research will evaluate the importance and the possible pathogenic or non-pathogenic role of phytoplasmas not typical of fruit trees encountered in some samples, since they were already reported on in vivo material (Lee et al., 1995; 2002).

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Tables

Table 1. Chloroform/phenol extraction: results of direct PCR on micropropagated samples with different primer pairs (a), results of nested PCR (b) and of bi-nested PCR (c).

Sample (a)	P1/P7	PA2f/r	R16F2/R2	M1/M2	R16(X)F1/R1	U3/U5
DO 13 (1)	-	-	-	-	-	a
DO 13 (3)	-	-	-	-	-	a
DO 31 (52-G)	-	-	-	-	-	a
DO 30 (16)	-	-	-	-	-	a
DO 30 (8)	-	-	-	-	-	a
DO 30 (9)	-	-	-	-	-	a

(b)	R16F2/R2 on P1/P7	M1/M2 on P1/P7	M1/M2 on PA2f/r	U3/U5 on P1/P7	R16(X)F1/R1 on P1/P7
DO 13 (1)	-	-	-	-	-
DO 13 (3)	-	-	-	W	-
DO 31 (52-G)	-	-	-	W	-
DO 30 (16)	-	-	-	-	-
DO 30 (8)	-	-	-	-	-
DO 30 (9)	-	-		-	-

(c)	M1/M2 on R16F2/R2	*Mse*I on M1/M2	R16(X)F1/R1 on R16F2/R2	U3/U5 on R16F2/R2	*Mse*I on U3/U5
DO 13 (1)	-			-	
DO 13 (3)	+	?		+	I
DO 31 (52-G)	-			d	
DO 30 (16)	+	V		+	XII-A
DO 30 (8)	-			-	
DO 30 (9)	-			-	

-, negative; +, positive; w, weak amplification; V=16SrV (elm yellows phytoplasmas); I=16SrI (aster yellows phytoplasmas); XII-A=16SrXII-A (STOL, stolbur phytoplasmas); a, aspecific.

Table 2. Silicagel extraction results of bi-nested PCR on micropropagated samples with different primer pairs.

	Last nested on R16F2/R2						
Samples	M1/M2	*Mse*I	R16 (X)F1/R1	*Rsa*I	*Ssp*I	U3/U5	*Mse*I
DO 10 (1)	-		-			-	
DO 10 (2)	-		-			-	
DO 10 (3)	+	V	-			-	
DO 10 (4)	-		-			w	
DO 13 (1)	-		-			-	
DO 13 (3)	+	I	-			+	a
VC 10	+	X	+	X-B	X-B	-	
K-Prunus	+	I	-			+	I
DO 31 (19-52G)	+	V	+	a		-	
DO 31 (20-2)	-		-			-	
DO 31 (21-2)	-		-			-	
DO 30 (15)	-		-			-	
VC 9 (1)	+	a	-			-	
VC 9 (2)	+	X	+	X-A	X-A	+	a
K Malus	-		-			-	
VC 12 (5)	+	V	-			w	a
VC 12 (37)	-		-			-	
VC 12 (12)	+	V	-			+	a

-, negative; +, positive; w, weak reaction; a, aspecific amplification; X-A=16SrX-A (AP, apple proliferation phytoplasmas); X-B=16SrX-B (ESFY, European stone fruit phytoplasmas); X=16SrX (apple proliferation phytoplasmas); V=16SrV (EY, elm yellows phytoplasmas); I=16SrI (aster yellows phytoplasmas).