PRELIMINARY EVALUATION OF ANTIMICROBIAL ACTIVITY OF SOME CHEMICALS ON *IN VITRO* APPLE SHOOTS INFECTED BY '*CANDIDATUS* PHYTOPLASMA MALI'

M. ALDAGHI[1,2], S. MASSART[1], Ph. DRUART[3], A. BERTACCINI[4], M.H. JIJAKLI[1] and P. LEPOIVRE[1,*]

[1] Plant Pathology Unit, Gembloux Agricultural University (FUSAGx)
Passage des Déportés 2, BE-5030 Gembloux, Belgium
[2] Plant Diseases Department, Plant Pests and Diseases Research Institute (PPDRI)
P.O. Box 19395-1454, Tehran, Iran
[3] Department of Biotechnology, Wallon Agricultural Research Centre (CRA-W)
Chaussée de Charleroi 234, BE-5030 Gembloux, Belgium
[4] Department of Agro-environmental Science and Technology, Plant Pathology
Alma Mater Studiorum, University of Bologna, Bologna, Italy
*E-mail: lepoivre.p@fsagx.ac.be

SUMMARY

Phytoplasmas are associated with several hundred plant diseases worldwide, including numerous ones with important economical impact. Control of epidemic outbreak of phytoplasma diseases can be theoretically carried out by antibiotics. However, they are expensive, not allowed or prohibited in several countries, and even not always efficient. Presently, effective but safe antimicrobial agents are needed to control severe phytoplasma diseases in field. The aim of the present study was to evaluate the susceptibility of '*Candidatus* Phytoplasma mali' to several chemical or synthetic antimicrobial agents. We tested nisin, esculetin, pyrithione and chloramphenicol as molecules having different target activities against micro-organisms. Because of their antimicrobial properties against fungi and bacteria, 4 phyto-essential oils (carvacrol, eugenol, terpineol, α-pinene) had also been tested. The activity of these molecules was compared with two antibiotics (tetracycline and enrofloxacin) used as control products. All these compounds were tested in *in vitro* culture of apples (MM106) infected by '*Ca.* P. mali'. All compounds were added to the proliferation medium (modified MS) after autoclaving at 3 concentrations (100, 500, 1,000 ppm), except nisin and pyrithione which were tested at 10, 100 and 500 ppm. Phytoplasma infection was quantified in plant materials by real-time PCR before their transfer and after one or two months of culture in the presence of antimicrobial agents. Primary results showed that phytoplasma were not detectable after one and two months in the presence of pyrithione (at 10 and 100 ppm). Moreover, some other products reduced the concentration of phytoplasma after two months. Shoots died or withered on media enriched with essential oils; that made them impossible to assess, especially when they were used at concentration of 500 and 1,000 ppm.

Key words: '*Candidatus* Phytoplasma mali', *in vitro* tissue culture, antimicrobial product, control, quantification.

INTRODUCTION

Phytoplasmas are wall-less plant pathogenic bacteria in *Mollicutes* class. They multiply in the phloem of their plant hosts, and in the haemolymph and salivary glands of their insect vectors, leafhoppers, psyllids, and cixiids. Phytoplasmas are associated with hundreds of plant diseases, several of which have world-wide agricultural significance (Blomquist *et al.*, 2001). Quarantine causal agent of Apple

Proliferation (AP) disease, '*Candidatus* Phytoplasma mali', is one of the most important applë diseases in Europe and causes considerable economic losses by decreasing size, quality and overall yield of the fruit (Frisinghelli *et al.*, 2000; Loi *et al.*, 1995). The failure in controlling this disease strongly increased the importance of finding new means of eradication or resistant genotypes.

Phytoplasmas are obligatory pathogens and, up to now, all attempts to culture them in cell-free media have failed. Hence, it has been difficult to find antimicrobial product to control them. These plant pathogens could be studied through micro-propagation of their host plant. Some phytoplasma species have been maintained in micro-propagated host plants for several years (Bertaccini *et al.*, 1992; Jarausch *et al.*, 1994, 1996; Wongkaew and Fletcher, 2004). No genetic difference was revealed between phytoplasma strains after 10 years of *in vitro* culture of *Malus pumila* apple trees (Jarausch *et al.*, 1996).

Since phytoplasmas were discovered, many methods have been tested for inhibiting them, but very few were practicable. Tetracycline antibiotics have a suppressive effect on phytoplasmas in both field-grown and tissue-cultured plants (Bradel *et al.*, 2000; Davies and Clark, 1994; Wongkaew and Fletcher, 2004), but due to the possibility of development of tetracycline resistant strains of bacteria important in human medicine, their repeated application in plants is less allowed or prohibited (Davies and Clark, 1994). Some other antimicrobial products can also suppress symptoms of phytoplasmas, but do not eliminate them. Since the growth and metabolism of *Mollicutes* are likely to be inhibited by antimicrobial molecules, this provides a possible approach for control of these pathogens in plants. Tissue culture would give us an easy and useful tool to test the efficiency of single molecules against phytoplasmas. In the present work, we describe the behaviour of AP-infected apple shoots on two proliferation media. Then, for first time we measured the effect of ten antimicrobial chemicals on phytoplasma concentration in apple shoots: nisin, esculetin, pyrithione, chloramphenicol, carvacrol, eugenol, terpineol, α-pinene, tetracycline and enrofloxacin. The first eight chemicals are tested for the first time on phytoplasmas. Nisin, as an antimicrobial peptide produced by *Lactococcus lactis*, can inhibit the growth of a wide range of gram-positive bacteria (Rollema *et al.*, 1995). Esculetin and pyrithione are antimicrobial plant-derived chemicals (Barabote *et al.*, 2003). Chloramphenicol is known as an antibiotic effective on bacteria as well as spiroplasmas (Davis, 1981). Carvacrol, eugenol, terpineol and α-pinene are plant essential oils having antimicrobial activities against phytopathogenes (fungi, bacteria) (Soto-Mendivil *et al.*, 2006; Soylu *et al.*, 2006; Vanneste and Boyd, 2002).

MATERIALS AND METHODS

Plant material, phytoplasma strains and plant micro-propagation

Apple materials infected by five phytoplasma strains (namely AP-N17, AT1-IDARED, AT1-proliferation, AT1-No.2 and AT2-SO8D) provided by Station de Quarantaine des Ligneux (Lempdes, France) were grafted on the common rootstock *Malus pumila* MM106, which is very susceptible to phytoplasma. For *in vitro* establishment, nodal segments from 4-5 month-old infected and healthy leafy shoots of MM106 apple trees were chosen. After a sterilisation treatment with formaldehyde (37%) vapour, the segments were cultured on modified MS (Murashige and Skoog, 1962) containing 0.4 mg/l thiamine, 0.05 mg/l indol-3-butyric acid, 1 mg/l 6-benzylaminopurine and 0.1 mg/l gibberellic acid, or 699 (Druart, 2003) proliferation media. Samples were

maintained from 2005 in growth chamber conditions (22°C day, 19°C night, 16h photoperiod). They were sub-cultured every 1-2 month(s), respectively, in tubes or jars, and checked from time to time for the presence of phytoplasma by molecular methods. The suitability of media, symptom expression and presence of phytoplasma at selected times were studied.

Chemotherapy of infected plants by antimicrobial products

AP-infected apple tissue cultures derived from axillary buds were treated with nisin, esculetin, pyrithione and chloramphenicol as substrates having different target activities in micro-organisms. Because of their antimicrobial properties on fungi and bacteria, 4 phyto-essential oils (carvacrol, eugenol, terpineol, α-pinene) were also tested. The activity of these products was compared to two control products (tetracycline and enrofloxacin). All compounds were added to the modified MS medium at 3 concentrations (100, 500, 1,000 ppm), except nisin and pyrithione which were tested at 10, 100 and 500 ppm. Antimicrobial products were added aseptically after autoclaving. After adding antimicrobial products, pH of all media was adjusted on 5.5. At least 10 micro shoots were transferred into medium containing each concentration-antibiotic combination. The shoots were maintained for one and two months, and then transferred to antibiotic-free medium. The phytoplasma concentration and the symptom expression were evaluated before and after antibiotic application (Table 2).

Phytoplasma detection and comparative quantification

Total DNA from *in vitro* healthy and diseased plant tissue was isolated using a crude DNA extraction method (Aldaghi *et al.*, 2008). A pooled mix of all tissues was used. For detection and quantification of phytoplasma, the AP-MGB probe was used in a '*Ca.* P. mali' optimized real-time PCR assay (Aldaghi *et al.*, 2007). The comparison of phytoplasma concentration between antimicrobial-treated and untreated samples was carried out by Ct values obtained from amplification curves. Statistical analyses were performed by the SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA). Statistical significance was tested at the $P < 0.05$ level

RESULTS

Micro-propagation, symptom expression and phytoplasma presence

Sprout and numerous shoots grew from the excised apple axillary buds within 2-4 weeks on both media. Diseased-buds developed a clump of numerous small shoots more rapidly than healthy ones. Although, no significant difference was recorded between culture media on the base of shoot growth or other morphological characteristics, MS medium was more suitable for culture maintenance and for subsequent serial transfers. Also, symptoms expression was more pronounced in jar than in tube cultures. The shoots derived from diseased explants displayed abnormal shoot proliferation, stunting, reduced leaf size and sometimes enlarged stipules as compared to the healthy ones. No shoots recovery during three years of *in vitro* maintenance of diseased-bud derived cultures was observed; moreover, the severity of symptoms expression did not change significantly after this period. The growth reduction and symptom expression indicate no less virulence of isolates had occurred after 3 years. The presence of '*Ca.* P. mali' in shoots derived from the

diseased-bud cultures was confirmed from time to time by real-time PCR. Except a few cases, the concentration of phytoplasma populations (as Ct value) was similar among subcultures. Although there is not significant difference in phytoplasma titre between strains, AP-N17 provided the lesser Ct value for the most subcultures (Table 1). This phytoplasma strain was therefore chosen for testing of antimicrobial products.

Table 1. Results of semi-quantification of phytoplasma concentration (as Ct value) in micro-propagated infected plant tissues with different strains of *'Ca. P. mali'*

Strain	Ct value (Mean of minimum 10 values)
AP-N17	23.31
AT1-IDARED	24.04
AT2-SO8D	24.08
AT1-No.2	25.18
AT1-Proliferation	25.68

Antimicrobial tests

In proliferation media supplemented even by control antibiotics, the shoots declined when chemical concentration increased.

The Ct value of samples selected for antimicrobial activity was checked before the treatments. All samples showed very similar phytoplasma concentration (Ct=23-25). Moreover, the symptom expression was very evident in all samples selected for antimicrobial tests. Esculetin and enrofloxacin in KOH, and chloramphenicol in ethanol were hardly soluble. Besides, all products except essential oils changed pH of medium, so after adding products, pH of all media was adjusted to 5.5. Despite problems in media preparation during addition of some of the tested products (solubility and pH), primary results showed that phytoplasmas were undetectable in presence of pyrithione (at 10 and 100 ppm) after one and two months treatment of shoots (Table 2). Moreover, some other products (nisin and terpineol) reduced the concentration of phytoplasma after two months (Table 2). In these cases, the symptom severity was lesser than in shoots cultured on media without supplemented molecule. The effect of the majority of essential oils was impossible to assess because shoots died or withered on treated media, especially in media supplemented with the product at concentrations 500 and 1000 ppm. After two months of antibiotic application, all alive samples from infected-bud cultures were transferred to basic media without any supply of antimicrobial products. The samples derived from pyrithione-supplemented media showed complete disappearance of symptoms, but the samples of media supplemented with other molecules started to re-express the symptoms, and their phytoplasma concentration reached to the rate of phytoplasma population in infected-control shoots sub-cultured successively on basic medium.

Table 2. Antimicrobial effect of some products on '*Ca.* P. mali'

Antimicrobial product	Ct values of infected samples						
	Control (without product)	One month after product application*			Two month after product application*		
		low	medium	high	low	medium	high
Nisin	24.60	27.16	27.01	25.61	28.15	28.33	28.38
Esculetin	23.09	25.44	25.19	26.57	25.52	-	-
Pyrithione	24.53	35.83	ND	-	35.44	ND	-
Chloramphenicol	24.37	27.24	-	-	27.51	-	-
Carvacrol	23.69	-	-	-	-	-	-
Eugenol	23.63	-	-	-	-	-	-
Terpineol	23.34	26.11	-	-	28.71	-	-
α-Pinene	23.73	26.68	26.01	-	26.26	27.12	-
Enrofloxacin	24.66	27.28**	27.78	-	31.00	-	-
Tetracycline	24.01	28.14	27.17	27.41	29.34	30.18	32.07

* Low, medium and high concentrations of all products were as 100, 500, 1,000 ppm, respectively, except nisin and pyrithione which were tested at 10, 100 and 500 ppm.

** In one shoot, phytoplasma was not detected.

\- All shoots died

ND Phytoplasma not detected.

DISCUSSION

Plant tissue culture has been previously described for long-term maintenance of plant pathogenic *Mollicutes* as well as for evaluation of possibility that phytoplasma could be eliminated from diseased plant tissues by tetracycline antibiotics. *In vivo* plant cultures has some problems for antimicrobial tests such as irregular distribution of pathogen within plant and variation of titre due to environmental or seasonal fluctuations, large quantity of antimicrobial products needed, not comparable condition and environment for plant samples, etc, so *in vitro* culture of diseased plant was privileged for antimicrobial tests. Although diseased explant cultures could be obtained with some other rootstocks and cultivars, long-term maintenance of '*Ca.* P. mali' is best achieved with rootstock MM106 (Jarausch *et al.*, 1996). Although MM106 is highly susceptible to phytoplasma *in vivo*, it remains a vigorous rootstock. In work of Kartte and Seemuller (1991), infected MM106 showed good symptom expression with high concentrations of phytoplasmas but no mortality *in vivo*.

The increasing of concentration of antimicrobial molecules had detrimental effect on shoots survival. This phytotoxic effect of the antibiotics confirmed previous observations (Wongkaew and Fletcher, 2004). Among tested products, only pyrithione was effective against phytoplasma infection. Pyrithione is a general inhibitor of membrane transport processes in fungi (Chandler and Segel, 1987). Carvacrol and eugenol at three tested concentrations, and terpineol, escluletin and chloramphenicol at 500 and 1,000 ppm were lethal for healthy and infected plants. α-pinene was no effective at all, and nisin and terpineol (at 100 ppm) could to some extent reduce the phytoplasma concentration after 2 months. Among the control products, tetracycline was effective to some extent, although enrofloxacin eliminated infection in a sample after one month. Both antibiotics used as control were reported in literature as products suppressing phytoplasma infections from *in vitro* shoots (Kaminska and Sliwa, 2003). Davis and Clark (1994) reported complete elimination of the pear decline phytoplasma from pear micro-propagated on

oxytetracycline amended medium. The effect of enrofloxacin on Mycoplasmas (Nir-Paz *et al.*, 2002) and other bacteria was also reported.

In the present work, a very sensitive method, the real-time PCR, was used to estimate the phytoplasma concentration in microshoots of apple. To our knowledge, this technique is used for the first time for assessment of antibiotic activity on phytoplasmas. End-point PCR, electron microscopy or tissue staining were previously used for estimation of phytoplasma presence in antibiotic-treated tissue cultures (Kaminska and Sliwa, 2003; Wongkaew and Fletcher, 2004).

Regarding to the problems in media preparation and also to the relative efficiency of both control products, more investigations by repeating the trials under optimized conditions to further validate the results reliability are in progress. Also, as essential oils are effective on wide range of pathogens including bacteria, testing the lower concentration of them on phytoplasmas, and also testing the products at longer periods probably result additional and perhaps useful data. If the efficiency of pyrithione is confirmed in future, it may be a hopeful means to control phytoplasmas.

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