

A PRELIMINARY SURVEY OF THE OCCURRENCE OF APPLE PROLIFERATION IN THE NORTH OF ROMANIA

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Abstract

Apple proliferation (AP), caused by the 'Candidatus Phytoplasma mali' ('Ca. P. Mali'), is considered one of the most important disease affecting apple. While different reports showed its largely spread in some European countries, very limited information about the occurrence of AP in Romanian apple orchards are available. To start to secure this missing data, a preliminary survey was performed in two apple orchards located in Bistrita area from Romania. Watching typical AP symptoms (witches' brooms, foliar reddening, dwarf fruits), twenty samples from symptomatic trees, belonging to three cultivars (Generos, Florina and Aura), were collected in autumn 2012. Serological DAS-ELISA using Bioreba monoclonal antibody, and nested-PCR using primer sets to detect AP group, were performed. ELISA results indicated that 17 out of 20 samples collected were positive, while nested-PCR revealed that all 20 samples were infected. Because plenty of trees showed similar symptoms like those sampled, this preliminary survey suggests a high incidence of AP in the two orchards. An extended study on surrounding areas of Bistrita, and then on regional and national level is necessary to provide relevant data about the AP occurrence in Romanian apple orchards and, subsequently, to recommend control measures, if will be needed.

Key words: apple proliferation, DAS-ELISA, nested-PCR, apple orchards, survey.

INTRODUCTION

Apple proliferation (AP) is considered one of the most important diseases that occur in almost all European countries, where apple is grown (Seemüller et al., 2011). Most of the apple cultivars are known to be susceptible to this disease. The causal agent of this disease is 'Candidatus Phytoplasma mali' ('Ca. P. Mali'), which belongs to the 16SrX phylogenetic group (Seemüller and Schneider, 2004). The first report of AP was made in Northern Italy (Rui et al., 1950). Apple proliferation can be transmitted by grafting and infected plant materials, but is not seed transmissible (Seidl and Komarkova, 1974).

Within the orchards, the most important natural spread of 'Ca. P. Mali' is made by insects vectors. Two *Cacopsylla* species, *C. picta* (Jarausch et al., 2003; Malagnini et al., 2010) and *C. melanoneura* (Tedeschi et al., 2002; Chireceanu and Fatu, 2012) were reported as 'Ca. P. mali' vectors. The pathogen is transmitted by both species in a persistent manner. Also, *Fieberiella florii* (Stal) was reported as vector of 'Ca. P. mali' in Germany

(Krczal, et al., 1988), and in Italy (Tedeschi and Alma, 2006).

The main symptoms of AP disease are foliar reddening, witches' brooms, enlarged stipules and small sized fruits with poor taste (Nemeth, 1986). At this time, there are a lot of possibility for detection of AP, from indexing on woody indicators and using electron microscopy (Seemüller, 1976), to serological methods (ELISA) using monoclonal antibodies (Loi et al, 2002), hybridization and molecular tests, using direct/nested-PCR and RFLP (Kison et al., 1994), or real-time PCR (Baric and Dalla-Via, 2004). Regarding the serological detection of AP group, Ploaie (2006) has showed that there is no serological difference between Apple proliferation, Pear decline and European stone fruit yellows.

The existence of AP in different orchards from Romania, based on symptomatology, was observed since 1958 (Pop, 1962; Pop et al., 1965, 1967). Along 15 years, Gheorghiu (1985) had studied the etiology, symptomatology, epidemiology and transmission of AP on two apple cvs., Jonathan and Red Delicious. During 1967-1973, Gheorghiu (1985) provided data on

AP monitoring performed in Romanian apple orchards, based on morphological symptoms. In the last thirty years there is a lack of information about AP incidence in Romanian apple orchards.

To date, there are no studies about the occurrence of AP in apple orchards from Romania based on serological and/or molecular tests. To start to secure these missing data, a preliminary survey was carried out in 2012, in two apple orchards located in Bistrita area, Romania, using serological and molecular assays.

MATERIALS AND METHODS

'Florina' cv., known to be one of the highly susceptible to AP (Loi et al. 1995) and another two Romanian cultivars, named 'Generos' and 'Aura', were the subject of this study. Twenty samples of symptomatic apple trees were collected in autumn 2012 from the three cultivars. Sampling was based on typical AP symptoms: witches' brooms, foliar reddening in late summer, enlarged stipules and dwarf of fruits (Figure 1).



Figure 1. Symptom of Apple proliferation disease: dwarf sized fruits (down) compared with healthy fruits (top) – original.

Photo 1. Symptom of Apple proliferation disease: dwarf sized fruits (down) compared with healthy fruits (top) – original.

Serological diagnoses were performed by Double Antibody Sandwich-Enzyme Linked

Immunosorbent Assay (DAS-ELISA) – (Clark and Adams, 1977), using a monoclonal antibody (Loi et. al., 2002) raised against AP phytoplasmas group, according to the manufacturer's instructions (Bioreba, Switzerland). Absorbance values were measured at 405 nm after 30 and 60 minutes, using a TECAN plate reader. Samples were considered positive if their absorbance values were more than twice those of negative control. Positive and negative controls were provided in AP kit (Bioreba), and used both in serological and molecular assay.

For molecular detection, total DNA was purified by using DNeasy Plant Mini Kit and the protocol recommended by manufacturer (Qiagen, Germany). DNA was extracted from leaf veins and phloem, which were prior grind to a fine powder under liquid nitrogen. Aliquots of DNA were then used in nested-PCR. A first round of amplification was made by using an universal primers pair P1/P7 (Deng and Hiruki 1991).

RESULTS AND DISCUSSIONS

Seventeen samples out of twenty reacted positively by DAS-ELISA, using monoclonal antibody provided by Bioreba, which specifically recognize AP (Table 1).

Nested-PCR, performed in parallel with serological detection, allowed us to detect 16SrX phytoplasmas group. All samples tested by nested-PCR reacted positively, both in the first and the second PCR round.

Consequently, "infected status" of all the 20 trees showing typical AP symptoms analyzed in the present work was confirmed in nested-PCR. However, three samples were found negative in DAS-ELISA. There is possible that the three isolates were not recognized by monoclonal antibody.

Table 1. Results of serological and molecular tests

Cultivar	Isolate code	DAS-ELISA	Nested-PCR	Status
Generos	G1	+	+	<i>infected</i>
	G2	+	+	<i>infected</i>
	G3	+	+	<i>infected</i>
	G4	+	+	<i>infected</i>
	G5	+	+	<i>infected</i>
	G6	+	+	<i>infected</i>
	G7	+	+	<i>infected</i>
	G8	+	+	<i>infected</i>

Florina	F1	-	+	<i>infected</i>
	F2	-	+	<i>infected</i>
	F3	+	+	<i>infected</i>
	F4	+	+	<i>infected</i>
	F5	+	+	<i>infected</i>
	F6	+	+	<i>infected</i>
	F7	+	+	<i>infected</i>
	F8	+	+	<i>infected</i>
Aura	A1	+	+	<i>infected</i>
	A2	+	+	<i>infected</i>
	A3	+	+	<i>infected</i>
	A4	-	+	<i>infected</i>
AP positive control		+	+	
AP negative control		-	-	

Because plenty of trees showed similar symptoms like those sampled, this preliminary survey suggests a high incidence of AP in the two orchards. An extended survey on surrounding areas of Bistrita, and then at regional and national level, focused not only on symptomatic trees, but also on asymptomatic, is necessary to provide relevant data about the AP occurrences in Romanian apple orchards. Subsequently, overall data will allow to establish control measures, knowing that this disease is included in the list of quarantine.

This work can be considered a first step in evaluation of incidence of AP in apple orchards from Romania, by using not only visual monitoring, but also serological and molecular assays.

CONCLUSIONS

The finding of AP in all twenty apple trees tested corroborated with plenty of symptomatic trees indicates a potential for a high prevalence of AP within and around the surveyed orchards. These results request an additional and exhaustive study at regional and national level. Subsequently, appropriate measures to reduce the impact could be recommended, if will be needed.

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