

THE RECOVERY PHENOMENON IN APPLE PROLIFERATION-INFECTED APPLE TREES

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SUMMARY

The presence and colonisation step of apple proliferation phytoplasma in recovered, symptomatic and never symptomatic apple trees were studied. Using serological and molecular techniques, it was demonstrated that recovered plants maintain infectivity in their roots while they lose phytoplasmas from shoots and leaves; in contrast, apple proliferation symptomatic trees are completely colonised. Several plants that showed no apple proliferation symptoms during twelve years of observation were found to be infected in their roots. Graft transmission experiments with apple proliferation phytoplasma, using both roots and buds collected from donor recovered and symptomatic trees, confirmed the results obtained by the analyses. The graft-inoculated plants did not recover and symptom severity was not influenced by the source of inoculum. To determine the health status of the apple trees as regards apple proliferation the roots must be tested.

Key words: diagnosis, phloem colonisation, phytoplasma, remission of symptoms.

INTRODUCTION

Apple proliferation (AP) is one of the most important vector-borne, graft-transmissible diseases of apple trees in Europe. The disease agent associated with it is a phytoplasma belonging to the apple proliferation (= 16SrX) group (Lee *et al.*, 1998; Seemüller *et al.*, 1998). Recently, the disease has spread epidemically in northern Italy (Loi *et al.*, 1995; Minucci *et al.*, 1996) and in Germany (Bliefernicht and Krczal, 1995), causing serious damage in several traditional apple-growing areas such as Trentino-Alto Adige (Springhetti *et al.*, 2002). Consequently, research on AP was intensified and important aspects of the disease were clarified, primarily

concerning the insect vectors. These are the psyllids *Cacopsylla costalis* (synonym *Cacopsylla picta*) and *Cacopsylla melanoneura* (Frisinghelli *et al.*, 2000; Carraro *et al.*, 2001; Tedeschi *et al.*, 2002; Jarausch *et al.*, 2003). Other points, such as the recovery phenomenon, have not been clarified up to now and have not been well investigated. Recovery, the spontaneous remission of symptoms in plants that had been symptomatically infected by a given pathogen, is also known to occur in trees infected by phytoplasmas such as AP, European stone fruit yellows, pear decline and grapevine yellows (Caudwell, 1961; Schmid, 1965; Kaminska and Zawadzka, 1973; Seemüller *et al.*, 1984; Osler *et al.*, 2000). In AP-infected trees, the development of symptoms is rather irregular. In the first year of their appearance, typical symptoms appear, but their severity varies over time (Seemüller, 1988). Symptoms remission can be transient or permanent and is influenced by host genotype and by environmental conditions. Different strains of the pathogen may also play an important role (Seemüller, 2002). The precise causes that induce recovery remain unknown.

Another important point related to recovery is the colonisation behaviour of the phytoplasmas in AP-infected trees. It is known that during winter, AP phytoplasma is almost always eliminated in the above ground parts of the trees because of the degeneration of the sieve tubes in the previous year's phloem. In spring, the upper part of the plant can be recolonised from the roots, where the phytoplasma persists throughout the whole year (Schaper and Seemüller, 1984; Seemüller *et al.*, 1984). It is known that variation in symptom expression is related to the presence/absence of the associated disease agent in the above ground part of the plants. Seemüller *et al.* (1984), using the DAPI technique, found a high percentage of AP asymptomatic trees colonised by phytoplasmas only in the roots while the symptomatic ones were also infected in the stem and trunk. Recovery and plant colonisation by phytoplasmas are important in the epidemiology of the disease. At present, it is unknown if the recovered tree can be a source of inoculum for AP transmission.

In the work reported in this paper, recovery was investigated by comparing experimental transmission of

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AP phytoplasma from recovered, symptomatic and never symptomatic apple trees. The colonisation behaviour of AP phytoplasma in the same trees was evaluated by serological and molecular diagnostic techniques.

MATERIALS AND METHODS

In 1988, an experimental apple orchard was established in an area of the Friuli-Venezia Giulia Region, northeast Italy, where serious epidemics of AP were known to occur. The orchard, containing 367 apple trees (cv Florina), was checked at least three times per year for the presence of AP symptoms. The symptoms considered were witches' broom, small leaves with enlarged stipules, bronze reddish leaves, flowers with an abnormal number of petals and pale flat fruits. The first symptoms of AP appeared in two plants during 1990; during the following years the disease spread quickly inside the orchard. The first recoveries were observed in 1992. From 1990 to 2000, 278 apple trees became symptomatically infected by AP and 196 (71%) recovered at least temporarily (Osler *et al.*, 2000). Knowing this, in 1999 a total of 30 trees were selected for analyses for the presence of AP phytoplasma. Of these, 10 plants recovered from AP for at least two years; 10 plants were symptomatically infected for at least two years and 10 never showed AP symptoms. The analyses were done in September 1999 and repeated in September 2000 on the same trees. The recovered and symptomatic trees selected in 1999 were also used for graft transmission experiments. In September 2000 other recovered, symptomatic or never symptomatic trees were chosen and analysed, repeating the analyses in September 2001.

Analyses on recovered, AP symptomatic and never symptomatic trees in the field. Roots (diam. 4-6 mm), shoots (diam. 4-6 mm) and leaves randomly collected from each recovered, AP symptomatic and never symptomatic tree were analysed for the presence of AP phytoplasma. The immunofluorescence (IF) technique was applied to roots and shoots. One cm long pieces of shoots and roots were fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline), and left overnight at 4°C. They were then cut longitudinally using a cryomicrotome (Leitz Jung 1500) to obtain sections 20-25 µm thick. The sections were treated with the AP monoclonal antibody tissue culture supernatant (Loi *et al.*, 2002) and incubated for 1 h at 37°C. After washing, FITC (fluorescein isothiocyanate) – antimouse conjugate (Sigma-Aldrich n. F1010, Milan, Italy) was added and they were incubated for 30 min at 37°C. The sections were then washed and observed under an epifluorescence microscope.

Both DAS-ELISA and PCR/RFLP were used on leaves. For DAS-ELISA, the commercial kit (BIOREBA

n. 151775, Basilea, Switzerland) was employed, following the manufacturer's instructions. For PCR/RFLP analyses, DNA was isolated from 1 g of leaf petioles and midrib tissues, following the modification of the phytoplasma enrichment procedure developed by Kirkpatrick (Malisano *et al.*, 1996). The presence of AP phytoplasma was determined using the ribosomal primers f01/r01 (Lorenz *et al.*, 1995). Five µl of the PCR products were analysed by electrophoresis in 1.5% agarose gel and visualised by staining with ethidium bromide and UV illumination; 10 µl of the PCR products were then digested with *SspI* and *BsaAI* (Lorenz *et al.*, 1995; Marcone *et al.*, 1996), following the manufacturer's instructions (Bio Laboratories, Beverly, MA, USA). Restriction fragments were resolved in 2% agarose gel.

Each selected tree was analysed twice, for two consecutive years, i.e. 1999 and 2000 or 2000 and 2001.

Graft transmission experiments using recovered and AP symptomatic trees as donor plants. Both the 10 recovered and the 10 AP symptomatic trees analysed during 1999 and 2000 were the donor plants for graft transmission experiments, using roots and buds randomly collected. One year old apple trees (cv Florina) grafted on M 9 were used as recipient plants; 100 similar trees, no graft-inoculated, were the negative controls. In March 2000, roots (0.6-1 x 6-8 cm) were collected from either recovered or symptomatic trees and grafted onto the roots of the test plants (1 graft per plant). The root-grafted test plants were transplanted into large pots (25 x 25 x 35 cm) and kept in an insect-proof greenhouse until October 2003. At least six recipient plants were graft-inoculated from each donor tree. During the following winter, the taking root of the grafts was checked, planting out temporarily all the plants.

In September 2000, buds were collected from the same donor trees used in March. They were grafted (2 buds per plant) onto the recipient potted plants. Six test plants were obtained from each donor plant. They were kept in the same way as the previously inoculated ones.

Test plants and controls were observed for AP symptom expression until October 2003. In September 2002, 20 control plants, all the asymptomatic test plants and some of the AP symptomatic ones were analysed as previously described.

RESULTS

Analyses on recovered, AP symptomatic and never symptomatic trees in the field. The results of the analyses carried out during 1999 and 2000 for the presence of AP phytoplasma on recovered, AP symptomatic and never symptomatic trees from the experimental orchard (cv Florina) are shown in Table 1. All the recovered trees were colonised by AP phytoplasma only in the

Table 1. Colonisation of apple proliferation phytoplasma in field apple trees (cv Florina). In September 1999 roots, shoots and leaves were randomly collected from 10 recovered, 10 AP symptomatic and 10 never symptomatic trees and analysed using immunofluorescence (IF), PCR and ELISA; the analyses were repeated on the same trees the following year.

Status of the trees	Year of analyses	IF on roots	IF on shoots	PCR on leaves	ELISA on leaves
Recovered	1999	10/10 ^a	0/10	0/10	0/10
	2000	10/10	0/10	0/10	0/10
Symptomatic	1999	10/10	9/10	10/10	9/10
	2000	10/10	9/10	10/10	8/10
Never symptomatic	1999	6/10	0/10	0/10	0/10
	2000	6/10	0/10	0/10	0/10

^a Nominator = number of apple proliferation positive trees; denominator = number of trees analysed.

roots; no phytoplasmas were detected in the above ground parts, shoots and leaves. The AP symptomatic plants in contrast were completely colonised. More than half (6/10) of the never symptomatic trees were colonised by AP phytoplasma in the roots. During 2000, all the trees maintained the status shown during 1999, recovered or AP symptomatic or never symptomatic, and the analyses carried out in 2000 confirmed the 1999 results.

The results of the analyses carried out during 2000 and 2001 on other recovered, AP symptomatic and never symptomatic apple trees are comparable with those obtained from the plants previously analysed. Among these plants, those that had recovered were colonised by AP phytoplasma in the roots and the symptomatic ones in roots, shoots and leaves. Five out of the never symptomatic trees (50%) were infected in the roots. During 2001, the analyses were repeated on the same trees. The results were confirmed with the exception of 3 recovered trees that changed their status from recovered (during 2000) to symptomatic (during 2001); the 3 “new symptomatic” plants were completely colonised by AP phytoplasma.

Graft transmission experiments using recovered or AP symptomatic apple trees as donor plants. The results obtained using recovered or AP symptomatic apple trees as donor plants (Table 2) showed, first of all, high transmissibility of AP phytoplasma using root grafting. In September 2000, 84% and 86% of the recipient plants inoculated by using roots, collected respectively from recovered or symptomatic trees, showed clear and severe symptoms of AP: witches’ broom and small leaves with enlarged stipules. These percentages increased to 97% and 98% up to 2002. During 2003 the situation did not change (data not shown). The minimum incubation period of AP in the test plants inoculated by root grafting was six months. The maximum incubation period was 16 months and 28 months on the test plants inoculated respectively from recovered and

Table 2. Transmission efficiency of apple proliferation (AP) phytoplasma by root or bud grafting from either recovered or AP symptomatic trees. Test plants were one year old apple trees (cv Florina) that were root-grafted in March 2000 or bud-grafted in September 2000. Symptom appearance was observed during 2000, 2001, and 2002.

Inoculum	Year	AP symptomatic plants	
		N. ^a	%
Root of recovered trees	2000	64/76 ^b	84
	2001	74/76	97
	2002	74/76	97
Root of symptomatic trees	2000	48/56 ^c	86
	2001	52/56	93
	2002	55/56	98
Buds of recovered trees	2000	0/60	0
	2001	0/60	0
	2002	0/60	0
Buds of symptomatic trees	2000	0/60	0
	2001	30/60	50
	2002	31/60	52

^a Nominator = number of AP symptomatic plants; denominator = number of grafted plants.

^b 4 out of 80 grafts failed.

^c 4 out of 60 grafts failed.

AP symptomatic trees (Table 3). The symptoms observed were always severe and diffused over the entire canopy regardless of the source of the inoculum. Recovery was never observed during the period of symptom observation (2000-2003). The analyses carried out during 2002 on roots, shoots and leaves of all the asymptomatic test plants and on 50% of the AP symptomatic trees showed complete colonisation by AP phytoplasma

Table 3. Incubation period of apple proliferation (AP) in one years old apple trees (cv Florina) infected by root or bud grafting using as donor plants either recovered or AP symptomatic trees.

Inoculum	Date of graft	Plants showing AP symptoms for the first time in					
		September 2000		July 2001		July 2002	
		N. ^a	%	N.	%	N.	%
Root of recovered trees	March 2000	64/74	86.5	10/74	13.5	0/74	0
Root of symptomatic trees	March 2000	48/55	87.3	4/55	7.3	3/55	5.5
Buds of symptomatic trees	September 2000	0/31	0	30/31	96.8	1/31	3.2

^a Nominator = number of AP symptomatic plants; denominator = number of AP infected plants.

in the symptomatic test plants and the healthy status of the asymptomatic ones.

The graft transmission experiments using buds gave different results (Table 2). None of the plants inoculated using buds collected from recovered plants showed AP symptoms. It was only possible to transmit AP phytoplasma to test plants by using buds collected from AP symptomatic donor plants, when 52% of the recipient plants reacted positively. The minimum incubation period in this case was 10 months while the maximum was 22 months (Table 3). The number of symptomatic and asymptomatic test plants did not change during 2003 and recovery was never observed. The analyses carried out in September 2002 on all the asymptomatic and on 50% of the AP symptomatic test plants confirmed the presence of AP phytoplasma in roots, shoots and leaves of the symptomatic plants. None of the controls were infected by AP.

DISCUSSION

The spontaneous remission of symptoms in plants previously symptomatically infected, a phenomenon known as recovery, was studied in apple proliferation-infected apple trees cv Florina. The precise causes that induce recovery still remain unknown, although recently Musetti *et al.* (2004) demonstrated that some components of the oxidant-scavenging system in leaves of recovered apple trees are not very active, leading to an overproduction of hydrogen peroxide. This appears to be involved in counteracting AP symptom severity.

In the present paper, other aspects related to recovery were investigated: the presence and the colonisation by phytoplasma in AP recovered trees and its transmissibility by grafting. The analyses carried out, using serological and molecular techniques on roots, shoots and leaves of recovered, AP symptomatic and never symptomatic apple trees showed that there is a complete correspondence between the presence of AP phytoplasma in

the above ground part of the apple trees and the development of symptoms. The AP symptomatic plants were colonised by the disease agent in roots, shoots and leaves. The recovered plants, on the other hand, were colonised by AP phytoplasma only in the roots. Similar results were obtained by Seemüller *et al.* (1984) using the DAPI technique as detection method. In addition, more than 50% of the trees that, during several years of observation never showed symptoms, was found to be infected in the roots. Clearly the presence of AP symptoms is correlated to the presence of the phytoplasma in the above ground part of the plants. Moreover the symptomless status of the upper part of the tree does not necessarily mean a healthy status of the entire tree. Consequently, it is possible to affirm that the presence of AP symptoms is sufficient for AP diagnosis in sensitive cultivars and the laboratory analyses can be useful in the doubtful cases. Similarly, in the absence of symptoms, constant or temporary, the diagnosis of AP must be done first on the roots of the trees.

True and complete recovery does not occur in trees because all of the recovered plants maintain the infection in the roots. Recovered plants can change their status becoming again symptomatic because the AP phytoplasma recolonises the aerial part of the plants. It is not known if recolonisation comes from the roots or from vector reinoculation or from both. In Germany, Schaper and Seemüller (1984) demonstrated that recolonisation from roots started in early Spring, as soon as newly differentiated sieve tubes were present in the apple trees.

The graft transmission experiments, using the recovered and the AP symptomatic trees as donor plants, confirmed the results obtained by analyses for the presence of AP phytoplasma. The transmission efficiency of AP phytoplasma was very high using donor roots collected from recovered trees. In contrast, no positive transmission was obtained using buds from the same plants. When donor plants were AP symptomatic apple trees, 98% and 52% respectively of the root-grafted and of the bud-grafted test plants showed symptoms

and were infected. Consequently, root grafting is more efficient than the bud grafting in transmitting AP phytoplasma.

The severity of the symptoms induced on the test plants infected by using roots of recovered trees was completely comparable to that observed in plants inoculated from symptomatic donor plants. Consequently, in the recovered plants the phytoplasma maintains its virulence. During the four years of observation, remission of symptoms was never observed in the potted test plants. Recovery therefore seems to occur only in the open field.

In conclusion, the spontaneous remission of symptoms in AP-infected plants is a common field phenomenon correlated to the absence of AP phytoplasma in the above ground part of the trees. In nature, AP-infected apple trees can be either completely colonised by AP phytoplasma, both in roots and in the upper part of the tree, in which case they are symptomatic, or infected only in the roots, in which case either the apple proliferation symptoms have disappeared in the canopy or the trees have been infected latently. We never detected apple trees infected only in the canopy and not in the roots. To correctly diagnose apple proliferation, it is necessary to test the roots using laboratory techniques or indexing.

REFERENCES

- Bliefernicht K., Krczal G., 1995. Epidemiological studies on apple proliferation disease in southern Germany. *Acta Horticulturae* **386**: 444-447.
- Carraro L., Osler R., Loi N., Ermacora P., Refatti E., 2001. Fruit tree phytoplasma diseases diffused in nature by psyllids. *Acta Horticulturae* **550**: 345-350.
- Caudwell A., 1961. Les phénomènes de rétablissements chez la flavescence dorée de la vigne. *Annales Epiphyties* **12**: 347-354.
- Frisinghelli C., Delaiti L., Grando M.S., Forti D., Vindimian M.E., 2000. *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. *Journal of Phytopathology* **148**: 425-431.
- Jarausch B., Schwind N., Jarausch W., Krczal G., 2003. First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. *Plant Disease* **87**: 101.
- Kaminska M., Zawadska B., 1973. Studies on apple proliferation in Poland. Observation on the disappearance of apple proliferation symptoms. *Acta Agrobotanica* **26**: 97-101.
- Lee I.M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M., 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* **48**: 1153-1159.
- Loi N., Carraro L., Musetti R., Firrao G., Osler R., 1995. Apple proliferation epidemics detected in scab-resistant apple trees. *Journal of Phytopathology* **143**: 581-584.
- Loi N., Ermacora P., Carraro L., Osler R., Chen T.A., 2002. Production of monoclonal antibodies against apple proliferation phytoplasma and their use in serological detection. *European Journal of Plant Pathology* **108**: 81-86.
- Lorenz K.H., Schneider B., Ahrens U., Seemüller E., 1995. Detection of apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non ribosomal DNA. *Phytopathology* **85**: 771-776.
- Malisano G., Firrao G., Locci R., 1996. 16S rDNA-derived oligonucleotide probes for the differential diagnosis of plum leptonecrosis and apple proliferation phytoplasmas. *EPPO Bulletin* **26**: 421-428.
- Marcone C., Ragozzino A., Seemüller E., 1996. European stone fruit yellows phytoplasma as the cause of peach vein enlargement and other yellows and decline diseases of stone fruits in southern Italy. *Journal of Phytopathology* **144**: 559-564.
- Minucci C., Navone P., Boccardo G., 1996. Presenza di scoppazi del melo (apple proliferation) in frutteti del Piemonte. *Informatore Fitopatologico* **46**: 47-49.
- Musetti R., Sanità di Toppi L., Ermacora P., Favali M.A., 2004. Recovery in apple trees infected with the apple proliferation phytoplasma: an ultrastructural and biochemical study. *Phytopathology* **94**: 203-208.
- Osler R., Loi N., Carraro L., Ermacora P., Refatti E., 2000. Recovery in plants affected by phytoplasmas. In: *Proceedings 5th Congress of the European Foundation for Plant Pathology, Taormina-Giardini Naxos, Italy 2000*, 589-592.
- Schaper U., Seemüller E., 1984. Recolonization of the stem of apple proliferation and pear decline-diseased trees by the causal organisms in spring. *Journal of Plant Disease and Protection* **91**: 608-613.
- Schmid G., 1965. Five and more years of observations on the proliferation virus of apples in the field. *Zastita Bilja* **16**: 285-291.
- Seemüller E., 1988. Colonization patterns of mycoplasma-like organisms in trees affected by apple proliferation and pear decline. In: Hiruki C. (ed.). *Tree mycoplasmas and mycoplasma diseases*, pp. 179-192. The University of Alberta Press, Edmonton, Alberta, Canada.
- Seemüller E., 2002. Apple proliferation: etiology, epidemiology and detection. In: Brunelli A., Canova A. (eds.). In: *Proceedings Giornate Fitopatologiche, Vol. I, Basiglio di Pinè (Trento) 2002*, 3-6.
- Seemüller E., Kunze L., Schaper U., 1984. Colonization behaviour of MLO, and symptom expression of proliferation-diseased apple trees and decline-diseased pear trees over a period of several years. *Journal of Plant Disease and Protection* **91**: 525-532.
- Seemüller E., Marcone C., Lauer U., Ragozzino A., Göschl M., 1998. Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* **80**: 3-26.

Springhetti M., Janes P.G., Dallago G., 2002. Diffusione degli scopazzi (AP) nel melo nelle valli del noce. In: Brunelli A., Canova A. (eds.). In: *Proceedings Giornate Fitopatologiche, vol. II, Baselga di Piné (Trento) 2002*, 599-606.

Tedeschi R., Bosco D., Alma A., 2002. Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in northwestern Italy. *Journal of Economic Entomology* **95**: 544-551.

Received 17 February 2004

Accepted 31 May 2004