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Oncopsis alni (Schrank) (Auchenorrhyncha: Cicadellidae) as a vector of the alder yellows phytoplasma of *Alnus glutinosa* (L.) Gaertn.

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Abstract

Alder yellows phytoplasma was detected by PCR in *Alnus glutinosa* trees in the Palatine and Mosel areas of Germany. The restriction profiles obtained by *Taq*I and *Alu*I digestion of a PCR amplified ribosomal DNA fragment from this phytoplasma and a periwinkle isolate of alder yellows from Italy (ALY) could not be distinguished while elm yellows isolates from Europe and North America led to different fragment patterns. Different restriction profiles for ALY and the German alder phytoplasma were obtained by *Tru*I digestion of a non-ribosomal DNA fragment. Phloem feeding insects were collected from infected alder trees. Phytoplasmas of the elm-yellows group were detected by PCR in psyllids and the leafhopper *Oncopsis alni*. These pathogens were indistinguishable from the phytoplasma found in alder. Only *O. alni* was able to transmit the pathogen to healthy alder seedlings. Thus, it is the first insect known to transmit this phytoplasma. This leafhopper could be responsible for the ubiquitous infection of *Alnus glutinosa* due to its close association with alder and its wide distribution in Europe.

Introduction

Common alder, *Alnus glutinosa* (L.) Gaertn. is frequently infected by alder yellows, a disease caused by phloem-inhabiting phytoplasmas (Lederer and Seemüller, 1991). Infected trees may exhibit symptoms such as yellowing, reduced foliage, or small leaves, but often they remain free from obvious symptoms. The alder yellows agent has been identified as a phytoplasma of the elm yellows group (Mäurer et al., 1993; Seemüller et al., 1994; Marcone et al., 1997a). In spite of the wide distribution of alder yellows no information was yet available about the epidemiology of this disease and disorders of other plants such as grapevine (Bianco et al., 1993; Maixner et al., 1995b), European field elm (Mäurer et al., 1993), eucalyptus (Marcone et al., 1996a) or *Spartium junceum* L. (Marcone et al., 1996b) that are also caused by elm yellows-group phytoplasmas. Only the vectors of two other diseases in Europe that are caused by members of the elm yellows phytoplasma group, rubus stunt (Mäurer and Seemüller, 1994) and flavescence dorée (Daire et al., 1993), are identified. They are *Macropsis fuscula* (Zetterstedt) (De Fluiter and Van der Meer, 1953) and *Scaphoideus titanus* Ball (Schvester et al., 1961), respectively. The distribution of the latter vector is restricted to certain southern European areas (Arzone et al., 1987) while *M. fuscula* is common all over Europe (Ribaut, 1952).

The objective of our study was to identify a vector of alder yellows. Information on the epidemiology of this disease is essential for the understanding of its almost ubiquitous distribution in *A. glutinosa* but is also necessary to assess the risk for other, particularly cultivated plants, to become infected by this phytoplasma. In this paper, we report the detection of the alder yellows phytoplasma in psyllids and leafhoppers feeding on *A. glutinosa* and the identification of the leafhopper *Oncopsis alni* (Schrank) as the first known vector of alder yellows.

Materials and methods

Plant samples

Leaf samples from 32 *A. glutinosa* trees were collected from June to September of 1996 and 1997 in the Palatine and Mosel areas of Germany. Samples were taken from trees with diameters of the trunks of at least 15 cm which usually did not exhibit any disease symptoms. Leaf samples were also taken from *A. glutinosa* seedlings after inoculation with insects in transmission trials.

Isolates of phytoplasmas maintained in periwinkle (*Catharanthus roseus* (L.) G. Don) were included in the study for comparison of the alder phytoplasma with other EY-group phytoplasmas. Strain ULW was isolated by G. Morvan (INRA, Avignon-Montfavet, France) from European field elm (*Ulmus minor* Mill. em. Richens), strain EY1 was isolated by W.A. Sinclair (Cornell University, Ithaca, NY, USA) from American elm (*U. americana* L.) (Lee et al., 1993), and strain ALY was isolated by C. Marcone (Universita de Napoli, Italy) from *A. glutinosa* in Italy (Marcone et al., 1997b). All periwinkle isolates were kindly provided by E. Seemüller, BBA, Dossenheim, Germany.

Insect samples

Insects were caught biweekly from phytoplasma-infected alder trees using a modified motorized leaf-blower. Individuals of species of interest were picked from the collection net with an aspirator and transported alive to the laboratory. They were either immediately stored in a freezer or used in transmission trials and were then frozen.

Identification of insects

Keys for the identification of psyllids and Auchenorrhyncha described by Haupt (1935), Ossianilson (1978, 1981, 1983) and Ribaut (1936, 1952) were used to identify the insects collected. Identification of Auchenorrhyncha species was based on preparations of male genitalia.

DNA isolation

DNA was extracted from 0.1 g of midribs of fresh alder or periwinkle leaves according to the procedure described by Maixner et al. (1995a). Insects were stored at $-20\,^{\circ}$C for not more than 90 days before their DNAs were extracted. DNA was extracted from single leafhoppers or from batches of three psyllids according to Maixner et al. (1995a). The pellet containing DNA of plant or insect samples was washed with 70% ethanol and resuspended in 80 µl (plants) or 40 µl (insects) of TE-buffer (10 mM TRIS, 1 mM EDTA, pH 7.6).

Primers

Three sets of primers were used. The primer pair fAY/rEY (Ahrens et al., 1994) allows the amplification of a 16S rRNA-gene fragment of approximately 300 bp from EY-group phytoplasmas. These primers were used for routine detection of elm-yellows type phytoplasmas in plants and insects. Primers P1 (Deng and Hiruki, 1991) and P7 (Schneider et al., 1995) were used for universal amplification of the 16S rRNA gene and the adjacent spacer region. Primers FD9f/r (Daire et al., 1997), derived from a cloned fragment of flavescence dorée phytoplasma, were used to amplify a 1300 bp fragment of non-ribosomal DNA from EY-group phytoplasmas.

PCR amplification

PCR was carried out in an OmniGene TR3 thermocylcer (Hybaid Ltd.) in 25 µl reactions. Reaction mixtures contained 2 µl of DNA template, 125 µM of each the four dNTPs, 0.5 µM of each primer, 0.7 U of Replitherm DNA polymerase (Biozym, Hameln, Germany) or *Taq* polymerase (MWG Biotech, Munich, Germany), and $1\times$ of the Replitherm reaction buffer for both polymerases. The concentration of $MgCl_2$ was adapted to the kind of template and primers. The $MgCl_2$ concentration was 4 mM with primers FD9f/r and 2.5 mM with plant samples or 1.25 mM with insect samples for the other primers. The mixtures were overlaid with mineral oil. The following incubations were used: FD9f/r: 40 cycles with 45 s of denaturation at 94 °C, 30 s of annealing at 51 °C, and 60 s of extension at 72 °C; P1/P7: 35 cycles with 60 s of denaturation at 94 °C, 75 s of annealing at 52 °C, and 90 s of extension at 72 °C; fAY/rEY: 35 cycles with 60 s of denaturation at 94 °C, 60 s of annealing beginning with 65 °C and decreasing to 58 °C within the first eight cycles, and 30 s of extension at 72 °C. Amplification products were electrophoresed in 1.2% horizontal agarose gels in TAE

buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.5 mg/ml ethidium bromide. DNA bands were visualized with UV light.

RFLP analyses

Amplification products obtained with primers P1/P7 or FD9f/r were digested by mixing 6 U of *Alu*I or *Taq*I or *Tru1*I (all MBI Fermentas, Vilnius, Lithuania) respectively, with 20 µl of reaction mixture according to the manufacturer's instructions. Restriction fragments were separated by electrophoresis of 15 µl of the mixtures on vertical 5% polyacrylamide gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide and the DNA fragments visualized in UV light. A 1 kb DNA ladder (BRL life technologies, Eggenstein, Germany) was used as a size standard.

Transmission trials

All insects used for transmission trials were caught from infected *A. glutinosa* in the field. Groups of psyllids or leafhoppers were fed on seedlings of *A. glutinosa* in cages containing a single plant. Psyllids were kept in groups of 20–100 individuals while the group sizes of leafhoppers varied between 5 and 20 individuals, depending on the number of insects available. The insects were kept in the cages for two weeks at 24 °C and a photoperiod of 16 h. Individuals that died during the transmission period were removed and stored at −20 °C like all other specimens after the end of the experiments. The seedlings were then sprayed with an insecticide and grown in an insect proof greenhouse. They were tested for phytoplasma infection approximately eight weeks after the end of the transmission trials and a second time four weeks later. They were then hibernated at a minimum temperature of 4 °C under natural light conditions and retested after dormancy.

Results

PCR detection of phytoplasmas in A. glutinosa

DNA was amplified with the group-specific primers fAY/rEY (Figure 1) from 19 of 22 alder trees (86%) collected at four locations of the Palatine area and from seven of ten trees (70%) collected at four sites of the Mosel valley. Most of the infected trees did not exhibit evident symptoms of phytoplasma infection. Some trees had small or slightly rolled leaves or stunted shoots. Fragments of the expected size were also amplified from reference strains EY1 and ULW.

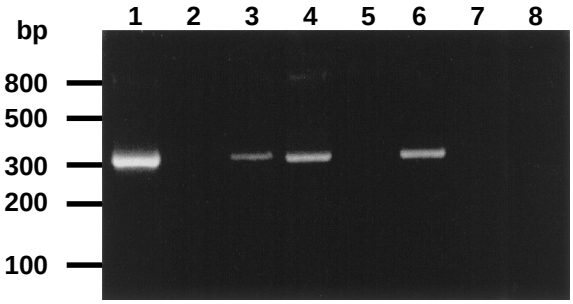

Figure 1. PCR amplification of a ribosomal DNA fragment from phytoplasmas of the elm yellows group with primers fAY/rEY. 1, *Alnus glutinosa* from the field; 2, Greenhouse grown seedling of *A. glutinosa*; 3–5, Insects caught on infected alder trees: 3, *Oncopsis alni*; 4, *Psylla* spp.; 5, *Idiocerus stigmaticalis*; 6–8, Alder seedlings inoculated with insects collected from infected alder trees: 6, Inoculated by *O. alni*; 7, Inoculated by *Psylla*; 8, Inoculated by *I. stigmaticalis*.

Homoptera found on alder

Only a few species of psyllids and Auchenorrhyncha were caught regularly on the infected alder trees. *Psylla alni* and a second psyllid, which has not yet been identified, were the most numerous Hemiptera on *A. glutinosa* throughout the season. Three species of parenchyma feeding Typhlocibinae (*Empoasca smaragdula* (Fallén), *Alnetoidea alneti* (Dahlb.), *Eupteyicyba jucunda* (Herrich-Schäffer)) as well as the xylem feeding Cercopid *Aphrophora alni* (Fall.) were not used for phytoplasma detection and transmission experiments since phytoplasmas are restricted to the phloem. Only the phloem-feeding *O. alni* and *Idiocerus stigmaticalis* Lew. could be collected in sufficient numbers for transmission trials. Another leafhopper, *Allygus mixtus* (F.), was found on alder only occasionally.

PCR detection of phytoplasmas in insects

PCR with EY-specific primers fAY/rEY led to the amplification of DNA from two groups of insects, psyllids and *O. alni* (Figure 1), while no positive results were obtained with *I. stigmaticalis* (Table 1). The positive *O. alni* were caught from June to August, while

Table 1. PCR detection of elm-yellows group phytoplasmas in insects collected from infected alder trees

Origin of insects	*Psylla* spp.			*Idiocerus stigmaticalis*			*Oncopsis alni*		
	No. tested[1]	No. positive[1]	% positive[1]	No. tested	No. positive	% positive	No. tested	No. positive	% positive
Palatine	114	42	37	7	0	0	38	3	7.9
Mosel	106	66	62	20	0	0	32	5	15.6
Total	220	108	49	27	0	0	70	8	11.4

[1]Batches of three psyllids each.

Table 2. PCR detection of elm yellows group phytoplasmas in *Psylla* spp. collected from infected alder trees. Seasonal variation of the proportion of positive samples

Collection in month	Batch samples of three psyllids			% positive insects[1]
	No. tested	No. positive	% positive	
May	40	0	0	0
June	73	20	27	10
July	84	68	81	43
August	23	20	87	49

[1]Estimation of the fraction of positive psyllids from the proportion of positive batch samples.

a substantial increase in the infestation of the psyllid populations could be observed through the season (Table 2). The proportion of positive samples, each prepared from three psyllids, increased from none in May to 87% in August. Thus, the infection of the psyllid populations estimated from this fraction of positive samples (Bhattacharyya et al., 1979) equals almost 49%.

RFLP analyses of PCR-products from plant and insect samples

Two types of restriction fragment profiles could be distinguished after digestion of amplification products obtained with primers P1/P7 with *Alu*I or *Taq*I. Reference strains ULW and EY1 showed the same profiles. They were different from the pattern obtained from all alders from the Palatine and Mosel area, from *O. alni*, and also from the Italian ALY isolate (Figure 2). A higher diversity of restriction profiles became evident by *Tru1*I digestion of non-ribosomal DNA that was amplified with primers FD9f/r (Figure 3). Four different patterns of restriction fragments could be distinguished. The American and European elm yellows strains were different from each other and from the alder phytoplasmas, but also the profiles produced from ALY and infected *A. glutinosa* from Germany were not identical.

Transmission trials

Attempts were made to inoculate seedlings of *A. glutinosa* with the alder yellows phytoplasma by psyllids and the two most common leafhoppers found on alder. Only *O. alni* was able to inoculate healthy seedlings with the phytoplasma (Table 3), although a high proportion of psyllids had been found to carry the pathogen. PCR with primers fAY/rEY led to the amplification of the target DNA (Figure 1) from samples prepared from three of 21 alder seedlings on which *O. alni* had been fed. In contrast, phytoplasmas were not detected in the test plants used to feed psyllids or *I. stigmaticalis* even ten months after the end of the transmission experiments.

The *Alu*I and *Taq*I restriction profiles of ribosomal DNA from alder seedlings inoculated by *O. alni* (Figure 2), but also *Tru1*I digestion products of non-ribosomal DNA amplified with primers FD9f/r (Figure 3) were always identical to the patterns achieved from field grown *A. glutinosa* from which the insects had been collected. The phytoplasma in naturally infected alder trees and the pathogen transmitted by *O. alni* to seedlings were indistinguishable. None of the infected seedlings developed symptoms so far.

Discussion

Most of the alder trees examined in the Palatine and the Mosel areas were infected by a phytoplasma of the elm yellows group in spite of the frequent lack of symptoms. This result is consistent with the report of Lederer and Seemüller (1991) who found almost all *A. glutinosa* infected by phytoplasmas. Since different elm yellows-group phytoplasmas are present in wild and cultivated plants in Europe (Bianco et al., 1993; Daire et al.,

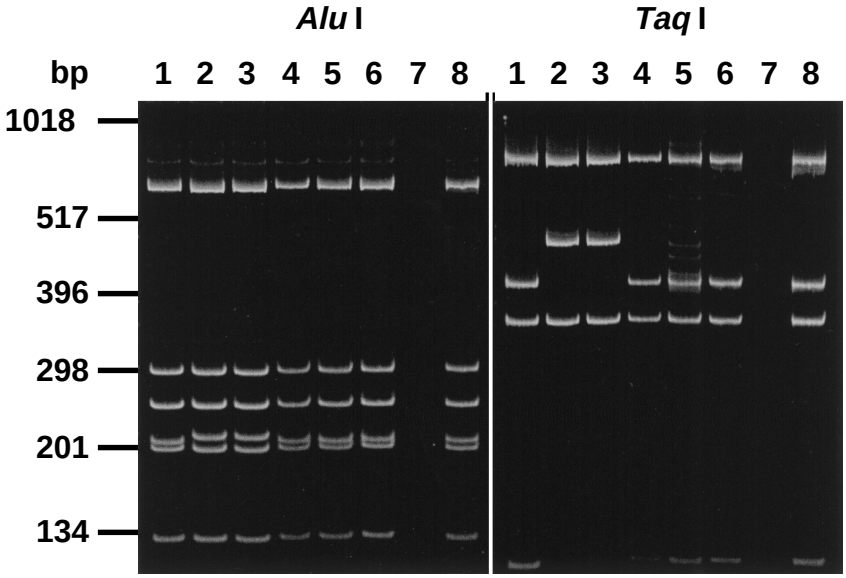

Figure 2. *Alu*I (a) and *Taq*I (b) restriction profiles of ribosomal DNA and 23S–16S intergenic spacer region from phytoplasmas that was amplified with primers P1/P7. 1, Periwinkle isolate ALY; 2, Periwinkle isolate ULW; 3, Periwinkle isolate EY1; 4, Field grown *A. glutinosa* from the Palatine area; 5, Field grown *A. glutinosa* from the Mosel valley; 6, *Oncopsis alni* collected from infected alder; 7, Greenhouse grown seedling of *A. glutinosa*, 8, Alder seedling inoculated with *O. alni*.

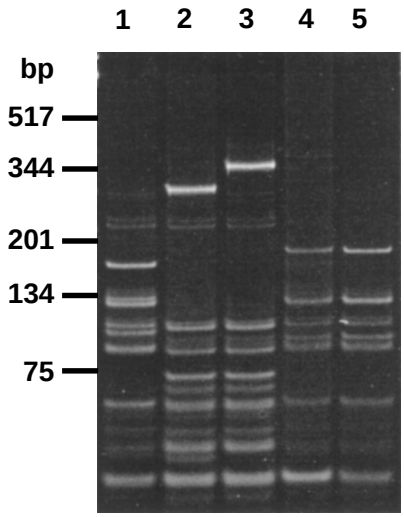

Figure 3. *Tru1*I restriction profiles of non-ribosomal DNA from phytoplasmas of the elm-yellows group that was amplified with primers FD9f/r. 1, Periwinkle isolate ALY; 2, Periwinkle isolate EY1; 3, Periwinkle isolate ULW; 4, Field-grown *A. glutinosa*; 5, Seedling of *A. glutinosa* inoculated by *O. alni*.

1993, 1997; Marcone et al., 1996a,b, 1997a; Mäurer et al., 1993; Mäurer and Seemüller, 1994) it was necessary to characterize the pathogens in our plants and to compare the phytoplasmas from field samples of alder trees, from insects and from alder seedlings that were inoculated experimentally.

Digestion of amplified fractions of ribosomal DNA led to identical restriction profiles with all alder samples. The patterns resemble those described by Marcone et al. (1997a,b) for alder yellows phytoplasma in Italy, but are different from the reference strains of elm yellows from European (ULW) and North American (EY1) elm. They are also different from the *Alu*I restriction profile of the P1/P7 fragment from rubus stunt phytoplasma (Reinert and Maixner, 1997). However, RFLP analysis of a non-ribosomal DNA fragment amplified with primers FD9f/r revealed that the Italian alder yellows isolate ALY is not identical to the phytoplasma that we detected in the alder trees examined in Palatine and the Mosel area. The restriction fragment pattern obtained from these trees, on the other hand, resembles one of the profiles described by Daire et al. (1997) from yellows infected grapevine collected from a vineyard in Palatine in close vicinity to the alder

Table 3. Results of transmission trials by feeding of insects on seedlings of *Alnus glutinosa*. PCR detection of elm-yellows group phytoplasmas in seedlings

Origin of insects	Inoculation by								
	Psylla spp.			*Idiocerus stigmaticalis*			*Oncopsis alni*		
	No. plants inoculated	No. plants infected	% plants infected	No. plants inoculated	No. plants infected	% plants infected	No. plants inoculated	No. plants infected	% plants infected
Palatine	30	0	0	6	0	0	10	1	10.0
Mosel	29	0	0	7	0	0	11	2	18.2
Total	59	0	0	13	0	0	21	3	14.3

trees. Recently, the other two profiles of grapevine yellows phytoplasma described by these authors were also detected in *A. glutinosa* (W. Reinert, unpubl.). The significance of the differences in RFLP profiles described here remains unsolved, as long as it cannot be linked to biological traits such as host range or vector specificity. It may indicate a geographic variability of alder yellows but it could also be due to strain variation within the same pathogen that is distributed all over Europe. A detailed comparison of more samples from different European areas may help to solve this question. However, the objective of the RFLP analyses in this study was not the characterization of alder yellows isolates but the confirmation of the identity of phytoplasmas in trees, insects, and inoculated plants. RFLP analysis of the non-ribosomal fragments amplified with primers FD9f/r proved to be an appropriate method for this purpose.

We concluded from the almost ubiquitous infection of *A. glutinosa* in Germany (Lederer and Seemüller, 1991) that the alder yellows phytoplasma should be transmitted by a widespread though monophagous vector. Two insects, *Psylla alni*, one of the two psyllids that were extremely abundant on alder, and the leafhopper *O. alni* comply with these requirements and both were found to carry the phytoplasma. However, in spite of the high proportion of infected psyllids, only *O. alni* was able to transmit the phytoplasma to healthy alder seedlings. RFLP analyses of amplified DNA fragments indicated identical profiles for naturally infected alders, *O. alni* and inoculated seedlings and confirmed the identity of the phytoplasmas in these hosts. These results reveal the ability of *O. alni* to transmit the ALY-phytoplasma. It is the first and only vector of this pathogen identified to date.

There is a good correlation between the distribution of *O. alni* and alder yellows. The disease has been reported from *A. glutinosa* and *A. incana* (L.) Moench collected from southern France and Italy to northern Germany, from lowlands up to mountain regions (Lederer and Seemüller, 1991). *O. alni* is distributed all over Europe (Ribaut, 1952) and was found up to subalpine regions (Wagner and Franz, 1961). This leafhopper is strongly restricted to *A. glutinosa* and *A. incana* (Ossianilson, 1981) but has not been reported from *A. viridis* (Chaix) Dc. Interestingly, Lederer and Seemüller (1991) failed to detected phytoplasmas in this latter *Alnus* species. These authors discuss a different suitability of *Alnus* species for phytoplasmas, but the difference is more likely due to the host preference of *O. alni*.

No information is yet available about the vector-efficiency of *O. alni*. The rearing of this species under controlled conditions could help to solve this and other questions, but it is hindered by its univoltism and its strongly monophagous feeding behavior. Although it is not possible to conclude from the results of this study that psyllids are not able to transmit the disease, the lack of transmission in 59 experiments with more than 2700 individuals allows the assumption that psyllids are not efficient vectors if they are able to transmit alder yellows at all.

It is interesting that the two European elm yellows-group diseases with known vectors, rubus stunt and alder yellows, are both transmitted by closely related leafhoppers of the subfamily Macropsinae, *Macropsis fuscula* (De Fluiter and Van der Meer, 1953) and *O. alni*, while grapevine Flavescence dorée, a disease of presumed North American origin (Caudwell, 1983) and the North American elm yellows are both transmitted by Deltocephaline leafhoppers of the genus *Scaphoideus*, *S. titanus* (Schvester et al., 1961) and *S. luteolus* van Duzee (Baker, 1949), that both are American species.

The infection of grapevine (*Vitis vinifera* L.) by an elm yellows-group phytoplasma has previously been reported from the Palatine area (Maixner et al., 1995b). The RFLP-profiles obtained from ribosomal (Reinert and Maixner, 1997) or non-ribosomal (Daire et al., 1997) DNA fragments of this organism are identical to those of the alder yellows phytoplasma. Therefore, it could be possible that infected alders and *O. alni* are involved in the epidemiology of this grapevine yellows disease although grapevine is not a host of the leafhopper. However, we found *O. alni* on grapevine in affected vineyards of the Palatine region to which it probably has been displaced by wind-drift from nearby alder trees growing along creeks. Another grapevine yellows phytoplasma is also transmitted by probe-feeding of a vector (*Hyalesthes obsoletus* Sign.) that feeds on grapevine only occasionally (Maixner et al., 1995a). However, the hypothesis that *O. alni* is involved in the epidemiology of elm yellows-group phytoplasmas of grapevine has to be proven by further transmission experiments using grapevine seedlings, too.

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