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New mealybug species vectoring Grapevine leafroll-associated viruses-1 and -3 (GLRaV-1 and -3)

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Abstract

Many grape viruses, such as filamentous *Grapevine leafroll-associated viruses* in the Closteroviridae family, are spread primarily through infected propagating material. However, there is increasing evidence that leafroll disease are spread in the field by insect vectors, namely mealybugs and other scale insects. This study was carried out in the northern wine-growing regions of France where *Grapevine leafroll-associated virus*-1 and -3 (GLRaV-1 and -3) are the most widespread grape *Ampelovirus* species. The vineyards were inspected for presence of mealybug and scale insects and grapes infected by GLRaV-1 and -3. Mealybugs, *Heliococcus bohemicus*, *Phenacoccus aceris* (Pseudococcidae) and the soft scale insect *Parthenolecanium corni* (Coccidae), were capable of a transmission efficiency of 14%, 23% and 29% respectively. GLRaV-1 and -3 infections that resulted from virus transmission were confirmed with DAS-ELISA using polyclonal antibodies. This is the first report of GLRaV-1 and -3 transmission by mealybug and coccid species in France, and the first report of the ability of *H. bohemicus* and *Phenacoccus aceris* to transmit these viruses to grapevines. The relevance of these findings with regards to maintenance of virus-free grapevine stocks and to control leafroll spread in commercial vineyards is discussed.

Introduction

Grapevine leafroll disease occur in all major grapevine-growing areas in the world. Eight grapevine-infecting phloem-restricted filamentous virus strains associated with leafroll are known, of these only *Ampelovirus* species, like *Grapevine leafroll-associated virus*-1, -3 and -5 (GLRaV-1, -3 and -5) have known vectors (Martelli et al., 2002). Among the Closteroviridae family, GLRaV-1 and -3 have the highest incidence and are widely distributed in Europe and around the Mediterranean basin (Martelli, 1986). Leafroll virus insect vectors for grapevine are known within two hemipteran insect families, mealybugs (Pseudococcidae) and soft scales (Coccidae). Mealybugs are known as vectors of grapevine viruses A (GVA) and B (GVB) (Rosciglione and Castellano, 1985; Engelbrecht and Kasdorf, 1990; Garau et al., 1995) of GLRaV-3 (Petersen and Charles, 1997), and of GLRaV-5 (Golino et al., 2002). Mealybugs are able to transmit viruses from grape to grape (Tanne et al., 1989; Belli et al., 1994) and to herbaceous plants (Rosciglione and Castellano, 1985; Garau et al., 1995). GLRaV-1 and -3, and GVA have been reported to be vectored by soft scales, such as *Pulvinaria vitis* for GLRaV-3 (Belli et al., 1994), and *Neopulvinaria innumerabilis* (Rathvon) (Coccidae) implicated in transmission of GLRaV-1 only when the latter was associated with GVA in vine donors whereas *Parthenolecanium corni* (Coccidae) transmitted GLRaV-1 alone (A. Fortusini, University of Milano, Italy, unpublished data).

In France, nine scale-insect species are known to develop on grapevine (Sforza, 2000). Of these, *Heliococcus bohemicus* is of particular interest for its large distribution in French northern vineyards and *Phenacoccus aceris* for its ability to transmit *Little cherry virus 2* in the family of Closteroviridae in North America (Raine et al., 1986), which makes this species a potential candidate for vectoring leafroll viruses to grape. These two species have been consistently found in vineyards infected with GLRaVs and showing symptoms or not, but they are considered secondary pests of grape in France since they rarely induce serious damage (Sforza, 2000). During the last decade in Europe, the palaearctic species *H. bohemicus* has become a significant pest of grape in central Europe (Camporese, 1994; Jakab and Szendrey, 1989) and was revealed as polyphagous (Kosztarab and Kozar, 1988). *Phenacoccus aceris* is distributed in both the palaearctic and nearctic regions. In Hungary, *P. aceris* and *H. bohemicus* occur on grapevines in the same localities (Jakab and Szendrey, 1989).

This is the first study on the role of natural vectors of GLRaVs in French vineyards. A survey was undertaken to determine natural vectors of the two most important leafroll-associated viruses in northern France, GLRaV-1 and -3. Transmission trials were carried out for these two viruses. The experiments tested the abilities of *H. bohemicus* and *P. aceris* to vector GLRaV-1 and -3 to grapevine and the ability of *P. corni* to transmit GLRaVs in similar conditions. Control of the natural spread of leafroll-associated viruses in the French vineyards is discussed.

Materials and methods

Insect collection

Field observations were carried out in 1999 and 2000 in five villages of three wine-growing regions of north-eastern France: Bourgogne, Alsace, and Champagne-Ardennes (Figure 1). One collection site was selected

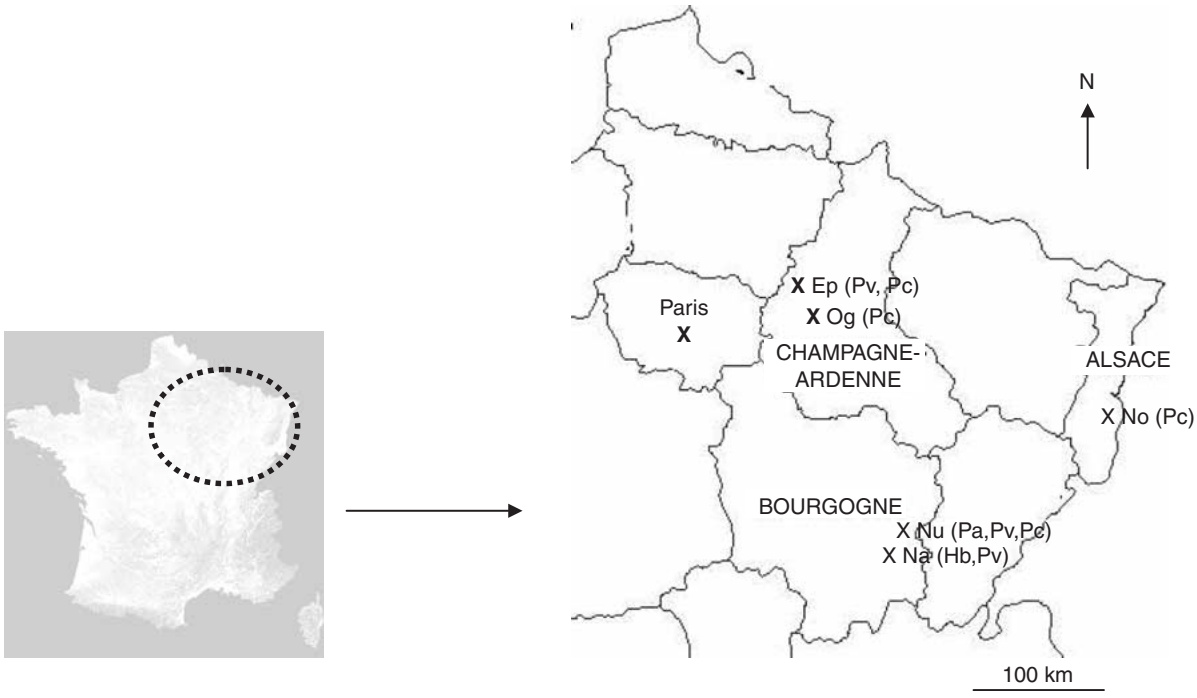

Figure 1. Geographical locations in France of field surveys with occurrence of mealybugs and soft scale insects in 1999. Hb: *Heliococcus bohemicus*; Na: Nantoux (Dept. Côte d'Or , alt. 305 m; cv. Pinot Noir); Nu: Nuits St-Georges (Dept. Côte d'Or, alt. 231 m; cv. Pinot Noir); No: Nothalten (Dept. Haut-Rhin, alt. 210 m; cv. Riesling); Ep: Epernay (Dept. Marne, alt. 122 m; cvs. Chardonnay, Pinot meusnier); Og: Oger (Dept. Marne, alt. 122 m; cv. Chardonnay); Pa: *Phenacoccus aceris*; Pc: *Parthenolecanium corni*; Pv: *Pulvinaria vitis*.

in each village on the basis of the presence of the disease caused by either GLRaV-1 or -3, or both. Each site was checked for the presence of mealybugs and scale insects as potential vectors. In 1999, weekly or monthly observations were made in the three regions, from March to October. Surveys were devoted to determine the natural diversity of the Coccoidea fauna in the five vineyards previously selected. In each of the vineyards, 10 grapevines in each of 10 rows, were randomly selected and observed. Leaves, trunk, and rootstock were checked. Insects were collected and stored in 70% alcohol and sent for identification to Y. Ben-Dov (Volcani Center, Bet-Dagan, Israel). Ants associated with scale insects were also collected and sent for identification to M. Martinez (Inra Montpellier, France). In addition, colonies of collected insects were established in the insectary on young grapevine seedlings and on potatoes.

Vector colonies

Insects were reared and maintained at 23 °C, 16/8 (L : D) using artificial fluorescent illumination, and 80% relative humidity in the insectary at Inra, Dijon. Insects were reared in a glass square cage of 50 cm wide. Field-collected gravid females of *H. bohemicus* were transferred onto virus-free potted rooted grapevine shoots of cv. Pinot noir and hybrid LN33, watered weekly, and onto sprouting potatoes.

Field-collected gravid females of *P. aceris* with ovisacs were transferred onto grapevine shoots cv. Pinot noir and hybrid LN33, watered weekly, and on sprouting potatoes and reared for several generations throughout the year; potatoes are not hosts of GLRaV-1 or -3. Virus-free insect colonies of the two latter insect species maintained on potatoes were used for controls in transmission trials. *Parthenolecanium corni* was not reared on but only maintained after collection on detached leaves of virus-infected *Vitis vinifera* cv. Chardonnay until virus transmission experiments. Potatoes used for insect rearing were removed every 2 weeks and replaced by fresh ones.

Virus acquisition sources

All transmission trials were performed from field-collected vine donors, which were cv. Pinot noir plants infected with GLRaV-1 plus -3 (collected in Nantoux and Nuits Saint Georges), and cv. Chardonnay plants infected with GLRaV-1 alone (collected in Oger). In each treatment, the same donors were used for insect acquisition.

Recipient vines

Virus-free grapevines derived from dormant cuttings of *V. vinifera* cv. Pinot noir and hybrids LN33 and Baco22A, known for their sensitivity to leafroll disease. The mother plants of these cuttings are regularly used in the laboratory as healthy controls in ELISA tests using polyclonal antibodies specific to grape viruses, among which GLRaV-1 and -3. Dormant cuttings approximately 20 cm long were stored at 4 °C until use. Canes were rooted in 12-cm pots with perlite and compost (20/80) and then grown in the glasshouse to the stage of four expanded leaves in average and then used for transmission experiment. The plants were sprayed monthly with insecticide (Dichlorvos, Bayer, Puteaux, France) to ensure the absence of insects. Insecticide spraying was stopped accordingly to their remanence before insect inoculation and plants were isolated in insecticide-free chamber.

Transmission experiments

For all insect species, the field collected batches used for transmission were of all stages when field collected from June to October. Leaf fragments collected in the field and bearing individuals of *H. bohemicus* (from Nantoux), *P. aceris* (from Nuits Saint-Georges) and *P. corni* (from Oger) were deposited onto virus-free recipient vines, assuming that the insects were naturally infected. Field donor leaves were selected among the most mature available to ensure a high level of viral infection in the leaf tissue. The detached leaf method was used for transmission trials and performed in the insectary. Insect-infested leaves were cut into sections carrying groups of 30–50 insects. Each group was transferred to a GLRaV-free grapevine cutting for the inoculation access period (IAP). After 5–7 days, all insects crawled off as the leaf section dried off. Test plants were placed into individual Plexiglas cages. Three to twelve replicates were used in each treatment. Each month period, from June to October, was considered a separate treatment. After an IAP of 1 month, insects were collected and stored in 70% alcohol. Test plants were sprayed with Dichlorvos and transferred to the glasshouse where they were maintained at 20–25 °C and checked periodically for symptom expression. After 5 months, the vines were pruned back

to two buds and stored outside under winter conditions for 2 months. Controls were test plants with mealybugs from healthy colonies maintained on potatoes. Two controls were maintained in the same conditions as recipient grapes within the insectary, the glasshouse, and then stored outside after pruning. After IAP, all test plants, including controls, were protected from insects until the end of the study.

Virus detection

Before transmission trials, recipient vines were regularly tested by DAS-ELISA for the absence of infection by GLRaVs. Two to three months after inoculation, recipient vines were tested by ELISA for the presence of GLRaV-1, -3. Second and third ELISA tests were carried out 3–5 months (December, 1999), and 9–12 months (July, 2000), after inoculation. ELISA tests were carried out using polyclonal antibodies raised against GLRaV-1 and -3 produced in the laboratory (Zimmermann et al., 1990).

Results

Survey of mealybugs and scale insects and rearing

Four species were collected: two species of Pseudococcidae (*H. bohemicus* and *P. aceris* collected in Bourgogne) and two species of Coccidae, *P. corni* collected in Bourgogne, Champagne-Ardennes and Alsace and *P. vitis* collected in Bourgogne (Figure 1). Mixed populations were not observed for mealybug species, whereas *P. corni* and *P. vitis* were occasionally collected on the same site with one or the other of the two mealybugs collected in the study. Additional locations found infested with *H. bohemicus* in Bourgogne, like Mercurey and Beaune (data not shown). In many sites, scale insects were associated with ants. In Nantoux, from May to October, the ant species *Tetramorium caespitum* (Myrmicinae) and *Lasius niger* (Formicinae) were closely associated with *H. bohemicus* and *P. vitis*. In Nuits Saint-Georges, the ant species *Lasius alienus* (Formicinae) was associated with *P. aceris*. In June and July, females of *P. aceris* were protected in clay nests made by *L. alienus* to lay their eggs.

Phenacoccus aceris could be reared on potatoes continuously throughout the year: thousands of mealybugs were obtained each month. *Heliococcus bohemicus* rearing was interrupted in autumn due to a diapause period lasting at least 6 months. Under laboratory conditions, *H. bohemicus* could be maintained on grapevine, potato, broadbean, hazelnut tree (*Coryllus avellana*), and chesnut tree (*Aesculus hippocastanum*). During their diapause on grapevine, insects sat along the stem in crevaces, under the earth surface in the pots, escaping from direct light.

Virus detection in donor vines

In the vineyards surveyed at Nantoux and Nuits Saint-Georges, the incidence of leafroll symptoms on cv. Pinot noir, characterized by leaf reddening and rolling, reached almost 100%. In Oger, leafroll symptoms on Chardonnay were characterized by leaf yellowing and slight rolling. Symptoms on Pinot noir started in mid June and increased sharply until harvest. ELISA tests carried out on randomly selected grapevine samples representing about 5% of the plants in every surveyed vineyards of each region showed that both GLRaV-1 and -3 could be consistently detected. The two viruses were present in the same vineyard, either separately or in mixed infection in the leaf samples collected (results not shown).

Transmission experiment and virus detection in recipient vines

Symptoms on recipient vines appeared 3–4.5 months after inoculation. Symptoms on recipient vines did not appear on all vines tested positive with ELISA. Coccidae and Pseudococcidae species were capable of transmitting both GLRaV-1 and -3 from grape to grape during the vegetative season (Table 1). All plants which tested positive for GLRaVs in 1999 also tested positive in 2000 after pruning and cold storage. The plants inoculated with *P. corni* in October, 1999 were not tested before cold storage; only new expanded leaves were checked in July, 2000. One vine out of seven inoculated at the end of September, 1999, tested ELISA-negative 3 months later, then ELISA-positive for GLRaV-1 in 2000 (Table 2).

In one case, a group of field collected *P. aceris* transmitted the two viruses to the same recipient vine. *Phenacoccus aceris* was also able to transmit these viruses independently. Using groups of 30–50 mealybugs, the relative transmission rates were 14% (4 infected out of 28 inoculated plants) for *H. bohemicus*, 23% (3 out of 13) for *P. aceris*, and 29%

Table 1. Recipient plants assayed by ELISA for GLRaV-1 and -3 in October, 1999, December, 1999 and July, 2000

Transmission trials		GLRaV-1			GLRaV-3		
Insect species	Treatment	Oct. 99	Dec. 99	July 00	Oct. 99	Dec. 99	Feb. 00
Heliococcus bohemicus	June 99	0/3[a]	0/3	0/3	**2/3**	**2/3**	**2/3**
	July 99	0/7	0/7	0/7	0/7	0/7	0/7
	Aug. 99	0/12	nt	0/12	0/12	nt	0/12
	Sept. 99	**2/6**	nt	**2/6**	0/6	nt	0/6
Phenacoccus aceris	June 99	**1/6**[b]	**1/6**[b]	**1/6**[b]	**2/6**	**2/6**	**2/6**
	Sept. 99	nt	0/7	**1/7**	nt	0/7	0/7
Parthenolecanium corni	Aug. 99	**3/9**	**3/9**	**3/9**	0/9	0/9	0/9
	Oct. 99	nt	nt	**2/8**	nt	nt	0/8

[a]First number indicates the number of GLRaV-positive plants, the second indicates the number of replicates in each treatment.
[b]Double infected vine (GLRaV-1 + GLRaV-3).
nt = not tested.

Table 2. Mean absorbance values of ELISA assays on leaf tissue of recipient grapevines in October, 1999, December, 1999 and July, 2000

Transmission trials		GLRaV-1 (405 nm)[a]					GLRaV-3 (405 nm)				
Insect species	Treatment	Oct. 99	Dec. 99	July 00	Control		Oct. 99	Dec. 99	July 00	Control	
					+	−				+	−
Heliococcus bohemicus	June 99	0.090[b]	0.060	0.040	1.150[c]	0.060[d]	**1.370**[e]	**1.070**	**1.225**	1.450	0.095
	July 99	0.060	0.060	0.060	1.150	0.060	0.095	0.095	0.095	1.450	0.095[a]
	Aug. 99	0.070	nt	0.070	1.180	0.070	0.105	nt	0.105	1.645	0.105
	Sept. 99	**0.830**	nt	**0.745**	1.180	0.070	0.135	nt	0.100	1.645	0.105
Phenacoccus aceris	June 99	**1.515**	**0.930**	**1.100**	1.150	0.060	**1.870**	**0.895**	**1.210**	1.450	0.095
	Sept. 99	nt	0.080	**1.100**	1.180	0.120	nt	0110	0.075	1.645	0.105
Parthenolecanium corni	Aug. 99	**0.925**	**0.735**	**0.630**	1.165	0.060	0.155	0.055	0.095	1.465	0.090
	Oct. 99	nt	nt	**0.835**	0.850	0.035	nt	nt	0.145	1.885	0.080

[a]Absorbance values (405 nm) have been adjusted for the absorbance of blank wells.
[b]Mean absorbance values (405 nm) in each treatment.
[c]Mean absorbance values (405 nm) of positive controls.
[d]Mean absorbance values (405nm) of virus-free controls.
[e]Values in bold indicate a positive result, i.e. values greater than two-times the negative control value.
nt = not tested.

(5 out of 17) for *P. corni*. A negative or a positive plant remained the same over time (Tables 1 and 2). The virus titer in recipient grapevines, estimated on the basis of ELISA mean absorbance values in parallel with the average values of positive and negative controls, did not change in value with time, even after a further vegetative cycle (Table 2). The two healthy controls in each treatment remained symptom-less and tested ELISA-negative. Two GLRav-1 and two GLRaV-3 control grapevines showed symptoms and tested positive in ELISA. No living insects were seen on test or control plants after IAP.

Discussion and conclusions

This study is the first report on transmission of GLRaV-1 by mealybug species in general. Mealybugs have previously been implicated only as vectors of GLRaV-3 and -5 in many countries around the world

(Rosciglione and Gugerli, 1989; Tanne et al., 1989; Engelbrecht and Kasdorf, 1990; Cabaleiro and Segura, 1997; Golino et al., 2002). Based on the results, it is clear that the species *H. bohemicus* and *P. aceris* collected in Bourgogne were capable of transmitting at least two of the viruses, GLRaV-1 and -3, that cause leafroll disease to grapevines, under experimental conditions. It is the first report of *H. bohemicus* as a vector of a plant virus, and of *P. aceris* as a virus vector to grapevine.

Until now, *P. aceris* was known as a species vectoring three different viruses among the Closteroviridae family, e.g. GLRaV-1 and -3 in France and *Little cherry virus* 2 in North America (Raine et al., 1986). This insect species is becoming a serious pest, that has to be surveyed accurately and controlled in vineyards and orchards. In addition, we showed the ability of *P. corni* to transmit GLRaV-1. *Parthenolecanium corni* is the most widespread soft scale insect in French vineyards. Already considered as an occasional grape pest in Europe (Pellizzari-Scaltritti, 1997), it is now recognized as a vector of GLRaV-1 in two European countries at least, France and Italy (A. Fortusini, University of Milano, Italy, unpublished data). Of the four insect species considered in this study, three were shown to be GLRaV vectors. Further studies in France should focus on *Planococcus* sp., *P. vitis*, and *N. innumerabilis*, already known as vectors of GLRaVs in neighbor countries (Belli et al., 1994; Cabaleiro and Segura, 1997; A. Fortusini, University of Milano, Italy, unpublished data). As reported by Garau et al. (1995), and here confirmed, there is no strict scale-insect family or species specificity in virus transmission. Thus, these insects are also good candidates for the missing vectors of the other GLRaVs. In addition, the results show that mealybugs can transmit viruses from June to September. Closteroviruses are semi-persistently transmitted which implies a constant virus acquisition on infected donor vines by these insects to remain viruliferous. As the vineyards surveyed were highly contaminated, it may be assumed that field contamination could start earlier in the season, since insects over-winter on grapevine and start feeding in early spring on infected plants.

Our data showed that transmission rates were low, even though groups of insects were taken from heavily contaminated vineyards. In fact, no differentiation was made between immature and adult stages in transmission trials. Petersen and Charles (1997) reported that young mealybugs were the only efficient vectors, which may explain our low transmission rates. Though percentages of transmission are an estimate since we used groups of insects, the latter data indicated the potential threat of the insect species tested. We cannot conclude whether one individual of *P. aceris* could transmit both GLRaV-1 and -3 or two individuals in the same group transmitted the two viruses separately. Though *P. aceris* is a monovoltine species, a permanent rearing was obtained and produced thousands of insects every month. Further studies might use this species as a model for studying the duration of inoculation and the acquisition access period, and the precise transmission efficiency for GLRaV-1 and -3, and perhaps for other GLRaVs.

Immunocapture-RT–PCR was also used to detect GLRaV-1 in the recipient vines. Primers were designed from the sequence of a cDNA fragment corresponding to the hsp70-like open reading frame of GLRaV-1 genomic RNA (a French isolate from Meursault, Burgundy; data not shown). Although the PCR procedure is recognized as more sensitive than ELISA for the detection of many grapevine viruses, we never detected more GLRaV-1-infected recipient vines than by ELISA, nor the virus detected sooner. In the frame of this study both techniques showed the same efficacy, thus ELISA was preferred due to its lower cost.

The epidemiology of leafroll is not yet fully understood, because spread by insect vectors often interferes with planting of infected material. The survey revealed the large distribution of each species tested, ascertained by recent observations for *H. bohemicus* and *P. aceris* on grapevines in Alsace (Sforza et al., 2003). Although field transmission by insects is suspected in leafroll-infected vineyards worldwide, this hypothesis is poorly documented (Cabaleiro and Segura, 1997). The vineyards in Bourgogne that contained populations of *P. aceris* or *H. bohemicus* reached 100% infection of GLRaV-1 and -3. Further studies are needed to understand natural spreading of the disease in the vineyards by dispersion of mealybugs by wind, human activities and in some cases by ants (Gullan, 1997). Our observations in the vineyards surveyed showed that ants cohabited with scale insects and that they probably acted both as carriers of crawlers and as protectors against predators and parasitoids, as reported on other crops (Rohrbach et al., 1988).

Although documented data about the history of the vineyards surveyed and the occurrence of mealybugs since plantation are lacking, it is essential that virus-free grapevine clones produced from French certification programme remain protected from natural

spread of the disease. In European vineyards, mealybugs are considered as seasonal problems. In France, they are controlled by insecticides, which have been reduced in the past decade. In the frame of Integrated Pest Management, a biocontrol strategy is advanced, following recent studies in South Africa (Walton and Pringle, ARC-Infruitec, unpublished data) and in the US (Geiger et al., 2001), and supported by numerous and promising field-collected mealybug parasitoids (Sforza et al., 2003).

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