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# Occurrence, distribution and epidemiology of Grapevine Yellows in Spain

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**Abstract**

The main viticultural production areas in Spain were surveyed in 1994, 1995 and 1996 for the occurrence and incidence of Grapevine Yellows diseases associated to phytoplasmas. Samples from 300 plants showing symptoms of phytoplasma infection were collected from grapevine fields in the Spanish regions of Aragón, Catalonia and Navarra and analysed by PCR with specific primers for a non-ribosomal DNA of stolbur/Bois Noir (BN) and of Flavescence dorée (FD) phytoplasma. Nested PCR with universal primers P1/P7 and fU5/rU3 was also used. In the survey conducted in 1994 and 1995 only BN/stolbur phytoplasma was detected. The incidence of symptomatic plants was low in five plots of Catalonia from 3% to 18% in 1994 and 1995, respectively, and high in two plots of Navarra, from 60% to 80%. In the survey conducted in 1996 the incidence of symptomatic plants in Catalonia increased (6–80%) due to the presence of FD in five plots in the Northeastern Catalonia. An epidemiological study was carried out in two BN-affected plots of two regions from 1994 to 1997, with the evaluation of potential vectors and of host plants. The stolbur phytoplasma was found in individuals from different insect species belonging to the families *Cicadellidae* and *Delphacidae*. Some wild plants naturally infected with stolbur phytoplasma around the infected grapevines were: *Convolvulus arvensis*, *Lavandula officinalis*, *Polygonum convolvulus*, *Solanum nigrum*, and *Thymus officinalis*. The incidence of the disease in one BN-infected grapevine plot increased from 3.4% in 1994 to 18.40% in 1997.

## Introduction

Different Grapevine Yellows (GY) diseases associated with phytoplasma are known in most grape growing countries to cause crop loss and depreciation of quality (Bertaccini et al., 1995; Daire et al., 1997a; Davis et al., 1997; Maixner et al., 1995a; Marcone et al., 1997a; Osler et al., 1993; Padovan et al., 1996; Terlizzi et al., 1994). The symptoms of infected plants are similar: leaf roll, vein chlorosis and necrosis, withering of flowers and bunches, and absence of lignification in the fall (Figure 2). The diseases mainly differ in their epidemiological expression, such as severity and progress of the attack in vineyards, vectors and source of inoculum. Among them Flavescence dorée (FD) and Bois Noir (BN) are the more frequent and cause the most serious damage in vineyards. FD is very epidemic, mainly because it is vectored by *Scaphoideus titanus*, which is frequent in Southern France, Northeastern Spain and Northern Italy. This leafhopper is well adapted to viticultural areas where the summer is long enough for the adults to lay their eggs. BN has been detected in many countries, such as France, Croatia, Greece, Israel, Italy, Slovenia and Spain, and is similar to Vergilbungskranheit (VK) in Germany (Bertaccini et al., 1996; Bianco et al., 1997; Boudon-Padieu 1996; Daire et al., 1997a; Davis et al., 1997; Koruza 1996; Laviña et al., 1995; Maixner et al., 1995a; Skoric et al., 1998). BN phytoplasma belongs to the stolbur group (Seemüller et al., 1998), also named 16S rRNA group 16S rXII-A (Davis et al., 1997) and is detected by PCR with universal and specific

primers (Daire et al., 1997b) or with specific antiserum (Kuszala, 1996). Stolbur phytoplasma appears to be a ubiquitous pathogen that can be hosted by plants of several families. Weeds, vegetable and trees such as *Convolvulus arvensis*, *Lepidium draba*, *Amaranthus retroflexus*, *Chenopodium album*, *Cirsium arvense*, *Lavandula officinalis*, *Lycopersicum sculentum* and *Prunus dulcis* (Fos et al., 1992; Maixner et al., 1995b; Marcone et al., 1997b) are known hosts for Stolbur phytoplasma, which is vectored by different leafhoppers and planthoppers: *Macrosteles laevis*, *Aphrodes bicincta*, *Euscelis plebejus* and *Hyalestes obsoletus*. In grapevine, only *Hyalestes obsoletus* sign has been found to be a vector of the phytoplasma (Maixner, 1994; Sforza et al., 1998).

Control strategies against phytoplasma-borne diseases should be based on sound knowledge of the phytoplasma infections present in the area, their population structure and their epidemiology. Results on the incidence and occurrence of grapevine phytoplasma infections in Spain and also the results of an epidemiological study of a BN/stolbur phytoplasma-affected plot in Catalonia are reported. The study includes an evaluation of the temporal evolution of affected plants over four years, the identification of potential vectors and of host plants present in the area.

## Materials and methods

### *Phytoplasma detection by PCR technique*

Total DNA was extracted from a phytoplasma-enriched fractions prepared from petioles, midribs and leaves from grapevine, weeds and shrubs (Ahrens and Seemüller, 1992). The phytoplasmas were detected by PCR amplification phytoplasma with the primer pair fU5/rU3 designed for the specific amplification of a region of 16S rDNA gene from most known phytoplasmas (Lorenz et al., 1995). In 1996 a nested-PCR procedure was utilised: the first primer pair was P1 (Deng and Hiruki, 1991) and P7 (Smart et al., 1996) and the second was the pair fU5/rU3. The amplification mixture contained 0.25 μM of each primer, 250 μM of dNTPs, 1 unit 100 $\mu l^{-1}$ of Taq DNA polymerase (Appligene) and Taq buffer (Appligene). Amplification was carried out in a total volume of 20 μl containing 5–10 ng DNA, for the first amplification. Two μl of the amplification product were used for the second step in a total volume of 40 μl of amplification mixture. Ten μl of the mixture containing the DNA amplified in the second step was directly digested overnight with 1 unit of enzyme *Tru 9*I. FD specific primers FD9f/r, derived from both ends of a cloned 1.3 kb fragment from non-ribosomal FD-DNA and BN/stolbur specific primers stol 4f/r, were also used for PCR on the same samples (Daire et al., 1997b). Native amplification products were electrophoresed in agarose (1.5%) gel and digested products were analysed using polyacrilamide (10%) gel electrophoresis, according to standard procedures. DNA was viewed under UV light following ethidium bromide staining.

### *Epidemiological studies*

Insects were captured on sticky yellow traps placed within or near the BN-infected vineyards and with an aspirator. Aspiration at the plot edges was performed at the rate of one aspiration per 10 $m^2$. There were 10 aspirations per sampling. Sampling was done at regular intervals during the period of May to September. DNA from insects was extracted by grinding 1–15 insects depending on the species as described by Daire et al. (1992). Weeds and wild plants were sampled in the vineyards and in the edges and analysed with the same technique.

The temporal evolution of the incidence of the BN/stolbur was studied in the vineyard located in Catalonia, planted with Chardonnay vine. A microplot of 500 plants in the middle of the field was selected and the disease incidence was evaluated yearly for 4 consecutive years.

## Results

### *Survey and phytoplasma detection*

A survey was conducted during the 1994, 1995 and 1996 growing seasons in three regions of Northeast of Spain (Aragón (Region 1), Catalonia (Region 2) and Navarra (Region 3)). In each region, vineyards were arbitrarily selected and inspected from September to November, according to the region, so that each field was surveyed at approximately the same phenological state, when the symptoms of phytoplasma were most apparent. Phytoplasma disease incidence (% of plants with phytoplasma symptoms) was estimated for each sampling site by visual inspection of 500–1000 plants (according to the incidence) following a W-shaped itinerary (Lin et al., 1979) and expressed as the percentage of symptomatic samples. Samples from plants

showing phytoplasma infection symptoms (up to a maximum of 10 per field) were collected in order to identify the phytoplasma involved. Samples were taken from symptomatic vines in the field belonging to cvs Chardonnay, Grenache and Tempranillo.

The results of the survey showed that the occurrence, distribution, and relative incidence of phytoplasma infections varied between the different growing regions (Table 1). Symptoms of phytoplasma infection were seen in 7, 9 and 14 fields in 1994, 1995 and 1996, respectively, in two regions (Catalonia, 2 and Navarra, 3). Incidence of symptomatic plants varied largely, not only between regions but also between fields. In the 1994 and 1995 surveys, only BN/stolbur phytoplasma was detected in five and six plots respectively out of fifteen in Region 2 (Catalonia) and in two and three respectively plots out of ten in Region 3 (Navarra). No phytoplasma infection was detected in Region 1 (Aragón). There were few symptomatic plants in the plots in Region 2 (3–18%) in 1994 and 1995, when only BN/stolbur was detected, and many in the two plots in Navarra (60–80%) in which only BN was also detected. In the 1996 survey, the incidence of symptomatic plants in Region 2 increased (6–80%) due to the presence of FD in five plots of the Northeastern Catalonia region.

With the PCR procedure with universal primers 45 symptomatic samples out of 50 in 1994, 52 out of 60 in 1995 and 103 out of 110 in 1996 were positive in Region 2. All samples with symptoms were positive in Region 3.

The stolbur group phytoplasma was the only one detected in the samples collected in 1994–1995. Both techniques used (PCR with specific primers and PCR with universal primers followed by RFLP) gave the same results.

In 1996, an EY group phytoplasma was detected using nested PCR followed by RFLP in 50 of 110 samples in Region 2 (Table 1). This phytoplasma was found in vineyards that had not been previously sampled. The same results were achieved using FD9f/r specific primers.

### *Epidemiological study of BN*

An epidemiological study of BN/stolbur was done in two vineyards from the two regions with more phytoplasma infected areas: Catalonia (Region 2) and Navarra (Region 3). Potential vectors and potential hosts plants were identified at the species level and tested for stolbur phytoplasma with specific primers. The species of insects captured and identified in Regions 2 and 3 are listed in Table 2. The species found infected by stolbur phytoplasma were: *Adarrus taurus*, *Aphrodes bicinctus*, *Agallia laevis*, *Macrosteles sexnotatus*, *Neoaliturus fenestratus*, *Psammotettix striatus* and *Zyginidia scutellaris*. Other species collected but not infected were *Allygus mixtus*, *Edwardsiana rosae*, *Empoasca decipiens*, *Eupelix cuspidata*, *Eupteryx rostrata*, *Euscelis incisus*, *Euscelidius variegatus*, *Laodelphax striatellus* and *Scaphoideus titanus*.

The wild plants collected and identified as stolbur positive in Regions 2 and 3 are listed in Table 3. The species found to be infected by stolbur phytoplasma were: *Convolvulus arvensis*, *Lavandula officinalis*, *Polygonum convolvulus*, *Rubus fruticosus* and *Solanum nigrum*. Other species found to be sporadically infected were *Plantago lanceolata* and *Thymus officinalis*.

The evolution of the disease in the plot of Region 2 (Catalonia) show that the infection in 1994 was 3.4%. In 1995 the incidence increased to 7.2%. In 1996 14% of the plants had BN/stolbur, and in 1997 BN infection reached 18.40% (Figure 1).

*Table 1*. Occurrence of BN and FD phytoplasma as determined by PCR on grapevine samples with symptoms collected from fields surveyed during 1994, 1995 and 1996 in Regions 1–3

| Region | No of fields with phytoplasma symptoms/ No of surveyed field | | | Percentage of symptomatic plants[2] | | | PCR detection[1] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | +BN/Total samples | | | +FD/Total samples | | |
| | 1994 | 1995 | 1996 | 1994 | 1995 | 1996 | 1994 | 1995 | 1996 | 1994 | 1995 | 1996 |
| 1 | 0/10 | 0/10 | 0/10 | 0 | 0 | 0 | — | — | — | — | — | — |
| 2 | 5/15 | 6/15 | 11/20 | 3–14 | 5–18 | 6–80 | 45/50 | 52/60 | 53/110 | 0/50 | 0/60 | 50/110 |
| 3 | 2/10 | 3/10 | 3/10 | 60–80 | 65–80 | 65–80 | 20/20 | 30/30 | 30/30 | 0/20 | 0/30 | 0/30 |

[1]Data are number of symptomatic plants positive in PCR for BN and FD.
[2]Estimated by visual inspection in fields from which symptomatic samples were collected.

*Table 2*. Leafhoppers and planthoppers (Homoptera: Auchenorrhyncha) captured in Northeast Spain with aspirators or on sticky yellow traps during 1997 and 1998 and detection of stolbur phytoplasm in them

| | Species | Region 2 | Region 3[1] | PCR[2] detection |
|---|---|---|---|---|
| Cicadomorpha | | | | |
| *Fam. Cicadellidae* | | | | |
| Sbf. Typhlocybinae | *Asymmetrasca decedens* (Paoli, 1932) | x | — | – |
| | *Edwardsiana rosae* (L. 1758) | x | — | – |
| | *Empoasca decipiens* (Paoli, 1930) | x | x | – |
| | *Erythroneura tamaricis* (Put, 1872) | x | — | – |
| | *Eupteryx rostrata* (R. 1936) | — | x | – |
| | *Fruitoidia bisignata* (Nast. 1987) | x | — | – |
| | *Hauptidia marocanna* (Melichar, 1907) | x | — | – |
| | *Typhlocyba aurovittata* (Dgl 1875) | x | — | – |
| | *Zyginidia scutellaris* | x | x | + |
| Sbf. Agalliinae | *Agallia laevis* (Rib. 1935) | x | x | + |
| | *Peragallia sinuata* (M.R., 1855) | — | x | – |
| Sbf. Aphrodinae | *Aphrodes* sp. (Rib. 1952) | x | x | + |
| Sbf. Eupelicinaee | *Eupelix cuspidata* (F. 1775) | — | x | – |
| Sbf. Deltocephalinae | *Adarrus taurus* (Rib. 1952) | x | x | + |
| | *Allygus mixtus* (F. 1794) | — | x | – |
| | *Circulifer haematoceps* (M.R. 1855) | x | x | – |
| | *Euscelidius variegatus* (Kbm, 1858) | x | x | – |
| | *Euscelis incisus* (Kbm, 1858) | — | x | – |
| | *Macrosteles sexnotatus* (Fallén, 1806) | x | x | + |
| | *Neoaliturus fenestratus* (H.S. 1834) | x | x | + |
| | *Opsius stactogalus* (Fieber, 1866) | x | — | – |
| | *Psammotettix striatus* (L. 1758) | x | x | + |
| | *Scaphoideus titanus* (Ball, 1932) | x | — | – |
| Fulgoromorpha | | | | |
| *Fam Delphacidae* | *Laodelphax striatellus* (Fallén) | x | x | – |

[1]x: present in the studied region; [2]positive in the PCR detection with specific primers stol 4f/r.

*Table 3*. Wild plants collected near a plot of grapevine infected by BN and found infected by stolbur phytoplasma after PCR test with specific primers stol 4f/r

| Species | Region 2 | Region 3 |
|---|---|---|
| *Chenopodium album* L. | x | x |
| *Convolvulus arvensis* L. | x | x |
| *Lavandula officinalis* Chaix | x | x |
| *Plantago lanceolata* L. | x | x |
| *Polygonum convolvulus* L. | x | x |
| *Rubus fruticosus* L. | — | x |
| *Solanum nigrum* L. | — | x |
| *Thymus officinalis* L. | x | x |

## Discussion

The results of the survey of the presence and incidence of phytoplasma infections conducted in the main vine-growing areas in Spain showed that the phytoplasma identified most often was the BN/stolbur. However, the presence of FD, although mainly restricted to the

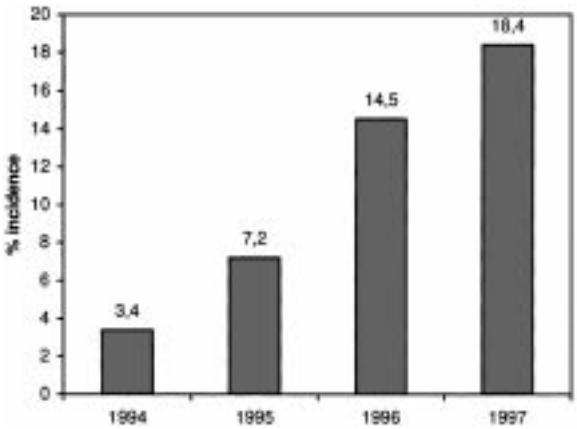


*Figure 1*. Evolution over 4 years of number of plants affected by BN/stolbur phytoplasma in a vineyard plot with 500 plants of the variety Chardonnay.

*Figure 2*. Symptoms (leaf roll and absence of lignification) caused by phytoplasma infection in cv. Grenache.

Northeast of Region 2 (Catalonia), is of particular concern, because FD phytoplasma is a quarantine organism and is regarded as one of the most destructive vine pathogens. The occurrence of the disease in Spain underlines the importance of starting control measures. Vector populations should be limited, since the leafhopper vector of FD (*S. titanus*) was present in all vineyards sampled (Batlle et al., 1997). The use of clean planting material (Caudwell et al., 1997) and the control of new foci of the disease by surveying GY in neighbouring viticulture areas are measures that should be implemented to contain the disease.

The study of BN/stolbur epidemiology indicated the presence of efficient vectors for BN in Region 3 (Navarra). In this area, plots with a high infection index of BN infection were found. However, in Region 2 (Catalonia) the dissemination index was low. Several leafhopper and planthopper species are suspected to be involved in the transmission of stolbur phytoplasma, however, only the cixiid *Hyalestes obsoletus* has been found to be a transmitter of stolbur in grapevine (Maixner, 1994). This vector is a polyphagous species which overwinters as larvae on the roots of several host plants (Glucu and Ozbek, 1988; Maixner, 1994; Sforza et al., 1998). However, this species was not found in the plots sampled in this work, suggesting that the vector in the studied area is one of the insects identified as positive for BN/stolbur phytoplasma. Transmission tests must be conducted with these species. *Psammotettix striatus* is the most frequent species. However, it has never been demonstrated to transmit phytoplasmas. At the sampling sites individuals of *Aphrodes* sp., *Euscelis incisus*, *Euscelidius variegatus*, *Macrosteles sexnotatus* and *Neoaliturus fenestratus* were identified. The same species or other species of these same genera have been reported as possible vectors of phytoplasma diseases (Cali et al., 1989; Marcone et al., 1997a; Valenta et al., 1961).

The majority of infected vectors were sampled from the area of the plot which was closer to the forest, where there are numerous wild plants which host the phytoplasma. Disease patterns are then influenced by the occurrence and distribution of alternative hosts of the pathogen. The most frequently infected wild plants were *Convolvulus arvensis* and *Polygonum convolvulus*, already reported by other authors (Maixner et al., 1995b; Marcone et al., 1997b), *Lavandula officinalis* and *Solanum nigrum* were two other species frequently found infected by stolbur. Other species found to be infected sporadically were *Plantago lanceolata*, *Rubus* sp, and *Thymus officinalis*.

The three-year survey of grapeyards in Spain showed that phytoplasma infection is widespread in this crop. Information from the epidemiological study such as the data on potential vectors and host plants present in the studied area provides basic information for the control of Grapevine Yellows in Spain.

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