

Experimental proofs regarding the association of cell wall deficient bacteria (mycoplasma-like organisms, phytoplasmas) with grapevine yellows disease in Romania

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Abstract

A study was performed on Grape yellows from 2008-2010, in five commercial grapevine growing regions in Romania. The presence of typical yellowing symptoms resembling *Flavescence dorée* and Bois noir diseases, described in West Europe, and on other continents, were detected on *Vitis vinifera* cvs. Chardonnay, Hamburg, Merlot, Fetească regală, Cabernet Sauvignon, and Pinot Noir. Natural foci with *Convolvulus arvensis*, with symptoms of stolbur, *Cichorium inthybus* and *Taraxacum officinale* with symptoms of yellowing and virescence were detected in South of Romania and *Trifolium repens* with symptoms of clover phyllody and clover dwarf (aster yellows) were detected in Transylvania. Insect vectors as *Hyalostyles obsoletus*, *Reptalus panzeri*, *Dictyophara europaea*, *Scaphoideus titanus*, *Macrostelus laevis* and *M. viridigriseus* were detected also in natural foci. The vector *H. obsoletus* collected from grapevine cultivars transmitted stolbur MLO to periwinkle plants. By electron microscopy, on ultrathin sections, structures resembling cell wall deficient bacteria (MLOs, phytoplasma) were detected in the sieve elements of grapevine, and in the sediment of purified material. Using a polyclonal antiserum raised in rabbits with Aster yellows antigen, growth in artificial media, an indirect dot blot immunoassay (ELISA) technique was used and etiologic agent as stolbur and aster yellows were identified. These results are first experimental proofs regarding the association of stolbur and aster yellows with grape yellows in Romania.

Key words: *Vitis vinifera*, grape yellows, insect vectors, electromicroscopy, serology

1. Introduction

Grapevine Yellows (GY) are known as catastrophic or destructive diseases in many grape growing areas over the world and are associated with a cell wall deficient bacteria, called formally mycoplasma-like organisms (MLOs) or phytoplasmas.

The GY is a syndrome that includes yellowing and reddening of leaves, depending of cultivars of grape, followed by downward curling of leaves, lack or incomplete lignifications and necrosis of shoots, abortion of inflorescences and shriveling of berries and degeneration of plants. The first symptom of disease was discovered in France in 1957, named *Flavescence dorée* (FD), suspected to be caused by a virus [1] and transmitted by insect vector *Scaphoideus titanus* Ball [2]. A few years later it was established that the MLOs is probable the cause of FD [3]. Similar diseases, with presumed MLOs etiology, were detected in Italy in 1973, called "Giallumi della vite" or "*Flavescenza dorata*" [4, 5, 6]. Since then GY was detected in all Europe, U.S.A [7], Chile [8, 9], Tunisia [10], South Africa [11] and Australia [12, 13].

In 1961 a new disease called *Bois noir de la vigne* (BN), different of FD, also attributed to a virus, was detected in France [14] and in Germany, named *Vergilbungskrankheit* [15]. This new disease agent is transmitted to grape by planthopper *Hyalostyles obsoletus* Sign., the vector of stolbur disease agent of solanaceous plants in

Europe [16, 17], also known as MLOs since 1968 [18], and then more investigated in Italy [19, 20], Germany [21], Spain [22], Serbia [23] and Canada [24]. Both diseases (FD and BN) have a similar symptomatology but different etiology and epidemiology [25]. In Europe, three distinct FD strains cluster and group 16SrV MLOs were detected [26]. Around of Romania, these diseases were detected in Bulgaria [27], Serbia [28], Hungary [29], Moldova [30] and Ukraina [31]. In Romania, symptoms of GY were described in different counties and attributed to a virus [32] or to stolbur MLO [33], but experimental investigations to proof the nature of the causal agent were not performed by the authors.

Since yellowing alterations can be induced also by other causes, a research project was initiated to investigate the symptoms on grape, natural foci associated with wild plants and insect vectors and to establish the nature of the etiological agent in Romania.

2. Material and methods

2.1. Selection of the grapevine diseased material

The study was performed during 2008-2011, in five grapevine growing regions: Banu Mărcăine (Oltenia), Bucharest area and Prahova Valley (Muntenia), Murfatlar (Dobrogea) and Blaj (Transylvania). Different cultivars of grape were carefully analyzed to establish the presence of typical yellowing or reddening symptoms resembling *Flavescence dorée*, *Bois noir* or *Aster yellows* diseases described in Europe, North and South America, Africa and Australia. The symptoms were recorded by Fujifilm Digital Camera *FinePixS5700* and shoots, bunch stems and pedicels were collected and used for MLOs purification, light and electron microscopy or serology examination.

2.2. Field Surveys of Leafhoppers and Planthoppers vectors

The insect vectors were detected by yellows sticky traps placed in all grapevine areas selected for investigations, from June to September. Vectors were also collected by sweep net method on the bindweed (*Convolvulus arvensis*) from inside of grape areas and used for experimental transmissions. Captured leafhoppers and planthoppers were counted and identified to species [34].

2.3. Experimental transmission by vectors

Adults of *Hyalesthes obsoletus* planthopper used in experimental transmissions were collected on the bindweed (*Convolvulus arvensis*) with symptoms in vineyards with symptoms of GY in Dobrogea region (South-East of Romania). Groups of five-seven adults were transferred on red periwinkle plants (*Catharanthus roseus* G.Don) before flowerage, under individual cages in controlled conditions. The insects remained on the plants until they died. The plants were maintained in the insect-proof room at 15–25°C, 8-16h photoperiod.

2.4. Purification of MLOs

To establish the morphological aspect of the disease agent, MLOs were purified only from petioles and midribs of infected grape using the methods described before [35, 36]. All green material along the midribs was removed with a razor blade and only shoots, without leaves, petioles and midrib were retained and sectioned in small discs on sterile filter paper and transferred in a sterile mortar with sterile MLOs buffer (0.1M phosphate buffer + 0.33 M NaCl) supplemented with 0.5% sodium sulfite to prevent the oxidation process.

Using sterile pestel, the discs were gently pressed and ruptured with small dust of carborundum and the MLOs were released into suspending buffer. The plant organelles

(chloroplasts, nuclei, mitochondria, microbodies from companion cells or phloem parenchyma) or other cell debris were removed by microcentrifugation for 5 min at 750g in microfuge G Force 13 (Denver Instrument, Colorado). The MLOs were sedimented from supernatant by centrifugation at 20.000g for 30 min in a Janetzki T 24D centrifuge, and the pellets were resuspended in MLOs buffer and clarified by centrifugation for 5 min at 750g. The purity of MLOs was appreciated by electron microscopy after negative staining with 3% ammonium molybdate in dd water and stored at -20°C [37].

2.5. Electron microscopy investigations

For electron microscopy analysis of the plant material, midribs and petioles of plants showing symptoms were double fixed with glutaraldehyde and osmium tetroxide, dehydrated in an acetone series (70-100%) and embedded in Epon 812. Ultrathin sections, cut with a Tesla 490A ultramicrotome, were transferred on 300 mesh copper grids and examined with Tesla BS 500 electron microscope [37].

2.6. Serological detection

An indirect dot blott ELISA method was performed on nitrocellulose membrane (SIGMA-N-8017) having a 0.2 µm pore size [36]. The purified MLOs were pipetted as 5µL/spot on nitrocellulose strips 8/0.8cm. The strips were transferred in 10 ml volume plastic tubes filled with blocking buffer warmed at 45°C (0,1M Tris-HCl, pH 8, supplemented with 4% bovine serum albumin -SIGMA B4287, 0.1% gelatin -Merck and 0.2% sodium azide) for 60 min. The blocking solution was removed and the strips were incubated in 1/10 dilution of primary polyclonal antiserum for 5-8 hours or overnight. The primary antisera were raised in rabbits using a culture on artificial media of *Aster yellows* MLO [38]. Negative controls were carried out by omitting the antigen or the primary antibody. The strips were washed several times (10-15 min) with buffer to remove unbound antibody.

To detect antibodies bound to MLOs, the strips were introduced in tubes filled with secondary antibodies (Goat anti-rabbit IgG - whole molecule) conjugated with Alkaline Phosphatase-AP (SIGMA A3937). The strips were incubated over night in diluted secondary antibodies (1/100). The membrane strips were thoroughly washed in dd water and the positive reactions were identified using SIGMAFAST-BCIP/NBT substrate for AP (product no. B0274, tablets substrate) for 5 min. A colorless zone appeared where the antigen was not spotted (blank). The staining reaction was stopped by washing the strips with dd water.

3. Results and discussions

3.1. Evaluation of GY symptoms

In vineyards of the South part of Romania all cultivars of grapevine such as *Chardonnay*, *Hamburg*, *Cabernet Sauvignon*, *Riesling* and *Merlot* were detected with symptoms of GY (figs.1-3).



Figure 1. Symptoms of yellowing on *Chardonnay* cv., (Tohani, Prahova Valey). Strong reduction and spoon like aspect of leaves were always visible



Figure 2. Symptoms of reddening and triangle aspect of leaves on *Hamburg* cv. (Bucharest)



Figure 3: a. Symptoms resembling of FD on *Chardonnay* with triangle aspect of leaves (Murfatlar); b. Magnification from a; c. FD like symptoms on *Cabernet Sauvignon* (B. Mărăcine).

3.2. Evaluation of populations of insect vectors

Our investigations have showed that the grape yellows is a disease of natural foci. The circuit in nature of GY MLOs is associated, in Romania, with two major components: insect vectors and weeds that have origin inside and outside of grape growing areas. In 2009 and 2010 the following specific vectors, planthopper and leafhopper species, associated with Yellows MLOs were collected on yellow sticky traps in grapevine with symptoms of yellowing and reddening: *Hyalesthes obsoletus*, *Scaphoideus titanus*, *Reptalus panzeri*, *Dictyophara europaea*, *Macrosteles laevis*, *Psamotteix striatus* and *Euscelis variegatus*. These vectors were detected in association with *Convolvulus arvensis* with symptoms of

stolbur, *Cichorium intibus*, and *Taraxacum officinale* with symptoms of virescence and proliferation and *Trifolium repens* with symptoms of clover phyllody and clover dwarf. These plants can be donors or acceptors of MLOs through intermediate vectors, in natural foci. The host range of MLOs coincides with the food and breeding plants of their insect vectors. Expansion of these natural foci creates the possibility of mixed infection in grape with different European Aster Yellows MLOs.

3.3. Experimental proof of transmission of stolbur agent to periwinkle plants

Several series of experimental transmissions were performed in 2009 and 2010 using the stolbur vector, *H. obsoletus*, collected in grapevine from Murfatlar vineyard, Dobrogea zone. Periwinkle (*C. roseus*) plants were used for detection of stolbur agent. Typical symptoms for stolbur disease appeared on periwinkle plants two months from contact of vector with healthy plants, in comparison with uninfected plants (Fig. 4a). The symptoms evolved from normal plant (a) to chlorosis (b), shortening of plant (c), hypertrophy of calyx and virescence of petals (d).



Figure 4.

a. Normal flower and leaves of periwinkle

b. Strong yellowing and spoonlike leafs (arrow)

c. Shortening of stem (arrow)

d. Hypertrophy of calyx and green petals (arrow)



Figure 5. Left: Normal leaf and flower. Right: Yellowing and leaf reduction with zigzag nerve; hypertrophy of calyx and green petal (arrows) produced by stolbur to periwinkle

After two or three months, the leaves with strong symptoms of chlorosis become wilt and fell down (fig.4c arrows). The modification of dimensions, color of leaves and alterations of flowers are serially illustrated in figure 5.

3.4. Electron microscopy detection

To establish the nature of etiologic agent, electron microscopic examination was performed on ultrathin sections across shoots and petiole of grape showing symptoms of GY and on purified pellets. Structures resembling mycoplasma were purified from symptomatic plants shoots and from leaf midribs of different grapevine. Typical MLO cells (figure 6) were detected, similar with thus purified and visualized by Giannotti [3] and Caudwell [39] associated with *Flavescence dorée* in France. The purified MLO cells appear as round particles with or without filaments developed by budding. The morphology of the agent is better visible on purified material than on ultrathin sections (fig. 7).

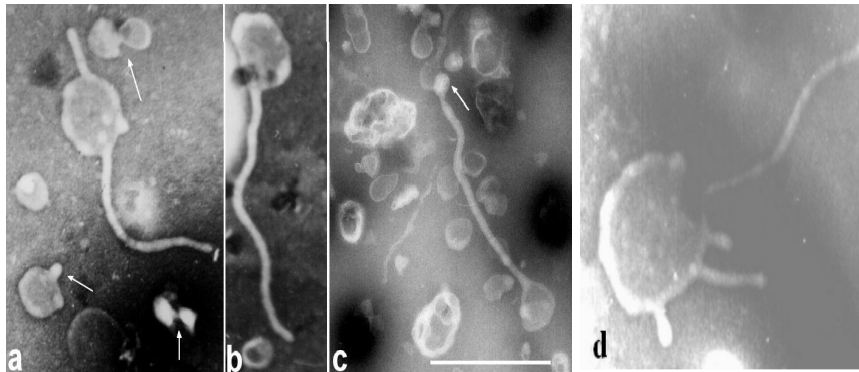


Figure 6. Different aspect of particles of cwdb-phytoplasmas purified from grape, *Chardonnay* with yellow symptoms: **a.** Round and filamentous particles, some in division or budding (arrows); **b.** A cell with long filament; **c.** Numerous round particles and a filamentous cell with elementary body at end (arrow); **d.** Development of filaments by budding from unit membrane of MLOs. Bar: 2 μ m.

The presence of cell wall deficient bacteria (cwdb) in the sieve elements of grapevine *Chardonnay* and *Hamburg* (fig. 7) and *Pinot Noir* were detected on ultrathin sections.

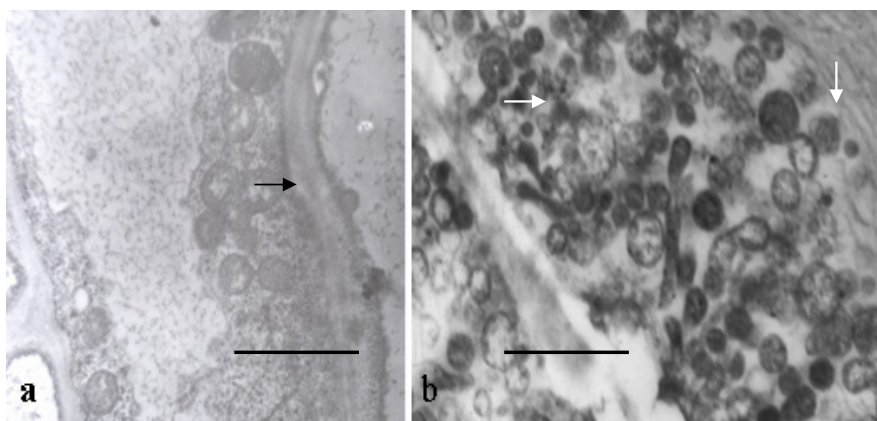


Figure 7. a. Ultrathin section through sieve elements of chlorotic shoots of grape vine *Chardonnay* with symptoms of yellowing illustrated in fig.1. Numerous particles of cwdb are visible in a sieve cell (arrow); **b.** A sieve element of *Hamburg* grape vine (fig. 2) filled with cwdb. Bar = 1 μ m. The small particles in image **b** (arrows) are cross sectioned filaments or elementary bodies.

3.5. Serological proofs for aster yellows MLO in grapevine

The serological tests were performed on purified MLOs from grapevine and *S. titanus* and visualized by electron microscopy. Indirect dot blot ELISA technique was performed and two antisera were used: a primary antiserum produced in rabbits with European Aster yellows, antigen isolated from barley [40] and growth in artificial media in our laboratory from 1975, and a secondary antisera (goat antirabbits antisera conjugate from SIGMA) as is detailed at material and methods.

The following grape cultivars were used for MLOs purification and detection by dot blot technique as is illustrated in figure 8. The purified MLOs were spotted on nitrocellulose strips as follows:

A: 1. *Pinot Noir*, Blaj; 2. *Hamburg*, Bucharest; 3. *Riesling*, Murfatlar; 4. MLOs from *Cacopsylla pyri* vector of pear decline in Bucharest area; 5. *Cabernet Sauvignon*, Banu Mărăciine; 6. Blank (without antigen); 7. *S. titanus*;

B: 1. Healthy periwinkle; 2. Stolbur periwinkle (fig. 4c); 3. Stolbur periwinkle (fig. 4d); 4. *Feteasca neagră*, Tohani; 5. *Chardonnay*, Tohani; 6. Blank (without antigen); 7. *Beaujoules* (original from France in Collection of UASVM Bucharest); 8. Aster yellows, cultivated on artificial media;

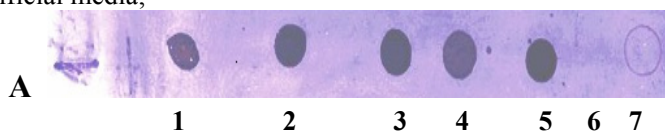


Figure 8A. Results of detection of Aster yellows MLO in grape

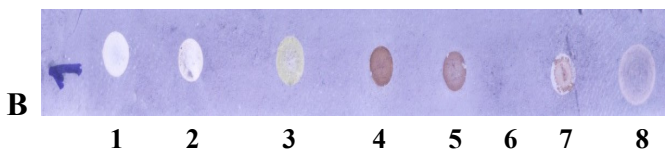


Figure 8B. Results of detection of Stolbur MLO in periwinkle

The strong reaction is evident in figure 8A, on strip **A**, in positions 1-5 where AY was detected in grape cultivars *Pinot noir* (1), *Hamburg* (2), *Riesling* (3) and *Cabernet Sauvignon* (5). Negative reaction was observed in position 6 where the antigen was not spotted and a weak reaction was obtained in position 7 with extract from *S. titanus*. Similar reaction appeared in position 4, where an extract from *C. pyri* was spotted. In figure 8B, position 1-3, the reaction was negative for extract of healthy periwinkle (1) and for stolbur (2, 3), but strong positive in positions 4 (*Feteasca neagră*) and 5 (*Chardonnay*). Position 6 was blank, position 7 was positive for *Beaujoules* and position 8 for AY.

These results are the first experimental and clear proofs on the association of the AY (16SrI) and stolbur (16SrXII) with symptoms of grape yellows-type diseases in Romania. The Aster yellows (AY) MLO isolated in artificial media and used by us to produce polyclonal antisera in rabbits is identical with American Aster yellows (Tulelake strain) examined by Dr. Ploaie in California, in laboratory of Prof. Freitag [41] and European Aster yellows observed at Bratislava, in laboratory of Valenta [17]. These antibodies have been characterized not only by the double diffusion in agar gel and immuno dot blot method but also by immunogold labelling [42, 43] in periwinkle, on ultrathin sections.

The prevalence of AY MLO in grapevines with symptoms of GY was established after 1990 in Italy [44, 45], S.U.A. [46], Tunisia [10], South Africa [11, 47] and Australia [13]. The MLOs groups 16SrI (Aster yellows) and 16SrXII (Stolbur) are considered analogous [5] but are distinct by symptoms on periwinkle plants, in the same conditions of temperature and humidity in greenhouse [48].

Recent investigations based on heteroduplex mobility assay [49] and by RFLP analysis of the amplicons obtained by PCR [50] established that those grapevines are infected by numerous MLOs, including aster yellows. FD MLO is associated not only with grapevine but also with clematis (*Clematis vitalba*) in natural foci [51]. All MLOs have a name according to the symptoms. Transmission of these agents on specific host plants as *C. roseus* is recommended for comparative investigations in the same conditions, for characterization of so called MLOs or phytoplasmas.

Purification of GY and other MLOs is easily performed if the osmotic pressure of buffer is adjusted to osmotic pressure of sieve elements of phloem, where MLOs abundantly grow [3, 35, 36]. Electron microscopy is essential for visualization of MLOs in sediments, after purification from grape plants, and utilization in PCR of DNA purified from MLOs instead of total DNA, that is a source of false positive results [52].

4. Conclusions

Grape yellows is a disease of natural foci caused, in Romania, by solbur and AY.

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