WHAT ARE GRAPEVINE PHYTOPLASMAS?

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SUMMARY

Phytoplasmas, unicellular vegetable parasites, belonging to the class Mollicutes, which exhibit globular (spherical) or ellipsoidal form with polymorphism (cell wall-less prokaryotes), are present in the phloem cells of the plants which they parasite. Typically phytoplasmas are detected and characterized using the molecular methods PCR-RFLP.

The symptoms induced by phytoplasmas in grapevines, specifically transmitted by some Cicadelloidea, are primarily associated with delayed bud-burst, curling and the appearance of yellows or reddish leaves in summer. Among the several characterized phytoplasmas in Europe the disease of interest is known as grapevine yellows disease - "Flavescence dorée" (FD), is specifically transmitted through the vector *Schaphoideus titanus.*

The control of phytoplasmas in grapevines is done mainly by avoiding infected vegetable material propagation and controlling the insect vector.

Keywords: phytoplasmas, MLOs, "flavescence dorée", "grapevine yellows"

PHYTOPLASMAS: WHAT ARE THEY?

Phytoplasmas are unicellular vegetal parasites belonging to the class Mollicutes. They exhibit globular (spherical) or ellipsoidal form with polymorphism due to the non-existence of a cellular wall. These obligate parasites are present in the plant's phloem (Caudwell, 1988; Agrios, 1997).

Many plant diseases of unknown origin associated with "yellows", "deformations" and/or "decline" and normally transmitted by insects were for many years considered possible viruses. In 1967, Doi *et al*. (citados by Borges, 1975) observed for the first time, with transmission electron microscopy, polymorphic bodies 80-800 nm in diameter in a phloem cell preparation of several plants with "yellows" and also in insect preparations of known vectors of those diseases. These organisms were then called MLOs – "mycoplasm like organisms", for their similarity to mycoplasms already identified as pathogenic agents of several animal and human diseases. In 1994, the "Subcommittee on the Taxonomy of Mollicutes" (Avinent and Llácer, 1994) decided to adopt the name phytoplasma in substitution of MLOs for these vegetal parasites, thus initiating studies of phytoplasmas molecular characterization (Davis e Sinclair, 1998). Cousin (1995) point out the principal differences between phytoplasmas, classic bacteria, bacteria present in conducting tissues, virus, and all vegetal parasites that can trigger "decline" and "yellows" in plants.

PHYTOPLASMAS: WHAT ARE THE SYMPTOMS?

The given name to phytoplasmosis is associated with the morphologic symptoms of the host plant. Therefore, leaf color changes are frequent in diseases caused by phytoplasmas. In the vine (Vitis *vinifera* L.) the symptoms are mainly associated with late bud burst followed by leaf curling and chlorosis (or redness in red varieties) of summer leaves.

All the cultivated grape varieties are susceptible to different degrees of FD, the world's most spread phytoplasma, (Torrubiano, 1998). The characteristic symptoms are shown in picture 1 and picture 2 (Caudwell, 1988). When infected, bud burst is late in the spring and the

leaves stay small. After summer, leaves discolor, curl downward and harden (picture 1). In white varieties the disease is characterized by a yellow-gold color in angular spots through the main veins, sometimes with necrosis (picture 2). In red varieties there is often generalized or sectional early redness.

It is important to not confuse FD symptoms with direct damage from cigarrinha verde (*Empoasca vitis* Gothe, *E. solani* Curtis, *E*. *decepiens* Paoli *and Jacobiasca lybica* Bergevin & Zanon) plague very common in vineyards in Portugal (Freitas and Sobrinho, 1999; Amaro, 2001). The phytotoxicity of this piercing-sucking plague of the phloem causes mainly at the edge of the leaf, red spots (or yellows in white varieties), well limited by the secondary veins, resulting in leaves at the end of the vegetative cycle with a "burnt" look. The intensity of this damage depends of the affinity of cigarrinha for several vine varieties. It is also important, not to confuse FD with the damage caused by the leafroll virus, (*Grapevine leaf roll associated virus* 3 - GLRaV-3), the most prevalent virus in Portuguese vineyards. In addition to leaf early redness (or yellow in white varieties), the symptoms also include a pentagonal image throughout the remaining green color in the main veins (picture 4). Grapevines with phytoplasmas can present non-woody stems with black spots. If the symptoms appear before or during flowering, all the inflorescence dries up.. If the symptoms appear at the end of summer, the stems dry up and the grapes corrugate and retain a fibrous pulp. (Caudwell, 1988).

It is important to note that the simple presence of phytoplasmas in the grapevine does not establish the disease since we can find phytoplasmas in plants with no symptoms. Therefore, the onset of the disease can only be established after the achievement of the transmission of the phytoplasma by a vector insect, or graft, from the infected plant to the new host plant.

PHYTOPLASMAS: WHAT KIND OF DAMAGE DO THEY CAUSE?

Asphytoplasmas multiply in the grapevine phloem, the products resulting from photosynthesis accumulate in leaves and are not used by the plant.(Barrios *et al*., 1998). A strong decrease in production and progressive degeneration of the plant results in killing the vine in a few years.

Of the several grapevine phytoplasmas, FD (Flavescence Dorée) is the most economically significant because it is associated to a common vector of *S. titanus.* This leafhopper originated from North America but is well spread throughout Southern Europe and Eastern European Countries (Boudon-Padieu, 2000).

PHYTOPLASMAS: HOW ARE THEY TRANSMITTED?

In Nature, phytoplasmas are transmitted from plant to plant specifically by *homoptera* insects from the *Cicadellidae* (Leafhopper) family and also by insects from the *Psyllidae* and *Fulgoridae* families, Currently the leafhopper *Scaphoideus titanus* (Boudon-Padieu, 2000) and the plant hopper *Metcalfa pruinosa* recently introduced in Europe (Guadagnini *et al*., 2000) are known as FD vectors. The insect fulgoromorpha polífago, *Hyalesthes obsoletus* Signoret is the vector of another phytoplasma in grapevines, responsible for "black wood" disease - BN (Sforza and Boudon-Padieu, 1998; Maixner *et al*., 2001)

Studies made in France and cited by Avinent and Llácer (1994) show that *S. titanus* only have an annual generation: the adult deposit eggs in the cortex and in the winter buds; the eggs hatch in April-May, succeeding through five larva states that colonize quickly on the leaves (mainly on the lower surface) until the death of the adults in September – October. It is during summer that the plant can be infected. Phytoplasmas grow and multiply in the alimentary canal, hemolymph, salivary glands and intercellular spaces of their insect vectors. The pick-up period of phytoplasmas by the vectors (nymphs and adults) it is generally longer than 24 hours and they have a latency period of 10-45 days depending on the temperature. The latency period is shorter with high temperatures (Agrios, 1997; Avinent e Llácer, 1994). This period of incubation is needed for the multiplication and distribution of the phytoplasma throughout the insect's body then passing to the hemolymph and infecting several organs

including the salivary glands. When the insect pricks a new plant to feed itself, the phytoplasma is transferred through the saliva. Once infectious, the vector transmits the pathogenic agent during its remaining lifecycle. After being affected by the vector's inoculation, the FD symptoms in the grapevine resulting from the phytoplasma, are only apparent normally in the following year (Rivenez and Bonjotin, 1997) and possibly, due to the long potential incubation period, as long as three years (Boudon-Padieu, 2000). Avinent and Llácer (1994) determined that phytoplasmas are not transmitted through mechanical, pollen, or seeds.

As with all infectious diseases, grafting also transmits phytoplasmas, even if the percentage of transmission is conditioned by grafting timing, due to irregular distribution of phytoplasmas in lapsed leaves species (Avinent e Llácer, 1994). Therefore, the primary infection in a vineyard can have its origin caused by infected vegetal material or have been introduced by insect vectors. The natural spreading from vine to vine is essentially made by the insect vectors. Regional spreading of the disease can be the result of transportation of infected material.

In Portugal, the leafhopper *S. titanus* was identified for the first time in 2000 in samples collected from the regions Entre Douro e Minho - Arcos de Valdevez e do Douro - Vila Real (Quartau *et al.*, 2001). Picture 5 is an adult and a nymph of *S. titanus* captured in the Douro region in July 2001 (Quinta de Prados, Vila Real). It is important to point out that several species of green leafhoppers, nominated *Empoasca vitis*, *E. solani*, *E. decepiens* and *Jacobiasca lybica* (Freitas and Sobrinho,1999; Amaro, 2001) are not vectors of phytoplasmas in grapevines.

PHYTOPLASMAS: HOW TO IDENTIFY THE DISEASE?

Works of data collecting and studies of epidemiology of a disease have different specificities. Detecting the disease can be enough for quality control of the vegetative propagation material, but identification and characterization of the pathogenic agent will need more specific analyses. Therefore, the procedure for detecting and identification of grapevine phytoplasmosis depends on the objective.

The several methods of detecting and identification of grapevine phytoplasmosis are:

- detection by the symptoms. The observation in Spring-Summer of typical symptoms associated with the capture of vectors in the vineyard can give the indication of possible infection by phytoplasmas, but it is not a diagnostic procedure since a complementary laboratory test is necessary. The symptoms normally only appear one to three years after the implantation of infected material (Boudon-Padieu, 2000).
- detection by biological indexing. Consists of testing vegetative material propagation in sensitive indicator plant varieties to the phytoplasma. For grapevine FD the indicator plant commonly used is the hybrid Baco 22A as is the *V.vinifera* cv. Chardonnay and cv. Aramon. Nevertheless, regarding to the irregular distribution of phytoplasmas in the grapevine, it is not possible to use dormant canes for this artificial transmission; they must be used from graftings in May because during the vegetative rest phytoplasmas concentrate themselves in the roots.(Caudwell and Martelli,1993; Avinent and Llácer, 1994).
- detection *in situ* with staining methods. For example, the DAPI method; DAPI (4,6-diamidino-2-phenylindole) is a nucleic acid specific stain that will stain bacteria in the phloem. This technique does not allow us to distinguish phytoplasmas from bacteria, causing grapevine diseases such as Pierce's Disease.
- detection by transmission electron microscopy. The phytoplasmas can be observed in cuts of infected phloem tissues and preparations of infected insects
- ELISA identification. This diagnosis – Enzyme Linked Immunosorbet Assay – is possible for some phytoplasmas of fruit plants and several ornamental plants. The identification of phytoplasmas by ELISA, can be done in the vegetative material and in the vectors. Normally it has been difficult to obtain enough purified material for the

production of good antibodies for the grapevine phytoplasmas, due to their low numbers and irregular distribution in the plant and during the vegetative cycle (Boudon-Padieu, 2000). In France specific antibodies have been developed for FD and “Black Wood” since ELISA technique is routinely used for the French protection of plants services for the detection of these phytoplasmas (Boudon- Padieu, 2000).

- Identification by PCR-RFLP. Diagnosis based in the PCR analysis (polymerase chain reaction), followed by RFLP (restriction fragment length polymorphism), has been, just after the development of universal primers for the amplification of ribosomal DNA of phytoplasmas, the more commonly used method in molecular identification and characterization of phytoplasmas (Davis and Sinclair, 1998; Lee *et al*., 1998).
- Also the use of PCR for amplification of no ribosomal DNA has been efficient for the study of variability among isolates (Boudou-Padieu, 2000). "Nested" - PCR and construction of highly specific primers for several grapevine phytoplasmas are in study (Boudou-Padieu, 2000). Firrao *et al.* (cited by Boudou-Padieu, 2000) developed a "dot-blot" with a primer from PCR products from rDNA amplified with universal primers. Grapevine samples to be tested by PCR - RFLP must be harvested by September-October. However, there is a reference of detection done in May in plants with symptoms (Barrios *et al.*, 1998).

PHYTOPLASMAS: WHICH ONES ARE ASSOCIATED WITH GRAPEVINES?

In grapevines, the economically most important phytoplasma has been “flavescence dorée”, with the vector although originally from North America now being well established in Europe. In the last meeting of ICVG, Boudon-Padieu (2000) identified the following phytoplasmas associated to grapevines:

- FD - "flavescence dorée". The disease was detected in France since the 50’sand considered a virus until 1986. Phytoplasma classified in EY group or 16SrV (Elm yellows group). Its geographic distribution currently seems to be the south of France, Northern Italy, Northern Spain and Germany. The major vector is *Schaphoideus titanus* and *Metcalfa pruinosa* has also been implicated.
- PGY- "palatinate grapevine yellows". Phytoplasma also from the EY group, but molecularly distinct from FD. Its vector is *Oncopsis alni*. For now, this phytoplasma seems restricted to the Palatinate region in Germany.
- BN - "Black Wood" and VK - "vergilbungskrankheit". Phytoplasmas from the Stolbur (16SrXII) group found in France, Germany, Switzerland, Italy, Hungary, Croatia and Israel. Are transmitted not by a leafhopper but by the insect *Hyalesthes obsoletus* (Sforza and Boudon-Padieu, 1998; Maixner *et al*, 2001).
- Phytoplasmas from the AY (aster yellow) group identified in grapevines in Italy, Slovenia, Croatia and Israel.
- Phytoplasmas from the WX (western-X phytoplasmas) group identified in grapevines in the north of Italy, Israel and also in the USA (Virginia grapevine yellows -VGYIII).
- Another phytoplasma still under characterization and detected in Australia is now known as "Australian grapevine yellows" (AUSGY – 16rXII group).

PHYTOPLASMAS: HOW TO FIGHT THE DISEASE?

Controlling phytoplasmosis in grapevines is not easy. Currently, it seems that FD is only economically important in France and Northern Italy, but its presence and the presence of its efficient vector, already detected in several countries, indicate the possibility of a fast spreading problem.

The long incubation period of this disease (up to 3 years) makes real the possibility of planting a vineyard with infected material and only identifying the disease several years later. In France, INRA and ENTAV are using thermotherapy as a way of controlling FD and BN by

immersing the rootstocks and the scions in water at a temperature of 50ºC for 45 minutes, (Geoffrion, 1998).

Monitoring the nurseries by looking for the disease symptoms followed by laboratorial confirmation should be a regular practice. Plants present in the vineyards that can be transmitters of phytoplasmas and/or vector insects (Maixner *et al*., 2001) should be also be monitored especially in nurseries.

PHYTOPLASMAS: WHAT IS THE SITUATION IN PORTUGAL?

Phytoplasmas in Portugal are organisms in quarantine. Until this date, no phytoplasma has been identified in Portuguese grapevines, but as far as we know this issue has not been studied in the laboratory. Adults and nymphs of *S. Titanus*, the efficient vector of FD were found in the North of Portugal (in the Entre-Douro and Minho and in Douro regions) in 2000 and 2001 (Quartau *et al*., 2001), It is advisable to evaluate if this appearance was sporadic or if *S. titanus* was already established. Regarding the economic importance of grapevines in Portugal, the facility of circulation of vegetative material and the potential danger of FD, since its vector was detected should warrant more intense investigation of the vector and of the pathogenic organism followed by laboratory tests for identification of grapevine phytoplasmas.

PHYTOPLASMAS: PICTURES

Picture 1 - Chlorosis and leaf curl in Baco 22 A leaves infected with "Flavescence dorée" (A. Caudwell, 1988).

Picture 2 – Angular spots through the main veins in Baco 22 A leaves infected with "Flavescence dorée" (A. Caudwell, 1988).

Picture 3 – Spots very clearly limited to the secondary veins as a result of phytotoxic pricking by the leafhopper Empoasca vitis (A. Caudwell, 1988).

Picture 4 – Leaf roll and early leaf reddness and maintenance of green main veins as a result of infection by GLRaV3 (photo from M. Carvalho, Vila Real,1997).

Picture 5 – Adult and nymph of S. titanus (photo from L. Torres, Vila Real, 2001).

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