Research Note

Detection of flavescence dorée phytoplasma in grapevine in northern Spain

A. Batlle[1]), A. Laviña[1]), C. Kuszala[2]), D. Clair[2]), J. Larrue[2]) and E. Boudon-Padieu[2])

Summary: Grapevine yellows diseased stocks were observed in vineyards of the Alt Empordà area, northern Spain. Phytoplasma was detected and characterized by PCR-RFLP in the region of the 16S rDNA of phytoplasmas or by ELISA. For the first time a FD phytoplasma was detected in Spain.

Key words: phytoplasma, grapevine yellows, detection, flavescence dorée, bois noir.

Introduction: Phytoplasma diseases of grapevine, called grapevine yellows (GY), have already been found in many vine growing areas of America, Australia, Europe and in Israel (Daire *et al.* 1997 a). In Europe, flavescence dorée (FD) and bois noir (BN) are most frequent; they cause most serious damage. FD and BN-associated phytoplasmas belong to the elm yellows (EY) group and the stolbur group, respectively, and are transmitted by different insect vectors. In the last 5 years serial samplings in vineyards of north-eastern Spain, indicated that several diseased plants presented the symptomatology associated with phytoplasmas. The symptoms were: mosaics limited by the leaf veins, leaf roll, absence of lignification in autumn, withered flowers and dried berries. Up to now, only the phytoplasma associated to BN had been detected in samples with yellows symptoms (Laviña *et al.* 1995). However, *Scaphoideus titanus* Ball. the leafhopper vector of FD, is present in all vineyards. Thus the occurrence of FD was kept under a constant watch because the leafhopper species is a highly efficient vector of FD, which in turn is a very dangerous disease because of its quick spreading when the vector is present.

In the fall of 1996 in the Alt Empordà (Catalonia) viticulture area near the French border several vineyards were found with a high level of GY-diseased stocks. As FD is causing a severe epidemic on the French side of the border, our attention was directed to this new occurrence of a GY in Spain. This paper reports on the first detection of FD in Spain.

Material and methods: Tissue samples were collected in September and October 1996 at different plots of the Alt Empordà viticultural area from Grenache gris and Carignan grapevines with typical symptoms of GY. Control samples of FD or BN-infected grapevines and of healthy cuttings were included.

Total DNA was extracted from a phytoplasma-enriched fraction prepared from grapevine main leaf veins as previously described (Daire *et al.* 1992). A nested-PCR procedure was used with primers specific for a 16S rDNA region in all known phytoplasma: the first primer pair was P1 (Deng and Hiruki 1991) and P7 (Smart *et al.* 1996) and the second was the pair fU5/rU3 (Lorenz *et al.* 1995). The amplification mixture contained 0.375 µM of each primer, 150 µM of dNTPs, 1 unit·100 μl^{-1} of Taq DNA polymerase (Appligene), Taq buffer (Appligene), overlaid with mineral oil. Amplification was carried out in a total volume of 20 µl containig 5-10 ng DNA, for the first amplification. 2 µl of the amplification product were used for the second step in a total volume of 40 µl of amplification mixture. 10 µl of the mixture containing the DNA amplified in the second step was directly digested overnight with *Tru* 9I restriction enzyme. The RFLP of the fragment allows to distinguish EY and stolbur phytoplasmas (Daire *et al.* 1997 a). FD specific primers FD9f/r (Daire *et al.* 1997 b) derived from both ends of a cloned 1.3 kb fragment from non-ribosomal FD-DNA, were also used for PCR on the same samples.

An indirect ELISA sandwich procedure was applied according to Caudwell and Kuszala (1992). Samples were extracted as described, including the addition of CHAPS (SIGMA) in the grinding buffer. Plates were coated with polyclonal rabbit anti-FD antibodies and a cocktail of monoclonal anti-FD antibodies was used as second antibody, followed by alkaline phosphatase antimouse conjugate. Each sample was assayed in 4 replicates and the OD 405 nm values were recorded after 150 min of incubation with the enzyme substrate.

Results and Discussion: In 7 samples out of 9 from diseased grapevines, an EY group phytoplasma was detected in the nested PCR procedure followed by RFLP analysis (Figure). The same samples were positive when FD9f/r primers were used, and a 1.3 kb amplification product was ob-

Table

ELISA readings of grapevine samples harvested in 2 localities of Alt Empordà. Infected controls were samples from FD- and BN-diseased grapevines from France. Healthy cuttings were ground in greenhouse. Samples 2 and 6 were considered negative for FD

Sample	Origin	Variety	ELISA readings OD 405 nm
1	Locality 1	Grenache gris	0.377
2	(Alt Empordà)		0.078
3			0.207
4	Locality 2	Carignan	0.664
5	(Alt Empordà)		0.330
6			0.093
7			0.525
8			0.288
FD control	Languedoc	Carignan	0.283
	(France)		0.594
BN control	Vallée du Rhône	Chardonnay	0.036
	(France)		0.056
Healthy control	greenhouse	Chardonnay	0.027
			0.037

[1]) Departament de Patologia Vegetal, IRTA, 08348 Cabrils, Barcelona, Spain. Fax: +34-3-7533 954. E-mail: batlle@cabrils.irta.es

[2]) Station de Recherches sur les Phytoplasmes, INRA, Dijon, France.

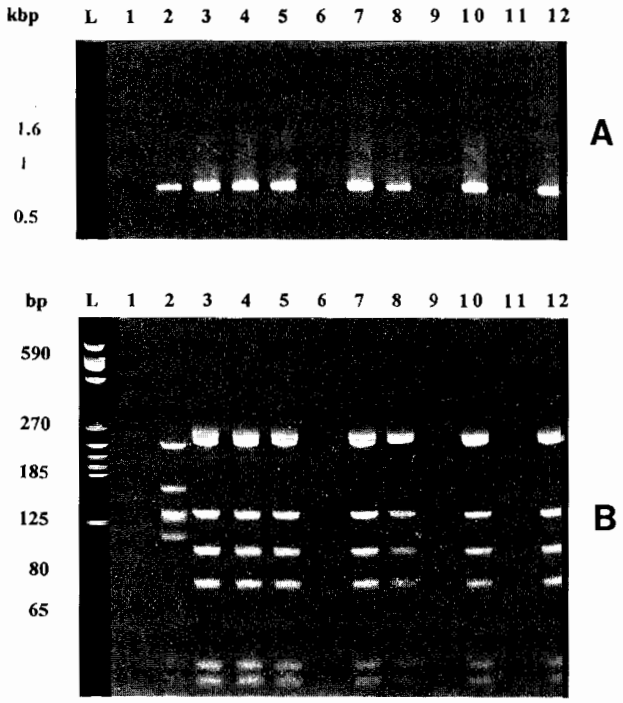

Figure: **A**: agarose gel (1.2%) electrophoresis of 16S rDNA nested-PCR amplified fragments (860 bp). **B**: polyacrylamide gel (10%) electrophoresis of the amplified products shown in A after *Tru* 9I restriction. (1) healthy Carignan, (2) Chardonnay infected with stolbur (BN), (3) Alicante naturally infected with FD from France, (4, 5, 6, 12) Grenache gris from Alt Empordà, (7, 8, 9, 10, 11) Carignan from Alt Empordà. L. Molecular weight marker: **A**, 1Kb DNA (GIBCO BRL); **B**, pBR 322/*Hae* III (Appligene).

tained (data not shown). With the two procedures one of the samples gave only a weak response (lane 6). With the use of FD antibodies in ELISA, 6 samples out of 8 and the FD control gave positive results; healthy and BN infected controls gave negative results (Table).

This shows that a EY-group phytoplasma is also associated with grapevine yellows in Spain. Further restriction analysis showed that this phytoplasma isolate is similar to the FD isolate detected in grapevines in southern France (DAIRE *et al.* 1997 b). The role of *S. titanus* will be checked in the next seasons by transmission trials and direct analysis on wild specimens of the leafhopper species collected in affected vineyards. The occurrence of the disease in Spain underlines the importance to start control measures by limitation of vector populations and the use of cleaned planting material (CAUDWELL *et al.* 1990). Careful watch of possible new foci of the disease by a survey of GY occurring in neighbouring viticulture areas seems to be appropriate.

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