Nematode vectors, grape fanleaf virus (GFLV) incidence and free virus vine plants obtaining in « Condado de Huelva » vineyards zone

Vecteurs de nématode, incidence du virus du court-noué (GFLV) et obtention de plants de vignes exemptes de virus dans la zone viticole « Condado de Huelva »

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Abstract: The « Condado de Huelva » Registered Appellation Origin Mark (RAOM) is located in the Province of Huelva, in the southwest of Andalucía (Spain), being limited by the Atlantic Ocean and the Province of Sevilla. « Zalema », a white high productive grapevine plant is its major cultivar. The predominant rootstocks used are « Rupestris du Lot », « Castel 196-17 », « Couderc 161-49 », Couderc 33-09 », « Richter 110 » and « Millardet 41-B ». Traditionally, « Zalema » cv. has been dedicated to the elaboration of amber, bouquet-flavoured wines and in the last years mainly to young, fruit-flavoured white table wines.
The presence and distribution of *Grapevine fanleaf virus* (GFLV) and *Xiphinema index* and *X. italiae*, the main nematode-vectors of GFLV, were determined by ELISA and soil analysis, respectively. Samples were collected according to a stratified random model. The number and distribution of the samples were related to the size of each area (county) of the RAOM and dispersion of the results represented by the standard deviation (S.D.), being 2.500 and 210 the total vines and soil samples analysed in two years in the 16 counties considered.
From the results, an erratic distribution of healthy plants was found, ranging from 37% (63% of infected plants) in the most attacked county to 87% of free-virus plants in the less affected. The average was close to 27%, considering the surface of vineyards and incidence in each county.
There were also high variations in the nematodes distribution, existing counties without presence of them and others with high number of populations. In average, a 6.2% of soil samples with *X. index* and 20.5% with *X. italiae* were detected. There was no relationship between the number of nematodes and the number of GLFV-infected plants in each county. Nevertheless, if the nematode free zones are not considered, the results indicate a small but appreciable relationship. The use of non-controlled GFLV-infected scions for grafting was considered as the most important way for virus transmission.
The in vitro culture of apical meristems was a good method for the obtaining of free-virus plant material, reaching even a 100% of healthy plants and the non-infected plant material grew better *in vitro* than the infected one. When this free-GFLV plant material was used as scion for grafting in field, an increase of plant growth and production was obtained.

Key words: *Xiphinema index, Xiphinema italiae, in vitro,* Zalema

Introduction

The « Condado de Huelva » vineyard zone (south-western Andalusia-Spain) is characterized by the white wine production mainly from « Zalema » c.v. but also from other varieties, like « Palomino Fino », « Listán de Huelva », « Garrido Fino », « Moscatel de Alejandría » and « Pedro Ximénez ». Normally these vines are grafted on « Rupestris du Lot », « Castel 196-17 » rootstocks and to a lesser extent on « Couderc 161-49 », Couderc 33-09 », « Richter 110 » and « Millardet 41-B »ones. The RAOM is divided in 16 areas (counties) (fig 1). According to Papadakis classification (FAO 1981) the RAOM is characterized by Subtropical Warm thermal regime and Mediterranean Humid humidity regime. The main soil types are Calcic Cambisol and Chromic Vertisol.

From several years the *Grapevine fanleaf virus* (GFLV) (Weiland and Pérez-Camacho, 1993) and its vectors, the soil dagger nematodes *Xiphinema index* and *X. Italiae,* in the « Condado de Huelva » vineyards (Weiland and Pérez-Camacho, 1995) have been detected. Under severe environmental conditions (shallow soils, hot and sunny climate) fanleaf virus may kill the vines. However, affected vines usually remain alive for a long time, but becoming less and less productive provoking the no cultivation of the infected vineyards for economic reasons. Between *X. index* and *X. italiae* GFLV-vectors the last one is considered less effective as virus giving in field. It has been verified that the nematode can receive the virus from an infected plant after only five minutes of contact. In adult nematodes, the presence of active virus particles can be maintained from several weeks to more than 9 months and even, up to two years (Arias and Andrés, 1988). The easy virus acquisition and retention could facilitate the disease dispersion, although nevertheless the reduced displacement in field of these nematodes (1-1.5 m/year) limits this event.

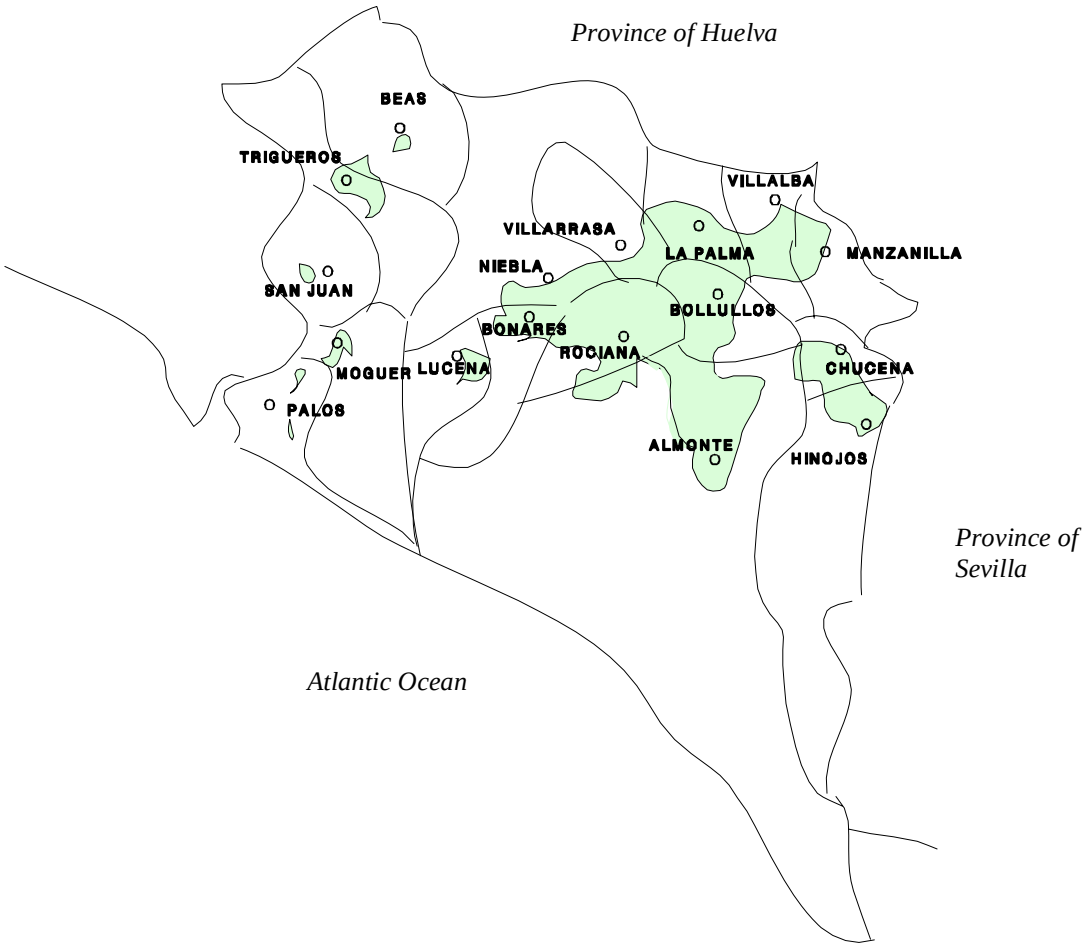


Figure 1 - The « Condado de Huelva » Registered Appellation Origin Mark (RAOM)

When a high level of the GFLV infection exists, it would be advisable to have the possibility of the obtaining of virus free plants. Thermoterapy (Vuittenez, 1962), chemotherapy (Kummer y Semal, 1970) and *in vitro* culture (Gifford *et al.*, 1961; Harris and Stevenson, 1979) are suitable methods. Cantos *et al.* (1993) and Weiland and Cantos (2004) obtained very good results by combining both thermoterapy and *in vitro* culture. Then the goal of this work was to determine the degree of GFLV-infection in the « Condado de Huelva » RAOM, the possible relationship between virus infection and presence of nematodes *X. index* and *X. italiae* and the obtaining of free virus ones by combining thermotherapy and *in vitro* meristem culture.

Materials and methods

To determine the GFLV, *X. index* and *X. italiae* distributions in the « Condado de Huelva » RAOM a stratified random sampling soil model was applied. For that both geographical distribution of the vineyard in the 16 counties and standard deviation (SDi) previously determined, were considered. According to these parameters the following formulas were applied:
With the results obtained in each pilot-sample, the sample size is « n »:

$$n = \frac{\sum_{i=1}^{i} W_i P_i Q_i N_i/(N_i-1)}{(e_\alpha^2 / \lambda_\alpha^2) + (1/N) \sum_{i=1}^{i} W_i P_i Q_i N_i/(N_i-1)}$$

n = total sample (ha); Ni = « i » zone grapevine surface (ha); N = « Condado de Huelva » grapevine surface (ha); $W_i = N_i/N$; P_i = pilot-sample, zone « i » level GFLV-affected or Xiphinema index or X. italiae (%); Q_i= pilot-sample, zone « i » level GFLV-non affected or soil without Xiphinema index or X. italiae (%); e_α= sample error (5 %); λ_α= confidence coefficient (1,96); α=level coverage for mean confidence intervals (95 %).

Once known the « n » value, it is distributed proportionally to grapevine surface (N_i) and to the standard deviation in each zone (SD_i), as it appears next:

$$\frac{n_1}{N_1 \times SD_1} = \frac{n_2}{N_2 \times SD_2} = \frac{n_3}{N_3 \times SD_3} = \ldots = \frac{n_i}{N_i \times SD_i} = \frac{n}{\sum_{i=1}^{i} N_i \times SD_i}$$

N_i = « i » zone grapevine surface (ha); SD_i = pilot sample, zone « i » standard deviation; n_i = surface to analyze in each zone(ha.).

In order to determine the relation between GFLV affected plants and soil with vectors nematodes percent the correlation coefficient « r » (Pearson) was calculated.
To determine the level of GFLV infection one adult leaf per plant of each vineyard-plot was sampled at the end of spring. Every leaf was washed gently first with tap water and then with distilled water. After that, the mesophyll of each leaf was cut into small pieces and analized by ELISA test according to Gugerli *et al.*, (1984).
The isolation of Xiphinema was reached by Flegg (1967) method and their identification by Thorne and Allen (1950) and Martelli and Lamberti (1967) ones.
For the in vitro experiments GFLV-affected and non-affected uninodal explants of near two cm length were used. The explants had been previously disinfected by immersion for a few seconds in 70% ethanol and another immersion in 12% sodium hypochlorite (3,5% active chlorine) plus some drops of Tween 20 (20 min. at 35 ºC with stirring). After the disinfection, the explants were placed individually in sterile test tubes containing 10 ml of VID culture medium (Troncoso *et al.,* 1990). Once covered with plastic caps and sealed with parafilm, the tubes were then placed in a growth chamber at 23±1 ºC, 30 $\mu Em^{-2}s^{-1}$ of light intensity and 16 h. photoperiod. After 90 days of in *vitro* culture, the number and development of the infected and non-infected *in vitro* new plants was compared. From these plants, new explants were prepared and *in vitro* cultured (subculture) in the same manner as mentioned above.
For the *in vitro* obtaining of free virus plants, meristems of 0.1-0.2 mm by cutting the apical end of *in vitro* cultured virus infected plants were prepared. The meristems were cultured *in vitro* as previously indicated but during meristem *in vitro* development the temperature was increased up to 37 ºC. This process was repeated several times. The regenerated plants were analyzed by the ELISA test.

Results and discussion

The first pilot-sample results indicate 18.09 % GFLV-affected plants and 0.39 standard error and considering the results in each county the surface to sample is 209,82 ha. This data are proportionally distributed to the

grapevine surface (N_i) and to the standard error of each zone (SD_i). The final results indicate 27,49 % GFLV-affected plants (table 1). These results indicate a dangerous situation since 1 out of 4 plants (approx.) cultivated in the « Condado de Huelva » RAOM are GFLV-affected, and in some counties like Bonares, Moguer, Palma, Palos and Trigueros the proportion is even higher. The second pilot-sample results indicate 1.61 % and 20.97 % of soil with *X. index or X. italiae*, respectively. With these data the surface to sample is 210 ha. This data are proportionally distributed to grapevine surface (N_i) and to standard error of each zone (SD_i). The definitive results indicate 6.19 % and 20.48 % of soil with *X. index* or *X. italiae*, respectively (table 2).

Table 1 - Results of the first pilot-sample and proportional sample of GFLV-affection (%) in « Condado de Huelva » RAOM.

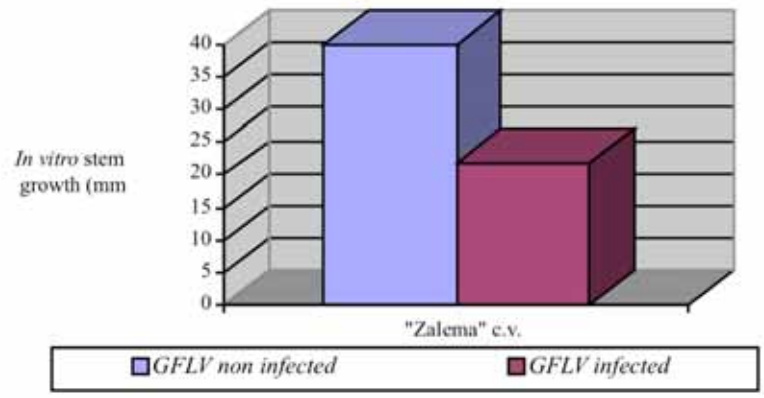
		Pilot-Sample			Proportional Sample		
County	N_i (ha)	0,5 % (ha)	GFLV (%)	SDi	n_i (ha)	GFLV (%)	SD_i
Almonte	2.398	11,99	10,00	0,30	36,90	23,02	0.42
Beas	300	1,50	20,00	0,41	6,29	15,79	0.38
Bollullos	2.356	11,78	15,92	0,37	44,22	29,57	0.16
Bonares	343	1,72	8,69	0,29	5,05	40,98	0.50
Chucena	321	1,61	26,09	0,45	7,37	13,48	0.34
Hinojos	214	1,07	46,67	0,52	5,65	29,41	0.46
Lucena	171	0,86	15,38	0,38	3,28	27,50	0.45
Manzanilla	643	3,22	9,30	0,29	9,66	12,93	0.34
Moguer	171	0,86	66,67	0,49	4,30	63,46	0.49
Niebla	342	1,71	21,74	0,42	7,37	22,47	0.42
Palma	514	2,57	16,67	0,38	9,93	54,17	0.50
Palos	26	0,13	50,00	0,71	0,94	58,33	0.52
Rociana	1.713	8,57	23,68	0,43	37,40	21,60	0.41
Trigueros	428	2,14	20,69	0,41	9,02	37,61	0.49
Villalba	685	3,43	24,44	0,43	15,22	27,87	0.45
Villarrasa	373	1,87	16,67	0,38	7,18	29,88	0.46
TOTAL	10.998	54,99	18,09	0,39	209,82	27,49	0.45

Table 2 - Results of the second pilot-sample and proportional sample of soils with X. index or X. italiae (%) in « Condado de Huelva » RAOM ».

		Pilot-Sample					Proportional Sample		
County	N_i (ha)	0,5 % (ha)	*X. index* (%)	SD_i	*X. italiae* (%)	SD_i	n_i (ha)	*X. index* (%)	*X. italiae* (%)
Almonte	2.398	12	0,00	0,00	25,00	0,45	45	4,44	24,44
Beas	300	2	50,00	0,71	50,00	0,71	6	16,66	16,66
Bollullos	2.356	12	0,00	0,00	25,00	0,45	44	2,27	15,00
Bonares	343	2	0,00	0,00	0,00	0,00	7	14,28	28,57
Chucena	321	2	0,00	0,00	50,00	0,71	6	0,00	16,66
Hinojos	214	2	0,00	0,00	0,00	0,00	4	0,00	0,00
Lucena	171	1	0,00	0,00	0,00	0,00	4	0,00	0,00
Manzanilla	643	4	0,00	0,00	50,00	0,58	12	8,33	41,67
Moguer	171	1	0,00	0,00	0,00	0,00	4	0,00	50,00
Niebla	342	2	0,00	0,00	0,00	0,00	7	0,00	0,00
La Palma	514	3	0,00	0,00	0,00	0,00	10	0,00	16,66
Palos	26	1	0,00	0,00	0,00	0,00	1	0,00	0,00
Rociana	1.713	9	0,00	0,00	11,11	0,33	32	3,12	21,87
Trigueros	428	3	0,00	0,00	0,00	0,00	8	25,00	0,00
Villalba	685	4	0,00	0,00	50,00	0,58	13	7,69	15,38
Villarrasa	373	2	0,00	0,00	0,00	0,00	7	42,85	14,28
TOTAL	10.998	62	1,61	0,13	20,97	0,41	210	6,19	20,48

The correlation analysis indicates almost no relation between % GFLV and % soil with *X. index* (r = -0,119) or *X. italiae* (r = 0,111). Nevertheless, if the nematode free zones are not considered, the coefficient correlations change to 0.322 and 0.405, respectively. The two last correlations indicate a small but considerable relation.

Figure 2 - Influence of the GFLV-infection on *in vitro* 50 days) stem-growth of « Zalema » grapevine explants.



These lack or low correlation between GFLV-infection and nematode presence indicated that other factors can be responsible for the virus spreading. The use of infected plant material as rootstock or mainly scion for grafting can be one of the first causes of disease extension.

Combining plant meristem *in vitro* culture and thermotherapy a 90-100% of « Zalema » virus free vine plants were obtained indicating the suitability of the method. When GFLV-infected and non infected « Zalema » explants were cultured in vitro there were a significant lower growth of the infected one (fig. 2).

On the field experiment, the grafted in vitro obtained « Zalema » free virus plant material, showed during the first year, a juvenile-like development: The growth of the free virus scions was clearly higher than the normal one, and their leaves much bigger, with red colour along the nerves and pubescences, but there was no production. After the first year the symptoms of juvenility disappeared and the shoot and leaf growth continued to be higher, but with less differences and the production was practically two-fold than that of the normal field surrounding plants. In consequence, the in vitro obtained free-virus plant material offered interesting field culture conditions.

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