Investigations on Grapevine Fanleaf Disease and Relationships Grapevine Fanleaf Virus (GFLV) With Vector Nematode *Xiphinema spp.* at Vineyards in the Trakya Region of Turkey*

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ABSTRACT

Grapevine fanleaf disease caused by *Xiphinema* nematode transmitted Grapevine fanleaf virus (GFLV) has been the most important disease on grapes (*Vitis vinifera* L.) in the vineyards of the World. In order to determine Grapevine fanleaf disease caused by GFLV on grapevine stocks and the relationships between virus and vector nematodes *Xiphinema spp,* this research study was performed during the years of 2013 and 2014. This study was implemented by examining infected stocks and collecting samples in villages and districts of Tekirdag, Kırklareli, and Edirne provinces in the Trakya Region. During the survey visits grapevine leaves, shoots, pediols and cambium tissue samples were collected from virus infected stocks. Beside tissue samples, soil samples with hairy roots were collected from rhizosfer around each infected vine grape stocks. For the identification of GFLV Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) test was implemented to tissue samples as morphological and morphometrical measurements were applied to soil samples for the identifications of nematode species. As a result of DAS-ELISA tests 35 out of 152 tissue samples revealed the presence of GFLV. Nine soil samples of 35 GFLV infected stocks revealed the presence of *X. index* vector. Thirteen soil samples from GFLV infected stocks, however had *X. pachtaicum* as 13 soil samples were lack of any *Xiphinema* species at all. Those GFLV infected stocks without nematode vector probably got virus during the grafting and pruning or at the vineyard establishment with infected saplings. Another results of this study also implied that *X. pachtaicum* has been more widespread nematode vector species than *X. index* for GFLV in the Trakya Region.

Keywords: *Vitis vinifera,* (GFLV), DAS-ELISA, *Xiphinema spp.*, Trakya Region

ÖZ

Türkiye'nin Trakya Bölgesi Bağlarında Kısa Boğum Hastalığı Grapevine Fanleaf Virus (GFLV) ile Vektör Nematod *Xiphinema* spp. İlişkilerinin Araştırılması

Dünya'da üzüm (*Vitis vinifera* L.) bağlarında görülen en önemli virüs hastalığı *Xiphinema* nematod türleri tarafından taşınan Grapevine fanleaf virus (GFLV)'nün neden olduğu kısa boğum hastalığıdır. 2013 ve 2014 yıllarında yürütülen bu çalışmada, Trakya Bölgesi Bağları'ndaki asmalarda ve omcalarda kısa boğum hastalığına neden olan GFLV ile bu virüsün vektörü olan *Xiphinema spp.* nematod türlerinin yaygınlık durumunun belirlenmesi, vektör nematodlar ile GFLV arasındaki ilişkinin saptanması amaçlanmıştır. Araştırma ile Trakya Bölgesi'nin Tekirdağ, Kırklareli ve Edirne illerinde, ilçe ve köylerdeki bağ alanlarında gözlemler yapılmış ve örnekler alınmıştır. 2013 ve 2014 yıllarında yapılan sürveylerde, virüs hastalık belirtileri gösteren omcaların yaprak, sürgün,

* This study is master science thesis.

petiol ve kambiyumlarından örnekler toplanmıştır. Ayrıca hastalıklı omcaların her birinin kök bölgesinden, kılcal kökleri ile birlikte rizosferinden toprak örnekleri de olmak üzere toplam 152 adet örneği sağlanmıştır. Alınan bitki doku örnekleri, Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) yöntemi ile serolojik teste tabi tutulurken; toprak örneklerine morfolojik ve morfometrik ölçümler yapılarak, toprak bünyesindeki nematod türleri saptanmıştır. DAS-ELISA testi sonucu elde edilen bulgulara göre, 152 adet yaprak ve doku örneğinin 35 adedi, GFLV ile enfekteli olarak saptanmıştır. GFLV ile enfekteli olarak saptanan omcaların 9 adedinin toprak örneğinde *Xiphinema index*, yine GFLV enfekteli 13 omcanın toprak örneğinde ise *Xiphinema pachtaicum* nematod türü tanılanmıştır. Öte yandan GFLV ile enfekteli 13 omcanın kök bölgelerinde ise herhangi bir *Xiphinema* türüne rastlanmamıştır. GFLV saptanan omcalara virüsün aşılı bağ fidanı üretimi esnasında bulaşmış olabileceği gibi budamalarda mekanik inokulasyonla ya da hatalı fidan seçiminden kaynaklanmış olabileceği düşünülmektedir. Sürvey yapılan bağ alanlarında *X. pachtaicum*'un baskın olduğu saptanmış olup enfekteli omcaların kök bölgesinde tek başına bulunması bu nematod türünün de GFLV vektörü olabileceğini düşündürmektedir.

Anahtar kelimeler: *Vitis vinifera***,** GFLV, DAS-ELISA, *Xiphinema spp.,* Trakya Bölgesi

INTRODUCTION

Grape vine (*Vitis vinifera* L.) had been obtained by selection of wild *Vitis* species and used as fresh fruit, raisin and the production of an alcoholic beverage vine and vinegar since 6 000 BC. By having the historical past, grapevine has been cultivated in most of temperate region. Archeological investigations by using radiocarbon techniques on old grape seeds in Turkey revealed the presence of grapevine cultivation and its usage 10 000 years ago (Ağaoğlu 1999). Having a past of thousands of years and a number of forms and cultivars, grapevine (*V. vinifera*) production has expanded in Northern Hemisphere in the World in between North latitudes of 10° - 52°. So Turkey has the best agro-ecological conditions with it's the best location of 36° - 42° North latitudes (Oraman 1965). Due to the suitable geographical position and being the germplasm center of grapevines Turkey has become the fourth grade in vineyard area and sixth grade of grape production in the World (Anonymous, 2011).

Grapevine pests and diseases are the most important factors reducing grape yield and quality. Pearson and Goheen (1981) listed 43 abiotic stress factors and pathogenic diseases including eight virus infections. Soil-borne grapevine pests like Phylloxera grape root aphid (*Dactylosphaira vitifolia*) and nematodes are important in vineyard in the World. Martelli and Boudon-Padieu (2006) compiled 58 grapevine virus diseases and viruses as the member of 21 genera on grapevines in the World. Among them, soil-borne grapevine fanleaf virus disease had been known as one of the oldest disease described and identified in 1950's. Previously, nematode transmission feature of Grapevine fanleaf virus (GFLV) and its vector *Xiphinema index* were determined and identified by Hewitt et al. (1958). After discovery of this virus and nematode relationship has leaded to discovery of other nematode transmissible plant virus diseases. Thus far, 32 Nepovirus (Genus of nematode-transmitted polyhedral shape viruses) and NETU (Nematode transmitted tubular shape plant viruses) disease have been reported. At least 12 of them had been proved to be transmitted as none-persistent and semi-persistently by the exoparasitic species of 3 nematodes genera of Longidoridae family (Brown and Weischer 1998, MacFarlane et al. 2002, Andret-Link et al. 2004). In spite of the first determination of GFLV and its vector of *X. index* in the USA, Bashir et al. (2007) and Bashir and Khabbazi (2009) claimed that this disease had been spread from Erdebil province of eastern Azerbaijan to all over the World.

GFLV has been known since 1965 in the Trakya Region of Turkey (Akdoğan 1965). Yüksel (1966) observed GFLV infections and identified its vector nematode *X. index* in Manisa vineyards in Turkey. Akbaş and Erdiller (1993) listed 15 virus diseases and their vectors on grapevine in Turkey, whereas the GFLV disease was the most widespread one. Official plant protection regulations and standards required that at least 7 of those viruses must be avoided in nurseries. These virus diseases and viruses were listed as Grapevine fanleaf (Grapevine fanleaf virus ,GFLV), Grapevine mosaic disease (Arabis mosaic virus, ArMV), Grapevine latent ringspot disease (Strawberry latent ringspot virus, SLRSV): Grapevine ringspot disease (Tomato ringspot virus, ToRSV), Grapevine necrotic

ringspot disease (Raspberry ringspot virus, RpRSV), Grapevine leafroll virus complex (Grapevine leafroll associated virus, GLRaV 1-9) and Grapevine A virus disease (Grapevine vitivirus A, GVA). Beside, GFLV, ArMV and ToRSV Sertkaya et al (2013) identified *Tomato black ring virus* (TBRV) in Hatay Province of Turkey as Kepenekçi et al (2014) identified 22 nematode species belonging to 16 genera in Central Anatolian vineyards.

In addition to color and shape changes of leaves and shoots GFLV infections have shortened the productive life span of vineyards, reduced grape yield up to 80 % and resulted in important economic losses. The GFLV vector nematode *X. index* presents almost all the vineyards of the world. Mixed infections of GFLV with other *Nepovirus*es like ArMV, RbRsV, and TmBRV cause much more severe symptoms and dramatic yield loses in vineyards. GFLV infection reduces the vegetative and generative growth of stocks makes them susceptible to adverse climatic conditions, which cause sudden death. (Raski et al. 1983, Martelli and Savino 1990, Andret-Link et al. 2004). (Andret-Link et al. 2004). Therefore, GFLV has sustained universally with its hosts and efficient vectors has continued being an important health problem for all vineyards. Almost all the *V. vinifera* species and cultivars were found susceptible to GFLV. Therefore, there is no tolerant grapevine cultivar to this virus including the hybrits breed from American grapevine stocks *V. labrusca, V. riparia, V. rupestris, V. berlandieri, V. aestivalis*, and *V. candicans.* GFLV was also transmitted by mechanical inoculation tests to 30 indicator plant species belong to 7 families (Vuittenez and Martelli 1988). Recently Izadpanah et al. (2003) found a perennial Poaceae weed (*Cynodon dactylon* Persoon) as the natural host of GFLV in the Fars Region vineyards of Iran.

Brunt et al. (1996) defined GFLV virions as isometric, icosahedral particles with 25-30 nm in diameter, having two types of single stranded ssRNA. Purified samples of virus has three bands; 50S (T), 86S (M) and 120S (B) according to their sedimentation coefficient (Vuittenez ve Martelli 1988). Virions in the T band do not have any RNA molecule, as virions in the middle M band has 1400-kDa RNA2 with 3774 nts. GFLV virions in B band however has 2400-kDa RNA2 molecules with 7342 nts the genome of the virus. According to the recent virus taxonomy, GFLV has taken place as a species in the Nepovirus genus of Secoviridae family, Comovirinae subfamily (King et al. 2012). RNA1 is responsible for coding synthesis of 58 kDa protein subunit molecules as well as coding RNA viral proteinase enzyme (Margis et al. 1991). Both RNA molecules of GFLV virion is capsulated by 60 protein subunits in the icosahedral arrangement (Chandrase and Johnson 1998). Depending on their genomes, different GFLV isolates were reported from France (Vigne et al. 2004), Iran (Bashir et al. 2007), Slovenia (Pompe-Novak et al. 2007), Tunisia (Fattouch et al. 2005) and USA (Naraghi-Arani et al. 2001).

GFLV may be sustain in the infected stocks and transmitted by *X. index* from infected stocks to healthy ones. Grapevine is propagated by vegetative means by cuttings or grafting. Therefore, infected cuttings and samplings have become sources of inoculum of GFLV for spreading everywhere on earth. After the determination of nematode *X. index* transmission characteristics of GFLV in soil by Hewitt et al (1958) virus–vector relationship has considered as reality (Brown and Weischer 1998). During the GFLV infection, vector nematodes act as ectoparasite pest on the root tips of stocks causing nodulation, folding, rotting, decay and death of them. For this reason, it is necessary to control both, virus diseases and its vector, which they increase, cost of implementation. In order to control GFLV diseases, the following measures can be taken; i) virus and vector nematode-free cuttings and nurses must be produced and used for newly establishments of vineyards, ii) crop rotations with cereals must be implemented for 2-3 years during the reestablishments of vineyards, iii) all kinds of sanitation and sterilization methods should be used during planting, pruning, harvesting during the implementations of other cultural practices (Brown et al 1993).

Previously single and mixed infections of GFLV with Rugose wood complex, Rupestris stem pitting-associated virus (RSPaV), Grapevine leaf roll associated virus (GLRaV)'es and Grapevine fleck virus (GFkV) diseases were reported in Trakya (Savino et al. 1987, Köklü et al 1998). Recently Öztürk et al. (2016) also reported substantial fruit losses up to 80 % in vineyards of Tekirdağ by determining 36 % of GFLV infection in relation with vectors of *X. index* and *X. italiae.* In order to determine the rate of incidence and the severity of GFLV disease in relation with vector *Xiphinema spp* nematodes in the vineyards of Trakya Region of Turkey. This study was carried out during the years of 2013-2014.

MATERIAL and METHODS

The surveys were carried out in October and November 2013 as well as in May 2014. At least 832 plots were examined, representing 7.850 ha vineyard land in 8 out of 29 districts of three provinces at Trakya Region as exhibited in Figure 1. At least 152 symptomatic grapevine leaf samples and 152 soil samples were taken from the same vineyards as listed in Table 1. Soil samples, one kg each were taken from the 0–80 cm depth of soil profiles from the rhizosphere sections of the infected grapevine canes over 7 years old vineyards. Grapevine stocks exhibiting chlorosis, mosaic, short internodes and zigzag shootings, fanleaf and unmarketable fruit set were determined and recorded with color pictures.

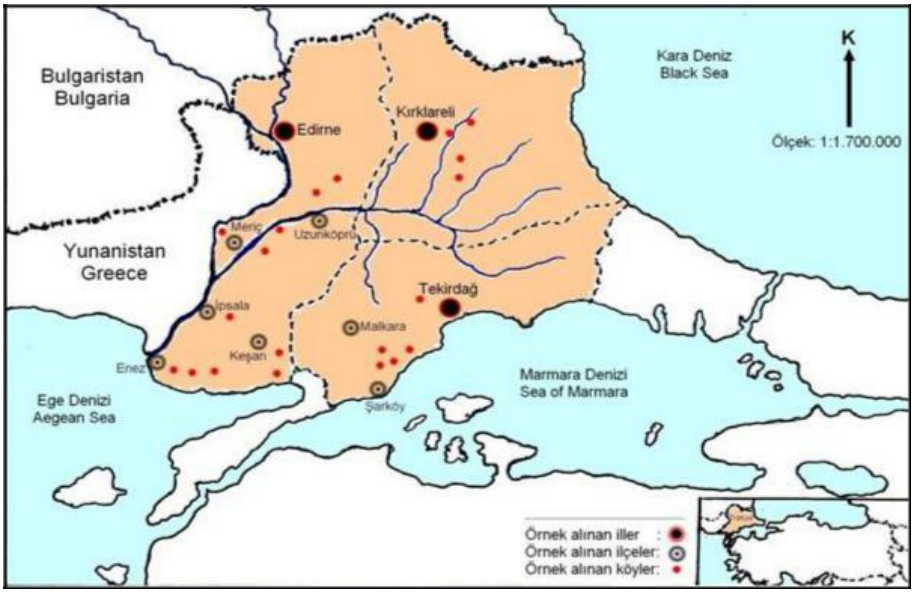

Figure 1. Survey area of *Grapevine fanleaf virus* (GFLV) disease and vector *Xiphinema spp.* in the Trakya Region of Turkey

All of the leaf samples were kept in deep freeze until they were used for Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) tests. Implementation of this serological test, ELISA test kits were obtained from BIOREBA AG (Reinach, Switzerland) firm. DAS-ELISA test methods of Clark and Adams (1977) and BIOREBA AG procedures were used. Absorbance values were recorded with Thermo Scientific Multiskan FC, USA plate reader.

Soil samples were evaluated by employing Murray (2011)'s pH analysis. For this aim Hanna Instrument HI-221 Model Microprcessor Woonsocket-RI, USA pH meter was used. Soil texture analyses were made by using Carpenter (2003)'s sedimentation method. Nematode isolations however were performed by employing Sucrose Centrifugation method with the usage of Hettich Universal 320 R model centrifuge as suggested by Hafez (2006). For the measurement and identification of isolated nematodes, their permanent slides were made. Nematodes were killed by keeping them for 1 minute in incubator adjusted to 55 º C and fixed in TAF solution [7 ml formalin (% 40 formaldehyd) + 2 ml triethanolamin + 91 ml distilled water] (Hooper 1986). Fixed nematodes were transferred into the gliserol on plates, covered and fasten (Seinhorst 1959). Plates were examined under Leica Research Microscope. Morphological and Morphometrical measurements of nematodes were made as suggested by (Loof and Luc 1990, Loof and Luc 1993, Siddiqi 2000). Nematode identification results were confirmed at pH meter at the Department of Plant Protection, Faculty of Agriculture, Çukurova University Adana/Turkey.

Table 1. Grapevine fanleaf fanleaf virus disease survey in the Trakya Region in 2013 and 2014, distribution of locations, grapevine leaf and soil samples collected from vineyards and their cultivars older than 7 years old.

Province	Name of District	Location & village	Number of Samples	Grape Cultivars
Edirne	Enez	Çavuşköy	4	Ata sarısı Erenköy Beyazı
		Hasköy	2	
		Büyükevren	1	
	İpsala	Sarpdere	4	Sauvigron blanc Merlot
	Keşan	Yenimuhacir	6	Ata sarısı Erenköy Beyazı
		Gökçetepe	2	
		Sazlıdere	14	
	Meriç	Nasuhbey	3	Cabernet S. Merlot Kalecik karası
	Uzunköprü	Yeniköy	5	Cabernet S. Kalecik karası Hamburg misketi
		Kırcasalih	3	
		Salarlı	1	
		Çobanpınar	5	
Kırklareli	Center	Kızılcıkdere	6	Cabernet S. Merlot Alphonse I.
		Üsküp	12	
		Bayramdere	1	
		Deveçatağı	2	
Tekirdağ	Süleymanpaşa	Süleymanpaşa	66	Cabernet S. Cardinal Merlot Cinsaut Clairette Trakya İlkeren
		Nusratlı	2	
	Şarköy	Merkez	6	Merlot Gamay Cinsaut Semillion Yapıncak Çavuş Alphonse I.
		İğdebağları	2	
		Çengelli	1	
		Yukarı Kalamış	2	
		Mürefte	2	
Total	**8 distrcts**	**23 village and location**	**152**	**16 grape cultivars**

RESULTS and DISCUSSION

During the survey visits in 2013 in the Trakya vineyards, characteristic GFLV disease symptoms were observed. One of the severe symptom was on grape bunch with small uneven and unmarketable berries of Müşgüle cultivars as exhibited in Figure 2. Another symptom of GFLV was death of whole stock and canes as shown in Figure 3, which reduce grape yield and quality as well as cause loss of productivity of vineyards. This confirms results of Digiaro et al. (1997)'s findings of Red Globe and King's Ruby cultivars, as well as Tarla and Yılmaz (2004)'s observations on local grape cultivars in Eastern Mediterranean Region of Turkey. These observations on the symptomatology of disease confirmed the previous descriptions of GFLV infections by Akdoğan (1965), Savino et al. (1987) and Köklü et al. (1998) in Trakya province of Turkey.

Figure 2. Grapevine fanleaf virus (GFLV) infected table grape cultivar Müşgüle with small unmarketable berries in in vineyard in Tekirdağ

Figure 3. GFLV infection caused death canes and stocks vineyard in Tekirdağ.

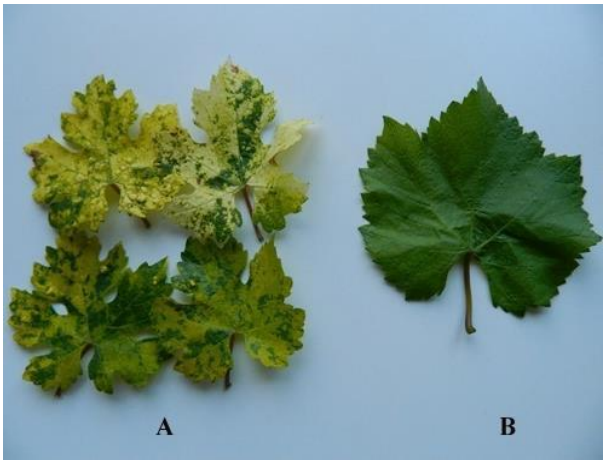

Figure 4. Mosaic and fanleaf symptoms on GFLV infected Clairette grape cultivar leaves in vineyard in Tekirdağ (A), and (B) healthy leaf.

Figure 5. GFLV infected Cardinal grape symptoms mosaic, yellowing and leaf deformations.

During the survey visits in May 2014, characteristic GFLV infection symptoms on Clairette cultivar grape leaves exhibit severe mosaic and fan shape leaves with deep lobes as observed in Figure 4. Öztürk et al. (2016) reported similar symptoms on other grape cultivars in vineyards in Tekirdağ. Other severe symptoms of GFLV

infection were revealed as chlorosis, yellowing, mosaic and degeneration patterns on Cardinal cultivar grapevine leaves as exhibited in Figure 5. These findings confirmed the similar fanleaf infections caused by different GFLV isolates on different grapevine cultivars as described by Oliver and Fuchs (2011).

The results of nematode identification studies depending on morphological and morphometrical measurement averages of 30 female nematode specimens, at least 3 suspected vector *Xiphinema* species were identified as suggested by Loof and Luc (1990), Loof and Luc (1993) and Siddiqi (2000). Namely those identified nematode species were *Xiphinema index* Thorne and Allen 1950, *Xiphinema pachtaicum* Tulaganov 1938 and *Xiphinema italiae* Meyl 1953. All *Xiphinema spp* were found in Tekirdağ province *X. pachtaicum* was being dominant one especially in the Tekirdağ districts of Süleymanpaşa and Şarköy as well as Keşan district of Edirne. Among them 2 soil samples had all 3 *Xiphinema spp.* together. Another 2 soil sample had *X. index* and *X. pachtaicum* with *Longidorus sp.* Beside them 23 out of 152 soil samples had 2 *Xiphinema spp* together, visited vineyards Meriç district of Edirne however was found free of nematodes. So tested 65 soil samples of 152 however did not contain any nematodes at all. These findings in Trakya Region confirmed the previous findings of *X. index* by Yüksel (1966)' in Manisa vineyards, *X. index* findings of Gözel et al. (2011) in Çanakkale vineyards. Rare species of *X. italiae* found only in 2 soil samples confirmed the findings of Kepenekçi (2012), Kepenekçi et al. (2014) in Central Anatolian vineyards and Peneva et al. (2012)'s finding of *X. italiae* in the Bulgarian vineyards where just western border of Turkey.

As a result of DAS-ELISA tests as exhibited in Table 2, totally 35 out of 152 symptomatic plant samples at the rate of 23.02 % were found infected with GFLV. The distribution of GFLV infections and their vectors in the region were determined at the rate of 38.27% that indicated 31 out of 81 grapevine samples in the districts of Tekirdağ as parallel with vector *Xiphinema spp.* These results and high incidence rate of GFLV confirmed findings of Teliz et al. (2007) in Spain and presence of both GFLV and vector nematode species were in harmony with the reported results of Nogay et al. (1995) in Marmara Region, Tarla and Yılmaz (2004) in Çukurova Region and recent findings of Öztürk et al. (2016) in Tekirdağ. GFLV infected samples were found only 6% in Edirne and 5% in Kırklareli vineyards. In order to determine the reality of both DAS-ELISA and RT-PCR tests, Erilmez and Kaya (2016) compared them to each other on the identification of grapevine viruses from Manisa vineyards. They determined that DAS-ELISA test were as reliable as much with RT-PCR tests. DAS-ELISA tests also revealed the incidence of GFLV in some vineyards where there was not any vector nematodes. This means that GFLV may disseminated to those vector nematode free vineyards by infected nurseries or by mechanical transmission of pruning equipment.

Table 2. Distribution of GFLV diseased samples and *Xiphinema spp* nematodes and disease free samples in provinces of Trakya Region of Turkey.

Name of Province	Number Samples	GFLV Samples	*Xiphinema* species				Number of Disase & vector free samples
			X. index	*X. pachtaicum*	*X italiae*	*longidorus sp*	
Tekirdağ	81	31	15	54	3	2	27
Edirne	50	3	7	21	-	-	29
Kırklareli	21	1	-	11	-	-	9
Total	152	35	22	86	3	2	65

The results revealed that 9 out of 35 GFLV infected samples were together with *X. index.* At least 14 of 35 GFLV infected samples were not with any vector nematode species. Finally, 11 GFLV infected samples were with *X. pachtaicum* as, only 1 GFLV infected sample was with *X. italiae.* Our results confirmed the results of Pearson and Goheen (1981), Griffiths et al. (1983) and Brunt et al (1996) reports.

In order to get rid of both GFLV and its vector *Xiphinema spp.* nematodes, usage of virus-free saplings was recommended in the CABI/EPPO (2000) report. Before the reestablishment of old GFLV infected and nematode infested vineyards, 3 to 5 years of rotation was suggested with cereals (Brown et al. 1993). As long as GFLV host range includes new weed species like *Cynedon dactylon* as reported by Izadpanah et al. (2003) weed control measures by using suitable herbicides may help to combat with GFLV disease. Finally, as a new control measure of *X. index* infested vineyards Hoa et al. (2012) recommended usage of local and systemic mycorrhiza which induced protection for GFLV disease.

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