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Study of distribution and analysis of the transmission of *Grapevine fanleaf Virus* in northern Tunisian vineyards

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Abstract –*Grapevine fanleaf virus* (GFLV) is to be the most serious virus disease affecting grapevines and is spread by infected plant propagation material and by dagger nematode, *Xiphinema index*. The objective of this work is to improve knowledge on the etiology of the *Grapevine fanleaf virus* in the northern Tunisia. The serological analysis showed that GFLV is widespread in vineyards surveyed (northern Tunisian regions in 2016-2017) with a prevalence of 36%. A relationship between the symptoms observed in the field in the spring and the serological diagnosis of this disease was observed. We have also shown that, pollen is a very efficient carrier of GFLV viral particles under natural conditions. Vine-Vector interaction study showed that the ectoparasitic nematodes belonging to the *Xiphinema* genus, have a homogeneous distribution in the different vineyards. As for Virus-Vector interaction study, GFLV was detected by DAS- ELISA in its nematode vector isolated directly from the rhizosphere of infected vine.

Keywords: *Fanleaf degeneration diseases, symptoms,* GFLV, *Xiphinema index* and pollen.

1. Introduction

In Tunisia, viticulture is one of the most significant economic sectors. Despite its importance, the average yield per hectare is insufficient and is evaluated to 30 tons per hectare for table grapes and only 3,5 tons for not irrigated grapes (Anonymous 2017). Several constraints affect the vine productivity, such as vineyards ageing and phytosanitary problems, notably virus diseases. Over 55 viruses or strains, belonging to 20 different virus genera, are able to infect this crop (Martelli 2003). The oldest known virus disease of grapevine is infectious degeneration or fanleaf. The affected plant shows widely opened petiolar sinuses and abnormally gathered primary veins, resulting in the appearance of an open fan of the leaf (Martelli et al., 2001). The economic loss induced by the disease varies depending on grapevine cultivars. Yield losses of up to 80%, progressive decline of the vines, low fruit quality, reduction in vineyard longevity, low proportion of graft take and reduced rooting ability of propagation material.(Martelli and Savino, 1990).

The principal causal agent of fanleaf, *Grapevine fanleaf virus* (GFLV), which is a member of the genus *Nepovirus* within the family *Comoviridae* (Fauquet et al., 2005). GFLV has polyhedral particles with a size of approximately 30 nm in diameter (Martelli et al., 2001). Its genetic information is divided over two single-stranded positive sense RNA molecules, RNA-1 and RNA-2, coding for two polyproteins (Serghini et al., 1990; Ritzenthaler et al., 1991). This virus is distributed in most vineyards worldwide and can infect almost all Vitis species (Raski et al., 1983; Martelli and Savino, 1990). Distorting and chromogenic strains of GFLV have been described based on leaf symptoms they induce on grapevines (Martelli and Savino, 1990). Spread of this virus over long distances can be occur by transfer of infected propagation material (Martelli and Savino, 1990) but it possible to be transmissible by mechanical way (Martelli et al., 2001). In the short distances, GFLV can be transmissed from vine to vine under natural conditions occurs through the Longidorid nematode *Xiphinema index* (Martelli et al., 2001). *Xiphinema italiae* Meyl. and *Xiphinema vuittenezi* Luc, Lima, Weischer &Flegg, were also reported as possible vectors of GFLV (Cohn 1977) but the transmission by these two species has never been confirmed.

The contribution of pollen to the spread and distribution of *Grapevine fanleaf virus* has not been proven until now. At this level, deeper scientific investigations focused on the study of the interaction between GFLV and his nematode vectors or the transmission through sexual forms (e.g. pollen) are needful, toward developing better control measures and limiting the spread of the disease in the Tunisian vineyards.

2. Materials and methods

2.1 Surveyed regions and sampling

This study was carried out in the main vineyards located in northern Tunisia, especially in the vineyards of Nabeul (Gromlbalia and Takelsa), Ben Arous (Mornag) and Bizerta (Rafraf) governorates (Fig. 1).

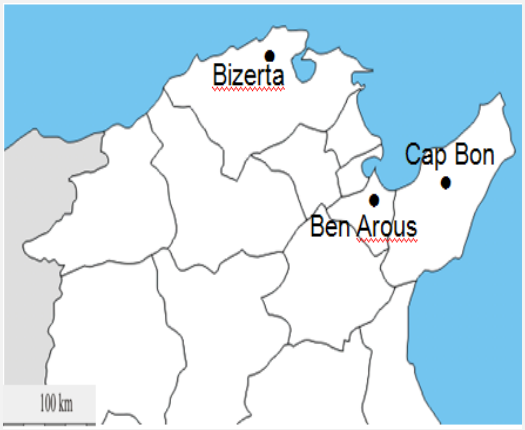

Fig 1. Map of northern Tunisia illustrating the main vineyard regions covered by this study.

Samples were collected from economically important cultivars. For visual symptoms study of fanleaf degeneration diseases, 295 vines (tab.1) were surveyed during the spring 2017; optimal period for the expression of symptoms. For *Grapevine fanleaf virus* (GFLV) incidence study, 178 vine phloem samples were collected in winter 2016 during vegetative dormancy (November-December); period which the viral concentration is maximal (Tab. 1).

For GFLV detection in pollen from vine tested infected with this virus, pollen collection was carried out at the flowering stage just at the opening of the anthers during May 2017. A total of 110 pollen samples were collected (Tab. 1) at reason of the mixture of pollen from at least 4 different inflorescences per vine (0,5 g).

To assess the specificity of the virus-nematode interaction and the spatial distribution of nematode vectors of GFLV, nematode surveys were conducted in 2017 during the springing season. 80 samples at reason of 20 samples per region (Tab. 1) were collected with a shovel from the rhizosphera of plants GFLV infected at a depth of 50-80 cm.

Table 1. Samples number for symtomatology and serology stadies of GFLV in northen Tunisia.

Object	Target	Organs	Period	Total samples number
Symtomatology stady	Fanleaf degeneration diseases	Leaf, stem and whole plant	March and April 2017	295
Prevalence of GFLV	GFLV	Phloem	November and December 2016	178
GFLV detection in pollen	GFLV	Pollen	May 2017	110
***Xiphinema* genus distribution**	*Xiphinema* genus	Soil	March and april 2017	80
GFLV detection in *X. index*	GFLV	*Xiphinema* index	March and april 2017	18*

* One sample consists of 20 individuals of femal adult of *Xiphinema index*.

2.2 Visual diagnosis

Symptoms were carried out by an observation of the different symptoms on all the visible organs of the plant (leaves, branches, space between two successive nodes ...) and the general appearance of the whole plant. These symptoms have been well described, photographed and statistically analyzed.

2.3 Nematode extraction

Nematodes were extracted from 1000 cm3 of soil by centrifugal flotation (Coolen, 1979) and a modification of Cobb's decanting and sieving (Flegg, 1967) methods.

Prevalence of infestation and population density of *Xiphinema* genus nematodes was determined. Prevalence of infestation was calculated as the percentage of samples in which at least one species of *Xiphinema* genus was diagnosed with respect to total number of samples. Nematode population density in soil was assessed for each sample and calculated as the average of the count population of *Xiphinema* genus.

Xiphinema genus identification is based on morphological characters of adult females observed under binocular loupe (x60) based on the identification key of major genera as described by Cobb in 1913.

The morphological identification of *Xiphinema index* species was carried out according to the taxonomic keys elaborated by Luc and Dalmasso (1975) and Siddiqui (1974). Identification was performed using photonic immersion microscope *Olympus* C40. An estimation of the population was also determined.

2.4 Serological assays

The interaction study between vine and GFLV was performed by the DAS-ELISA methods (Double Antibody Sandwich Enzyme Linked Immuno Sorbent Assay) as described by Clark and Adams (1977). Polyclonal antibodies (Bioreba, Switzerland) were used to detect GFLV in leave, phloem and pollen samples. The fluorescence obtained in each well were measured at a wave length of 405 nm using spectrophotometer (LabSystem) and the final interpretation of the results was made 2 hours after deposition of the substrate based on the calculation of a positivity threshold which is twice the mean O.D of healthy controls (2 x mean optical density of healthy controls).

Detection of GFLV in *Xiphinema index* was performed using 20 females per sample. In total, 18 samples were analyzed (Tab. 1). Nematode were collected under binocular microscope and gently crushed with a hand homogenazer in the eppendorf tube containing 100 µl extraction buffer (Bioreba), prior to be analyzed by DAS-ELISA.

2.5 Statistical analysis

Symptoms observed on grapevine in the various locations, viruse detected and prevalence of *Xiphinema* genus on the rhizophera formed a matrix, whereby several contingency tables crossing the different parameters were established with the SPSS 16.0 software. These various contingency tables were submitted to correspondence analysis by the STATISTICA software in order to identify the regional characteristics. The matrix formed by the different symptoms, virus and *Xiphinema* sp. density per kg of soil was also subjected to the discriminate analysis by the STATISTICA software. Three discriminate analyses were performed according to the different regions and varieties.

3. Results and discussion

3.1 Symptomatology

Discriminant Analysis (DA) highlighting observed symptoms made in the main wine-growing regions in northern Tunisia; Rafraf, Grombalia, Takelsa and Mornag, extract 3 axes that explain 100% of the total variability (Tab. 2).

Table 2. First 3 canonical discriminant functions (% of variance, Cumulative % and Canonical Correlation) used in the discriminant analysis (AD) linking the different symptoms of the grapevine fanleaf diseases observed in the different vineyard located in northern Tunisia

% of Variance	Cumulative %	Canonical Correlation
68,4	68,4	0,694
25,1	93,6	0,504
6,4	100,0	0,283

The first axis explains 68.4% (Tab. 2 and Fig. 3) of the total variability correlated with the symptoms of short internodes (ENR) and leaf yellowing (JF) (Tab. 2 and Fig. 3). The second axis explains 25.1% (Tab. 2 and Fig. 3) of the total variability which is more related to leaf veins clarification (ECN), abnormal branching with double nodes (END) and the fasciations of the branches (FAS). The last axis accounts for only 6.1% (Tab. 2 and Fig. 3) of the total variability correlated with variegation (PAN) and acute indentations (EV).

The discriminate analysis in the Cartesian coordinate system (Fig. 2) formed by axes 1 and 2 shows the existence of three distinct regions. The first region is Mornag in which observed symptoms are different from those observed in the other three sites (Grombalia, Takelsa and Mornag) (Fig. 2). The second region is Grombalia characterized by symptoms different from those observed in Rafraf and Takelsa (Fig. 2).

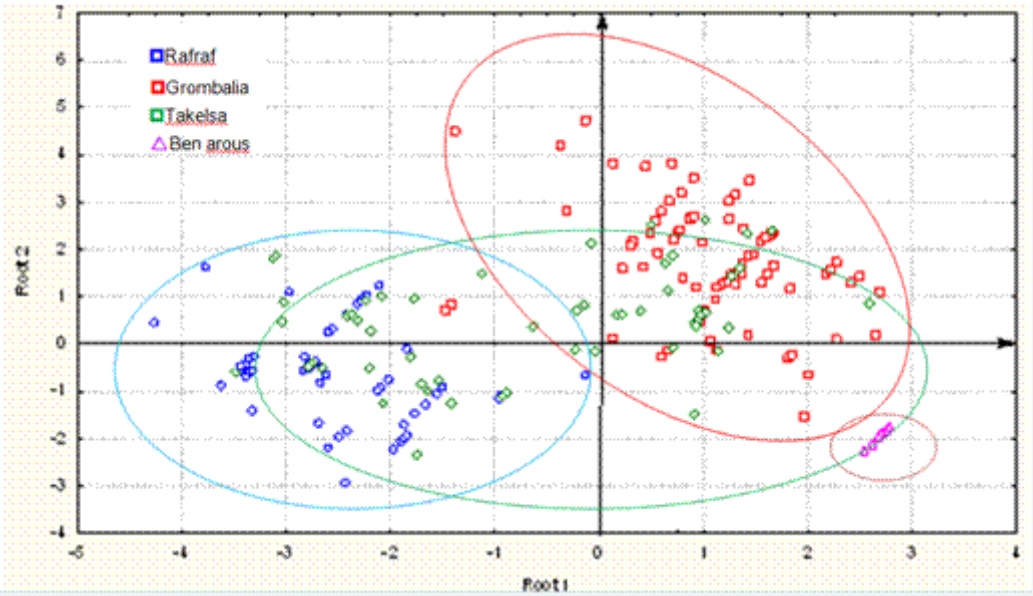

Fig. 2. Discriminate analysis (DA) relative to symptoms of fanleaf degeneration disease observed in the different wine regions prospected.

The third region includes the vineyards of Rafraf and Takelsa and show similar symptoms.It also appears from this visual diagnosis that the fanleaf degeneration disease is characterized by the presence of two types of symptoms; staining and growth abnormalities. Examination of the distribution of these two types of symptoms shows that in the region of Rafraf, a typical general appearance characterized by a remarkable reduction of the vegetation, an acute weakening of vines, an accentuated shortening of the internodes, leaves yellowing and rarely a flattening of the shoot and splitting of the nodes were observed (Fig. 2 and 3). Unlike Rafraf, aspects related to fanleaf degeneration disease are less frequent in Grombalia. However, aspects of leaf yellowing, limb deformation, fan leaves, leaf veins clarification, flattening and fasciation of twigs and doubling of nodes are the most common in this region (Fig. 2 and 3).

Takelsa region, although geographically near Grombalia, has symptoms intermediate to those seen in Grombalia and Rafraf (Fig. 2 and 3), characterized by the appearance of leaf yellowing and variegation and leaf veins clarification.

However, in Ben Arous region, the typical symptoms of the fanleaf degeneration diseases are very rare or even negligible in the vineyards prospected (Fig. 3).

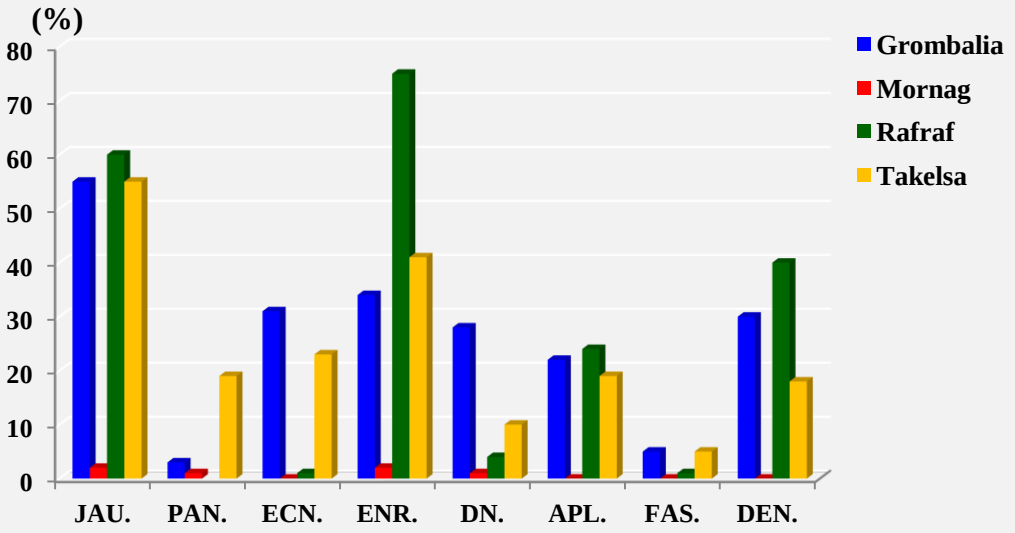

Fig. 3. Distribution of the main symptoms of fanleaf degeneration diseases observed in the different vineyards in northern Tunisia. Staining anomalies: yellowing "YAU", variegation "PAN", and veins clarification "ECN". Formation anomalies: "ENR" short internodes, double nodes "DN", flattening of the branch "APL", fasciation of the branch "FAS" and acute dentations of leaves "DEN".

3.2 Serological detection of GFLV on grapevine

The prevalence study of GFLV shown that this virus is widespread in the main vineyard in northern Tunisia. Indeed, 50 asymptomatic samples of vine wood reacted positively with the polyclonal antibodies by means of the serological DAS-ELISA technique on a total of 178 samples analyzed, which represents an overall average prevalence of around 36%. GFLV was present with 57% of incidence in Cap Bon (Grombalia and Takelsa) and with 45% in Rafraf. However, Ben Arous region was free from GFLV and no positive sample was found by serology essai. Distribution of GFLV infections varied not only between regions but even within vineyards from the same region. Indeed, in the Cap Bon, this virus is present with very high rates in Grombalia (71%) and with smaller rates (42%) inTakelsa. The study of the GFLV sensitivity of the various varieties cultivated shows a variation between 10% and 67%. Mourvèdre and Carignan cultivars are highly susceptible to the virus for infection rates 71% and 61% respectively. Muscat of Alexandria (Muscat de Rafraf), Superior seed less and Syrah have average infection rates of 49.5%, 49% and 35.7% respectively. Whereas, Farrani cultivar cultivated in the Rafraf region in the same conditions as Muscat of Alexendria, has the lowest infection rate (15%).

3.3 Evaluation of the nematode population

Counting and identification of nematodes in the surveyed vineyards showed that the *Xiphinema* genus was prevalent with an average density ranging between 1-4 specimens/kg of soil (Fig. 4a).

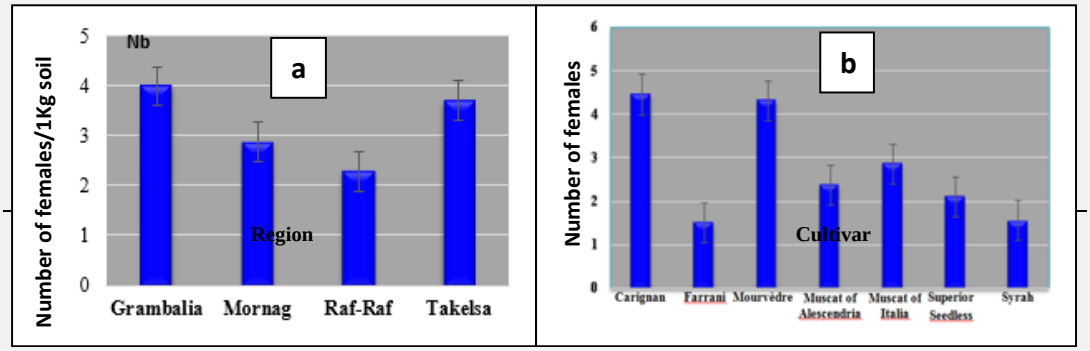

Fig 4. Infestation level of females of *Xiphinema* sp.: (a) by region, (b) by different cultivars. Interval confidence in different histogram are at $\alpha = 0.05$.

Throughout the analysis of nematofauna parasitizing the different cultivars, both Muscat of Alexandria and Mourvedre appeared to be the most infested by *Xiphinema* sp.. In fact, more than 4 specimens /kg

of soil were recovered. Similarly to viral infections, lower nematode infestation levels (1 specimens/kg) of soil) were obtained from grapevines of Ferrani cultivar although they were grown in the same plots with Muscat of Alexandria (Fig. 4b).

3.4 Serological detection of GFLV on the *Xiphinema index*

In Rafraf vineyards, GFLV was detected in 13 out of 16 *X. index* population tested samples for a prevalence of 92.8%. In Grombalia region, only two nematodes samples extracts tested and were free from GFLV.

3.5 Serological detection of GFLV on pollen

Serological analyzes of the 110 pollen samples collected from vines naturally infected with GFLV indicate that this nepovirus is present in pollen with high infection rates, regardless of their geographical origin. Indeed, the highest GFLV pollen infection rate was recorded in the Rafraf region followed by the Takelsa and Grombalia region for infection rates respectively in the order of 96%, 77% and 73%. These results suggest that pollen are very effective carriers of GFLV.

This study shows that in northern Tunisia vineyards two types of syndromes caused by Grapevine fanleaf virus are present. The first one consisted of an abnormal vegetative growth of the vine, i.e. shortening of the nodes, bifurcation, flattening, indentation and asymmetry of leaves and the second one affects the leaves color. The type of symptoms produced by GFLV is highly variable from one region to another and from one cultivar to another. Indeed, the discriminated analysis proved that the symptoms observed in Rafraf are different from those observed in Grombalia. However, Takelsa, geographically nearest to Grombalia shows intermediate symptoms that those observed in Rafraf et Grombalia.

These observations confirm those described by Martelli (1985) and by Chabbouh et al (1993) who showed the presence of two different strains of Fanleaf diseases in Tunisia, one chromogen and the other malformant. Variation in the distribution of Fanleaf disease symptoms from one vineyard region to another appears to be influenced by several factors. Since 2001, Naraghi-Arani *et al*. proves that the genetic factor could be responsible for this variation.

Four years later, Demangeat et al. (2005) correlated this variation rather with pedoclimatic conditions, the nature of the grape cultivar and the viral strain itself. This was confirmed later by an experiment conducted by Vigne et al. (2013) between 2007 and 2012. This experiment consisted of inoculating two different genotypes of vines (Chardonnay-Ch and Gewurztraminer-Gw) with 5 strains of GFLV (B844, F13, GHu, CO1 and CO2) and to follow the symptoms evolution. This revealed the stunting of Gw's feet infected with the B844 strain, whereas those of Ch showed discreet symptoms of variegation with the F13 strain (Vigne et al., 2013). The other strains show only discrete symptoms on both grape varieties (Vigne et al., 2013).

Viral infection with GFLV confirmed by serological test has been reported in several countries around the world with varying prevalence. It is estimated at 96% in Spain (Bertolini et al., 2010), 50% in Switzerland (Reynard and Gugerli, 2012), 24% in Croatia (Poljua et al., 2010) and 6.9% in Chile (Fiore et al. ., 2011). In Tunisia, Mahfoudhi et al. (1998) detected GFLV in 18.2% of cases. Our results show that 19 years later, this rate has almost doubled (36%). This increase may be due either to the high epidemiological potential of the virus, or to the increase of the vector nematode populations in the rhizospher of vine stock, or it is aggravated by the use of non-certified propagation material.

This work shows also that the majority of vineyards surveyed located in northern Tunisia (Rafraf, Grombalia Takelsa and Mornag) are infested by at least one species of the *Xiphinema* genus, for which reason these species of nematodes are considered as one of the 10 the most economically important nematode groups (Sasser and Freckman, 1987). *Xiphinema* genus distribution was also influenced by the nature of the vine cultivar. Farrani cultivar seems to be the most tolerant variety unlike Mourvedre and Carignan

The serological detection of GFLV in the samples of *Xiphinema index* population of Rafraf proves that this nematode is at the origin of the transmission and dissemination of this nepovirus from an infected vine plant to another breast next to vegetative propagation. On the other hand, the non-presence of GFLV in the samples of Xiphinema index population of Grombalia does not confirm the non-transmission of GFLV by this nematode but may be due either to the loss of the viral load after moulting or to the capacity of the nematode to retain GFLV effectively (Demangeat, 2007).

According to Brown and Weischer (1998), the viral particles are ingested by the nematode with food, then retained specifically in the food apparatus and finally released during the flow of secretions produced by the salivary glands.
The effectiveness of these three steps, and in particular that of the step of adsorption and release of viral particles, determines the ability of the nematode to be an effective vector or not virus (Brown and Weischer, 1998).During this work we have shown that pollen is a very efficient carrier of virus particles of the Grapevine fanleaf virus, whatever the geographical origin. Our results further confirm the work done in 1967 by Cory and Hewitt who detected the presence of GFLV in pollen grains of vine plants as well as herbaceous plants with regard to *Chenopodium aramanticolor* and *Chenopodium quinoa*. Since that date, the reporting of the presence of GFLV in pollen has been reported by many other authors (Lazar et al., 1990; Marica et al., 2016; Gasparro et al., 2017).

4. Conclusion

It is accepted that phylogenetically, the majority of grapevines have hermaphroditic flowers whose preferred pollination route is self-fertilization. But pollen can also be carried by wind and insects, after opening the flower, the allogamy is not a phenomenon to exclude. On the other hand, the transmission of GFLV is also done in a horizontal mode through the vector nematodes of *Xiphinema* genus and in a vertical mode *via* the vegetative multiplication organs with regard to cuttings and grafts. However, investigations on other vertical means of transmission of GFLV through pollen are very little discussed which deserve further work to further confirm this hypothesis.

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