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Role of Overwintering Forms of *Erysiphe necator* in Epidemiology of Grapevine Powdery Mildew in Palestinian Vineyards

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Abstract: The overwintering modes of *E. necator* were studied on Palestinian vineyards, through observations on the differentiation and maturation of cleistothecia and on the occurrence of flag-shoots (deriving from overwintering mycelium) in vineyards. Field surveys were carried out in 17 vineyards for the presence of Flag shoots and cleistothecia, both forms were not observed. Genetic structure and composition of *E. necator* populations were investigated by application of already available SCAR (Sequence Characterized Amplified Region) primers specific for the "flag-shoot" and "ascospore" biotypes. These primers were used to evaluate the dynamics of the spatial and temporal distribution of the two biotypes, into fungal populations present in 8 vineyards, with different cultivars and spray histories, in various Palestinian districts (Hebron, Bethlehem, Jerusalem, Ramallah, Jericho, Nablus, Jenin, Tulkarm). 397 samples were analyzed by the uses of the primer pairs UnE-UnF in PCR reactions. All samples were found to be of the ascospore biotypes. This finding shows that the “flag shoot” biotype, appears soon after bud breaking and disappears later, while the “ascospore” biotype is more frequently associated to later infections and bunches damages. Such information would be helpful to understand the reasons underlying possible temporal evolution of the pathogen's populations in vineyards, and can have important implications for powdery mildew rationale control strategies.

***Keywords*:** PCR, Powdery Mildew, Identification, Palestine

Introduction

Grapevine is the second important fruit crop in Palestine after olive, covering about 11 thousand hectares. It is grown in many climatic regions in Palestine, but the majority of commercial vineyards are distributed in the southern part of the West Bank. This is mainly due to the more favorable climatic conditions of this region as well as to the traditional experience of the farmers. Hebron and Bethlehem are the main grapevine-growing areas where grapevine covers more than 75% of the grapevine total area. According to FAOSTAT, the average production of grapes in the last ten years in Palestine was 49435 thousand tons (FAOSTAT 2013).
Powdery mildew, caused by the ascomycete *Erysiphe necator* Schw., is one of most severe diseases of grapevine in the region, and growers are forced to a very intensive usage of fungicides for its control, due to lack of knowledge on the pathogen's biology and on disease epidemiology.

E. necator is the causal agent of grape powdery mildew, it present in all grape-growing areas all over the world. The fungus, indigenous to North America, was introduced in Europe in 1845 following the increase in commercial exchanges, but its cleistothecia, fruit bodies deriving from the sexual process, were first observed only 40 years later (Yossifovitch, 1923). Likely, this was due to the initial introduction of just one of the two mating types of the fungus. The pathogen's mating system is indeed a bipolar heterothallism governed by a single gene, named MAT1, and its two alleles (idiomorphs) MAT1-1 and MAT1-2 (Smith, 1970; Wicks and P. Magarey, 1985; Gadoury and Pearson, 1991; Miazzi et al., 1997, 2001, 2002, 2003; Hajjeh, 2000). Therefore, for a long time, cleistothecia were believed playing a marginal role in the disease epidemiology, also due to failures to reproduce the disease through artificial inoculations with ascospores (Yossifovitch, 1923; Weltzien and Weltzien, 1962).

The other form of overwintering, latent mycelium and conidia into dormant buds of vines (Sall and Wrisinsky, 1982; Pearson and Gartel, 1985; Cortesi et al., 1997, 2001) determines, in spring, the development of shoots that become precociously covered with white mycelium and abundant conidia and, due to their peculiar features, are called "flagshoots" (Pearson and Gartel, 1985).

Recently, the application of molecular techniques, such as RAPD (Random Amplified Polymorphic DNA) (Délye et al., 1996; Miazzi et al., 2002; 2003; 2008; Hajjeh et al.,2005), RFLP (Random Amplified Length Polymorphism) and microsatellites (Evans et al., 1996; Stummer et al., 2000), suggested the existence of sub-specific entities in *E. necator* (biotypes) that seem ascribable to the two different overwintering forms and are therefore indicated as the "flag-shoot" biotype and the "ascospore" biotype. The former seems only responsible of precocious infections on leaves and shoots and it would not be responsible of infections on bunches, because it disappears later in the season when the latter becomes prevalent. It has been hypothesized that the two biotypes are genetically separated because inter-sterile in sexual crosses (Délye et al., 1996, 1998). The two biotypes have been detected also among isolates of *E. necator* collected in Italy, but they showed inter-fertility in appropriate mates, thus evidencing the absence of any genetic barrier to the sexual process (Miazzi at al., 2002, 2003; 2008; Hajjeh et al., 2005; 2008). More recently, an analysis of the multilocus structure of a "flag shoot" subpopulation in a single vineyard submitted to an intensive sampling over height consecutive years was carried out (Cortesi, 2004). Results indicated a population structure determined by the co-existance of sexual reproduction and clonal propagation. Furthermore, it seems that ascospores released early in the season (Cortesi et al., 1995, 1997; Jailloux, 1999) can infect buds from which flag shoots will raise on the next year(Cortesi, 2004).

More and deeper studies are needed to clarify these aspects, defining the role of the two overwintering forms and their contribution to the beginning and evolution of powdery mildew epidemics on grapevine, and the biological and epidemiological roles of the two biotypes "flag-shoot" and "ascospore". For example, the characterization of the biotypes for their thermo-hygrometric requirements, virulence on various vine organs (shoots and leaves, bunches), and baseline sensitivity to the fungicides more commonly used for grape protection, might explain the reasons of their behaviour in vineyards and define more rationale, environmental- and consumer-friendly crop protection strategies.

The research is aimed at acquiring new knowledge on the biology of *Erysiphe necator* and on the epidemiology of the grape powdery mildew in Palestine, which is essential to improve crop protection strategies, rationalize and to reduce the usage of fungicides.

Material and methods

Field surveys on occurrence of the overwintering stages of *E. necator*, cleistothecia and flag shoots, were carried out in 2012-2013, in vineyards located in various Palestinian districts (Hebron, Bethlehem, Jerusalem, Ramallah, Jericho, Nablus, Jenin, Tulkarm). Vineyards were selected as a representatives of the different grapevine-growing areas, cultivars, training systems and control strategies, including organic agriculture.

Observations on flag-shoots

The presence and distribution of flag shoots were surveyed at the beginning of growing season (April-May) in the vineyards. In each vineyard, infected vines are marked by plastic flagging tape, for further observations.

Observations on cleistothecia

The occurrence of cleistothecia were monitored in the vineyards in October-December.Samples of leaves, bark and soil near the vines werecollected in the vineyards. Sampling were carried out as follows: Five kg of senescent fallen leaves were collected in November-December from 100 random vines in each vineyard, In spring, just before bud break, 20 samples (100 g each) of bark were collected, separately, from the upper trunk or branches of randomly selected vines located along the diagonals of the vineyard. Ten samples of the upper 2-cm layer of soil were collected from beneath vines; samples were-dried in laboratory at room temperature (25°C) for 24 h before use.

To separate cleistothecia from the substrate. Three samples of 30 g of bark or leaves or 100 g of soil are placed in 2 L Erlenmeyer flasks containing 1.5 L of water (1 L for soil). The flasks were shaken vigorously by hand for 3 min and the suspension is filtered through 120-mesh (125 µm) and 150-mesh (106 µm) Cobb sieves. The process is repeated three times, but shaking time were reduced to 1 min. After the fourth rinse, cleistothecia collected on the 150-mesh sieve were recovered in 25 ml of water.

Three replicated aliquots of 1 ml of each suspension were spread on 10 cm^2 filter paper discs in Petri dishes. Cleistothecia on the paper were observed and counted with the aid of a dissection microscope.

To evaluate cleistothecia viability, 40-70 cleistothecia from each of three replicate of each substrate were observed. Cleistothecia are considered viable when contained at least one viable ascospore. Viability of ascospores were evaluated using fluorescein diacetate staining (Widholm, 1972). The fluorochrome is dissolved in acetone (2.5 mg/ml), and then the solution is added to water (0.25% v:v). Cleistothecia are picked up with a needle, crashed in a drop of the dye solution on a glass slide, and observed at the fluorescence microscope.

PCR assessment of the two biotype

The evolution of the composition of *E. necator* populations at different times in the growing season were assessed in vineyards previously monitored for the presence of the two overwintering forms. Infected tissues were sampled at 1-month intervals during the season to be submitted directly to DNA amplification.

The primer pairs UnE-UnF were used in amplification reactions to discriminate the “flag shoot” and “ascospore” biotypes among collected samples. Amplification products obtained using the primer pair UnE-UnF, produced two bands of 299 bp and 323 bp specific for “flag shoot” and “ascospore” isolates, respectively.

The PCR reactions were carried out in a volume of 25 µl containing: 50 ng of fungal genomic DNA, 1 U of Red Taq DNA polymerase (Sigma, St Louis, Missouri, USA), 0.25 µM of each primer, 75 µM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 2 mM MgCl2 , 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.1% gelatin. Reactions were carried out in a thermal cycler programmed as follows: 4 min at 95°C; 30 cycles of 30 sec at 94°C, 1 min at 58°C, 1 min at 72°C; and a conclusive extension phase of 7 min at 72°C.

Conidia and mycelium were collected from field samples using a sterile single eyelash glued to glass pipette, and added to the reaction mixture. The mixture was heated at 95°C for 5, 10, 15 or 20 min and then chilled on ice bath for few min. Red Taq DNA polymerase (1 U) is then added. Amplification were carried out as described above, with the only exception that PCR is performed with 40 (instead of 30) cycles.

Amplification products were separated by gel electrophoresis. Aliquots of 15 µl of reaction mixtures are loaded on 2% Amplisize agarose gel and run in 0,5xTBE buffer at 110 V for 120 min. A 100-bp DNA Ladder is used as a size standard. Gels were stained with 1 µg ml-1 ethidium bromide for 15-20 min.

Results

Observations on flag-shoots and Cleistothecia

Field surveys were carried out in 17 vineyards (Table1) for the presence of Flag shoots (April-May). Flag shoots were not observed in the surveyed vineyards. Even though farmers confirm the presence of flag shoots in other seasons, and they usually remove it manually.

For the occurrence and distribution of cleistothecia, 90 samples of detached leaves and bark were collected from the 17 vineyards (Table1)at the end of the growing season (November-December), Samples were assessed in laboratory; cleistothecia were not found.

Table 1 Vineyards monitored for flag shoots and cleistothecia.

Vineyard No.	LOCATION	Cultivar
1	Al-Arowb- Hebron	Dabogy
2	Beat Omar- Hebron	Zeany
3	Dora- Hebron	Halawany
4	Halhul- Hebron	Zeany
5	Beat Emra- Hebron	Halawany
6	Al-Makhror -Bethlehem	Dabogy
7	Alkhader1- Bethlehem	Biruty
8	Alkhader2- Bethlehem	Zeany
9	Jerusalem	Betuny
10	Beateen- Ramallah	betuny
11	Ramallah	Dabogy
12	Jeftlic 1- Jericho	SPS
13	Jeftlec2- Jericho	SPS
14	Alnasaria1-Nablus	SPS/undercover
15	Alnasaria2 Nablus	Red Glob
16	Tulkarm	saltanina
17	Jenin	barleat

PCR assessment of the two biotype

With slight modification on the procedure of PCR amplification with the primer pairs UnE-UnF, we manage successfully, in 85% of the assessed samples, to perform PCR from conidia or mycelium from naturally infected bunches and leaves (Figures 1 and 2).

Figure 1: Infected Bunches used to perform PCR amplification.

Figure 2: Infected Bunches and leaves used to perform PCR amplification.

PCR was performed directly from conidia and/or mycelium from naturally infected field samples, allowing the large scale monitoring of fungal populations: 397 samples from 8 vineyards were analyzed by the uses of the primer pairs UnE-UnF (Figure 3 and Table 2).

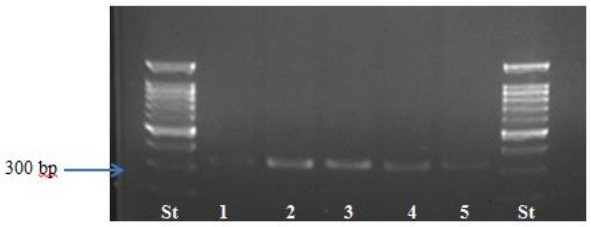

Figure 3: An example of results of DNA amplification reactions conducted with the primers UnE-UnF directly on field samples of *E. necator* conidia and mycelium. Lanes 1-5 "ascospore" isolates, St: Standard DNA ladder

In four vineyards (No 2, 5) and vineyards (No 13,14) it was possible to carry out only a single sampling in June and April respectively, because fungicide sprays prevented further spreading of the disease: a total of 102 samples were collected and analyzed, all were of the ascospore biotypes (Table 2). In vineyards (No. 3, 4, 7 and 15), several collections were done from April to September, a total of 295 samples were collected and analyzed, all were of the ascospore biotypes (Table 2).

Discussion

Observation and samples collection carried out in Palestinian vineyards dose not confirm the presence of either of *E. necator* overwinter formers.Flag shoots presence were not found in vineyards early in the season, even though many of the visited farmers confirm its appearance early on the season, but as a culture practice they remove it manually, and apply fungicide.

Table 2 Variation in the presence of the two biotypes of *E. necator* in vineyards assessed by PCR with the primers UnE-UnF directly on conidia and mycelium.

Vineyard No.	Sampling		No. of samples and biotypes		
	Date	Source	Total	Flag shoot	Asco-co-spore
2	20-06	Bunches	15	--	15
3	20-06	Leaves	10	--	10
	25-07	Leaves	12	--	12
	25-08	Bunches	7	--	7
	20-09	Leaves	15	--	15
4	29-05	Leaves	21	--	21
	20-06	Leaves	14	--	14
	25-07	Bunches	20	--	20
	25-08	Bunches	11	--	11
	20-09	Leaves	11	--	11
5	20-06	Leaves	34	--	34
7	29-05	Leaves	21	--	21
	20-06	Bunches	14	--	14
	10-07	Bunches	15	--	15
	25-07	Bunches	13	--	13
	25-08	Leaves	6	--	6
	20-09	Leaves	7	--	7
13	23-04	Leaves	34	--	34
14	23-04	Leaves	19	--	19
15	23-04	Bunches	24	--	24
	20-05	Bunches	30	--	30
	24-06	Bunches	15	--	15
	17-06	Leaves	30	--	30
Total			397	--	397

Cleistothecia were not found in the surveyed vineyards, since the occurrence of cleistothecia at the end of growing season totally depends on the occurrence and severity of late infections and, the farmers in the surveyed vineyards used and intensive program of disease control during the seasons.

PCR reactions with the primer pair UnE-UnF allowed a large-scale survey on the composition of E. necator populations in Palestinian vineyards with concern to the "flag shoot" and "ascospore" biotypes during the grapevine-growing season. The technique allowed extending the survey to a very high number of samples collected during the whole growing season and under different field conditions. The results of monitoring programme shows that the fungal populations were made up exclusively by "ascospore" isolates in vineyards and flag shoots isolates were not detected.

It is likely that, as suggested by Délye and Corio-Costet (1998) the "flag shoot" biotype, appearing soon after bud breaking and disappearing later, infects new forming buds remaining there quiescent until the next spring. The "ascospore" biotype is more frequently associated to later infections on bunches and leaves. In addition to that resent studies shows that the "flag shoot" derived isolates is more sensitive, to certain types of systemic fungicides, than "ascospore" derived isolates, which favors "ascospore" biotype over the other affecting the evolution of the epidemics (Miazzi and Hajjeh 2011; Hajjeh, 2012).

Finally, in Palestinian vineyards flag shoots infections tend to remain localized without further disease spreading in vineyards, while cleistothecia constitutes an important source of infection. Further studies and observation is required to understanding the evolution of the epidemics in vineyards. Such information would be helpful to understand the reasons underlying possible temporal evolution of the pathogen's populations in vineyards and, consequently, to schedule more rationale crop protection strategies

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