Resistance of some olive (*Olea europaea*) cultivars and hybrids to leaf spot disease analyzed by microsatellites

Fethi Ouerghi[1], Mehdi Fendri[2], Jihène Dridi[3], Hedia Hannachi[4], Neila Rassa[5], Ali Rhouma[6], Bouzid Nasraoui[7]

[1]Office of Forestry and Pastoral Development of Northwest, Beja, Tunisia
Email: oufethi@gmail.com
[2,6]Olive institute, Sfax University, Tunis Station, Tunisia
Email: fendrimahdi@yahoo.fr, ali.rhouma@iresa.agrinet.tn
[5]El Kef High School of Agriculture, Jendouba University, El Kef, Tunisia
Email: neilarassa@hotmail.fr
[1,3,4,7]National Agronomic Institute of Tunisia, Carthage University, Tunis, Tunisia
Email: dridijihene@hotmail.fr, nasraouibouzid2012@gmail.com, hannachi_hedia@yahoo.fr
Email:*Corresponding Author: oufethi@gmail.com

Abstract— *In order to investigate the resistance of some olive (Olea europaea L.) cultivars and hybrids to leaf spot disease caused by Venturia oleaginea, this study was conducted on high susceptible cultivar Meski and nine hybrids. Samples were collected from a field site located in Nabeul (North East of Tunisia) and evaluated for their susceptibility to leaf spot disease by means of visible and latent infection. Therefore, the studied plants were classified into three categories: very susceptible, intermediate and resistant. Meski cultivar and three hybrids (MxA) obtained through controlled crosses between Meski and Arbequina were the most susceptible to the disease. The hybrids MxC resulting from the crosses between Meski and Chétoui olive cultivars presented less severity. However, the hybrids obtained through crosses between Meski and Picholine cultivars showed the lowest incidence of infection. Microsatellites were used as markers to analyze the genetic relationships between parental olive cultivars and hybrids and the effects of crossing on the disease resistance. Cluster analyses, using the SSR data, showed that olive cultivars and hybrids obtained by controlled cross between MeskixPicholine, Meski x Arbequina and Meski ×Picholine were related to Picholine cultivar. The hybrid Meski x Chétoui was more related to cultivar Meski. Data analyses revealed that the GAPU101 showed the highest number of alleles (8) followed by the tow loci UDO99 and GAPU71 with 6 alleles. The DCA18 locus showed 5 alleles. Genetic variability was wide as indicated by the values of observed heterozygosity as noted 1.00 at locus of the four studied loci. Polymorphic information content (PIC) varied from 0.669 to 0.776. The gene diversity values were higher than 0.53. Genetic distances were determined based on the SSR genotype data and component principal analysis were used for finding possible correlation between severity disease, Meski cultivar and hybrids.*

Keywords— Olive, Venturia oleaginea, microsatellites, Genetic relationships, Disease resistance.

I. INTRODUCTION

Olive tree (*Olea europaea* L.) is the most important tree cultivated in Tunisia. Therefore, it contributes by 11% of the total value of agricultural production and by 33% of the wholesale value of agricultural exports. More than 1.76 million hectares planted with 74 million olives. The olive production rate over the last five-year was more than 792 thousand tons but the export rate during the same period was about 145 thousand tons of oil worth 590 million dinars (Tunisian Ministry of Agriculture, 2014). The great damage induced by fungal diseases and especially the olive leaf spot (OLS) disease caused by the fungi *Venturia oleaginea* has an important incidence (Rhouma *et al.,* 2013).

In recent years, alternative techniques have been applied for the control of the disease. Genetic resistance represents an effective, economically feasible and ecologically sustainable mean to control OLS (Rhouma *et al.,* 2013; Sanei and Razavi, 2011; Zine El Aabidine *et al.,* 2010). However, the level of susceptibility of olives to OLS is widely variable (Graniti, 1993; Sutter, 1994). Several varieties (eg. Leccino and Valatolina) are resistant to *V. oleaginea* (MacDonald *et al.,* 2000; Sanei and Razavi, 2011).

In order to exploit genetic diversity, many cross breeding programs were carried out and several Tunisian research teams have used PCR-based markers which include SSR for basic and applied research to assess the genetic diversity of Tunisian olive cultivars (Taamalli *et al.,* 2007; Hannachi *et al.,* 2008; Rekik *et al.,* 2008) and SNP (Rekik *et al.,* 2010).

The microsatellite technique is one of the most reliable methods used in olive cultivar characterization. It has revealed a high informative level because the markers are polymorphic, multiallelic, and codominant. Moreover, it consists of a relatively simple methodology that permits an easy interpretation of results (Rafalski *et al.,* 1996). SSR markers have been successfully used in germplasm bank classification and contributed to better management of several olive collections around the world (Khadari *et al.,* 2007; Muzzalupo *et al.,* 2010).

OLS of olive trees is quite prevalent in Tunisia. However, no reports on *V. oleaginea* fungal phytopathogens causing this disease on olive cultivars and hybrids obtained through cross breeding program.

The aim of this work was to characterize the resistance of a susceptible olive cultivar and hybrids to OLS disease, grown in Tunisia and to contribute in the study and development of OLS-resistant hybrids. SSR markers were used for the establishment of a relationship between Meski cultivar and hybrids in order to produce superior progeny. Genetic distances might be used to revealed differences between hybrids and cultivars in the resistance to OLS disease. So, that resistant cultivars or hybrids can be identified and thus used for replanting, or as sources for resistance in future breeding programs. This study was conducted to characterize the genetic resistance of some hybrids and olive cultivars with disease of OLS based on four SSR markers referring to their polymorphism and reproducibility.

II. MATERIALS AND METHODS

2.1 Plant material

Evaluation of observed was carried out on Meski cultivar and 9 hybrids collected from groves located in the experimental station of the National Institute Engineering and Forestry of Tunisia (INGREF) at Oued Souhil (latitude NR 36 (27′ 22″, E10 longitude (42′ 02″) which was characterized by a humid climate. Molecular analysis was performed on 13 trees belonging to four Tunisian cultivars and 9 hybrids. The collection includes parent's cultivars and hybrids resulting from crosses of 'Meski' (the main table olive cultivar in Tunisia) with pollinating cultivars 'Arbequina', 'Picholine' and 'Chétoui'.

2.2 Evaluation of infection of cultivars and hybrids

The infection degree of four cultivars and nine hybrids was evaluated. A total of 100 leaves with and without visible symptoms were randomly collected from three trees of each sample and then stored at 4°C in the laboratory. To evaluate whole leaf infection, leaves with and without symptoms were immersed in 5% NaOH for 2 min at 50-60°C. This treatment makes visible any latent symptomless infection (Shabi *et al.,* 1994). This infection was then evaluated by the multiplication of the incidence (rate of infected leaves) and the severity (rate of the leaf surface covered with lesion), carried back to a general rate of the whole infection (in percent).

2.3 SSR characterization

2.3.1 Extraction of DNA:

Extraction of DNA was performed on fresh young leaves in parental cultivars and hybrids (Table 1). Genomic DNA was extracted by ammonium bromide hexadecyltrimethyle (CTAB) according the method described by Murry and Thompson (1980) with modifications described by De la Rosa *et al.,* (2002).

2.3.2 SSR markers

Four microsatellite primers labeled with one of two fluorescent dyes 6-FAM or HEX (Sigma) (Table 2) ssrOeUA-DCA 18 (Sefc *et al.,* 2010), UDO99-043 (Cipriani *et al.,* 2002), ssrOeUA-GAPU 101, GAPU71b (Carriero *et al.,* 2002) were used. Studies carried out by Fendri *et al.* (2014) demonstrated that using few high polymorphic SSR markers can provide an efficient identification of olive tree.

Amplification reactions were carried out in final volumes of 10 µl using a thermal cycler (Biometra®). The reaction contained 1X PCR buffer, 0.75 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of forward and reverse primers, 0.5 unit/µl Taq DNA polymerase (Gotaq, Promega) and 50 ng/µl templates DNA.

PCR amplification was completed at the following profile with one cycle of 94 °C of initial denaturation for 5 min, followed by 35 cycles of 95 ºC for 20 s, 52 ºC for 30 s and 72 ºC for 30 s, and followed by a final extension for 8 min. The amplified products were tested on 1.2 % agarose gel to check for the amplification of the PCR products, finally, an amplification step to 4° C (hold temp) corresponding to the storage temperature of the amplification products.

PCR product (0.5 µl) was mixed with a 12 µl of deionised formamide and 0.5 µl Gene Scan 500 (LIZ) size standard marker. The resulting mixture was heated for 2 min at 95°C and then quickly cooled on ice. Each sample was loaded and run in a HITACHI 3130 automatic sequencer Genetic Analyzer (Applied Biosystems, USA). Gene Mapper software was used to determine the size of the alleles of the four markers analyzed for each sample.

2.4 Data analysis

Data on the percent of infected leaves were Log-transformed. All data were analyzed by Analysis of Variance (ANOVA). Significant differences among treatments were computed after SAS analysis at $P < 0.001$.

SSR fragments data were scored using Genomapper softwares. The expected heterozygosity (He) of each microsatellite was calculated according to the formula $He = 1- \Sigma (p_i)^2$ (Nei, 1979) using the GDA program (Weir, 1996). Polymorphic information content (PIC) values was calculated according to the formula $1-\Sigma(p_i)2-\Sigma\Sigma2(p_i)^2(p_j)^2$ using the CERVUS v.2 software (Marshall *et al.*, 1998).

The number of alleles for each SSR locus, information content (PIC), the observed heterozygosity (Ho) and the expected heterozygosity (He) were calculate with CERVUS v.2 software.

Genetic relationships among accessions were calculated on the basis of a similarity matrix analysis according to Dice's coefficient (Sneath and Sokal, 1973). A dendrogram was generated based on the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis and grouping using NTSYS software ver. 2.11a (Rohlf, 1998) using the PowerMarker V3.25 software employing the coefficient of similarity Nei (1983). The heterosis effect of infection degree was subjected to one-way analysis of variance.

III. RESULTS AND DISCUSSION

3.1 Evaluation of infection of hybrids and cultivars

Latent infection intensity showed three different clusters. The first group (Meski, Arbequina and MeskixArbequina) was very susceptible to OLS disease and the percentage of infection was ranged between 60 and 83%. The second group contains Chétoui and ChétouixMeski was susceptible to the disease but infection did not exceed 50% (Fig. 1). The last group was more resistant then the others. The cultivar Picholine and hybrid PicholinexMeski were the most resistant to the disease since their infections were less than 20%. The analysis of variance and the mean comparison revealed significant differences between the hybrids and cultivars (Table 1).

3.2 Molecular characterization

Data of analysis applied on SSR loci are shown in table 2. The GAPU101 showed the highest number of alleles (8), followed by the tow loci UDO99 and GAPU71 with 6 alleles. The DCA18 locus showed 5 alleles. Genetic variability was wide as indicated by the values of observed heterozygosity as noted 1.00 at locus of the four studied loci. Polymorphic information content (PIC) varied from 0.669 to 0.776. The gene diversity values were higher than 0.53 (Fig 2).

The dendrogram generated by UPGMA algorithm using SSR data of olive cultivars and hybrids showed that the hybrids obtained through controlled crossing between Meski x Arbequina and between Meski x Picholine were related to the Picholine cultivar. The hybrids Meski x Chétoui were more related to the Meski cultivar (Fig 3).

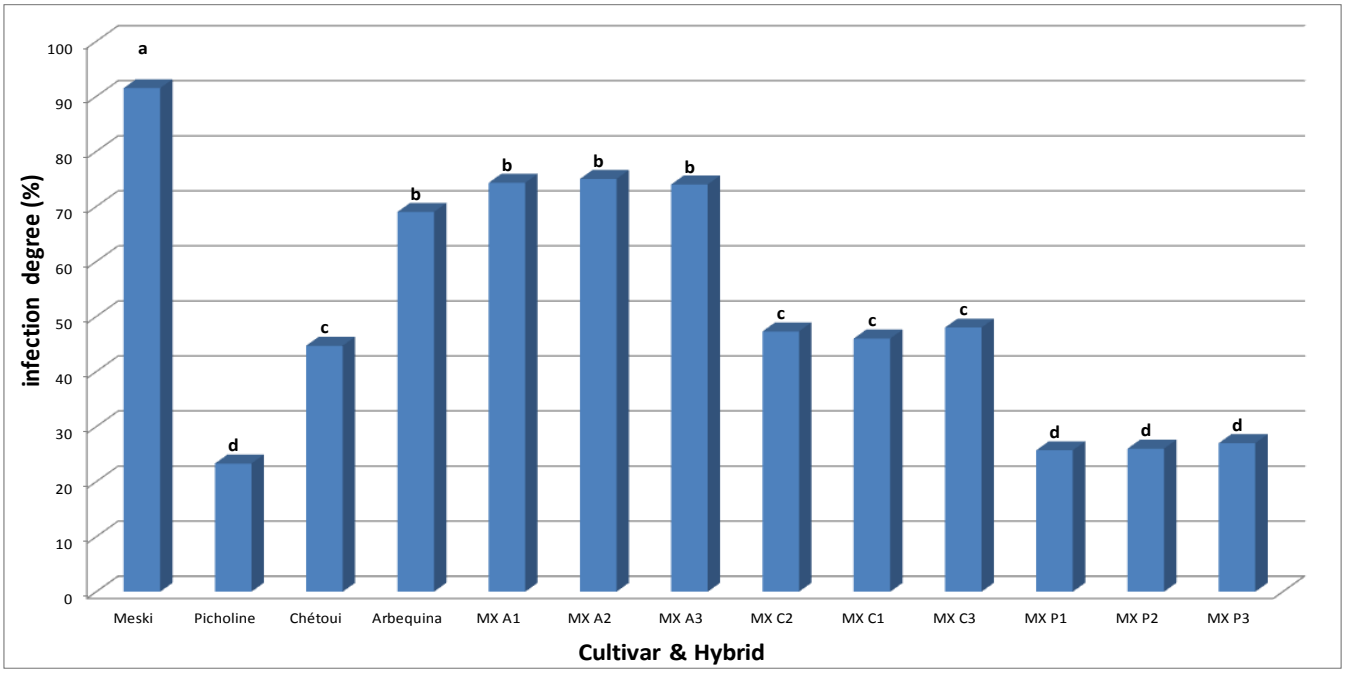

FIGURE 1 COMPARISON OF INFECTION OF HYBRIDS AND CULTIVARS BY OLIVE LEAF SPOT DISEASE

TABLE 1
ANALYSIS OF VARIANCE (ANOVA) APPLIED ON INFECTION DATA OF OLIVE CULTIVARS AND HYBRIDS

	Code	Infection degree (%)
Meski	Msk	91.533 ± 1.501^{A}
Picholine	Pchl	23.333 ± 2.113^{D}
Chétoui	Cht	44.667 ± 3.512^{C}
Arbequina	Arbqn	69.000 ± 3.606^{B}
MeskixArbequina	MXA1	74.333 ± 4.041^{B}
MeskixArbequina	MXA2	75.000 ± 2.645^{B}
MeskixArbequina	MXA3	74.000 ± 3.605^{B}
MeskixChétoui	MXC1	47.333 ± 5.571^{C}
MeskixChétoui	MXC2	46.000 ± 6.000^{C}
MeskixChétoui	MXC3	48.000 ± 4.917^{C}
MeskixPicholine	MXP1	25.667 ± 2.082^{D}
MeskixPicholine	MXP2	26.000 ± 4.000^{D}
MeskixPicholine	MXP3	27.000 ± 3.464^{D}

Superscript letters with different letters respectively indicate significant difference ($P < 0.05$) analyzed by Duncan's multiple range test.

TABLE 2
GENETIC DIVERSITY PARAMETERS OF OLIVE CULTIVARS AND HYBRIDS BASED ON SSR MARKERS

	No Alleles	Observed heterozygosity	Expected heterozygosity	PIC	Range size
ssrOeUA-DCA 18	5	1	0.788	0.719	167-175
UDO99-043	6	1	0.837	0.776	172-214
ssrOeUA-GAPU101	8	1	0.818	0.759	180-214
GAPU71B	6	1	0.772	0.669	117-138
Total	25				
Mean value	6,25	1	0,803	0,730	

No Alleles. : Number of alleles; PIC: Polymorphic information content

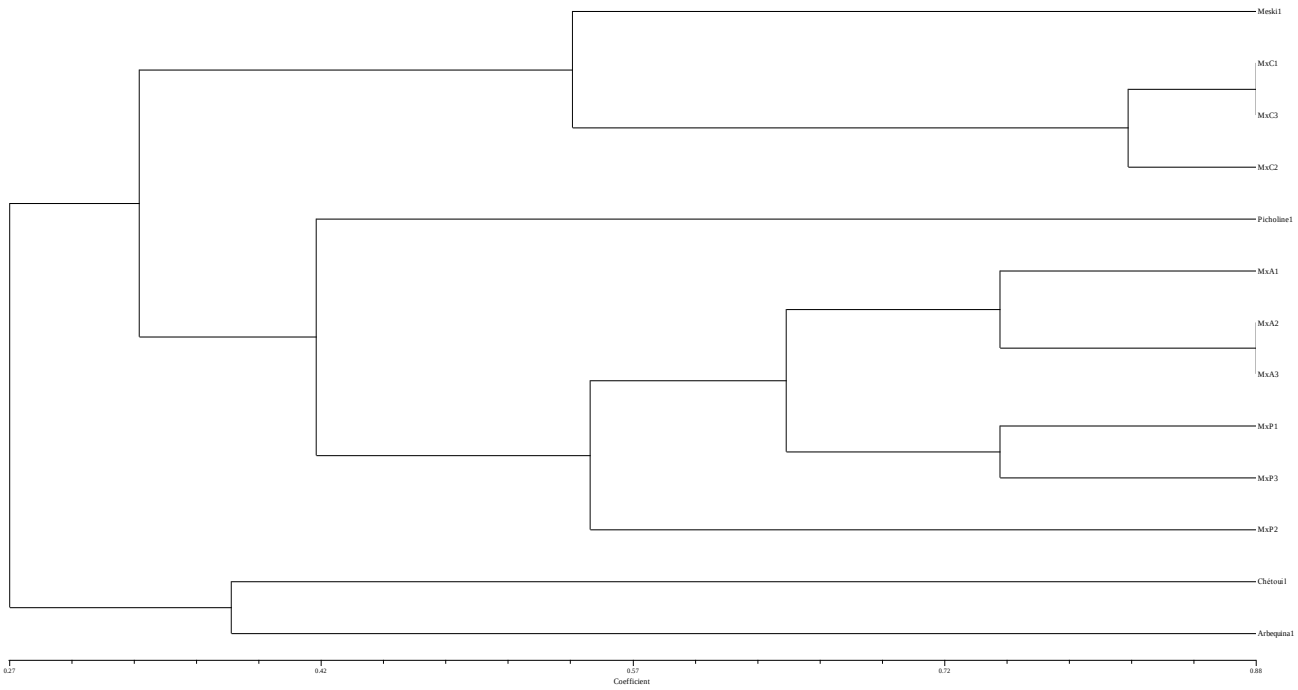

FIGURE 2 SSR DENDROGRAM OF OLIVE CULTIVARS AND HYBRIDS BASED ON SSR DATA GENERATED BY UPGMA ALGORITHM.

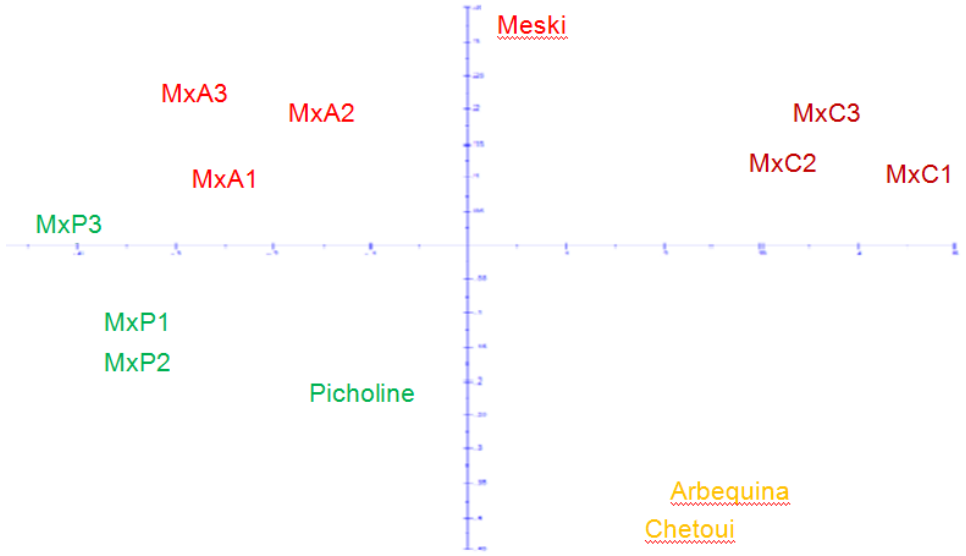

FIGURE 3 FACTORIAL CORRESPONDENCE ANALYSES OF OLIVE CULTIVARS AND HYBRIDS BASED ON SSR DATA

Our study was carried out to verify crossover effect directed between cultivars with different levels of susceptibility to OLS, a prerequisite for constructing genetic mapping of the olive tree and detection of QTL Loci related to this resistance. Meski is the olive of the most important table in Tunisia (Ben Amar *et al.*, 2010). Despite its importance, this variety is very susceptible to OLS disease (Triki *et al.*, 2003). The attacks of the fungus *V. oleaginea* cause significant production losses in this variety. Other varieties grown in Tunisia are resistant or insensitive such as the variety Picholine (Rahioui, 2007). The Arbequina variety is susceptible to the disease (Barranco *et al.*, 2002). The introduction of resistant varieties for genetic improvement requires knowledge of the phenotypic characteristics of the OLS disease resistance.

Mekuria *et al.* (2001) used RAPD PCR based on the method applied by Fabri *et al.* (1995) and Wiesman *et al.* (1998) to identify genetic markers linked to resistance to OLS disease by segregation of a population from a crossover trial. The DNA of these individuals was used to identify certain molecular markers associated with resistance to the disease by adopting RAPD (Mekuria *et al.*, 2011).

In our analysis, the total number of amplified alleles was 25 alleles with an average of 6.25 alleles per locus (Table 2). The number of alleles per locus varied from 5 until 8. These results were conform to the alleles found by Abdelhamid *et al.* (2013) with a total of 73 alleles identified for 10 microsatellite markers in a group of Tunisian trees. The heterozygosity values found at each locus was comparable to those reported in several studies performed by microsatellite markers, such as the genetic material of Moroccan olive (Khadari *et al.*, 2007; Charafi *et al.*, 2008). The observed heterozygosity rate is 1 but the calculated heterozygosity is 0.803.

Rare cases have been found in our study as allele 214 bp at the locus SSR-UDO43 and SSR-GAPU101 for variety Meski and Meski x Arbequina hybrid. The allele 117 bp (minimum) was present in the locus SSR-GAPU71B for the variety Chétoui and PicholinexMeski hybrid. The allele 125 bp is exclusive to the MeskixArbequina hybrid. The allele 171 bp is most present for common 9 hybrids and cultivars. Allele frequency ranges from 0.038 at 0.364.

Even though the number of identified accession is relatively limited, our present study is largely consistent and comparable to previous studies (Baldoni *et al.*, 2009; Bracci *et al.*, 2009; Muzzalupo *et al.*, 2010) using SSR as a method of genetic analysis to assess variability genetics of Italian olive cultivars, including some minor exceptions. PIC values for some varieties, in accordance with many previous studies (Taamalli *et al.*, 2008), showing that these memberships are a good source of diversity and the four loci used are adapted to the mapping (Poljuha *et al.*, 2008). The primers produced simple banding patterns, showing a degree of differential amplification. They were used for genotyping and will be useful for identifying varieties of olive and perform genetic studies that is of particular interest to proceed with the breeding and conservation program. In our study, it was only possible to choose four loci available (ssrOeUA-DCA18, ssrOeUA-GAPU101, UDO99-043 and ssrOeUA DCA-17) for rapid identification (Table 1).

Our data confirm that the set of microsatellite markers selected for this work is actually efficient in identifying parental and hybrid accessions showing a high polymorphic content. Thus, molecular data can be used to confirm the previously described study of susceptibility of hybrid accessions to OLS disease.

The results obtained by analysis of DNA microsatellites revealed a clear separation of most olive cultivars and hybrids studied and have shown a significant degree of genetic diversity. The construction of the genetic map of individual's olive based on the segregation of the population is essential to detect and identify in *V. oleagina* resistance locus. The high utility of SSR markers in providing grouping varieties is consistent with previous studies of a large number of Tunisian olive cultivars (Rekik *et al.*, 2008; Taamalli *et al.*, 2006).

A dendrogram was made using genetic distances between pair's accessions to visualize the genetic similarity between the accessions (Fig 2). A high range of similarity has been found among them from 0.15 to 1. For example, three main groups were observed. The dendrogram generated by UPGMA algorithm using the SSR data cultivars and hybrids olive showed that hybrids obtained by controlled cross between Meski×Picholine, Meski × Arbequina and Meski ×Picholine were related to Picholine cultivar. The hybrid (Meski × Chétoui) was more related to cultivar Meski.

The analysis of the dendrogram showed that studied hybrids and cultivars were classified into three different main groups (clusters). The first group contains MxC1, MxC2, MxC3, Arbequina1 and Chétoui1. The second group comprises MxA1, MxA2, MxA3 and Meski1 and the last group of MxP1, MxP2, MxP3 and Picholine1. The hybrids derived from crossing between Picholine and Meski showed significant resistance to disease, hence the possibility to transfer these resistance genes in cultivar Picholine to susceptible Meski. These results are consistent with those obtained by Rhouma *et al.* (2013) and Benitez *et al.* (2005) which demonstrated that resistance was controlled by several genes. But this susceptibility was affected by the environment (temperature, relative humidity, light) (Trapero and Blanco, 2008).

IV. CONCLUSION

Multi-factorial analysis carried out in this work has been conducted to characterize clustering tendencies among the identified cultivars according to their resistance to the disease (Fig. 2). The analysis revealed 36.96 and 22% of clustering according to two principal components. Such percentages allow considering the groups as clustering tendencies and not as a clear separations. The test ended with the clustering of the MxP1, MxP2, MxP3 and Picholine1 olive accessions which are resistant, MxA1, MxA2, MxA3 and Meski1 olive accessions which are not resistant and MxC1, MxC2, MxC3, Arbequina1 and Chétoui1 accessions with no clear separation. Thus, it seems that clustering tendencies correspond to the resistance to OLS disease, but resistant and susceptible parents might be in the same separation groups. This is probably a result of

grower's selections during the olive cultivation history considering the resistance to OLS disease which must be confirmed by further analysis using a wider collection of olive accession.

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