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Phenolic Compounds of Olive-Tree Leaves and Their Relationship with the Resistance to the Leaf-Spot Disease Caused by *Spilocaea oleaginea*

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Abstract: Phenolic compounds are associated with the olive tree resistance to the leaf-spot disease caused by *Spilocaea oleaginea* were studied in different resistant, susceptible and intermediate cultivars. The HPLC analysis highlights 33 phenolic compounds distinguished according to their chromatographic and spectral characteristics into five phenolic families (hydroxycinnamic derivatives, flavonoids, verbascoside derivatives, tyrosol derivatives, oleuropein derivatives). The phenolic extract of the olive-tree is dominated by ten major compounds identified as rutin, luteolin-7-glucoside, oleuropein, versbacoside, tyrosol, apigenin and four other phenolic compounds not completely identified (oleuropein derivative, hydroxycinnamic derivative and two flavonol monoglucosides). No qualitative difference was observed between cultivars. However, the principal components analysis highlights two multifactorial components distinguishing the various cultivars according to their behaviour to the disease. The first component, identified as oleuropein aglycone, a hydroxycinnamic derivative and a flavonol monoglucoside contents, clearly distinguished the resistant cultivars from the susceptible and intermediately resistant cultivars. The resistant cultivars contain higher contents. The second component, identified as tyrosol derivative and an oleuropein derivative contents, distinguished the susceptible cultivars from the intermediately resistant cultivar which presents the highest contents. The role of these phenolic compounds in the defense and their use as biochemical markers in olive-tree resistance to *S. oleaginea* is discussed.

Key words: *Olea europaea* · *Spilocaea oleaginea* · Phenolic compounds · Resistance

INTRODUCTION

The olive-tree constitutes the principal fruit tree cultivates in Morocco (more 50% of the surface of the fruit trees) and it has a major socio economical role since it contributes to the maintenance of the rural populations [1]. The "Moroccan Picholine" is the most dominant cultivar; more than 98% of the olive growing orchards are planted by this cultivar [2]. Despite of its adaptation to the Moroccan orchards, this cultivar is susceptible to the principal fungal diseases particularly to the leaf-spot disease caused by *Spilocaea oleaginea* which represents the most widespread fungal disease of olive-tree in the world [3]. This disease is caused by *Spilocaea oleginea*, specific biotrophic pathogen of olive-tree presenting a sub-cuticles development [4,5]. The disease appears by circular tasks on the leaves and the fruits leading to their fall and the general weakening of the olive-tree [6]. The economic damage is considerable when the conditions are favourable to the disease development [6,7]. The chemical treatment by fungicides containing copper appears rarely effective because of the appearance of resistant pathogen

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races to copper and of the disturbance of the plant metabolism following copper accumulation in the soil [8,9]. The genetic resistance represents currently the only effective mean to stop this disease [3]. The resistance of the olive-tree cultivars is largely studied [10,11], but the defence mechanisms of olive-tree are not well-known [7,12,13]. The phenolic compounds constitute the chemical molecules often implied in defence to the plant pathogens and associated with the plant host resistance [14,15].

The present work aimed to characterize the principal phenolic compounds of the olive-tree leaves and to find out if these compounds could be associated with the olive-tree resistance to *S. oleaginea*.

MATERIALS AND METHODS

Plant Materials: Seven olive-tree cultivars from the French olive germplasm maintnaid as ex-situ collection in Porquerolles island (southern of French) were studied. These cultivars displayed differential behaviours to *S. oleaginea* after four years of evaluation; three resistant cultivars (Gardisson, Colombale, Verdale), three susceptible cultivars (Moroccan Picholine, Berdaneil, Rabeyrolle) and a cultivar showing an intermediate behaviour (Languedoc Picholine). The phenolic compounds analysis related to leaves collected in March, period preceding the attack by *S. oleagina*. For each cultivar, the leaves from five olive-trees and for each tree, five samples were taken. The collected leaves are freeze-dried and then stored under vacuum.

Extraction of Phenolic Compounds: Extraction of phenolic compounds was carried out as previously described [16]. The freeze-dried leaves (25 mg) are crushed in 1.5 ml of methanol-water (4: 1, v/v) added with 10^{-4} M of D-glucoronique lactone acid for to prevent the hydrolysis of the glucosidic fraction and 10^{-4} M of 5-méthoxyflavone used like internal standard. The extraction is carried out in a vat ultrasound for 20 min at 20°C. The extract is then centrifuged at 5.000 g for 10 min and the supernatant obtained constitutes the phenolic extract.

Identification of Phenolic Compounds: The phenolic compounds were characterized according to the techniques previously described [17] by high-performance liquid chromatography (HPLC), their *Rf* values in thin-layer chromatography (TLC) in various solvents and their colour in the presence of the mixture of ferricyanide potassium-ferric chloride revealing the phenolic molecules and their UV fluorescence (254 nm and 366 nm) in the presence of the ammonia vapor [18], the Benedikt reagent distinguishing monophenols/ orthodiphenols [19], the *p*-toluene sulphonic acid highlighting the radical secoiridoide [20] and the Neu reagent revealing the flavonoids and the caffeic acid derivatives [21]. Each spot characterized in TLC is then isolated for determinate the peak corresponding in HPLC and to provide additional information for the identification (retention time, absorption spectrum). The phenolic moiety was determined after enzymatic hydrolysis (β-glycosidase, phosphate buffer, pH 6.8, for 4 h), acid hydrolysis (HCl 2 N for 1 h at 100°C) and alkaline hydrolysis [22]. The phenolic compounds identification is supplemented by co-chromatography in TLC, co-injection in HPLC and by comparing their chromatographic and spectral characteristics to phenolic standards (Sigma-Aldrich chimie S.a.r.l., St-Quentin Fallavier, French).

Quantification of Phenolic Compounds: Phenolic contents were determined by HPLC according to the technique previously described [16,22]. The HPLC (Waters 600 E with a photodiode bar detector Waters 2996) was performed on Spherisorb C18 column (250 X 4 mm, 5 μm) and μBondapack C18 Waters Guard PAK precolumn. Samples were eluted with a solvent consisting of acetonitrile and water acidified by acetic acid following a gradient of 5-37% of acetonitrile within 40 min with 1 ml. min^{-1} flow rate. The phenolic compounds contents compounds is expressed in equivalent 5-methoxyflavone, phenolic compound used like internal standard. For each cultivar, all data correspond to means of five replicates with samples of five olive-trees.

Statistical Analysis: The variance analysis to only one factor according to a random model (variation between cultivars) is carried out using the Statitcf software. The differences between the means (five replicates with samples of five olive-trees) are determined by the Duncan's range test at $P < 0.05$. The principal components analysis is carried out using the software Statistica (version 6).

RESULTS

Characterization of Phenolic Compounds of Olive-tree Leaves: The analysis of the phenolic extract by HPLC

Table 1: Phenolic compounds identified in olive-tree leaves

Number of compounds in HPLC	Identification	Phenolic families
5	Chlorogenic acid	
9	p-Coumaric acid	
11	Caffeic acid	
13	Hydroxycinnamic derivative	Hydroxycinnamic derivatives
21	Hydroxycinnamic derivative	
25	Hydroxycinnamic derivative	
26	Hydroxycinnamic derivative	
15	Monoglucoside de flavonol	
16	Rutine	
18	Luteolin-7-glucoside	
19	Monoglucoside de flavonol	Flavonoids
20	Apigenin-7-glucoside	
23	Monoglucoside de flavonol	
24	Monoglucoside de flavonol	
31	Apigenin	
6	Verbascoside derivative	
7	Verbascoside derivative	
10	Verbascoside derivative	
12	Verbascoside derivative	Verbascoside derivatives
14	Verbascoside derivative	
17	Verbascoside	
22	Verbascoside derivative	
28	Verbascoside derivative	
1	Hydroxytyrosol	
2	Tyrosol derivative	
3	Tyrosol	Tyrosol derivatives
4	Tyrosol derivative	
8	Tyrosol derivative	
27	Oleuropein	
29	Oleuropein aglycone	
30	Oleuropein derivative	Oleuropein derivatives
32	Oleuropein derivative	
33	Oleuropein derivative	

shows the presence of 33 phenolic compounds (Fig. 1) distinguished in five phenolic families according to their chromatographic and spectral characteristics (Table 1).

The hydroxycinnamic derivatives are represented by seven compounds (peaks 5, 9, 11, 13, 21, 25 and 26). They show a blue fluorescence which intensifies after pulverization of the Neu reagent. The co-chromatography in HPLC with standard phenols shows the presence of chlorogenic acid (compound 5), *p*-coumaric acid (compound 9) and the caffeic acid (compound 11).

The flavonoids are represented by eight compounds (peaks 15, 16, 18, 19, 20, 23, 24 and 31). These compounds are dark nonfluorescent in TLC and give fluorescent in yellow or orange after pulverization of the ammonia vapour or the Neu reagent. The glycosylated forms are very abundant in the phenolic extract of the olive-tree leaves. Thus, the TLC on polyamide in the separation system "water-ethanol-acetylacetone" distinguishes the monoglucosides (peaks 15, 18, 19, 20, 23, 24) and a diglucoside (peak 16). The comparison of the chromatographic (time of retention, *Rf* values, fluorescence) and spectral characteristics as well as the co-chromatography with the phenolic standars allows to identify the luteolin-7-glucoside (compound 18), the apeginin-7-glucoside (compound 20) and the rutin (compound 16). The flavonoides aglycones are represented by the apigenin (compound 31).

Table 2: Phenolic compounds contents of olive-tree leaves of resistant (Gardisson, Colombale, Verdale), intermediately (Languedoc Picholine) and susceptible (Moroccan Picholine, Berdaneil, Rabeyrolle) cultivars. For each phenolic compound, the values followed by a common letter do not differ significantly at $P = 0.05$ according to Duncan's range test

Number of compounds	Olive-tree cultivars						
in HPLC	Gardisson	Colombale	Verdale	Languedoc picholine	Moroccan picholine	Berdaneil	Rabeyrolle
1	3.52a	3.83a	3.28a	2.80a	3.05a	3.11a	3.90a
2	9.85ab	14.64a	7.07b	6.57b	6.20b	10.07ab	11.04ab
3	32.13b	16.19c	11.02c	38.97a	29.70b	17.94c	15.83c
4	8.40c	11.61bc	5.29d	17.61a	12.10bc	8.22cd	13.29b
5	2.27ab	2.01ab	5.71a	4.8a	3.18b	2.61b	1.13b
6	4.98a	3.37ab	2.67ab	2.54ab	3.01ab	2.65ab	1.44b
7	7.29ab	1.28b	1.43b	0.99bc	9.71a	7.11ab	0.68bc
8	10.61a	7.86ab	7.46ab	4.96b	2.85b	3.78b	11.69a
9	5.31a	4.63a	4.86a	3.42a	5.03a	4.54a	2.44a
10	3.64a	0.72a	0.59a	0.42a	4.61a	3.80a	1.76a
11	11.11a	9.43ab	9.01ab	6.08b	8.31ab	1.77c	7.66ab
12	0.81b	3.27a	3.34a	2.48a	2.13a	2.66a	1.58ab
13	10.43a	6.76a	8.94a	2.52b	5.53ab	0.62c	3.14b
14	2.49a	1.53a	3.08a	1.04ab	0.88b	1.59a	1.29a
15	7.80a	4.26b	7.59a	0.52d	2.67c	2.57c	1.07cd
16	16.44a	17.71a	4.91c	11.11b	13.23b	6.95c	5.42c
17	35.07bc	42.99b	66.25a	16.40c	41.03b	23.25bc	25.00bc
18	99.8a	75.86b	50.96c	44.94c	54.92c	33.21c	41.66c
19	0.42c	4.68ab	7.1a	1.76b	2.08b	3.31ab	0.42c
20	8.74a	9.09a	3.84b	7.99a	7.04a	7.95a	4.95b
21	12.8ab	8.5bc	13.79a	8.7b	8.90b	4.78cd	5.00c
22	3.60a	2.41a	0.92a	1.31a	0.87a	0.94a	2.13a
23	90.87a	62.01b	61.24b	55.33c	36.23cd	0.43e	44.35c
24	18.47a	35.40a	17.64a	12.81a	36.36a	41.30a	19.32a
25	26.80a	5.24d	18.5b	2.43d	0.45d	13.26c	1.4d
26	3.69a	2.19a	2.13a	2.55a	5.47a	1.29a	0.42a
27	46.77a	59.46a	38.41a	33.01a	30.44a	46.52a	57.12a
28	5.15a	5.56a	3.13a	2.12a	2.37a	0.99b	4.04a
29	13.04a	11.68a	9.70a	3.37c	7.26ab	7.2ab	4.2c
30	119.44abc	113.38bc	101.48c	130.86ab	116.5abc	120.8abc	138.27a
31	21.4de	24.31cd	13.56e	39.95a	31.04b	13.04ef	29.7bc
32	13.90bc	9.79c	2.22d	24.98a	18.8b	2.16d	14.82bc
33	9.26bcd	6.71cd	3.32d	19.92a	14.2abc	7.81cd	15.8ab

The verbascoside derivatives are represented by eight compounds (peaks 6, 7, 10, 12, 14, 17, 22 and 28). Only compound 17 could be identified to the verbascoside according to the chromatographic and spectral characteristics.

The tyrosol derivatives are represented by five compounds (peaks 1, 2, 3, 4 and 8). The hydroxytyrosol (compound 1) gives fluorescence blue which extinct in the presence of the Benedikt reagent indicating the presence of a function ortho-diphenol and a red fluorescence after pulverization of the *p*-toluene sulphonic acid confirming the absence of a structure secoiridoide. The compound 3 is identified as tyrosol by chromatographic and spectral characteristics.

The oleuropein derivatives are represented by five compounds (peaks 27, 29, 30, 32 and 33). The compound 27 gives an orange fluorescence in the presence of the *p*-toluene sulphonic acid. Its time of retention in HPLC, its *Rf* values in TLC and its UV absorption spectrum are similar to those of the oleuropein. The acid hydrolysis of

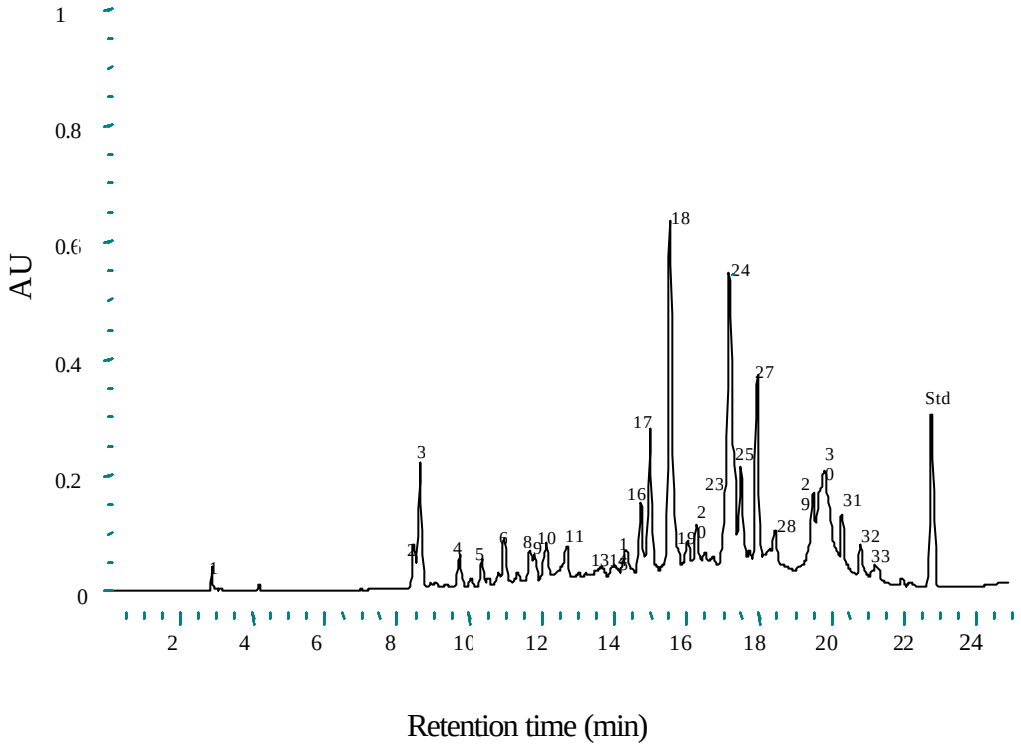

Fig. 1: HPLC Chromatogram of phenolic compounds of olive-tree leaves. 1 to 33: Phenolic compounds (for identification, see Table I), STD: 5-methoxyflavone (internal standard)

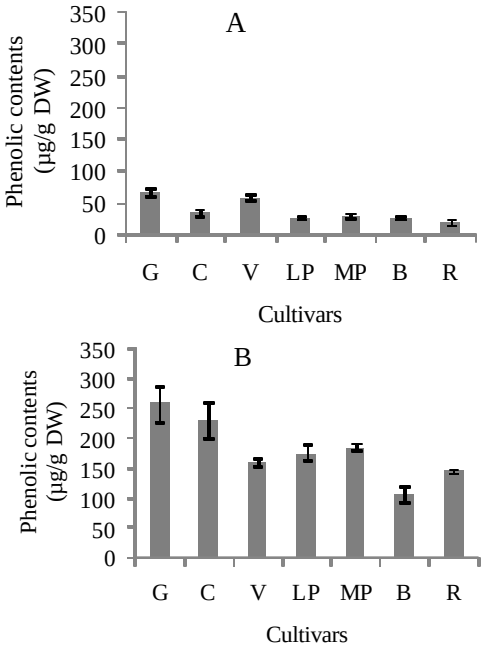

Fig. 2: Contents of phenolic families in olive-tree leaves of resistant (G, C, V), intermediately (LP) and susceptible (MP, B, R) cultivars. G: Gardisson, C: Colombale, V: Verdale, LP: Languedoc Picholine, MP: Moroccan Picholine, B: Berdaneil, R: Rabeyrolle. A: Hydroxycinnamic derivatives, B: Flavonoids, C: Verbascoside derivatives, D: Tyrosol derivatives, E: Oleuropein derivatives

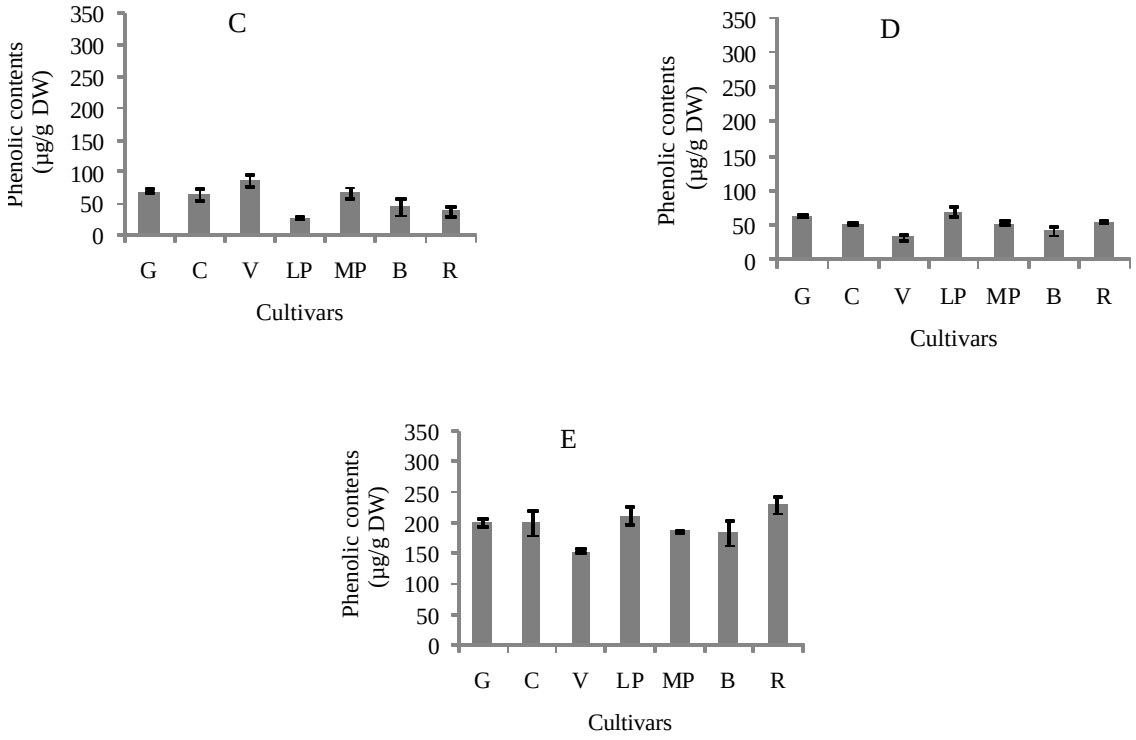

Fig. 2: Contents of phenolic families in olive-tree leaves of resistant (G, C, V), intermediately (LP) and susceptible (MP, B, R) cultivars. G: Gardisson, C: Colombale, V: Verdale, LP: Languedoc Picholine, MP: Moroccan Picholine, B: Berdaneil, R: Rabeyrolle. A: Hydroxycinnamic derivatives, B: Flavonoids, C: Verbascoside derivatives, D: Tyrosol derivatives, E: Oleuropein derivatives

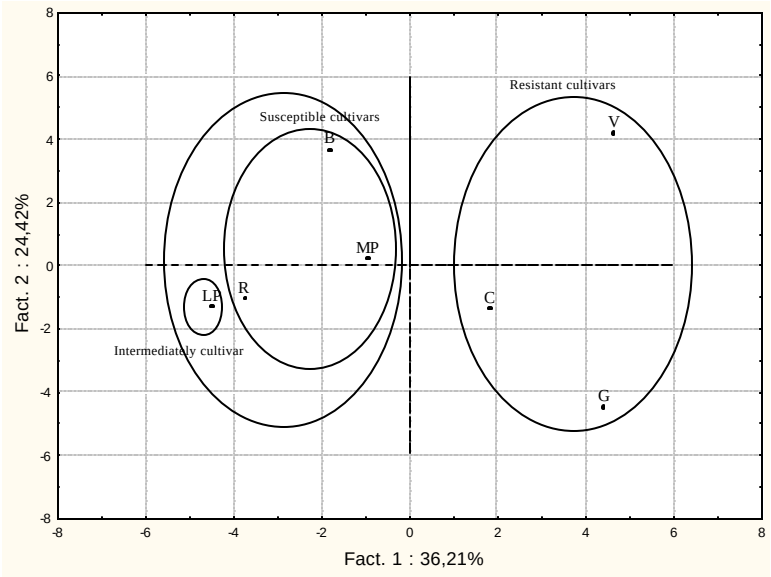

Fig. 3: Distribution by the Principal Components Analysis of resistant (G, C, V), intermediately (LP) and susceptible (MP, B, R) cultivars according to phenolic compounds and phenolic families. G: Gardisson, C: Colombale, V: Verdale, LP: Languedoc Picholine, MP: Moroccan Picholine, B: Berdaneil, R: Rabeyrolle

this compound releases the oleuropein aglycone which corresponds to compound 29.

At the quantitative level, the phenolic extract of the olive-tree leaves is dominated by 10 major phenolic compounds identified to rutin (compound 16), luteolin-7-glucoside (compound 18), oleuropein (compound 27), versbacoside (compound 17), tyrosol (compound 3), apigenin (compound 31), a hydroxycinnamic derivative (compound 25), an oleuropein derivative (compound 30) and two flavonol monoglucosides (compounds 23 and 24) not completely identified.

Relationship Between Phenolic Compounds and Resistance to the Leaf-spot Disease: All the characterized phenolic compounds were present in each of the seven studied cultivars, no qualitative difference was detected. However, the quantitative differences allowed distinguishing the various cultivars according to their behaviour to the leaf-spot disease. Thus, the contents of phenolic compounds described individually (Table 2) or gathered in phenolic families (Fig. 2) show that the resistant cultivars (Gardisson, Colombale, Verdale) were richer than the susceptible cultivars (Moroccan Picholine, Berdaneil, Rabeyrolle) and the intermediately cultivar (Languedoc Picholine) in flavonol monoglucosides (compounds 15 and 23), oleuropein aglycone (compound 29) and hydroxycinnamic derivatives particularly compound 13. The intermediately cultivar was distinguished from the susceptible cultivars by higher contents of tyrosol derivatives (compounds 3 and 4), chlorogenic acid (compound 5), apigenin aglycone (compound 31) and oleuropein derivatives particularly compounds 32 and 33.

To examine the distribution of the cultivars based on quantitative phenolic data, we carried out a principal components analysis by taking the contents of individual phenolic compounds and the contents of phenolic families as active variables. This analysis allowed identifying two main axes explaining 60.63% of the total variability including 36.21% for the first axis and 24.42% for the second axis (Fig. 3).

The first axis, determined on the positive side by the contents of compound 13 (hydroxycinnamic derivative), compound 15 (flavonol monoglucoside) and compound 29 (oleuropein aglycone) and on the negative side by the contents of compound 4 (tyrosol derivative) and compound 33 (oleuropein derivative), distinguishes two cultivars groups. The first group contains the resistant cultivars (Gardisson, Colombale, Verdale) presenting high contents of 13 (derivative hydroxycinnamic), compound 15 (flavonol monoglucoside) and compound 29 (oleuropein aglycone) and the second group contains the susceptible cultivars (Moroccan Picholine, Berdaneil, Rabeyrolle) and intermediately cultivar (Languedoc picholine) with contents weaker. The contents of compound 4 (tyrosol derivative) and compound 33 (oleuropein derivative), having the most weight in the determination of axis 1 on the negative side, distinguishes the intermediately cultivar and the suceptible cultivars. The intermediately cultivar presents the contents higher than the susceptible cultivars (Fig. 3).

The second axis, determined on the positive side by the contents of compound 19 (flavonol monoglucoside) and the compound 22 (derivative of the verbascoside) on the negative side, does not have an effect on the distribution of the various cultivars in relation to their behaviour to the leaf-spot disease.

DISCUSSION

The analysis of the phenolic extract of the olive-tree leaves shows a great phenolic diversity since it contains 33 compounds belonging to several phenolic families. Several previous studies focused on phenolic compounds analysis of olive and the olive-oil because of their antioxydant, sensory and organoleptic properties [23,24,25,26]. In contrast, leave analysis was the subject of less studies which remain fragmentary [27,28,29]. Our work characterized the principal phenolic compounds of the olive-tree leaves which some were completely identified whereas for others, only the phenolic moiety was identified and allows to characterize the phenolic family. The various phenolic compounds highlighted were distinguished in five phenolic families (8 flavonoids, 8 verbascoside derivatives, 7 hydroxycinnamic derivatives, 5 tyrosol derivatives and 5 oleuropein derivatives). At the quantitative level, the phenolic extract of the olive-tree leaves is dominated by 10 major phenolic compounds identified to rutin (compound 16), luteoline-7-glucoside (compound 18), oleuropein (compound 27), versbacoside (compound 17), tyrosol (compound 3), apigenin (compound 31), a hydroxycinnamic derivative (compound 25), an oleuropein derivative (compound 30) and two flavonol monoglucosides (compounds 23 and 24) not completely identified. Several works reported the abundance of these phenols in the olive-tree leaves, particularly the luteolin-7-glucoside, the verbascoside and the oleuropein [27,28,29,30].

The various phenolic compounds characterized were highlighted in all cultivars studied; no qualitative

difference was observed between the resistant, the intermediately and the susceptible cultivars as it was reported in other olive-tree cultivars [30,31,32,33]. However, the quantitative differences distinguish the cultivars studied according to their behaviour to the leaf-spot disease. Thus, the resistant cultivars (Gardisson, Colombale, Verdale) are richer than the susceptible cultivars (Moroccan Picholine, Berdaneil, Rabeyrolle) and the intermediately cultivar (Languedoc Picholine) in flavonol monoglucosides (compounds 15 and 23), oleuropein aglycone (compound 29) and hydroxycinnamic derivatives particularly compound 13. The intermediately cultivar is distinguished from the susceptible cultivars by higher contents of chlorogenic acid (compound 5), apigenin aglycone (compound 31), tyrosol derivatives (compounds 3 and 4) and oleuropein derivatives particularly compounds 32 and 33. The principal components analysis confirms these results and releases two principal multifactorial components having the most effect in the distinction of the cultivars studied according to their behaviour to the leaf-spot disease. The first component, determined by an oleuropein aglycone, a hydroxycinnamic derivative and a flavonol monoglucoside contents, clearly distinguishes the resistant cultivars from the susceptible and intermediately resistant cultivars. The resistant cultivars contain higher contents. The second component, determined by a tyrosol derivative and an oleuropein derivative contents, distinguishes the susceptible cultivars from the intermediately cultivar which presents the highest contents.

These results show that the hydroxycinnamic derivatives, the oleuropein derivatives, the tyrosol derivatives and the flavonol monoglucosides are in relation to the resistance of the olive-tree to the leaf-spot disease and could play a role in defense to *S. oleaginea*. The implication of the phenolic compounds in the plant resistance is largely reported [14,34] and their mode of action in defense to the pathogen micro-organisms is very variable and can go from a direct antimicrobial effect until the modulation and the induction of the mechanisms of defense [15,35]. The phenolic compounds are also precursors of the lignin whose role in defense is largely shown [36,37]. In olive-tree, the studies showed that the oleuropein, the tyrosol, the hydroxycinnamic derivatives and flavonol glucosides expresses an effect fongitoxic [38,39]. The oleuropein glucoside seems to be implied in the defense of the olive-tree by inhibiting the pectinases of *S. oleaginea* [7] and by constituting a precursor of phytoalexins [12,40], strongly toxic molecules induced in response to the pathogen infections [41,42,43]. The oleuropein aglycone, nonglucosidic form of the oleuropeine which is more hydrophobic, expresses an activity antioxydant [44] and a great cytotoxicity [45]. This property could confer on the oleuropein aglycone an important role in the hypersensitive reaction often associated to defense of the leaves in response to the pathogen aggressions [46]. The contents of orthodiphenols of the olive-tree, particularly luteolin-7-glucoside, the oleuropein, the cholorogenic acid and the rutin are implied in the tissue browning resulting from their oxidation [47]. The oxidation of these orthodiphenols, the preferential substrates of the polyphenol oxidase [48,49], give orthoquinones and melanins [15], very toxic products to the pathogen micro-organisms [14,46,50] and which appear in the form of brown pigments during the hypersensitive reaction [15,46]. In addition, the studies of the interaction olive-tree - *Verticillium dahliae* suggest a strong implication of the phenolic compounds in the olive-tree defence particularly, oleuropein, luteolin-7-glucoside, tyrosol and *p*-coumaric acid [51].

Our study suggest that the constitutive phenolic compounds, in particular the hydroxycinnamic derivatives, the oleuropein derivatives, the tyrosol derivatives and the flavonol monoglucosides, could play a role in the defence of the olive-tree to *S. oleaginea* and seem to constitute potential markers of the resistance of the olive-tree to this disease. These studies must be confirmed on other cultivars while taking account of the variation factors related to the modification of the phenolic metabolism.

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