Moroccan Journal of Chemistry
ISSN: 2351-812X
http://revues.imist.ma/?journal=morjchem&page=login
Zied Ben Abdeljelil / Mor. J. Chem. 5 N°1 (2017) 96-104

Impact of season and foliar fertilisers on phenolics of leaves of Chemlali olive cultivar

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Received 17 Feb 2017, Revised 03 Mar 2017, Accepted 01 April 2017

Abstract

The olive leaves are known as a source of nutraceuticals due to their richness in phenolics. Numerous studies explored the effect of several factors on leaf phenolics but few studies focused on the impact of foliar fertilizers on leaf phenolics. In this context, our study aimed to explore the effect of season and foliar fertilisers on phenolics in leaves of "Chemlali" olive cultivar. Four foliar fertilisers were sprayed separately at different seasons. Total polyphenol, total flavonoid *ortho*-diphenol and oleuropein contents were assessed in fresh olive leaves.Our results showed that polyphenols and *ortho*-diphenols reached their maximum in April, decreased in August and rose again in November. Flavonoid showed an inverted trend and were the most affected by the different foliar fertilisers. Oleuropein content had two peaks in April and November and was not affected by foliar fertilisers. Taking into consideration these results, the optimal periods to collect olive leaves are April and November. Our study brought out evidences about the impact of foliar nutrition on olive leaves.

Keywords: *words: Chemlali, olive leaves, polyphenols, foliar fertiliser, season*

Introduction

Olive cultivation is the most important agriculture activity in Tunisia. About 80 million olive trees, dominated by "Chemlali" cultivar, are planted in an area of 1.803.300 hectares, which represents 34% of the arable land in Tunisia [1-3]. Olive leaves are considerable solid by-products of olive cultivation and olive oil industry. They represent about 10% of the total weight dedicated to olive oil extraction and 25% of the total weight of by-products after olive tree pruning [4]. They may represent an environmental damage when they are thrown away.In Tunisia and the Mediterranean region, the use of olive leaves is common in popular folk therapy to treat and prevent hypertension and they are known for their hypoglycaemic, antiseptic and diuretic properties [5-7]. These therapeutic capacities of olive leaves are due to their interesting and rich phenols content. Many studies confirmed that olive leaves polyphenols have antioxidant, anti inflammatory, anti-atherogenic [8] and antimicrobial activities and even possible anti-cancer effect [9]. Among phenols, oleuropein, a specific compound for the Oleaceae family, is considered to be the main phenol compound in olive leaves and it is known for its important biological activities [10-13]. Thus, the olive leaves could be valorised as functional foods and as a source of nutraceuticals [14]. Indeed, the amount and the composition of polyphenols in olive leaves vary according to many factors: sampling time, water deficiency, salinity, geographical zone and light exposure are the widely reported abiotic factors to affect the phenolic composition in olive leaves. Nevertheless, bibliography reported less data about the impact of mineral fertilisation on the phenolic composition in this natural matrix [4]. Moreover, fewer data reports focused on the impact of foliar fertilisation on "Chemlali" olive leaves.In this context, our study aims to assess the impact of foliar nutrition on the phenolic composition olive leaves of the Tunisian olive cultivar "Chemlali".

Materiel and Methods

Field study and sampling

The experimental field study was conducted in 2013 in an orchard situated in the Region of Monastir (on the Mid-eastern Coast of Tunisia, 35°40'N, 10°40'E). The chosen geographical site ensures that the experimental field study is far from industrial and urban emissions and discharges. All the olive trees in the field belong to "Chemlali" variety and they were implanted in the same period. There is no implanted irrigation system in the field. The monthly variations in temperature and rainfall during the study period are shown in Fig.1 and Fig.2.

The physicochemical characteristics of the soil at this site were as follows: sand: 690 g kg^{-1}; clay: 140 g kg^{-1}; silt: 170 g kg^{-1}; pH: 8; Electrical conductivity: 0.82 m^{-1} cm^{-1}; organic C: 8.7 g kg^{-1}; N: 7.3 g kg^{-1}; Olsen P: 5 mg kg^{-1}.

In this experiment, foliar fertilization followed the stages of the development cycle of olive trees. Fertiliser spraying was always conducted early in the morning. The experimental trees were arranged in a randomized block design with three blocks and four treatments:

- F1: consisted of olive trees exposed to a foliar fertiliser rich in nitrogen (Table 1) and sprayed 3 times, at 10 days intervals, during the vegetation stage. F1 was sprayed at 5L ha^{-1}. A sample of olive leaves (S1) was collected 2 weeks after the last fertilizer spraying, in February 2013.
- F2: consisted of trees exposed to a foliar fertilise rich in boron, magnesium, sulphur and manganese (Table 1) and sprayed 3 times, at 10 days intervals, during the flowering stage. F2 was sprayed at 3L ha^{-1}. A sample of olive leaves (S2) was collected 2 weeks after the last fertilizer spraying, in April 2013.
- F3: consisted of trees exposed to a foliar fertiliser rich in phosphor and potassium (Table 1) and sprayed 3 times, at 10 days intervals, during the stage of fruit growth. F3 was sprayed at 3L ha^{-1}. An olive leaf sample (S3) was collected 2 weeks after the last fertilizer spraying, in August 2013.

- F4: consisted of trees exposed to a foliar fertiliser rich in phosphor and calcium (Table 1) and sprayed 3 times, at 10 days intervals, during the ripening stage of olive fruits. F4 was sprayed at 3L ha^{-1}. An olive leaf sample (S4) was collected 2 weeks after the last fertilizer spraying, in November 2013.
- C: Control trees: No foliar fertiliser was sprayed in this block of trees. Leaf samples were taken from this block after every fertilizer spraying: (C1), (C2), (C3) and (C4) samples were collected respectively in the same sampling campaign of (S1), (S2), (S3) and (S4).

In every sampling campaign, homogenous and not wounded leaves were carefully collected early in the morning from all sides of olive trees. The samples were immediately transferred to the laboratory and roughly rinsed with ultrapure water and air dried for one hour.

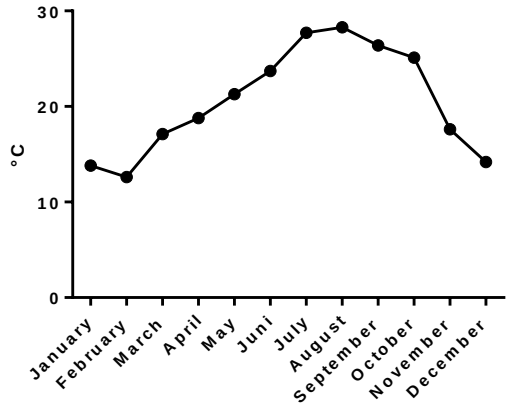

Figure 1. Monthly variations of temperature during the study period.

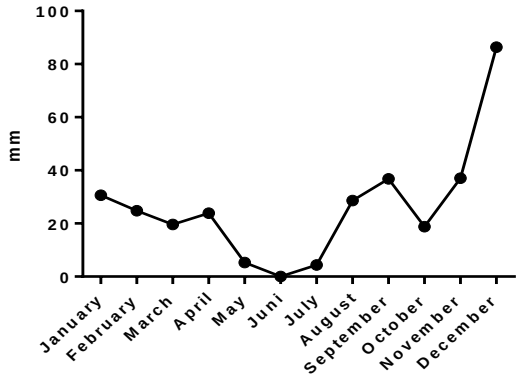

Figure 2. Monthly variation of rainfall during the study period

Table 1. Mineral composition of the different foliar fertilisers [g/l]

Foliar fertilizers	N	P_2O_5	K_2O	MgO	SO_3	CaO	B	Cu	Fe	Mn	Mo	Zn
F1	355	-	-	-	-	-	0.215	0.085	0.500	0.530	0.020	0.410
F2	-	-	-	50	111	-	27	-	-	10	-	-
F3	-	240	318	-	-	-	8	-	-	-	-	-
F4	-	60	-	-	-	186	-	-	-	-	-	11

Extraction and determination of total phenols, ortho-diphenols and flavonoids.

Samples of 0.5 g of fresh olive leaves were transferred into Falcon tubes and then 10 ml of methanol was added in each tube. After overnight shaking in darkness, the methanolic extracts were filtered through 45µm filters and transferred into amber glass tubes. The tubes, hermetically closed, were conserved at -20°C until analysis.

The total content of phenols was determined by the Folin–Ciocalteu method [15]. The *ortho*-diphenols content was determined colorimetrically according to the method described in [16]. The contents of polyphenols and *ortho*-diphenols were expressed as mg of gallic acid equivalent per g of fresh leaves. The total flavonoids content was determined colorimetrically according to the method described in [17]. The flavonoids content was expressed as mg catechin equivalent per g of fresh leaves.

Oleuropein quantification by HPLC

HPLC separation was carried out on a Hewlett-Packard system (Waldbronn, Germany) composed by a HP-1100 pump, a Rheodyne model 7725 injector (Cotati, CA, USA, loop volume 20 ml), an UV detector (280 nm) and a C_{18} Technochrom Eurosphere 100 analytical column (250 mm X 8 mm). Volumes of 20µl of the methanolic extracts were directly injected into the HPLC. The HPLC separation was running for a total time of 24min and the flow rate was maintained at 0.8ml.min^{-1}. The mobile phases for chromatographic analysis were: (A) Acetonitrile and (B) sulphuric acid/water (2:98) (v/v). A linear gradient was run from 15% (A) and 85% (B) to 40% (A) and 60% (B) during 12 min; it changed to 60% (A) and 40% (B) in 2 min; after 4 min it changed to 80% (A) and 20% (B); and then to 90% (A) and 10% (B) after 2 min; finally it becomes 100% (A) during 4 min. The data were stored and processed by HPLC Chemstation (Dos Series) (HewlettePackard). Cinnamic acid (purchased from Sigma Aldrich, St. Louis, France) was used as internal standard for the quantification of oleuropein, and the results were expressed as mg of cinnamic acid per g of fresh leaves.

Statistical analysis

Values are expressed as mean ± standard deviation of three measurements. One way ANOVA tests with Test student were performed to examine differences between means. Significant differences were considered at $P<0.05$. High significant differences were considered at $P< 0.01$. Statistical tests were performed using SPSS Release 11.0 for Windows.

Results and discussion

Effects of seasons on leaf phenols content

The total phenols, *ortho*-diphenols and flavonoids contents are detailed in table 2.

Table 2. Effects of foliar season on leaf contents of total phenols, ortho-diphenols, flavonoids and oleuropein during the year

Sampling Period	March 2013	April 2013	August 2013	November 2013
Samples	C1	C2	C3	C4
Total phenols (mg/g)	67.91 ± 9.36 (b)**	76.19 ± 0.04 (d)**(e)*	14.81 ± 0.56 (b,d,f)**	62.14 ± 5.19 (f)** (e)*
Orthodiphenols (mg/g)	9.27 ± 0.38 (b)*	8.75 ± 0.28	6.57 ± 0.21 (f)** (b)*	10.87 ± 1.91 (f)**
Flavonoids (mg/g)	21.55 ±0.96 (a,b,c)**	7.34± 1.69 (a,d,e)**	49.54 ± 0.18 (b,d,f)**	34.86 ± 1.69 (c,e,f)**
Oleuropein (mg/g)	2.31 ± 0.19 (a,c)**	4.42 ± 0.32 (a,d)**	3 ± 0.16 (d,f)**	4.20 ± 0.51 (c,f)**

C1, C2, C3 and C4: Control olive leaf samples in March, April, August and November respectively. Results are expressed as means ± standard errors (n=3). a, b, c, d: Control Values in the same row with the same letters showed statistically significant differences according to one way ANOVA analysis. The symbol "*" referred to ($P< 0.05$) and the symbol "**" referred to ($P< 0.01$).

Significant variations in total phenols content in control leaves were observed according to the different seasons. It decreased significantly from 67.91 mg/g in March (C1) and 76.19 mg/g in April (C2) reaching only 14.81 mg/g in

August (C3). Then a significant rise (62.14 mg/g) was noticed in November (C4). Similarly, the *ortho*-diphenols content in "Chemlali" fresh leaves reached its lowest level in August (6.57 mg/g) and its highest in November (10.87 mg/g). In a recent study, the total phenols content and the flavonoid content in the leaves of two Turkish olive tree varieties "Kilis Yaglik" and "Gemlik" were assessed monthly from January to December [18]. The authors report that the content of phenols reached the lowest level in summer (July and August) in both varieties; these results are in agreement with ours. Indeed, the summer increase of temperature induces an abiotic stress for the olive trees. This may lead to an increase of the activity of polyphenol oxidases, enzymes that catalyze the oxidation of phenols to quinones and are involved in the resistance against biotic and abiotic threats in plants [19]. Thus, this summer increase of the polyphenol oxidase activity may explain the decrease of polyphenols and *ortho*-diphenols observed in the present study in the olive leaves in August. On the other hand, the decrease of the contents of total phenols and *ortho*-diphenols in summer can also be explained by the inhibition of phenylalanine ammonia-lyase due to the elevated temperature of this season. This enzyme catalyzes a reaction converting L-phenylalanine to ammonia and *trans*-cinnamic acid. This is considered as the first step in the phenylpropanoid pathway and is therefore involved in the biosynthesis of most polyphenol compounds [20]. This hypothesis was demonstrated by the inhibition of this enzyme in apple leaves due to high temperatures [21]. On the other hand, the increase of the concentrations of polyphenols and *ortho*-diphenols from August to November can be explained by the stimulation of the phenylalanine ammonia-lyase activity during ripening. Ripening process occurs starting from September-October for "Chemlali" olive trees in Tunisia. The increase in total phenols content and of the phenylalanine ammonia-lyase enzyme activity was also observed in the leaves of the "Picual" olive cultivar during the ripening stage [22].

However, in the present study the flavonoids content showed a different trend through the vegetative cycle, compared to the total phenols content. In fact, it decreased from (21.55 mg/g) in March to reach its lowest level in April (7.34 mg/g). In August, the flavonoid amount reached its maximum level (49.54 mg/g), then it decreased again (34.86 mg/g) in November. It should be noted that the fluctuation of the flavonoid content through the vegetative cycle had an opposite dynamic compared to that of the total polyphenols content. This finding is agreement with the results of a study conducted on shoots of common walnut (*Juglans regia L.*) [23]. This study showed that the phenols content reached its maximum during spring and a mimimum in summer, while flavonoids content had the opposite curve. Moreover, In the one year study conducted on the Turkish olive cultivars "Kilis Yaglik" and "Gemlik"[18], the flavonoids content in the olive leaves showed a similar fluctuation as observed in our study. The lowest flavonoid levels were detected in April for "Gemlik" cultivar and in May for "Kilis Yaglik" one, while the highest levels were detected in July for "Gemlik", and in August for "Kilis Yaglik". The flavonoid amounts remained at high levels during autumn for both cultivars, which is in agreement with our results. Flavonoids are known to confer a protection for plants against ultraviolet irradiation stress [24-26]. Thus it can be hypothesized that their increase in summer may be due to a stimulation of their biosynthesis in leaves to ensure a protection against stress induced by ultraviolet irradiations. Although there was not an important fluctuation in temperature from March to April (Graph 1), the flavonoid content decreased drastically between these two sampling points. In fact, for "Chemlali" cultivar, the flowering stage occurs in April. Flavonoid compounds are essential constituents of the pollen coat in plants and have an important role in pollen fertility and in conferring a protection against biotic and abiotic threats, mainly the above mentioned ultraviolet irradiation [27, 28]. Probably, an inhibition of flavonoid biosynthesis may have been occurred in olive leaves while an enhancement of the flavonoid biosynthesis in anthers has taken place during the flowering stage in April. Further investigations are required to confirm this eventual spatial difference in flavonoid biosynthesis between leaves and anthers in "Chemlali" cultivar.

Effects foliar fertilization on leaf phenols content

To the best of our knowledge, no previous studies have been performed to evaluate the effects of foliar mineral nutrition on the phenols content in the leaves of "Chemlali" cultivar. The results showing the effect of foliar fertilisers are detailed in table 3. The use of (F1), the nitrogen-based fertilizer sprayed in March, at the start of vegetation decreased the *ortho*-diphenols content in (S1) which compared to the control sample (C1) ($P<0.05$) (table 2). On the contrary, the content of total polyphenols and *ortho*-diphenols were not significantly affected by the other foliar fertilisers. Interestingly, the flavonoid content in "Chemlali" leaves seemed to be more sensitive to mineral foliar nutrition. Foliar fertilization affected negatively the flavonoid content in leaves of fertilized trees comparing to control ones, by about 19.72%, 70.02%, 31.59% and 12.22% under the effect of (F1), (F2), (F3) and (F4), respectively.

The present results are in agreement with a recent study that described that the foliar fertilisation with nitrogen on "Picholine" olive leaves caused a decrease in the total phenols content [29]. Similarly, other studies conducted on other crop species, concluded that the use of nitrogen enriched fertilisers resulted in a decrease of the contents of polyphenols, *ortho*-diphenols and flavonoids in leaves and fruits [30], further confirming our results with the F1 foliar fertiliser. Recently, it was demonstrated that the decrease of the different phenolics was associated with the inhibition of the phenylalanine-lyase activity and the inhibition of the expression of its coding genes after the use of nitrogen fertilisers in many crop plants [31, 32]. In fact, phenylalanine is a common precursor for the phenols and protein biosynthesis and there is a competition for this precursor between these pathways [33]. Thus, the activation of the protein biosynthesis, in spite of the polyphenol biosynthesis, under a condition of nitrogen bioavailability due to the use of F1 may explain the decrease of the amounts of *ortho*-diphenols and flavonoids. On the other hand, phenylalanine-lyase activation occurs in a condition of nutrient depletion [34], hence a nutrient availability after the use of fertilisers may inhibit this enzyme, and thereby inhibits polyphenol biosynthesis.

Table 3. Effects of foliar fertilization on leaf contents of total phenols, ortho-diphenols, flavonoids and oleuropein during the year

Sampling Period	March 2013	April 2013	August 2013	November 2013
Samples	S1	S2	S3	S4
Total phenols (mg/g)	89.95 ± 0.56	77.200 ± 0.714	12.71 ± 3.81	61.38 ± 4.6
Orthodiphenols (mg/g)	8.355 ± 0.159 x	8.364 ± 0.036	5.57 ± 1.50	9.05 ± 0.52
Flavonoids (mg/g)	17.3 ± 0.8 xx	2.203 ± 0.730 x	33.89 ± 1.84 x	30.6 ± 0.80 x
Oleuropein (mg/g)	2.48 ± 0.11	4.184 ± 0.110	2.75 ± 0.69	3.79 ± 0.15

S1, S2, S3 and S4: leaf samples from olive trees fertilized with F1, F2, F3 and F4 foliar spray respectively. Results are expressed as means ± standard errors (n=3). x, xx: Sample Value showing statically significant difference with the corresponding control in table 2, according to test student. The symbol "x" referred to (P<0,05) and the symbol "xx" referred to (P<0.01)

After the spraying of F2, rich in boron, magnesium and manganese, the flavonoid content showed a significant decrease. Our results are in agreement with a previous study, which reported a decrease of the phenols content in olive fruits after the use of the same foliar fertiliser [35]. In the same sense, Liakopoulos and co-workers, reported that boron deficiency enhanced the total phenols and flavonoid contents in young and mature olive leaves and the boron availability caused the opposite effect [36]. It was also reported that boron availability induced the decrease of phenols in various plant species [37]. Nevertheless, the foliar fertiliser F2 also contained in its formulation magnesium and manganese, which are known to enhance the activity of phenylalanine lyase and thus increase the phenols content [38]. The decrease observed in the present study may be explained by an eventual high uptake of boron and a lower

uptake of manganese and magnesium by olive leaves. Indeed, differences in foliar uptake and foliar leaching between elements have been described [39, 40]. After the spray of F3, a foliar fertiliser enriched with potassium and phosphorous, only the flavonoid content was affected. The same finding was noticed after the use of F4, a foliar fertiliser enriched with phosphorous and calcium. This lowering effect is probably due to phosphorous. In fact, many studies reported that the administration of enriched phosphorous fertilisers decreased the flavonoid level in the different organs of crop plants [41]. Our results are in good agreement with these findings. The key enzymes in the flavonoid biosynthesis pathway are phenylalanine lyase, by converting L-phenylalanine to cinnamate, which is then converted to *p*-coumaroyl-CoA, and chalcone synthase, which initiate the flavonoid biosynthesis using *p*-coumaroyl-CoA and malonyl-CoA [42]. The activities of these enzymes increased for a nitrogen and phosphorous depletion [43]. Interestingly, our results also showed that the flavonoids content was more sensitive to all the foliar mineral formulations than the phenols and the *ortho*-diphenol ones in "Chemlali" leaves. To explain this finding, we suggest that chalcone synthase may be more sensitive to nutrient bioavailability/depletion than phenylalanine-lyase in "Chemlali" leaves. Recent molecular investigations highlighted that chalcone synthase was more sensitive to wounding and salinity than phenylalanine-lyase in safflower seeds, *Carthamus tinctorius*, supporting this hypothesis [44]. Further investigations are required to assess the eventual expression differences between these two key enzymes in olive trees under exogenous nutrient supply.

Seasonal variation of oleuropein content in olive leaves

Oleuropein content was analysed in fresh leaf methanol extracts by HPLC. Results are detailed in table 2. The oleuropein content rose significantly from 2.31 mg/g in March (C1) to reach a maximum peak of 4.42 mg/g in April (C2). Then a sharp decrease occurred, and oleuropein content reached the level of 3 mg/g in August (C3). In November (C4), oleuropein content increased significantly to reach another maximum peak of 4.2 mg/g. A previous report conducted on "Chemlali" olive leaves, showed that the oleuropein level did not vary significantly across the harvesting points, from July 2003 to March 2004 [45], which is not in agreement with our results. On the other hand, Ryan and collaborators, working on the "Hardy's Mammoth" cultivar [46], found that the oleuropein content reached a maximum in April in old and young olive leaves. Mert and collaborators observed that during the "Off" year, the oleuropein content in "Gemlik" olive cultivar had two maximum peaks [47]: the first one occurring at the beginning of May and the second one in November. The above results are in good agreement with our findings. Nevertheless, other studies report different dynamics of the oleuropein content in olive leaves. Fabbri and co-workes, showed that oleuropein content in olive leaves belonging to the Italian cultivar "Maurino" [48] reached a maximum peak in May and December and a minimum level in July. Işin and co-workers showed that oleuropein content in the olive leaves of the Turkish cultivar "Memecik" reached its peak during summer and in olive trees subjected to water stress [49]. In the same sense, another investigation found out that oleuropein content reached its lowest levels in April and November [50]. A recent assessment of the phenols compounds in leaves sampled from the Italian olive cultivars "Dolce Agogia", "Moraiolo", "Leccino" and "Frantoio", showed that the dynamic of Oleuropein content through the seasons varied between cultivars and there was not a unique pattern describing this variation [51]. The main finding from all these reports is that oleuropein is sharply affected not only by season, but also by cultivar and water supply.

Impact of foliar fertilisation on oleuropein content in olive leaves

Regarding the effects of foliar fertilization, our data (table 3) showed that the tested fertilizers did not significantly affect the oleuropein content in "Chemlali" leaves. Indeed, our findings were in agreement with the above reported data about the impact of foliar fertilisation on total the phenols content, which were also not affected by the foliar

mineral nutrition. Some previous studies documented the effects of mineral nutrition on oleuropein content. Liakopoulos and al. study [36] showed that boron supplementation increased oleuropein content olive in leaves of the "Manaki" cultivar. Similarly , the Tekaya and al. investigation [29] showed that oleuropein content in olive leaves was negatively affected by various foliar fertiliser formulations.

Conclusion

Our results highlighted the impact of season on the phenolic content and volatile fraction of olive leaves. Provided that total phenolics and oleuropein reached highest levels in April and November, we can propose that the best sample time to ensure high amounts of phenolics are April and November. Foliar fertilisation affected negatively the flavonoid content. Total phenolic and *ortho*-diphenol levels were less affected.

Acknowledgment

The authors would like to thank the Ministry of Higher Education and Scientific Research of Tunisia for funding this work. The authors are also thankful to Mme. Samia Dhaou Agir from the research laboratory LR12ES05 in the medicine faculty of Monastir and Mr. Fouad Labidi from the olive institute in Sousse for technical support.

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