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ECOSYSTEMS

Influence of the drought on antioxidant and enzymatic activities of two *Pinus* species in humid and sub-humid climate

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Abstract. *Pinus g*enus is widespread in the Mediterranean region and the most common in Tunisia. The impact of high temperatures in the mid-summer period (July 2015 and July 2016) on phenols and biological properties were examined *in vitro*. The study was carried out in two arboreta, in humid and sub-humid climates, dry needles of *Pinus pinea* and *Pinus pinaster* were used for secondary metabolites, antioxidant activities, and enzymatic inhibitory activities. The amount of all measured parameters increased from 2015 to 2016 in the two pine species in the two arboreta. *Pinus pinea* produced more phenols and showed higher antioxidant activity and α-amylase and lipoxygenase inhibitory activities than *Pinus pinaster*. Besides, both species generally presented better biological activities and higher phenol amounts in 2016 than in 2015, being 2016 warmer and drier than 2015 in both harvest zones. It was evident that both species in every bioclimatic zones adapted to the new climate conditions producing more secondary metabolites that rendered better antioxidant and inhibitory enzymatic activities. These findings confirmed the impact of mid-summer water deficits on pine species in the context of climate change and help to select the most resistant species for future reforestation.

Key words: antioxidant, enzymatic contents, *Pinus pinaster, Pinus pinea,* Tunisia.

INTRODUCTION

Pinus L. the genus is found naturally in the northern hemisphere conifers (Gernandt et al. 2005, Price et al. 1998), especially in the Mediterranean region, Asia, Europe, North, and Central America (Graikou et al. 2012, Petrassi et al. 2000).

Several studies have shown that the *Pinus* genus is a source of secondary metabolites. These metabolites are indeed known for their diverse biological activities and pharmacological properties. Among these properties found anti-allergic, anti-inflammatory, and antimicrobial effects. Moreover, they proved to have memory-enhancing and mood-boosting properties (Ait Mimoune et al. 2013, Calamassi & Ioana 1986, Dob et al. 2007, Naeem et al. 2010, Ustun et al. 2012, Yesil-Celiktas et al. 2009).

The needles of the genus *Pinus* are widely used in folk medicine and as food additives due to their anti-aging, anti-inflammatory effects, and for treating liver and skin diseases, and hypertension (Kim & Chung 2000, Lee et al. 2003, Watanabe et al. 1995).

Pinus pinea L. and *P. pinaster* are the most common resinous species in Tunisia. They have a great interest in terms of ecological functions (reforestation program) in Tunisia (El Khorchani et al. 2007) and economic importance since its management renders a variety of valuable

products: nuts, wood, firewood (Chouiter 2007, Génova et al. 2013). Due to their high economic and ecological importance, pines have been extensively studied (Fady 2012). However, few studies have been done concerning the biological activities of the extracts from the needles of these species when submitted to drought conditions.

Environmental stresses, such as drought and high temperatures are the main factors that restrain the plant distribution and generate secondary stress (osmotic and oxidative) causing changes in its development and metabolism (Bohenert et al. 1995, Kranner et al. 2010) and, therefore, influencing the contents of active substances.

The present work aimed to investigate the influence of environmental factors on the active substance contents and antioxidant activity of *P. pinea* and *P. pinaster* collected from different regions of Tunisia in July 2015 and 2016. Three parameters were studied: (i) Quantities of total phenolic and flavonoids in the extracts; (ii) Antioxidant activities (radical scavenging on ABTS and ferrous ion chelating assays, FIC); (iii) Inhibitory effects of α-amylase and lipoxygenase; (iv) Correlation between secondary metabolites and antioxidant activities; and (v) Correlation between temperature and antioxidant activity.

MATERIALS AND METHODS

Study site

The study was carried out in two arboreta: The first is Souiniet "SNT" in northwestern Tunisia. The second is Jebel Abderrahmane "JAB" in north-eastern Tunisia. Characteristics of each site and the monthly distribution of precipitation and temperature in 2015 and 2016 are resumed in (Table I, Fig. 1).

Sampling and extracts preparation

Sampling of *Pinus pinea* and *Pinus pinaster* was carried out in July 2015 and 2016. The diameter of the trunk and height was 16 cm and 3.5 m, respectively. Three branches from three trees of each species were cut and transported to the laboratory. Once in the laboratory, needles were dried in the shade for ten days. They were ground into a fine powder and stored in separate screw cap bottles before analysis. For each powder sample (2 g), 20 mL of distilled water was added. After 22 h of shake, at 30 °C, solutions were centrifuged/centrifuges at 5,000 rpm for 10 min. The supernatant was pulled out and kept in sterile plastic tubes (10 mL) at 4°C.

Table I. Geographical characteristics of two studied sites.

Sites	Climate	Latitude (N)	Longitude (E)	Altitude (ma.s.l)	Vegetation
Souiniet	humid	36°47'920''	8°48'495''	492	*Arbutus unedo*, *Erica scoparia E. Arborea, Myrtus communis, Phillyrea media, Halimium halimofolium, Cistus salvifolius* and trees of *Quercus suber*
Jebel Abderrahmane	Sub-humid	36°40'086''	10°40'582''	255	*Quercus coccifera*, *Erica arborea*, *Calycotome intermedia*, *Halimium halimofolium*, *Pistacia lentiscus* and *Phillyrea* sp.

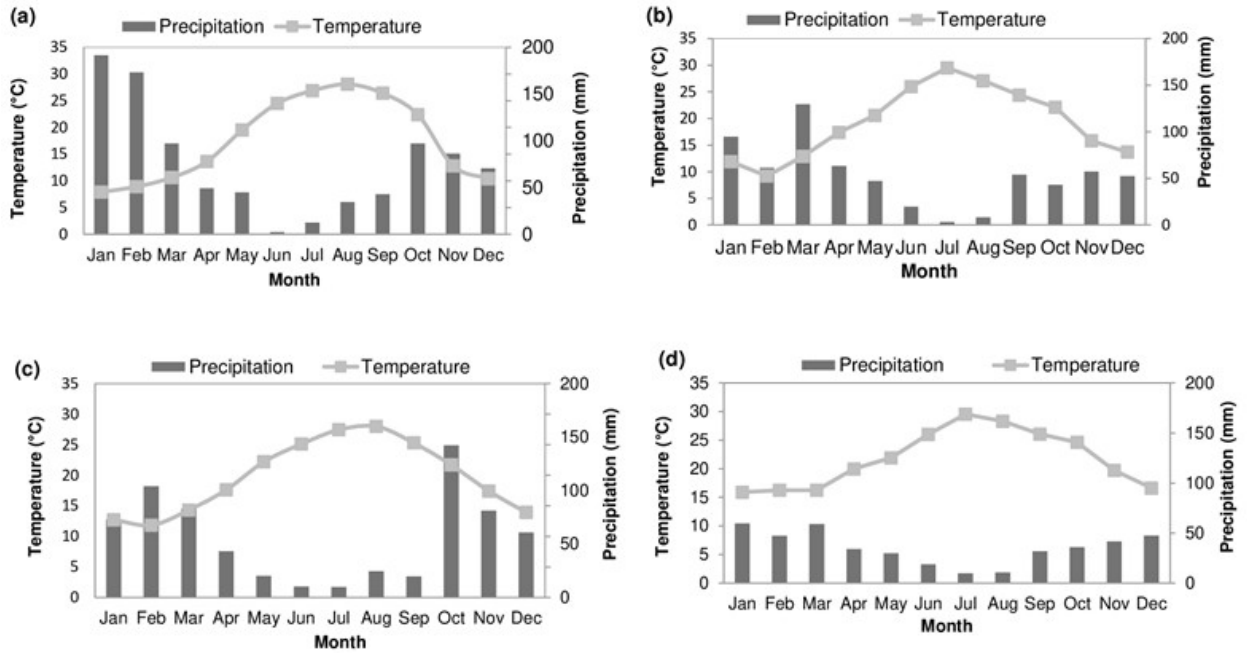

Figure 1. Climatograph of study site: (a) Souiniet site in 2015 (humid climate), (b) Souiniet site in 2016 (humid climate), (c) Jbel Abderrahmane in 2015(sub-humid) and (d) Jbel Abderrahmane in 2016 (sub-humid). The climatograph illustrates the monthly distribution of precipitation (P) and temperature (T) in 2015 and 2016.

Determination of total phenols (TPC) (Folin-Ciocalteau)

Total phenol content was determined using the Folin-Ciocalteau reagent as described by Singleton & Rossi (1965) using gallic acid as standard. In brief, a serial of dilutions of aqueous extracts (0.05 mL) was prepared and mixed with 0.25 mL Folin-Ciocalteau reagent 10% (v/v) and 0.2 mL of sodium carbonate (75 g/L). The reaction mixtures were then incubated at room temperature for two hours. The absorption was measured at 765 nm versus a blank prepared without extract. The standard curve was prepared using 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL solutions of gallic acid. Tests were carried out in triplicate. Total phenols content of the extracts was expressed as milligram of gallic acid equivalent per gram dry weight (mg GAE/g DW).

Determination of total flavonoids content (TFC)

Flavonoids content of the extracts was evaluated by the aluminum chloride colorimetric method reported by Sancho et al. (2016). The extracts (0.3 mL) were mixed with (0.15 mL) of sodium nitrite (1%). After 5 min of incubation, 0.15mL of aluminum chloride (20%) was added to 0.30 mL of sodium hydroxide (4%), the mixture was allowed to stand for incubation for 1h and, the absorbance was read at 510 nm. The flavonoids content of the extracts was expressed as milligram of quercetin equivalents per gram dry weight (mg QE/g DW).

Antioxidant activities

Capacity for scavenging ABTS free radicals

The ability of samples for scavenging 2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid ($ABTS^{+}$) radical cation was studied as reported previously by Miguel et al. (2010). Briefly, the

ABTS radical was generated by the reaction of ABTS aqueous solution (7 mM) with potassium persulfate ($K_2S_2O_8$) (2.45 mM) in the dark at room temperature, for 12-16 h. The $ABTS^+$ solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.05. Then, 0.1 mL of samples were mixed to 0.9 mL of $ABTS^+$ solution and the absorbance was read at 734 nm after 6 min of reaction. The ABTS radical cation scavenging activity was determined as:

$$[(A_0\text{-}A_1/A_0)\ x100]$$

A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the extract. ABTS activity of the extracts was expressed as micromoles of Trolox equivalents (TE) per gram dry weight (µmol TE /g, DW).

Ferrous chelating metal ions assay (FIC)

Ferrozine can chelate Fe^{2+} and forms a complex with a red color, which can be quantified. The ferrous ion-chelating effect of all extracts was estimated according to Aazza et al. (2013) with slight modifications. Briefly, the reaction was initiated by mixing 0.1mL of samples with 0.05 mL of $FeSO_4{\cdot}4H_2O_2$ mM, and 0.2 mL of ferrozine 5 mM. The absorbance of the reaction mixture was read at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was expressed as follows:

$$[(A_0\text{-}A_1/A_0)\ x100]$$

A_0 denotes the absorbance of the control, and A_1 denotes the absorbance of the test sample. Results were expressed as micromoles of EDTA per gram dry weight (µmol EDTA /g DW).

Enzymatic activity

α-Amylase inhibition assay

α- Amylase inhibition assay was performed as described by Uddin et al. (2014) with some modifications. The total assay mixture consisting of 0.1 mL sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM sodium chloride), 0.05 mL of α-amylase (0.02 units) and sample (0.05 mL) was incubated at 37 °C for 10 min. After the incubation, 0.2 mL of soluble starch (1%, w/v) was added to each test tube, and the mixture was reincubated for 20 min at 37 °C. About 0.3 mL 10% HCl was added to stop the enzymatic reaction, followed by the addition of 0.3 mL of iodine reagent (5 mM I2 and 5 mM KI), and after that, 8 mL of distilled water was added. The absorbance was read at 620 nm. Sample, substrate, and blank were undertaken under the same conditions. Each experiment was done in triplicate. The inhibition percentage of the enzyme was calculated using the following formula:

$$\%\ \text{of}\ \alpha\text{-amylase inhibition: } [1\text{-}\ [(A^-_{control}) - (A^+_{control})] - (A_{sample})]/\ (A^-_{control}) - (A^+_{control})]\ *100$$

Where $A^-_{control}$ is the absorbance of 100% enzyme activity (ethanol 70% with enzyme), $A^+_{control}$ is the absorbance of 0% enzyme activity (ethanol 70% without enzyme), and A_{sample} is the absorbance of the sample. The α-amylase activity of the extracts was expressed as micromoles of acarbose per gram dry weight (µmol acarbose /g DW).

Lipoxygenase (LOX) inhibition assay

The inhibition of the lipoxygenase enzyme was measured by following the previous method described by El-Guendouz et al. (2016) with some modifications. The reaction consisted on the mixture of 0.01 mL of each sample and 0.005 mL of enzyme solution (0.054 g/mL) and 0.05 mL of linoleic acid (0.001 M) and borate buffer 0.937 mL (0.1 M, pH 9). The measurement of the

absorbance was read at 234 nm. The analyses were carried out in triplicate. The percentage of inhibition was determined from the formula:

$$[(A_0-A_1/A_0) \times 100]$$

Where A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the sample solution (presence of the extract). Lipoxygenase activity of the extracts was expressed as micromoles of ibuprofen equivalent per gram dry weight (µmol IE /g DW).

Statistical analysis

Statistical analyses conducted using the Xl STAT V.2015. The data analyzed with ANOVA and Tukey's multiple range tests at a level of $p < 0.05$ to compare sample means. Pearson correlation coefficients determined at a significance level of 95% to investigate the relationships between phenolic and antioxidant activity. All values presented as the mean ± standard error.

RESULTS AND DISCUSSION

Secondary metabolites and antioxidant activities

Total phenolic content (TPC) and Total Flavonoid Content (TFC)

Table II depicts the total phenol (TPC) and flavonoid content (TFC) of pine extracts. The amount of TPC and TFC increased from 2015 to 2016 in the two pine species. TPC analyses revealed that *P. pinea* extracts have higher amounts of phenols in the two years under two climates than *P. pinaster* extracts. An increasing amount of TPC in *Pinus pinea* extracts was observed in 2016 in humid (31.59 mg/g) and sub-humid climates (16.94 mg/g). TPC amount tested in *P. pinaster* reached 12.09 mg/g in humid and 8.95 mg/g in sub-humid climates in 2016.

The phenol amounts obtained for *Pinus pinea* extracts were in the range of those previously reported (Uzel 2018), but significantly inferior to those described by Guri et al. (2006). The values of total phenols found for *P. pinaster* in the present work were also higher than those cited by Kang & Howard (2010), who found 4.4 mg/g.

The analyses of TFC revealed that *P. pinea* has higher amounts of flavonoids in the two years under two climates than *P. pinaster* (Table II). An increase in the level of TFC was observed in 2016, in humid climates (0.26 mg/g) and sub-humid climates (0.16 mg/g).

Kang & Howard (2010) reported that in Forest Farm, the flavonoid content in *P. pinea* and *Pinus pinaster* were respectively 0.5 mg/g and 2.4 mg/g. Karapandzovaa et al. (2015) reported

Table II. Contents of total phenolic (TPC) and total flavonoid (TFC) of aqueous extracts of needles of two pine species PP (*P. pinea*) and PM (*P. pinaster*) in two sites Souiniet, Jebel Abderrahmane in July 2015 and July 2016 expressed as milligram of standards per gram of dried weight (mg/g DW).

Variable	Species	Site 1: Souiniet		Site 2: Jebel Abderrahmane	
		July 2015	July 2016	July 2015	July 2016
TPC (milligram gallic acid/g DW)	PP	25.15 ± 0.69^{b}	31.59 ± 0.08^{a}	10.81 ± 0.01^{d}	16.94 ± 0.045^{c}
	PM	10.29 ± 0.39^{ab}	12.09 ± 0.05^{a}	7.57 ± 0.11^{c}	8.95 ± 0.07^{bc}
TFC (milligram quercetin/g DW)	PP	0.22 ± 0.01^{a}	0.26 ± 0.02^{a}	0.092 ± 0.12^{c}	0.16 ± 0.01^{b}
	PM	0.20 ± 0.00^{c}	0.24 ± 0.01^{b}	0.14 ± 0.12^{d}	0.25 ± 0.01^{a}

DW: Dry Weight. Each value represents the mean of three replicates ± SEM (standard error of means). Values with different letters in the same row are significantly different at *p < 0.05.

that in Kozuf, Pelister and Nidez Mt (Macedonia, Albanian), TFC in 3 species of *Pinus* were higher than those found in the present work: 3.3 mg/g in *Pinus nigra*, 3.7mg/g in *P. sylvestris* and 4.3 mg/g in *Pinus peuce*.

The increase of phenolics and flavonoids content in July 2016 compared to July 2015 were most likely also related to adverse stress, particularly stress water, which promotes the production of phenolic and flavonoids contents in *P. pinea*, especially in a humid climate (Table II). Our results were in agreement with Boscaiu et al. (2010), who reported that in the province of Valencia, *Thymus vulgaris* and *Rosmarinus officinalis* have a positive correlation between the stress caused by environmental factors (combining aridity, limited nutrients and salt toxicity in the soil) and the level accumulated of phenolic compounds. In our case, TPC and TFC highly correlated with temperature and humidity, which can confirm the importance of these environmental factors on the accumulation of secondary metabolites.

A positive correlation between temperature in *P. pinea* and *P. pinaster* in the two climates (r = 0.99) was observed (Table III). A high positive correlation was found in 2016 between TPC and TFC for *Pinus pinea* (r=0.99) in humid climate and *P. pinaster* (r=0.83) in the sub-humid climate (Table IV). In addition, statistical analysis showed a significant difference of TPC and TFC between species ($p < 0.001$), year ($p < 0.001$) and sites ($p < 0.001$).

Table III. Correlation between temperature in July 2015 and July 2016 , secondary metabolites (TPC and TFC), antioxidants activities (ABTS and FIC) and enzymatic inhibitory activities (α-amylase and LOX) in individual pine species PP (*P. pinea*) and PM (*P. pinaster*) in two sites "SNT" (Souiniet), "JAB" (Jebel Abderrahmane).

Variables	Species	Site 1 SNT		Site 2 JAB	
		Temperature Temperature			
		July 2015	July 2016	July 2015	July 2016
TPC	PP PM	0.51 0.98	0.97 0.99	-0.35 0.99	-0.35 0.86
TFC	PP PM	0.99 -0.48	0.99 -0.49	-0.18 0.52	-0.44 0.99
ABTS	PP PM	-0.71 -0.29	0.93 -0.92	0.14 -0.85	-0.35 -0.03
FIC	PP PM	-0.99 0.98	-0.92 0.54	0.53 -0.06	-0.98 -0.64
α-amylase	PP PM	0.35 0.17	-0.04 -0.77	0.93 0.61	0.75 -0.60
LOX	PP PM	0.48 0.97	0.99 0.68	-0.65 -0.65	-0.48 0.77

Table IV. Correlation between secondary metabolites (TPC and TFC), antioxidants activities (ABTS and FIC) and enzymatic inhibitory activities (α-amylase and LOX) in individual pine species PP (*P. pinea*) and PM (*P. pinaster*) in two sites "SNT" (Souiniet), "JAB" (Jebel Abderrahmane) in July 2016.

		TPC	TFC	ABTS	FIC	α-Amylase	LOX
PPSNT	TPC	1	0.99	0.84	-0.97	0.157	0.99
	TFC	0.99	1	0.91	-0.94	0.02	0.99
PPJAB	TPC	1	-0.68	0.99	0.50	0.36	-0.65
	TFC	-0.68	1	-0.68	0.29	-0.92	0.99
PMSNT	TPC	1	-0.56	-0.95	0.62	-0.71	0.61
	TFC	-0.56	1	0.80	-0.99	-0.17	0.30
PMJAB	TPC	1	0.82	-0.54	-0.94	-0.10	0.99
	TFC	0.83	1	0.017	-0.60	-0.64	0.74

ABTS scavenging and chelating activities

The ABTS scavenging activity increased from 2015 to 2016 in the two pine needle extracts (Table V). In 2016 and the humid climate, the highest activity was detected in *Pinus pinea* extract, while the highest ferrous ion chelating (FIC) activity was detected in *Pinus pinaster* (Table V).

Statistical analysis showed a highly significant difference of ABTS scavenging activity and chelating activity between species ($p < 0.001$), year ($p < 0.001$) and sites ($p < 0.001$). The interaction was also significant ($p < 0.001$). In both climate types, *P. pinea* revealed a high level of ABTS scavenging activity in the two years. In 2016, an increase in the level of ABTS activity observed in humid (55.25 µmol of TE/g) and sub-humid climate (47.39 µmol of TE/g). The amount was lower for *Pinus pinaster* in 2016.

Pinus pinaster showed a high activity of chelating in 2016 in a humid climate (38.78 µmol EDTA/g and sub-humid 31.53 µmol EDTA/g climates). Additionally, *Pinus pinea* showed an important chelating activity in 2016 in sub-humid climates (30.41 µmol EDTAE/g) and humid climates (20.54 µmol EDTA/g). High positive correlations observed between secondary metabolites content and antioxidant capacity determined by the ABTS assays in *Pinus pinea* or *Pinus pinaster*. Moreover the phenolic compounds may contribute to the radical-scavenging activity of the needles of *Pinus* species. Gülçin et al. (2010) showed that ABTS scavenging activity was associated with phenolic and flavonoid contents due to the redox properties that make them a good reducing of scavenging radicals.

In a humid climate, ABTS high positively correlated with temperature in 2016 in *Pinus pinea* (r = 0.93) and high negatively in *P. pinaster* (r=-0.92) (Table III). Additional, chelating activity highly negatively correlated with temperature in *P. pinea* (r = -0.92) for the same climate. These results may indicate that phenols are not responsible for these activities, therefore other non-phenolic compounds can be accountable for the chelating activity that increased with a temperature rising, and it was not determined in the present work. Another hypothesis is the possible increase of some phenolic compounds that do not possess chelating activity such as naringin, pelargonidin, phloridzin, and hesperitin (Miguel et al. 2014). In 2016, a high positive correlation was observed between phenolic content and ABTS activity in *Pinus pinea* in a humid (r = 0.85) and sub-humid climate (r=0.99), while a negative correlation was

observed in *Pinus pinaster* in humid (r = -0.95) and sub-humid climate (r = -0.55) (Table IV). A high positive correlation was found between flavonoids and ABTS especially, in *Pinus pinea* (r = 0.91) and *P. pinaster* (r = 0.80) in the humid climate. A high negative correlation was found between chelating activity and phenol / flavonoids in two pine species, except in *Pinus pinaster* in a humid (r = 0.62) and *P. pinea* in sub-humid climates (r = 0.50) (Table IV). These results are difficult to explain. The chemical structure of the compounds may have a significant role in these results, which needs to be exploited.

Enzymatic activity (α-amylase and lipoxygenase)

The α-amylase inhibitory activity increased from 2015 to 2016 in the two- needle extracts of pine species. In 2016, the highest α-amylase inhibitory activity detected in *Pinus pinea* extract in sub-humid climates. It was highly correlated with temperature (r = 0.75), and for *P. pinaster*, in a humid climate, the correlation was negative (r =-0.77).

Statistical analysis showed a significant difference of α-amylase and lipoxygenase inhibitory activities between year of collection with respectively ($p < 0.05$; $p < 0.001$), sites ($p < 0.05$; $p < 0.001$) and species ($p < 0.001$) except for α-amylase ($p = 0.55$). The highest lipoxygenase inhibitory activity was detected in *Pinus pinea* needle extract in both climate types (Table VI).

For *P. pinea*, lipoxygenase high and positively correlated with temperature (r = 0.99) in 2016 in a humid climate. For *Pinus pinaster*, the correlation was negative (r = -0.77) in the sub-humid climate. For α-amylase, the only high negative correlation was between flavonoids and amylase in *Pinus pinea* (r = -0.93) and *P. pinaster* in sub-humid climate r = -0.64. For *Pinus pinea*, in a humid climate phenolic content, high positively correlated with lipoxygenase (r = 0.99) in 2016. For *P. pinaster*, the correlation was high and positive (r = 0.98) in two climates.

In our results, enzymatic inhibitory activities increased in the two pine species in 2016. The highest activity was registered in *P. pinea* in the sub-humid climate (5.41 µmol/g). The lowest activity was observed in a humid climate (3.55 µmol/g). The results revealed a remarkable increment in the two pine species, mainly in *Pinus pinea* under sub-humid climate. An increment of 14-fold in July 2016 was observed when compared to July 2015, whereas, for *P. pinaster* an approximate increment of two-fold was observed in humid and sub-humid climates.

The utilization of these extracts for inhibiting inflammation or for combating diabetes remains possible due to the results obtained in the present work, nevertheless it is impaired by the high variation depending on the climate conditions.

CONCLUSIONS

The total amount of phenolic compounds and flavonoids accumulated in *P. pinea* and *Pinus pinaster* depended mostly on the geographic location of species and collection year.

Environmental factors (an increase of temperature and decrease of precipitation) exert a selective effect toward those that have a better adaptation. *Pinus pinea* showed the highest values of antioxidant and inhibitory enzymatic activities when compared to *Pinus pinaster*. It seems that *P. pinea* is better adapted to humid and sub-humid climate and *Pinus pinaster* more adapted to humid climate.

A positive correlation noticed between the degree of environmental stress and the level of phenolic and flavonoids compounds

Table V. Antioxidants activities (ABTS and FIC) of aqueous extracts of needles of two pine species PP *(P. pinea)* and PM *(P. pinaster)* in two sites Souiniet and Jebel Abderrahmane in July 2015 and on July 2016 expressed as micromoles of standards per gram of dried weight (µmol/g DW).

Variable	Species	Site 1: Souiniet		Site2: Jebel Abderrahmane	
		July 2015	July 2016	July 2015	July 2016
ABTS (µmol of trolox/g DW)	PP	26.91±0.49^{d}	55.25±0.13^{a}	30.93±8.67^{c}	47.39±2.76^{b}
	PM	15.40±0.88^{b}	22.16±2.58^{a}	19.77±0.37^{a}	20±4.41^{a}
FIC (µmol of EDTA/g DW	PP	10.95±0.23^{d}	20.54±0.03^{c}	25.78±0.44^{b}	30.41±0.085a
	PM	30.52±0.11^{c}	38.78±0.08^{a}	28.74±0.54^{d}	31.53±0.00b

DW: Dry Weight. Each value represents the mean of three replicates ± SEM (standard error of means). Values with different letters in the same row are significantly different at *p < 0.05.

Table VI. α-Amylase and LOX inhibitory activities of aqueous extracts of needles of two pine species PP (*Pinus pinea*) and PM (*P. pinaster*) in two sites "SNT" (Souiniet), "JAB" (Jebel Abderrahmane) in July 2015 and July 2016 expressed as micromoles of standards per gram of dried weight (µmol/g DW).

Variable	Species	Souiniet		Jebel Abderrahmane	
		July 2015	July 2016	July 2015	July 2016
α-amylase (µmol acarbose/g DW)	PP	3.55±0.045^{b}	3.76±0.09^{b}	0.77±0.045^{c}	5.41±0.14^{a}
	PM	0.70±0.07^{c}	4.05±0.6^{b}	4.47±0.09^{b}	5.37±0.03^{a}
LOX(µmol ibuprofen/DW)	PP	18.98±0.24^{c}	172.55±4.99^{b}	26.80±0.94^{c}	391.51±4.99^{a}
	PM	22.25±0^{d}	26.79±6.80^{b}	48.82±0.41^{c}	163.84±1.88^{a}

DW: Dry Weight. Each value represents the mean of three replicates ± SEM (standard error of means). Values with different letters in the same column are significantly different at* p < 0.05.

accumulated in the plants, suggesting the role of these secondary metabolites in the defense mechanisms against stress. These compounds appear to be suitable markers of stress in *Pinus* species. Therefore, the biosynthesis of phenolics induced by ecological parameters should examine in further studies.

These findings emphasize the impact of drought in mid-summer yearly of 2015 and 2016 in Mediterranean pines and their potential responses to future climate regimes.

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