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Research Article

Geographic variation in phytochemical constituents and allelopathic potential of *Pinus halepensis* barks

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ABSTRACT

Aqueous extracts (10, 20, 30 and 40 g/L) of *Pinus halepensis* barks, collected from Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) to elucidate the influence of ecological sites on allelopathic potential. Aqueous barks extracts from (S) and (O) have revealed a higher rate of phenolic acids than those from (T) and (B), respectively 13.23, 13.8, 11.63 and 10.37 mg/mL. Alep pin barks were analyzed using HPLC/UV for the identification and quantification of the phenolic compounds, among which in particular the catechin acetate, the gallic acid, the rutine hydrate, luteolin 7 glucoside and the cinnamic acid. In fact, the aqueous extract of barks from (S) revealed a highest level, respectively 2.61, 1.74, 1.61, 1.36, and 1.21 mg/mL. The *Pinus halepensis* barks was analyzed by GC and GC-MS. As a result, 29 compounds were identified representing 89% made up basically by β-caryophyllene, α-humulene. As for allelopathic activity, aqueous extracts of barks significantly delayed germination, reduced its rate and affected the seedling growth mainly the (S) and (O) extracts. The root growth of the two targets has shown a high sensibility compared to the shoot lengths. Pot cultures were conducted by the incorporation of barks powder (50 and 100 g/kg) or the irrigation with their aqueous extracts at 20 and 40 g/L. *Pinus halepensis* barks and its extracts have shown a high herbicide potent, particularly the one collected from (S) and (O), may be favorably used for incorporating in agricultural systems for sustainable weed management.

Keywords: Allelopathic potential, Barks, Phenolic acids, Phytochemical content, *Pinus halepensis*.

1. INTRODUCTION

Conifer forests are allelochimical-producing, and have a strong allelopathic potential [1]. *Pinus halepensis* Mill is one of the major conifers in Algeria, Morocco and Tunisia covering approximately 1.3 million hectares, one of the principal essences given the zone it covers [2]. Continually

expanding, heliophilous, invasive, and rich in secondary metabolites, *Pinus halepensis* could influence the secondary succession because of its great allelopathic potential [2-5]. Indeed this potential is influenced by the abiotic factors such as the high temperatures, hydrous stress, light, soil characteristics (pH, the structure and the state of the nutrients, texture, the presence of contaminants), altitude and the latitude [6-9]. This allelopathic potential depends on abiotic factors such as the edaphic microclimate, the intensity and the duration of rainfall [10]. These secondary metabolites are of great importance for the relations between the plant and its environment [11].

The bark of pine was a bothersome residue for the wood industry, abundantly available and cheap [12, 13], rich in polyphenols, phenolic acids fatty, aliphatic, and resinic acids [14-16]. Those secondary metabolites show an important ecological role in the allelopathic processes [5].

Many plants use chemical interactions, such as allelopathy [8], a principal factor in the management, implementation and growth of plants [17]. They have a negative impact on the surrounding plants under natural conditions [18], like in the agrosystems [19]. Many plant-derived compounds, [20] have herbicide effects without causing damage to the environment [21]. The use of secondary metabolites could be effective in the management of weeds [22]. Indeed the improvement of the agricultural output depends partly on weeding [23]. The development of natural pesticides would make it possible to decrease the use of chemical pesticides [24] and their negative impact on the environment [25].

We conducted the work to evaluate the herbicide potential of the pine barks of Alep and to explore the influence of the ecological sites Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) on the production of allelochemicals.

2. Materials and methods

2.1. Sampling Sites

The barks of *Pinus halepensis* were randomly collected from 20 trees in a 10×10 m^2 area in the Tunisian pine forests of Bizerte, Tabarka, Seliana, and Oueslatia, in January 2012. The samples were dried in a ventilated and lit place. Forty grams of each dried and grinded biomass was tempered in 1 L distilled water at ambient temperature for 24 h. The extracts were filtered through a paper filter (Whatman N°1) 3-5 times and saved at 4 °C in the dark until use [22].

2.2. Climatic data

The climatic data displayed in Table 1; were provided by the weather services (The Tunisian National Institute of Meterology).

Table 1. Climatic data of the four stations of sampling (According to the National institute of Meteorology).

Climatic data		**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
Rainfall		450-1500 mm/an	450-1500 mm/an	150-450 mm/an	100-400 mm/an
Altitude		21m	5 m	560 m	654 m
Location		37°14'N 9°45'E	36°56'N 8°46'E	35°57'N 9°28'E	35°52'N 9°30'E
Temp (°C)	Max(August)	30.3	34.7	36.3	31.6
	Min (December)	8.9	7.8	7.2	8

2.3. Bioassays with aqueous extracts

Barks aqueous extracts were prepared by soaking 40 g of dried biomass for 24 h in 1L of sterilized distilled water, diluted to give 10, 20 and 30 g/L [6]. They were tested on *Raphanus sativus* L. (radish) and *Triticum aestivum* L. (wheat), used as model plants in the studies on the allelopathy at the laboratory. Target seeds were surface sterilized with 0.525 g/L sodium hypochlorite for 15 min, then rinsed four times with deionized water, imbibed in it at 22 °C for 12 h and carefully blotted using a

folded paper towel [26]. Twenty imbibed seeds of target species were separately placed on filter papers in Petri dishes, 5 mL of each extract per treatment. Seeds irrigated with distilled water were used as controls. The seeds were germinated in a growth chamber with 400 µmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) at 22/24 °C for 14/10 h light and dark periods, respectively [6].

2.4. Phytochemical screening

2.4.1. Total phenolic content (TPC) determination

TPC in the extracts were estimated by a colorimetric assay based on the procedures described by Paras and Hardeep; Reis et al. [27, 28]. Basically, 1 ml of sample was mixed with Folin-Ciocalteu reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were mixed in vortex for 15 s and kept aside for 30 min at 40°C for color development. Absorbance was measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). TPC was expressed as mg gallic acid equivalent /g dry matter (mg GAE/g dw) using gallic acid calibration curve ($R^2 = 0.985$).

2.4.2. Total flavonoid content (TFd) determination

TFd were determined according to the method of Zhishen et al. [29] with some modifications. The extract (250 µl) was mixed with 1.25 mL of distilled water and 75 µl of a 5% $NaNO_2$ solution. After 5 min, 150 µl of 10% $AlCl_3 \cdot H_2O$ solution was added. After 6 min, 500 µl of 1 M NaOH and 275 µl of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (+)-Catechin was used as standard and the results were expressed as mg of (+)-catechin equivalents (CE) per g of the dry matter.

2.4.3. Condensed tannins content (TPA) determination

TPA was determined according to the method of Julkunen-Titto [30]. An aliquot (50 µL) of each extract or standard solution was mixed with 1.5 mL of 4% vanillin (prepared with methanol) and then 750 µL of concentrated HCl were added. The well mixed solution was incubated at ambient temperature in the dark for 20 min. The absorbance against blank was read at 500 nm. The results were expressed as mg of (+)-catechin equivalents (CE) per g of the dry matter.

2.4.4. Determination of o-diphenols

1 ml of a solution of HCl (0.5 N), 1 ml of a solution of a mixture of $NaNO_2$ (10 g) and $NaMoO_4 \cdot 2H_2O$ (10 g) in 100 ml H_2O, and finally 1 ml of a solution of NaOH (1 N) were added to 100 µl of each aqueous extract. After 30 min, o-diphenols were read at 500 nm. The o-diphenols were expressed on a dry weight basis as mg tyrosol equivalents per g of the dry matter [31].

2.4.5. Identification of phenolic compounds (HPLC/UV) in the extracts

The presence and amount of phenolic compounds in the extracts were studied by reversed phase HPLC analysis using a binary gradient elution. The phenolic compounds analysis was carried out by Usingan Agilent Technologies 1100 series liquid chromatography (HPLC, Palo Alto, CA) coupled with an UV-vis multiwavelength detector. The separation was carried out on a 250 mm × 8 mm, particle size 5 µm Eurospher-100C_{18} reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.8 ml min^{-1}. The gradient program was as follows: 15% A/85% B, 0-12 min; 40% A/60% B, 12-14 min; 60% A/40% B, 14-18 min; 80%A/20% B, 18-20 min; 90% A/10% B, 20-24 min; 100% A, 24-28 min. The injection volume was 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Peaks were identified by congruent retention times compared with standards.

2.4.6. Volatile compound analyses

Supelco (Bellefonte, PA, USA) SPME devices coated with polydimethylsiloxane (PDMS, 100 µm) were used to sample the headspace of two date seeds inserted into a 10-mL glass vial and allowed

to equilibrate for 30 min. After the equilibration time, the fibre was exposed to the headspace for 50 min at room temperature. Once sampling was finished, the fibre was withdrawn into the needles and transferred to the injection port of the GC-MS system. GC-EIMS analyses were performed with a Varian (Palo Alto, CA, USA) CP 3800 gas chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm; Agilent, Santa Clara, CA, USA) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperatures were 250 and 240 ^{o}C, respectively; oven temperature was programmed from 60 to 240 ^{o}C at 3 ^{o}C min^{-1}; carrier gas was helium at 1 mL min^{-1}; splitless injection. The identification of the constituents was based on a comparison of their retention times with those of authentic samples (Collection of volatile compounds purchased from Sigma-Aldrich Italia and ⁄ or Carlo Erba Italia as pure compounds or analytical kits; except for the two 2-tridecenes that have been identified by mean of their mass spectral data), comparing their linear retention indices (LRI) relative to a series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and Adams) and homemade library mass spectra, and MS literature data [32, 33]. Moreover, the molecular weights of all the substances identified were confirmed by GC-CIMS, using methanol as ionizing gas. Results were expressed as relative percentages obtained by peak area normalization [34].

2.5. Effect of the aqueous extracts

2.5.1. Effect on germination

Germination was given including the number of seeds germinated at 24 hour intervals for 6 days. The length of the roots and the air parts of young seedlings of target species were measured 7 days after sowing [6]. The data were transformed into percentage of control for the analysis. The index of germination GI was calculated by using the following formula [35].

$GI= (N_1) x1 + (N_2 - N_1) x (1/2) + (N_3 - N_2) x (1/3) + •••$

Where N_1, N_2, N_3,…, N_n: percentage of germinated seeds observed after 1,2,3,…, N days. This index represents the delay in the germination induced by the extract [36]. The percentage of germination inhibition was determined according to the formula:

[% germination inhibition] = [% germination control - % germination extract]

The percentage of inhibition/stimulation was calculated under the terms of the formula of Chung et al. [37]:

[Inhibition (-) /Stimulation (+)] = [(Extracted - Control) /Control] x 100.

2.5.2. Effect on the growth

The effect of the aqueous extracts on the growth was estimated by measuring the length of the root and the principal stem 7 days after germination. The results were expressed as a percentage of the control. The percentages of inhibition or stimulation induced by the various extracts were calculated [37].

2.6. Pot culture assay

2.6.1. Powder incorporation in soil

The vegetal powder of the barks, taken from various sites was incorporated in soil sample to the proportions of 50 and 100 g/kg. The soil without powder was used as a control. The mixtures were placed in 10 cm diameter plastic pots, each containing 250 g [38]. The experiment was undertaken under a greenhouse. The length of the roots and the principal stem were measured at the end of day 20 of culture. The treatments were randomly laid out in a device with three repetitions and the data were transformed into a percentage of the control for analysis [6].

2.6.2. Irrigation with the aqueous extracts

The target plants were sown in pots of 10 cm in diameter filled with the same soil type. The pots were irrigated with the aqueous extracts prepared from the various types of biomass of pine of Alep at two concentrations (20 and 40g/l). The added volume was of 10ml and the ground was humidified each time it desiccates. The treatments were randomly laid out in a device with three repetitions and the data were transformed into percentage of the control for analysis [6].

2.7. Statistical analysis

The biological tests in the laboratory and the greenhouse were carried out with three repetitions and five times for the phytochemical analyses. All the data were reported on average ± standard deviation using SPSS 18 program. An ANOVA of LSD post hoc test was carried out with the same software in order to analyze the differences between the treatments. The Pearson correlation between the essays of the different sites having the same concentration was made for each species and each concentration. The averages were separated on the level of probability 0.05.

3. Results

3.1. Phytochemical screening

The contents of the *Pinus halepensis* barks collected from the four ecological sites Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) in total polyphenols (TPC), O-diphenols, flavonoids (TFd) and in condensed tannins (TPA), revealed significant differences depending on the origin of the biomass (Table 2). This production of allelochemicals is partially due to genetic factors and is partly determined by environmental conditions [39]. It partly accounts for the high TPC contents in the barks from (S) and (O) compared with those of (B) and (T), which are respectively 75.34 and 71.46 mg GAE/g dw (Table 2). Flavonoids can play a significant role in the protection of the plants against the UV-A and UV-B [40]. Their production varies according to the plant geographical site [41]. The highest content was recorded in the barks of the Seliana forest with 36.44 CEQ/g dw, an average of 28.79 CEQ/g dw for The three other aqueous extract. Covelo et al. [39] showed that the content of tannins, in the pine forests, strongly depends on the availability of light. Indeed, the biomass of source (O) and (S) presents the highest content, respectively 6.79 and 5.85 CEmg/g dw, however (B) and (T) aqueous extract revealed a less rate of TPA, an average of 5.29 CEmg/g dw. This dissimilarity could be explained by the effect of the climatic factors [42].

3.2. Identification of phenolic compounds (HPLC/UV) in the barks extracts

Phenolic acids (caffeic, ferulic and cinnamic acids), polyphenols, tannins, flavonols (quercetin) are inhibitors of germination [43]. The effect of the phenolics compounds on germination is related to the regulation of endogenous auxine, the permeability of the seed tegument and the procurement of oxygen to the embryo [44].

In this study, HPLC showed many phenolic acids in the aqueous extracts of the barks of *Pinus halepensis* (Table 3). Elevated levels of phenolic acids is related to the mechanisms of defense of the plant against a microorganism attack [45], involved in resistance to various types of stress [46]. Indeed, the aqueous extracts of *Pinus halepensis* bark from (S) and (O) revealed higher rate of phenolic acids than those from (T) and (B), respectively of 13.23, 13.8, 11.63 and 10.37 mg/ml; this may be explained by the low rainfall and high temperatures of the two harvesting site (S) and (O). In fact, the aqueous extracts from (S) and (O) revealed higher levels of gallic and cinnamic acids, catechine acetate, rutine hydrate and Luteolin 7 glucoside, compared to those from (B) and (T) this may be explained by the low rainfall and high temperatures of the two harvesting site Seliana and Oueslatia (Table 3). In fact, the aqueous extracts of barks from (S) revealed higher levels of gallic acid 1,74 mg/mL, cinnamic acid 1,21 mg/mL, catechin acetate 2,61 mg/mL rutine hydrate 1,6 mg/mL and luteolin 7 glucoside 1,36 mg/ml (Table 3). Such flavonoids have strong allelopathic potent [47]. This may explain the high potential inhibitory of aqueous extracts of the barks collected from (S) and (O) on the germination and growth of target plants.

3.3. Chemical composition

Twenty-nine compounds were identified (Table 4), accounting for 94, 6-99, 7% of the aroma extract. The biomass of *Pinus halepensis* accumulate aroma compounds differently according to the geographic area: barks from Seliana and Oueslatia produced a higher numbers of monoterpene hydrocarbons, 10.5% (Table 5).

The major constituents of the volatile fraction from Oueslatia were *β*-caryophyllene (66.3%),

α-humulene (7.3%) (Table 4). However, barks from Tabarka showed a low percentage of β-caryophyllene (58.4%), α-humulene (1.4%) (Table 4). These compounds have been reported to have herbicidal activities [48]. Wang et al. [49] showed that (*E*)-caryophyllene at the dose of 3 mg/L significantly inhibited the germination rates and seedling growth of *Brassica campestris* and *Raphanus sativus*. Singh have demonstrated that exposure of seedling to α-pinene act to inhibited seedling growth causing oxidative damage in root tissue [50]. Barks from Oueslatia was characterized by the highest amount of sesquiterpene hydrocarbons (84.9%), made up by β-caryophyllene and α-humulene, and lowest amount of hydrocarbons monoterpenes (10.5%) (Table 4).

However, barks collected from Bizerte and Tabarka showed a low percentage, an average of (58.5%) for β-caryophyllene, (8.85%) for α-humulene (Table 4).

3.4. Effect of the aqueous extracts of *Pinus halepensis* barks on germination

In Table 6, the percentage of inhibitions obtained in the presence of the aqueous extracts of barks from the four sites. A more or less similar effect was recorded for the seeds of radish and wheat. It was noted that the inhibition, induced by the aqueous extracts of the barks on the germination of radish and wheat, increased with the augmentation of concentration of these extracts. At 10 g/L of the aqueous extract, recorded inhibitions of the germination of the seeds of radish were of 11.7% (B), 10% (T), 21.7% (S) and 16.7% (O). However at 30 g/L, the herbicide effect of the aqueous extracts was more announced and the reductions were of 20% (B), 18.35% (T), 28.35% (S) and 33.35% (O) (table 6).Several studies have shown that the inhibition degree increases with the augmentation of concentrations of the extract [22]. For all the concentrations, the site effect was shown; the inhibition of germination was more important for the aqueous extracts from (S) and (O), while the weakest reduction was recorded in the presence of the aqueous extract of the barks from (T). At 40 g/L, the seeds of wheat had an almost similar sensitivity towards the aqueous extracts of the barks of Bizerte, Tabarka and Oueslatia, with an average inhibition of 28.9%, but Seliana aqueous extract induced an inhibition of germination of 35% (Table 6). The richness of TPC, TFd, TPA and O-diphenols of the aqueous extract from (S) could explain the effect observed. Indeed, Bais et al. announced that the flavonoids have allelopathic effects [51]. The qualitative differences of these compounds in the extracts could contribute to different phytotoxicity rates [21].

At 40 g/L, the germination indexes recorded in the presence of the four extracts were similar for wheat and for radish with respective averages of 55.52 and 55.77; 64.08 and 56.96 at 30 g/L (Table 7). The aqueous extracts of the barks do not affect only the rate of germination, but also the extension of germination over longer periods. Similar observations were noted by Tiger et al. [52]. The presences of allelochemicals involve a delay of germination by disturbing mitochondrial breathing and metabolic enzymes implied in glycolysis and oxidative pentose phosphate pathway (OPPP) [21, 22, 53]. In addition, allelochemicals disturb peroxidase, alpha-amylase activities, cellular division and differentiation and the metabolism of phytohormones [54].

3.5. Effect of the aqueous extracts of the *Pinus halepensis* barks on the growth

3.5.1. On root growth

The lengths of the air parts or the roots are parameters usually used for the determination of allelopathic effects on the development of plants [52]. The results show a very significant effect of the aqueous extracts of the barks from B, T, S and O, even at weak concentrations, essentially in the presence of Seliana extract (Fig. 1).

The reduction of the root growth of wheat seedlings, at 10 g/L, ranged between 75 and 85% and between 95 and 99% at the strongest concentration, 40 g/L (Fig. 2). The growth of the roots in the presence of the aqueous extracts of the barks from the four sites, showed high inhibitions proportional to the concentrations (Fig. 2). Similar results were reported by Ladhari et al. [22]. Radish was shown to be more sensitive to the aqueous extracts of the barks of *Pinus halepensis* (Fig. 3).

Table 2. The total polyphenols (TPC), O-diphenols, Flavonoids, condensed tannins (TPA) content in the barks of *P. halepensis* collected from the forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling	Bizerte (B)	Tabarka (T)	Seliana (S)	Oueslatia (O)
TPC (mg GAE/g dw)	$71.76^{b**} \pm 1.05$	$68.64^{a**} \pm 2.03$	$75.34^{c**} \pm 2.04$	$71.46^{b**} \pm 1.09$
o-diphenols (mg eq tyrosol/g dw)	$4.75^{a} \pm 1.33$	$5.01^{a**} \pm 0.30$	$5.37^{a} \pm 0.37$	$6.11^{a} \pm 0.51$
flavonoids ((CEQ) /g dw)	$29.97^{a**} \pm 2.04$	$26.69^{a**} \pm 3.04$	$36.44^{b} \pm 0.44$	$29.71^{a} \pm 1.12$
TPA (CE mg /g dw)	$5.55^{ab**} \pm 0.95$	$5.04^{a**} \pm 1.05$	$5.85^{ab**} \pm 0.54$	$6.79^{b**} \pm 0.25$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. ** indicates a significant Pearson correlation at the level 0.01 between TPC, o-diphenols, Flavonoids and the TPA of the barks of the same site.

Table 3. Phenolic acids contents (mg/mL) in the barks aqueous extracts of *Pinus halepensis.*

Compounds (mg/mL)	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
Gallic acid	$1.71^{b**} \pm 0,1$	-	$1.74^{b**} \pm 0.26$	$1.53^{b} \pm 0.43$
Catechin acetate	-	-	$2.61^{b} \pm 0.3$	$1.31^{c} \pm 0.69$
Catechine hydrate	$1.28^{b**} \pm 0.2$	$2.82^{c**} \pm 0.82$	-	-
Resorcinol	-	-	-	0.6
Chlogénic acid	-	0.91	-	-
Syringic acid	-	$1.67^{b} \pm 0.07$	-	$1.7^{b} \pm 0.4$
Hydroxy phenylacetate	-	-	2.09	-
Catechol	1.23	-	-	-
Rutine hydrate	-	-	$1.61^{b*} \pm 0.61$	$1.23^{b*} \pm 0.44$
Verbascoside	$1.12^{b**} \pm 0.07$	$1.45^{b**} \pm 0.31$	-	-
Luteolin 7 glucoside	$1.31^{a} \pm 0.63$	$1.45^{a**} \pm 0.1$	$1.36^{a} \pm 0.46$	$1.7^{a**} \pm 0.11$
Neringenin	-	$0.76^{b**} \pm 0.4$	-	$1.21^{c**} \pm 0.21$
Apegenin 7 glucoside	$2.73^{c**} \pm 0.94$	-	1.01	$1.31^{b**} \pm 0.31$
Fereulic acid	-	-	1.84	-
m-Coumaric acid	-	$1.54^{c} \pm 0.5$	-	$0.89^{b} \pm 0.2$
Phenylacetate	$0.15^{a**} \pm 0.1$	-	-	$0.61^{b**} \pm 0.4$
Resveratrol	0.13	-	-	-
Luteolin	$0.11^{b} \pm 0.06$	$0.07^{b} \pm 0.01$	-	-
Pinoresinol	$0.1^{a**} \pm 0.05$	$0.12^{a**} \pm 0.03$	$0.52^{a} \pm 0.48$	$0.31^{a} \pm 0.1$
Naphtoresorcinol	-	-	0.2	-
Cinamic acide	$0.65^{a**} \pm 0.3$	$0.5^{a**} \pm 0.29$	$1.21^{b**} \pm 0.21$	$1.34^{b**} \pm 0.43$
Apigenin	-	0.71	-	-
2,4.D Pestanal	-	0.15	-	-
Flavon	$0.21^{a**} \pm 0.01$	$0.02^{b**} \pm 0.02$	$0.05^{b**} \pm 0.03$	$0.06^{b**} \pm 0.04$
Total (mg/mL)	10.37	11.63	13.23	13.8

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different at P <0.05. * indicates a significant Pearson correlation o at the level 0.05 and ** at the level 0.01 between compounds of the barks collected from the forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Table 4. Composition of volatiles obtained from Barks of *Pinus halepensis* according to their different geographical origin (B.B: Barks from Bizerte; B.T: Barks from Tabarka; B.S: Barks from Seliana; B.O: Barks from Oueslatia) (L.R.I: Linear Index Retention).

Compound	L.R.I.	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
α-pinene	941	1.6	2.1	2.1	2.4
β-pinene	982	-	0.4	0.2	0.7
myrcene	993	1.2	0.8	1.3	1.8
δ-3-carene	1013	-	-	0.4	0.5
p-cymene	1028	0.4	0.5	0.4	0.4
limonene	1032	2.2	1.8	2	2.9
terpinolene	1090	1.2	1.1	1.1	1.8
linalool	1101	0.6	0.5	0.4	0.3
nonanal	1104	1.9	0.8	1.7	0.5
phenyl ethyl alcohol	1141	-	0.5	-	0.2
camphor	1145	0.6	0.4	0.2	0.2
4-terpineol	1178	0.8	0.9	0.9	0.8
isobornyl acetate	1287	-	0.4	0.3	0.4
α-cubebene	1353	1.3	1.4	1.1	0.8
β-copaene	1430	3.6	3.5	3.2	2.6
β-caryophyllene	1419	59.3	58.4	61.7	66.3
β-ylangene	1422	1.6	1.4	1.4	0.9
α-humulene	1455	8.4	9.3	10.4	7.3
(E)-β-farnesene	1459	-	-	0.3	0.3
alloaromadendrene	1462	-	-	0.3	0.2
γ-muurolene	1478	0.5	0.7	0.5	0.3
valencene	1493	1.6	1	1.4	0.5
α-muurolene	1501	1.7	2.9	1.4	0.9
δ-cadinene	1524	1.1	1.7	0.9	0.5
caryophyllene oxide	1582	1.2	3.6	3.3	1.9
humulene epoxide II	1607	0	0.7	0.5	0.2
γ-muurolene	1478	0.5	0.7	0.5	0.3
valencene	1493	1.6	1	0.4	0.5
α-muurolene	1501	1.7	1.9	1.4	0.9
Total identified		99.6	98.4	99.7	97.3

Table 5. Chemical composition groups of barks of *Pinus halepensis*

	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
Monoterpene hydrocarbons (%)	6.6	6.7	7.5	10.5
Oxygenated monoterpenes (%)	3.9	3	3.5	2.2
Sesquiterpene hydrocarbons (%)	82.9	83.9	84.9	82.3
Oxygenated sesquiterpenes (%)	1.2	4.3	3.8	2.1
Others (%)	0	0.5	0	0.2
Total (%)	94.6	98.4	99.7	97.3

Table 6. Summary table of the % of inhibition of germination of *Triticum aestivum* and *Raphanus sativus* in the presence of the aqueous extracts of the barks of *P. halepensis* from the four sources Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling		% inhibitions of germination			
		Bizerte	**Tabarka**	**Seliana**	**Oueslatia**
wheat	10 g/L	$11.7^{a**} \pm 1.02$	$10^{a**} \pm 1.81$	$21.7^{a**} \pm 0.87$	$16.7^{a**} \pm 0,95$
	20 g/L	$23.35^{a} \pm 2.39$	$23.45^{c**} \pm 0.43$	$30^{b**} \pm 2.93$	$28.34^{b} \pm 1.76$
	30 g/L	$20^{a*} \pm 1.6$	$18.35^{b*} \pm 0.97$	$28.35^{b} \pm 1.35$	$33.35^{c} \pm 3.36$
	40 g/L	$30^{b} \pm 2.9$	$28.35^{d} \pm 2.25$	$35^{c} \pm 2.31$	$28.34^{b} \pm 1.36$
radish	10 g/L	$11.67^{a**} \pm 0.67$	$10^{a**} \pm 0.78$	$16.7^{a**} \pm 2.34$	$13.35^{a**} \pm 0.35$
	20 g/L	$16.34^{b**} \pm 0.91$	$14.35^{b**} \pm 2.35$	$20^{b} \pm 1.43$	$18^{b} \pm 0.61$
	30 g/L	$23.35^{c} \pm 3.35$	$22.35^{c} \pm 0.42$	$26.7^{c**} \pm 0.72$	$28.34^{c**} \pm 0.83$
	40 g/L	$30^{d*} \pm 0.49$	$28.7^{d*} \pm 0.46$	$30^{d**} \pm 0.35$	$29.34^{c**} \pm 2.76$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having the same concentrations of each target species.

Table 7. Summary table of the indices of germination of *Triticum aestivum* and *Raphanus sativus* in the presence of the aqueous extracts of the barks of *P. halepensis* from the four sources Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling		Indices of germination (GI)			
		Bizerte	**Tabarka**	**Seliana**	**Oueslatia**
wheat	10 g/L	$74.53^{c} \pm 3.1$	$80.61^{c**} \pm 3$	$58.75^{b} \pm 3.04$	$65.55^{b**} \pm 2.04$
	20 g/L	$64.21^{b} \pm 2$	$66.27^{b**} \pm 2.7$	$60.27^{b} \pm 1.09$	$54.56^{a**} \pm 4.36$
	30 g/L	$67.49^{b} \pm 3.51$	$66.7^{b} \pm 1.07$	$59.85^{b} \pm 4.08$	$62.3^{b} \pm 0.7$
	40 g/L	$54.44^{a} \pm 4$	$58.6^{a} \pm 0.95$	$52.48^{a**} \pm 2.02$	$56.59^{a**} \pm 0.84$
radish	10 g/L	$81.93^{d**} \pm 1.05$	$75.56^{c} \pm 5.07$	$73.65^{c**} \pm 2.04$	$73.35^{a**} \pm 3.77$
	20 g/L	$52.88^{c} \pm 3.01$	$61.98^{b**} \pm 0.27$	$66.25^{b**} \pm 0.09$	$66.45^{a**} \pm 2.96$
	30 g/L	$61.66^{b**} \pm 2.05$	$54^{a**} \pm 5$	$55^{a**} \pm 2.04$	$57.2^{a**} \pm 1.63$
	40 g/L	$55.33^{a**} \pm 3.05$	$57.41^{a*} \pm 4.01$	$55.43^{a**} \pm 1.94$	$56.92^{b*} \pm 0.94$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having the same concentrations of each target species.

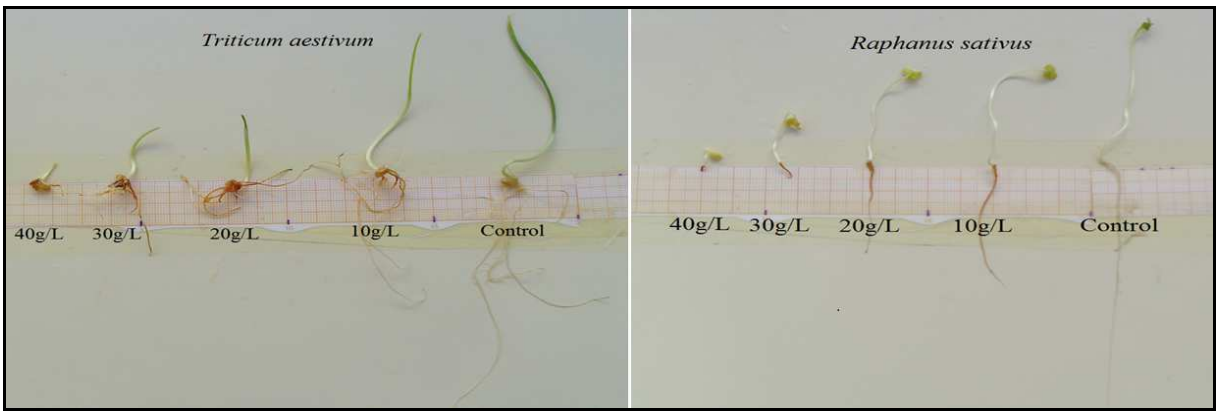

Figure 1. Effect of aqueous extract of *Pinus halepensis* barks from Seliana on the growth of *Triticum aestivum* and *Raphanus sativus.*

Indeed, Prati and Bossdorf indicated that the degree of allelopathic interference is specific to the species and can even vary within the same species [55]. At 10 g/L, the barks aqueous extract caused an inhibition ranging between 87 and 92% (Fig. 3). At 40 g/L, inhibitions exceeded 94% and reached 97% in the presence of the aqueous extract from (S). In all tests, the site effect was elucidated and a higher toxicity rate was attributed to the extracts from (S) and (O) (fig.1). These extracts are richer in TPC, TPA, TFd and o-diphenols. The allelopathic effect is due mainly to phenolic compounds [56].

3.5.2. On the air parts growth

At 10 g/L, the recorded reductions in the growth of the air parts of wheat, in the presence of the aqueous extracts of the barks of *Pinus halepensis*, were respectively of 6%, 4%, 20% and 14% for the extracts of the barks from Bizerte, Tabarka, Seliana and Oueslatia (Fig. 2). At 40 g/L, inhibitions of the seedlings growth ranged between 70% and 82%. Once again, the air parts of radish were more vulnerable, compared to those of wheat and inhibitions exceeded 40% at the weakest concentration (Fig. 2). It reached 59% in the presence of the extract from (S). At the highest concentration, the reductions of the air parts were between 91% and 97%. The roots of the two target species (wheat and radish) were more affected compared to the shoots (fig.1). Indeed, during the absorption of water, a low amount of the solution is available for the stem cells and the leaves [6], that's why they are less affected than the roots. The allelochemicals in the aqueous extracts reduce the length of the seedlings by the inhibition of the cellular division and elongation, acting on the expression and the synthesis of the DNA and the RNA [57, 58]. The aqueous extracts of the barks from (S) and (O) were most toxic on the air parts which can be partly explained by their richness in phenolic compounds compared to those from (B) and (T). Indeed the production and release of allelochemicals depend on temperature and rainfall [6]. These allelochemicals act on the meristematic cells by the reduction in lengthening [59].

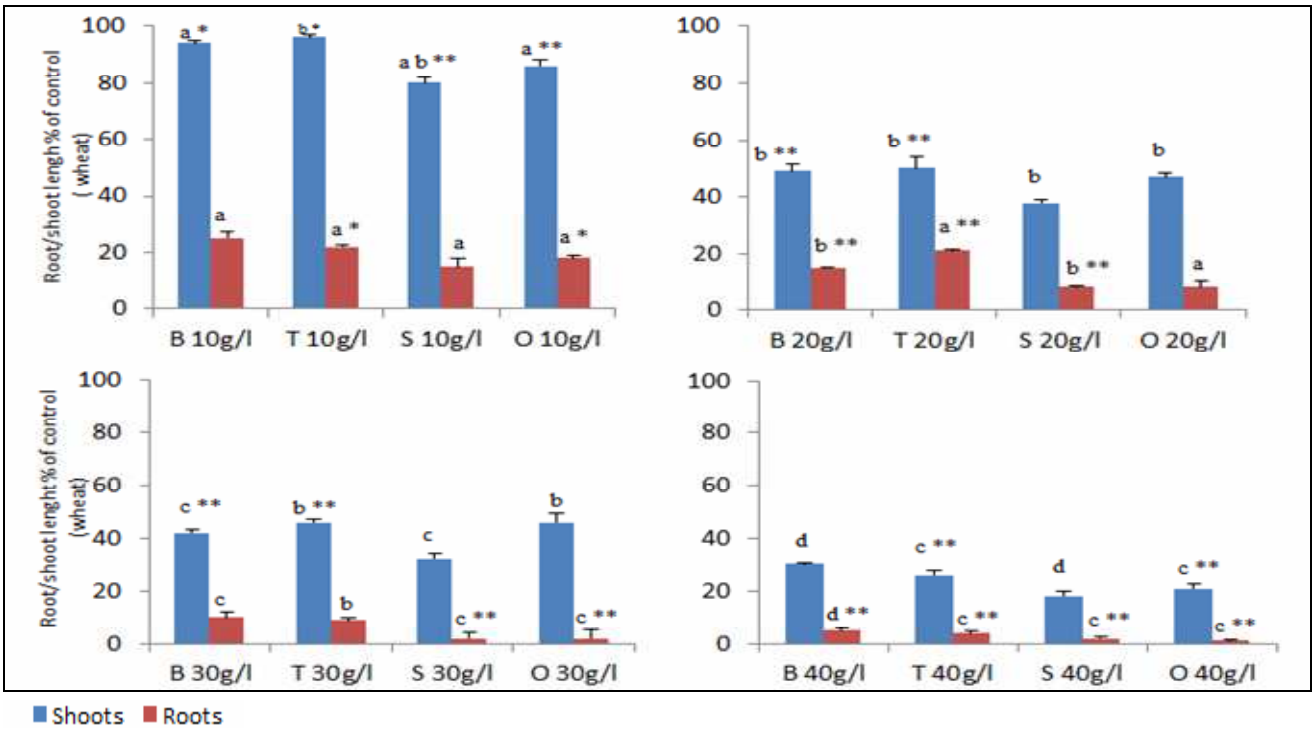

Figure 2. Summary table of the effect of the aqueous extracts of barks of *Pinus halepensis* from the four sources B, T, S and O on the growth of the roots and of the air parts of the seedlings of *Triticum aestivum*.

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having tests the same concentrations of each target species.

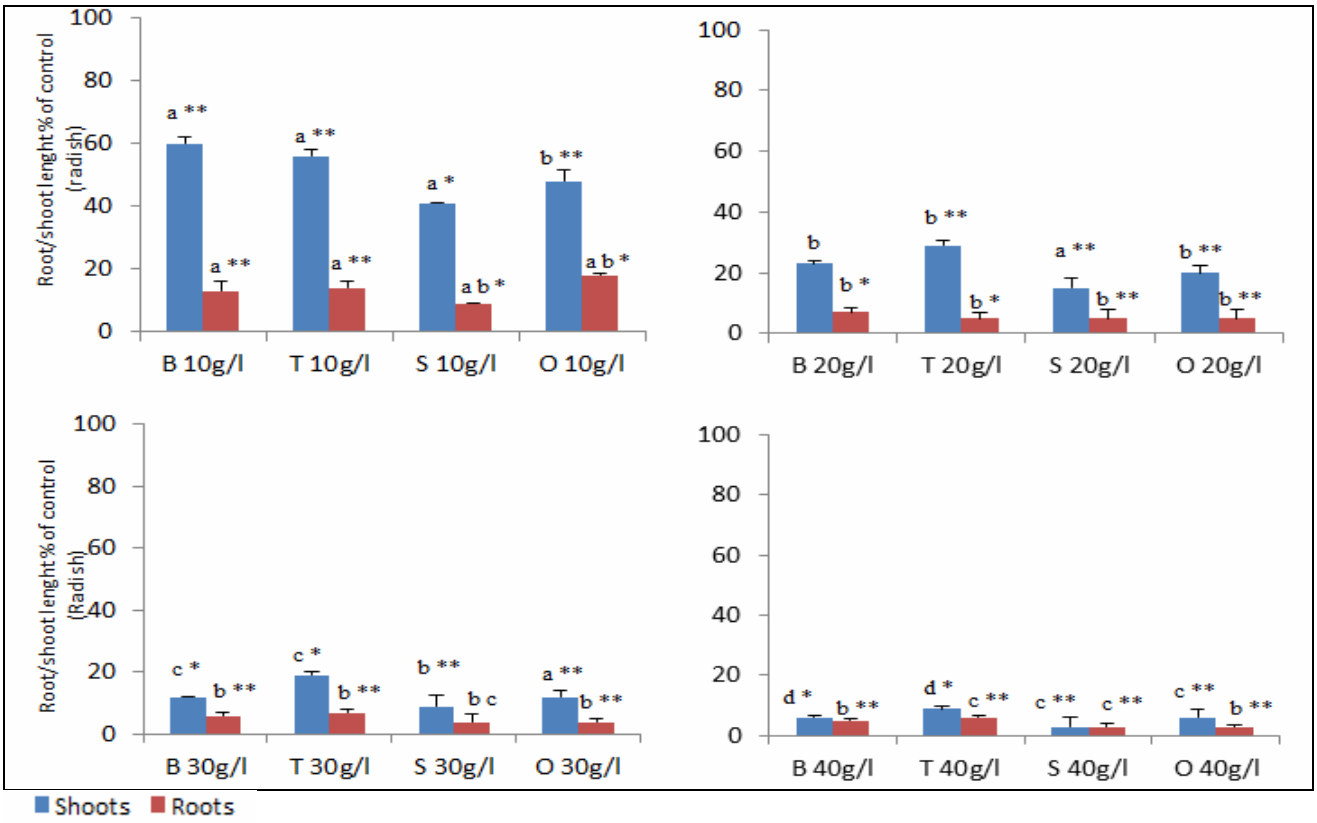

Figure 3. Summary table of the effect of the aqueous extracts of barks of *Pinus halepensis* from the four sources B, T, S and O on the growth of the roots and of the air parts of the seedlings of *Raphanus sativus*.
All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having tests the same concentrations of each target species.

3.6. Activity in soil

3.6.1. Effects of the irrigation with aqueous extracts from P. halepensis barks on seedlings growth

Aqueous extracts of the *P. halepensis* barks, from (B, T, S, and O), were prepared at two concentrations (20 and 40 g/L), and were used to irrigate the pots where the two target plants wheat and radish were cultivated. In fact, Omezzine et *al*. used cultures in pots in order to show the effects of aqueous extracts to show reproducibility of results under natural conditions and to evaluate the biological activity of allelochemical compounds released by the vegetal residues [6]. In our work, the irrigation with the barks aqueous extract involved a very marked reduction in the growth of the roots of wheat, especially at 40 g/L (Fig. 4). Inhibition increased proportionally with the augmentation of concentration of the extract. Indeed, the extracts of the barks at 20 g/L caused reductions in wheat roots growth ranging from 75.9% to 85.5% (Table 7). For the radish roots, which are more sensitive to the extracts, inhibitions were comprised between 94.5% and 99.2%, which proves a different behavior of the roots according to the species targets in the presence of the allelochemicals with a strong sensitivity of radish (table 7). The extract coming from Seliana was the most toxic and that of Tabarka was the least. At 40 g/L there was an almost total stop of radish root growth in the presence of the extracts from the four sites. At this concentration, the ecological site effect appeared in wheat and the extracts from (S) and (O) proved their higher toxic power higher. The respective length reduction of the roots of wheat and radish were of 95.1% and 92.4% (table 7). Compared to that of the roots, the growth of the shoots of wheat and radish were less affected by the irrigation with the aqueous extracts of the *Pinus halepensis* barks. At 20 g/L, the reduction did not exceed 24.6%, in the presence of the aqueous

extract from S and growth inhibition (10.8%) was induced by the extract from (B). At 40 g/L, the site effect of the aqueous extracts of the barks showed that the one prepared from the biomass of Seliana was most toxic on the growth with inhibition of 62.6%. An average of 51.5% was recorded for the extracts from the other sources. At the same concentration, the reduction of the growth of the radish shoots for the extract from (S) was of 84.7% compared to the average of 73.1% of the aqueous extracts of the barks from Bizerte, Tabarka and Oueslatia (Table 7). Our results are in agreement with those shown by Omezzine et al and Seal et al. [6, 60].

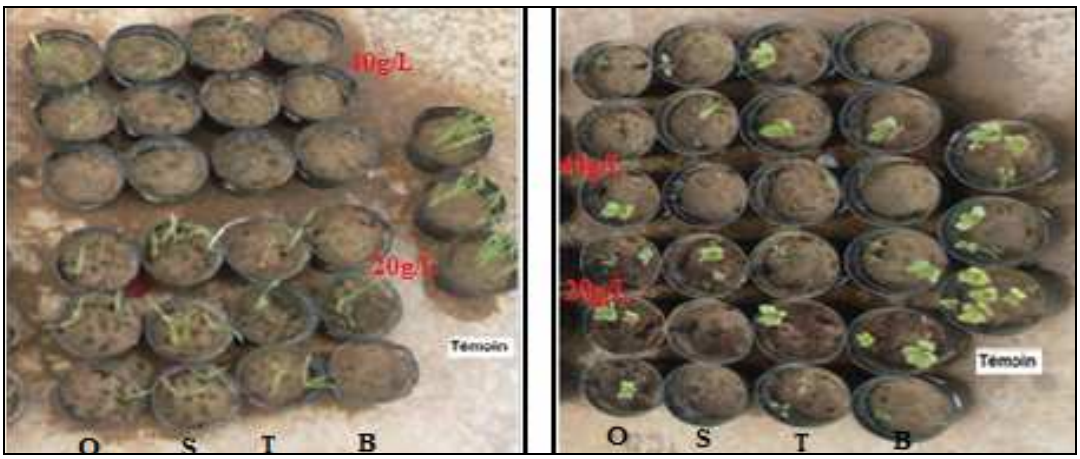

Figure 4. Culture of *Triticum aestivum* and *Raphanus sativus* on soil irrigated with aqueous extract of barks of *P. halepensis* from the four sites (B, T, S and O), 20 days after incorporation.

Table 7. Summary table of the effect of irrigation with aqueous extracts of the barks of *Pinus halepensis*, from the four sources, Bizerte, Tabarka, Seliana and Oueslatia incorporated in the soil at 20 g/L and 40 g/L, on the growth of the roots and the air parts of the seedlings of *Triticum aestivum* and *Raphanus sativus.*

Aqueous Extract (g/L)	% inhibitions of roots growth				% inhibitions of shoots growth			
	Bizerte	**Tabarka**	**Seliana**	**Oueslatia**	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
	Triticum aestivum							
20	75.9^{c**} ±2.4	78.7^{c**} ±1.5	85.5^{c**} ±1.4	82.1^{c**} ±1.2	10.8^{a*} ±0.4	16.4^{a*} ±1.2	24.6^{a**} ±1.3	20.9^{a**} ±0.9
40	85.3^{d*} ±2.9	79.7^{c*} ±0.9	95.1^{d*} ±0.9	92.4^{d} ±0.5	51.5^{b*} ±1.2	50.1^{b*} ±1.1	62.6^{b**} ±2.6	53.2^{b**} ±0.2
	Raphanus sativus							
20	96.6^{c**} ±2.4	94.5^{c} ±1.5	99.2^{c**} ±1.4	96.6^{c**} ±1.1	38.6^{a} ±0.4	44.5^{a**} ±1.2	58.9^{a**} ±1.3	38.6^{a**} ±0.9
40	97.3^{d} ±2.9	97.7^{c**} ±0.9	99.8^{d**} ±1.0	97.9^{d**} ±0.5	74.4^{b} ±1.2	70.9^{b**} ±1.1	84.7^{b*} ±2.6	74.4^{b**} ±0.2

All the analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. *indicates a significant Pearson correlation at level 0.05 and ** at level 0.01 between tests having t the same concentrations of each target species

3.6.2. Effect of the incorporation of P. halepensis bark powder in the soil on the growth of the target plants

The powder of the *P. halepensis* barks of the four sources (B, T, S, and O) was mixed with a soil sample in two amounts (50 g/kg and 100 g/kg) in order to see whether the effects recorded in bioassays are reproducible in experiments in pots. The same target plants were retained (wheat and radish).

The results related to the effect of the incorporation of the powder in the soil, on the growth of wheat and radish showed that the biomass

of the pine of Alep is very toxic (Table 8) with reductions ranging from 98.8% to 100% in the presence of the powders from B, T, S and O. The effects of the four types of biomasses are comparable for the two target plants and the sensitivity of the air parts was similar to that of the roots. The richness of the *Pinus halepensis* barks powder in polyphenols, flavonoids and allelochemical substances, explains the strong herbicide power of this biomass. Our results are in agreement with those reported in bibliography. Once in the ground, allelochemicals interfere with the neighbouring plants [61, 62] act in the stage the pre- and post-emergence of seedlings, and on the bank of seeds [11].

Table 8. Summary table of the effect of the powder of the barks of *Pinus halepensis* from the four sources, Bizerte, Tabarka, Seliana and Oueslatia incorporated in the soil at 50g/kg and 100 g/kg, on the growth of the roots and the air parts of the seedlings of *Triticum aestivum* and *Raphanus sativus*.

Dose (g/kg)	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
	% inhibitions of the shoots growth wheat				**% inhibitions of the roots growth wheat**			
50	99.9^{a}*±0,1	99.7^{a**}±0,2	99.8^{a**}±0.2	99.9^{a**}±0.2	99.8^{a*}±0.6	99.6^{a*}±0.0	99.7^{a*}±0.4	99.7^{a*}±0.4
100	100^{a}	99.5^{a**}±0,3	100^{a**}	100^{a**}	100^{b**}	99.6^{a}±0.18	100^{b}	100^{b}
	% inhibitions of the shoots growth radish				**% inhibitions of the roots growth radish**			
50	99.7^{a**}±0.3	100^{b**}	100^{a}	98.9^{a**}±0.0	99.8^{a*}± 0.8	100^{b}	100^{b}	98.8^{a*}± 0.7
100	100^{a}	100^{b}	100^{a**}	100^{a**}	100^{b}	100^{b}	100^{b}	100^{b}

All the analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. ** At the level 0.01 between tests having the same concentrations of each target species.

4. Conclusion

The objective of this study was to evaluate the allelopathic potential of the *Pinus halepensis* barks collected from the pine forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O). Indeed the two littoral sites (B) and (T) are characterized by a rainfall higher than 1100 mm/year, whereas the two other continental sites (S) and (O) receive only 400 mm/year. The average temperatures of Bizerte and Tabarka are of 11.7°C in winter and 24.6°C in summer whereas for the two other sites they are of 12.3°C and 28°C. The aqueous extract of the *Pinus halepensis* barks from (O), prepared at a concentration of 40 g/L, caused an inhibition of 33.35% of the radish seeds germination. At the same concentration the aqueous extract from (S) induced a reduction of 30% of the germination of the wheat seeds. The ecological site showed a high toxicity effect of the continental extracts (S and O) compared to aqueous extracts of the littoral sites (B and T). Aqueous extracts of the *Pinus halepensis barks* from (S) and (O) have a higher toxicity level and are richer in TPC, TPA, and TFd and in o-diphenols than those from (B) and (T). Phenols, the derivatives of the benzoic and cinnamic acids, flavonoids and tannins are substances having an allelopathic activity [63]. However these chemical products are not toxic for the donor plant [57]. Pine bark is rich in phenolic compounds [64]. The main tannin structures found in maritime pine bark are catechin/epicatechin, epigallocatechin and epicatechin gallate [65]. Indeed, several species of pine showed a strong allelopathic potential [1]. The results showed a very high allelopathic potential in the aqueous extracts of the barks of the pine of Alep from (O) inhibiting the root growth of wheat by up to 99% at a concentration of 40 g/L and of 97% for the roots of radish. The inhibitions induced by the aqueous extracts of the barks on the germination of radish and wheat increased with the increasing concentrations. The root growth is an excellent indicator of the phytotoxic effect of allelochemicals [6, 52]. Exposed directly to the aqueous extract, rich in allelochemicals, the root cells are more affected [21]. A higher permeability of the roots to

allelochemicals was demonstrated when compared to that of the air parts [66]. That's because they are the first to absorb environmental allelochemicals [67]. The air parts of the two target species (wheat and radish) were less affected, compared to the roots. At 40 g/L growth inhibitions of the air parts of wheat ranged between 70% and 82%, with a maximum reduction in the presence of the aqueous extract from (S). The same extract at the same concentration was the most harmful for the growth of radish with an inhibition of 97%. A wealth of rutin hydrate and luteolin 7 glucoside for aqueous extracts from (S) and (O), the average concentration was respectively 1.42, 1.53 mg/mL. Results of previous studies showed that the length reduction of the air parts and the roots was directly related to the action of allelochemicals, which is a proof of their effect [11]. This reduction can be attributed to the reduced rate of cellular division and the elongation of the cells due to the presence of allelochemicals in the aqueous extracts [22] which act on cellular differentiation, the absorption of ions and water, breathing, photosynthesis, enzymatic function, and the transduction of the signal as well as the form of the genes [54]. A strong inhibition of seedling growth by aqueous extracts from (S) and (O) may be due to the wealth of cinnamic and gallic acids. The average is respectively 1.63 and 1.27 mg/mL. The cultures in pots irrigated with the aqueous extracts of the *Pinus halepensis* barks from B, T, S and O, significantly inhibited the growth of the seedlings of Wheat and Radish. Cultures in pots were opted in order to demonstrate the effects which could be reproduced under natural conditions [68] and to evaluate the biological activity of allelochemical compounds released by the vegetal residues [21]. At 20 g/L, the aqueous extracts of the barks (S) induced inhibitions of the Wheat roots by up to 85.5%, it was of 96.6% for those of Radish in the presence of the extract from (O) compared to the controls. At the same concentration, the air parts were less affected by the irrigation with the aqueous extracts of the barks of the Alep pine. These results corroborate with those of Omezzine et al. [21] which proved that the roots are more sensitive than the air parts with a much higher phytotoxicity when the concentration increases. The high toxicity of the aqueous extract of the barks collected from the Oueslatia can be explained by the fact that the effectiveness of allelopathic compounds in the soil is very dependent on the biotic and abiotic conditions [69]. The results relating to the effect of the incorporation of the powder on the soil, on the growth of Wheat and Radish showed that the biomass of the Alep pine is very toxic with reductions ranging between 98.8% et 100% in the presence of the powders from B, T, S and O.

The richness of the *Pinus halepensis* barks residues in polyphenols and flavonoids, allelochemical substances, explains the strong herbicide power of this biomass. In fact our results are in agreement with those reported in the bibliography. Once in the soil, the allelochemicals interfere with the neighboring plants [64, 70] acting on the pre and post-emergence stages, and on the bank of seeds [11, 71]. Similar results showed that the incorporation of the residues in the soil or the irrigation with aqueous extracts of *Inula crithmoides* L. of *Pine Wollemi*, fruit peels of the coffee [6, 11, 60] led to the inhibition of the growth of several plant species. Compared with in vitro results, the allelopathic tests in vivo, in the soil, were less toxic and the growth of the target species was less affected. The soil micro-organisms can also play a part in the allelochemical released in the ground [69, 72]. The major constituents of the volatile fraction of green needles collected from Oueslatia were β-caryophyllene, α-humulene, compounds have been reported to have herbicidal activities, by reductions of the growth of the shoots and roots. Allelochemicals causes several damages [73, 74].

Our results showed the strong herbicide power of the aqueous extracts *in vitro*, which was proven by the test *in vivo* by the irrigation with the aqueous extracts or the incorporation in the soil of the vegetal powder which could be used like an organic herbicide.

Indeed, as a reaction to the increase in the resistance of weeds to the pesticides of synthesis [63], there has been a growing interest in the last decades in compounds having allelopathic properties [52] which can lead to the discovery of natural weed herbicides which do not damage to the environment [20, 75] but are effective against weeds that have become resistant to many synthetic herbicides [23].

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AUTHORS' CONTRIBUTION

RT: Acquisition of data, writing, analysis and interpretation of data of the manuscript. HC: Administrative, technical support. FG: GC-MS analyzes. AL: Administrative support. Ahmed NH: revision of the manuscript. All authors read and approved the final of the manuscript.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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