Research Article

Chemotaxonomic Study of Four Subspecies of *Pinus nigra* Arn. Grown in Common Garden Based on Essential Oil Composition

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The aim of this study was to investigate the chemical diversity of *Pinus nigra* Arn. essential oils. The research was carried out on the needles collected from eighteen provenances of black pine grown in common garden located in West-Northern Tunisia and belonging to four different subspecies (*Pinus nigra* subsp. *nigra*, *Pinus nigra* subsp. *salzmannii*, *Pinus nigra* subsp. *pallasiana*, and *Pinus nigra* subsp. *laricio*). Essential oil yields ranged from 0.19% to 0.68%. The obtained essential oils have been analyzed by GC-FID and GC-MS apparatus. Twenty-three constituents accounting about 98% of total essential oil composition were identified. The essential oil compositions appeared to be very different according to their origin. Thus, five main essential oil chemotypes were identified in *Pinus nigra* plants: caryophyllene oxide, camphene, β-caryophyllene, α-amorphene, and germacrene D. The chemotaxonomic value of the essential oil compositions was discussed in relation to the results of the multivariate statistical test, including a detailed survey of the available literature data.

1. Introduction

*Pinus nigra* Arnold (black pine) is a circum-Mediterranean species belonging to the *Pinaceae* family. The species is divided into six subspecies: *Pinus nigra* subsp. *nigra* (Arn.), *Pinus nigra* subsp. *salzmannii* (Dun.), *Pinus nigra* subsp. *dalmatica* (Vis.), *Pinus nigra* subsp. *pallasiana* (Lamb.), *Pinus nigra* subsp. *mauretanica* (Mair. & Pey.), and *Pinus nigra* subsp. *laricio* (Poir.) [1]. This species is discontinuously distributed from Southwest Europe to Asia Minor, extending to the Crimea, and is also found in North Africa (Morocco and Algeria). Pine essential oils were widely used as fragrances, flavoring, intermediates in the synthesis, and beverages. Traditional therapeutic and pharmaceutical uses of its oils have been recorded all around the world. Pine needle essential oils were mainly used in folk medicine for the treatment of cardiovascular and cholesterol-lowering benefits and respiratory infection, and they have powerful antioxidant and anti-inflammatory effects and the ability to enhance microcirculation by increasing capillary permeability [2]. Black pine was characterized by a genetic, morphological, phenotypic, and biochemical diversity. The chemical composition and the intraspecific variation of *Pinus nigra* essential oil have been the subject of numerous studies [3–11]. According to analysis of variance, these investigations indicated a significant variability in chemical composition of black pine essential oils between provenances and subspecies. In Tunisia, four subspecies from eighteen provenances of *Pinus nigra* have been introduced in a common garden in the northwest of the country since 1966.

Therefore, and based on the chemotaxonomic classification tool which was known as one of the most important guides for researchers in their studies for new industrial and medicinal plants, this work was conducted to study, for industrial and medicinal plants. This work was conducted to study, for the first time, the variability in essential oils obtained from the needles of 18 provenances of *Pinus nigra*...
growing under humid bioclimatic conditions in the northwest of Tunisia. This study could be helpful to highlight the variability of intraspecific terpene profile of Pinus nigra species growing in the southern limit of its range.

2. Materials and Methods

2.1. Plant Material. Eighteen samples of Pinus nigra needles were collected in April 2015 from the Souiniet common garden located in the Khroumirie region in West-Northern Tunisia (8°48'https://www.latency.com' W, 35°54'https://www.latency.com' N, 492 m). It is characterized by an annual temperature of 15.6°C and a mean rainfall of 1534 mm/year. The eighteen samples corresponding to eighteen provenances from different geographic origins have been planted since 1966 in provenance trials experimental sites (Table 1). The identification of the plant material was done by Professor Mohamed Larbi Khouja, and the voucher specimens (PN2020_i, where i represents the provenance number varied from 1 to 20) of the plants were deposited at the Herbarium of INRGREF (Tunisia).

2.2. Essential Oil Isolation and Analysis. Needles from the eighteen Pinus nigra provenances were subjected to hydrodistillation in a Clevenger-type apparatus for 3 h in order to isolate essential oils. The essential oils were measured directly in the extraction burette, and the amount of oil obtained (%) was calculated as volume (ml) of essential oils per 100 g of dry plant material. The essential oils were dehydrated over anhydrous Na2SO4 and kept in a cool and dark place prior to analysis. Analysis of the essential oils were carried out by combination of gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) according to a standard analytical procedure using a DB-5MS column (30 m, 0.25 mm, and 0.25 µm film thickness). The flow rate of the carrier gas (helium) was 1.0 ml/min. The GC oven temperature started at 100°C and then held for 1 min at 260°C and then for 10 min with a program rate of 4°C min

2.3. Chemometric Analysis. Principal component analysis (PCA) was performed on all the essential oil chemical compositions regrouped for the eighteen provenances (n = 20 replications). All the chemometric analyses were performed using XLISTAT software for Windows (v.2013.2.03, Addinsoft, New York, USA).

2.4. Statistical Analysis. Data were analyzed using the GLM procedure (general linear models) of the SAS (9.0) program.

An analysis of variance test of the studied parameters was performed. All values are the mean of three replications. Principal component analysis was evaluated with the R (version 3.1.1) program.

3. Results and Discussion

Statistical analysis showed a high variability between the four studied subspecies (p < 0.0001). The highest yield was recorded by needles of nigra subspecies (Table 2), while the lowest values were reached by both calabrica and salzmannii subspecies. Furthermore, a significant variability was recorded between the eighteen provenances. The most important oil yield (0.68%) was reached by needles from P6 (Pinus nigra austriaca; Puget-Théniers, France) while P2 (Pinus nigra calabrica; Trenta, Italy) showed the lowest oil yield with 0.19%.

The results of the identified compounds by GC-MS are shown in Table 3. Twenty-three constituents accounting about 98% of total essential oil composition were identified. The essential oil compositions appeared to be very different among provenances. The major essential oil components were especially variable in occurrence and concentration among the different provenances, ranging from almost absent in some samples to more than 90% of the total essential oil composition in others.

There appear to be five basic essential oil chemotypes in Pinus nigra plants investigated (Figure 1): (a) caryophyllene oxide as the major component (provenances Trenta (22.86%), Les Barres (41.53%), Cosenza (80.54%), Kustendil (88.51%), Alaçam (75.11%), Crimée (41.85%), St Guilhem (38.71%), Cazorla (66.49%), Olette (87.74%), Tavola (90.05%), and Marghese (90.85%)); (b) camphene as the major compound (provenances Brouzet-lès-Ales (19.95%) and Catanzaro (38.07%)); (c) β-caryophyllene (provenances Bois Frerot (42.82%), Grancia (32.93%), and Les Barres (51.10%)); (d) α-amorphene (Les Barres (leint) (26.04%)); and (e) germacrene D (Puget-Théniers (27.13%)). Only the essential oils from Brouzet-lès-Ales (P1), Puget-Théniers (P6), and Catanzaro (P10) provenances were more rich in monoterpenes than sesquiterpenes, while the oils from the other provenances (P2, P3, P4, P5, P8, P9, P11, P12, P13, P14, P16, P17, P18, P19, and P20) had more sesquiterpenes than monoterpenes.

The results of the principal component analysis showed that β-caryophyllene, caryophyllene oxide, linalool, myrcenol, and y-murolene were the most significant variables for the classification of the Pinus nigra essential oils. These parameters were considerably loaded into the two major principal components (Dim 1 and Dim 2) explaining more than 50% of the variance. According to the analysis, five different groups were revealed (Figure 2). The first group contained P6, P10, and P19 samples, which had the main concentrations of β-caryophyllene (15.65, 23.28, and 51.10%, respectively) and the lowest rate of caryophyllene oxide (1.23, 8.33, and 0.99%, respectively). On the other hand, the second group enclosed only P12 and P13 samples, which were characterized by its high amount of both β-caryophyllene (30.29–32.16%) and caryophyllene oxide...
Table 1: Geographic origin of the eighteen provenances of *Pinus nigra* Arn.

| Subspecies | Code | Provenances       | Country of origin | Altitude (m) | Latitude | Longitude |
|------------|------|-------------------|-------------------|--------------|----------|-----------|
| *salzmannii* | P1   | Brouzet-lès-Ales  | France            | —            | 44°07 N  | 4°05 E    |
|            | P12  | St Guilhem       | France            | 350–400      | 43°41 N  | 3°35 E    |
|            | P16  | Cazorla           | Spain             | 1500         | 37°50 N  | 3°00 O    |
|            | P18  | Olette (Pyr-Orient)| France            | —            | 42°36 N  | 2°14 E    |
|           | P2   | Trenta            | Italy             | 1050         | 39°25 N  | 16°35 E   |
|           | P3   | Les Barres        | France            | 150          | 47°50 N  | 2°45 E    |
|           | P4   | Cosenza           | Italy             | 1300         | 39°15 N  | 16°17 E   |
|           | P10  | Catanzaro         | Italy             | —            | 38°54 N  | 16°34 E   |
|           | P14  | Grancia           | Italy             | 850          | 39°41 N  | 16°58 E   |
|           | P17  | Tavola            | Italy             | 950          | 39°25 N  | 16°35 E   |
|           | P19  | Les Barres        | France            | 150          | 47°50 N  | 2°45 E    |
|           | P5   | Bois Frerot (Ardennes) | France      | 100          | —        | —         |
|           | P11  | Les Barres (leint) | France            | 150          | 47°50 N  | 2°45 E    |
|           | P20  | Marghese (Corse-du-Sud) | France        | 1100         | 41°39 N  | 9°12 E    |
| *laricio*  | P6   | Puget-Théniers    | France            | 1600         | 33°52 N  | 4°04 E    |
|            | P8   | Kustendil         | Bulgaria          | —            | 43°57 N  | 6°53 E    |
| *nigra*    | P9   | Alaçam            | Turkey            | 800–1000     | 39°35 N  | 28°35 E   |
|            | P13  | Crimée            | Russia            | 500          | 44°33 N  | 34°17 E   |
| *pallasiana* | P6   | Puget-Théniers    | France            | 1600         | 33°52 N  | 4°04 E    |
|            | P8   | Kustendil         | Bulgaria          | —            | 43°57 N  | 6°53 E    |

Table 2: Essential oil yields of *Pinus nigra* Arn. needles.

| Provenance | Oil yield (%) |
|------------|---------------|
| P1         | 0.244 ± 0.01  |
| P2         | 0.193 ± 0.01  |
| P3         | 0.306 ± 0.02  |
| P4         | 0.372 ± 0.01  |
| P5         | 0.415 ± 0.03  |
| P6         | 0.682 ± 0.05  |
| P8         | 0.384 ± 0.01  |
| P9         | 0.414 ± 0.02  |
| P10        | 0.513 ± 0.05  |
| P11        | 0.262 ± 0.04  |
| P12        | 0.472 ± 0.08  |
| P13        | 0.411 ± 0.01  |
| P14        | 0.462 ± 0.03  |
| P16        | 0.305 ± 0.01  |
| P17        | 0.665 ± 0.02  |
| P18        | 0.403 ± 0.01  |
| P19        | 0.273 ± 0.02  |
| P20        | 0.372 ± 0.01  |

Means with different letters in the same column were significantly different at *p* < 0.05. Results are expressed as mean ± standard deviation of 3 determinations.

(38.71–41.85%). The third group regrouped P1, P5, P11, and P14, which showed the most important amount of γ-muurolene (2.23–4.02%). The fourth group contained P2 and P3 oils characterized by the highest amount of linalool (5.83 and 2.97%, respectively). The fifth group regrouped all the other samples studied, which showed the highest rate of caryophyllene oxide.

When considering the variability between the four studied subspecies, statistical results showed that oils from *nigra* subsp. were the richest in α-pinene. This richness is related to the high amount found in P6 (Puget-Théniers, France) oil (19.34%).

The results of the principal component analysis revealed the presence of three groups (Figure 3)—the first group regrouped *laricio* and *salzmannii* subspecies, which showed the highest rate of camphene and limonene; the second group contained *pallasiana* subsp., which demonstrated the most important amount of caryophyllene oxide; and the third one enclosed only *nigra* subsp. representing the highest amount of α-pinene.

Various terpenoid compounds, which are characteristic constituents of conifers, have been reported in *Pinus nigra* needles. Several studies that noted the chemical composition of the essential oils extracted from needles of *Pinus* species growing in Tunisia (*Pinus halepensis, Pinus pinea*, and *Pinus pinaster*) found that β-caryophyllene, amorphene, limonene, and germacrene-D were the major components in all essential oils of pine needles [2, 12–14]. These results were supported by our study.

In our study, caryophyllene oxide was the major compound in oils of *pallasiana* subsp. from Turkey. These results were not similar to those found by Dogan and Bagci [15] that demonstrated that the main compounds of this subspecies were α-pinene, limonene, and β-caryophyllene. In the same context, Sezik et al. [11] proved that α-pinene and β-pinene were the main constituents of *Pinus nigra* essential oils from Turkey.

Several studies investigated the chemical composition of Italian essential oils. Macchioni et al. [10] indicated that α-pinene was the principal constituent. In the same context, Bader et al. [16] claimed that the essential oils from *laricio* subsp. were rich in α-pinene, β-caryophyllene, and germacrene-D. According to our results, the main compounds in Italian oils were β-caryophyllene, camphene, and caryophyllene oxide.

*Pinus nigra* subsp. *laricio* essential oils from Corsica were investigated by Rezzi and co-workers [9] who demonstrated that α-pinene, manoyl oxide, and germacrene-D were found
Table 3: Chemical composition of essential oils from *Pinus nigra* Arn.

| No. | Compounds (%) | KI | P1 | P2 | P3 | P4 | P5 | P6 | P8 | P9 | P10 | P11 | P12 | P13 | P14 | P15 | P16 | P17 | P18 | P19 | P20 |
|-----|---------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1   | α-Pinene      | 939 | —  | —  | —  | —  | —  | —  | —  | —  | —  | 19.34 | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  |
| 2   | Verbenol      | 952 | 0.40 | 2.17 | 0.81 | —  | —  | —  | 0.66 | —  | —  | 0.62 | —  | —  | 0.80 | —  | 0.15 | —  | —  | 0.03 | —  |
| 3   | Myrcenol      | 974 | 4.19 | 3.56 | 1.86 | —  | —  | —  | 0.80 | —  | —  | 1.41 | —  | —  | 1.60 | 1.05 | —  | —  | —  | 0.05 | 0.12 |
| 4   | 3-Udecyne     | 988 | 0.23 | 0.77 | 0.88 | —  | —  | —  | 0.98 | —  | —  | 0.30 | —  | —  | 0.36 | —  | 0.23 | —  | —  | —  | —  |
| 5   | Linalool      | 1082 | 0.45 | 5.83 | 2.97 | —  | 0.58 | 0.46 | 1.08 | —  | 1.06 | 0.43 | —  | 0.74 | —  | 0.16 | —  | —  | 0.16 | —  |
| 6   | Limonene      | 1089 | 1.82 | 1.49 | 0.91 | 2.53 | 0.47 | —  | —  | 1.33 | 1.71 | 2.96 | 1.64 | 2.85 | —  | —  | 0.12 | 1.40 | 0.21 |
| 7   | Geranyl acetate | 1101 | 3.51 | 1.20 | 0.73 | 0.87 | 1.20 | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  |
| 8   | Camphenol acetate | 1117 | 0.95 | 2.20 | 0.60 | 1.39 | —  | —  | 0.95 | 0.40 | 0.53 | —  | —  | 1.70 | —  | 0.31 | —  | —  | 0.07 | 0.11 |
| 9   | 3-Decyne      | 1152 | 0.89 | 2.17 | 8.19 | —  | 0.52 | 0.29 | 1.74 | 9.72 | 0.37 | 1.14 | 1.17 | 1.82 | 0.85 | 5.39 | 3.42 | 1.88 | 0.08 | 0.42 |
| 10  | Limonene oxide | 1168 | 14.21 | 8.14 | 6.88 | —  | 4.18 | 0.19 | 0.84 | 1.87 | 0.40 | 7.09 | 0.75 | 0.49 | 4.15 | 5.38 | 0.87 | 0.24 | 0.17 | 0.23 |
| 11  | β-Phenethyl butyrate | 1295 | 2.81 | 2.33 | 0.53 | —  | 0.89 | 23.17 | 0.48 | 8.32 | 5.39 | 2.61 | 0.83 | 4.82 | —  | 0.35 | 0.34 | 15.88 | —  | —  |
| 12  | 5,9-Tetradecane | 1328 | 0.53 | —  | 0.85 | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  | —  |
| 13  | β-Caryophyllene | 1418 | 13.72 | 4.75 | 1.27 | —  | 42.82 | 15.65 | 0.80 | 23.28 | 12.89 | 32.16 | 30.29 | 32.93 | 0.79 | —  | 1.28 | 51.10 | 0.70 |
| 14  | α-Amorphene   | 1422 | 6.69 | 7.38 | 0.59 | —  | 11.22 | —  | —  | 2.60 | 26.04 | 0.52 | 2.46 | 8.30 | —  | —  | —  | 3.98 | —  | —  | —  |
| 15  | Bicyclogermacrene | 1438 | 0.82 | 3.14 | 5.24 | —  | 1.44 | 0.21 | 1.05 | 0.65 | 1.21 | 2.20 | 3.33 | 3.80 | 0.72 | 1.25 | 0.57 | —  | 0.12 | 0.09 |
| 16  | γ-Murolene    | 1441 | 2.92 | 1.36 | 2.07 | 0.98 | 3.82 | 1.92 | —  | 0.90 | 2.16 | 4.02 | 1.49 | 1.44 | 2.23 | —  | —  | 1.53 | 2.08 | 0.43 |
| 17  | α-Caryophyllene | 1460 | 1.59 | 1.52 | 5.29 | 1.92 | 1.98 | —  | 1.79 | 0.64 | —  | 1.58 | 8.84 | 9.44 | 1.74 | 1.11 | 1.20 | —  | —  | 0.57 |
| 18  | Caryophyllene oxide | 1466 | 16.10 | 22.86 | 41.53 | 80.54 | 8.14 | 1.22 | 88.51 | 75.11 | 8.33 | 10.33 | 38.71 | 41.85 | 23.75 | 66.49 | 90.05 | 87.74 | 0.99 | 90.85 |
| 19  | Germacrene D  | 1488 | 3.28 | 3.09 | 2.46 | 2.51 | 0.77 | 27.13 | 0.76 | 10.89 | 0.41 | 1.08 | 0.86 | 0.44 | 7.11 | 0.16 | 0.16 | 23.75 | 2.46 |
| 20  | Farnesene epoxide | 1509 | 0.59 | 8.13 | 1.34 | —  | 0.64 | —  | —  | —  | 1.17 | 0.45 | —  | —  | —  | —  | —  | —  | —  | —  | —  |
| 21  | γ-Cadinene    | 1526 | 0.29 | 2.28 | 3.85 | —  | 0.64 | —  | —  | 0.94 | —  | 1.22 | —  | 0.46 | 1.01 | —  | —  | 0.20 | 0.25 | 0.05 | 0.12 |
| 22  | Camphene      | 1578 | 19.95 | 8.40 | 6.70 | 9.17 | 3.26 | —  | —  | 0.76 | 38.07 | 16.77 | 0.42 | —  | 5.08 | 5.61 | 0.15 | 0.17 | 2.06 | 0.43 |
| 23  | α-Cadinol     | 1632 | 2.05 | 5.23 | 2.80 | 0.62 | 13.23 | 8.41 | 1.02 | 2.34 | 0.64 | 1.72 | 0.77 | —  | 2.08 | 3.82 | 0.18 | 0.18 | 0.06 | 0.81 |

K1: Kovats indices calculated in regard to standards mixture of hydrocarbons (C8–C24) for 4.790–48.969min. %: percentage calculated by GC-FID on DB-5MS column.
to be the main constituents. These findings were not in accordance to that found by our research team. In our case, laricio subsp. essential oils showed three main compounds, which were β-caryophyllene, α-amorphene, and caryophyllene oxide.

According to Jurc et al. [17], the terpene profile of Pinus nigra subsp. salzmannii from France was characterized by a high amount in γ-cadinene and δ-cadinene. Our results differ considerably from those reported previously. In this study, oils from salzmannii subsp., France, were characterized by a significant rate of camphene and caryophyllene oxide.

In most previous literature that focused on Pinus nigra essential oils composition, α-pinene was mentioned as the major compound, while in our study, this compound was absent in almost all the studied provenances excepting nigra.
subsp. austriaca var. that was mainly composed of germacrene and α-pinene. This finding was supported by Jure et al. [17].

These comparisons with earlier studies indicated that, when planted in a common garden in Tunisia, where the pedoclimatic conditions were constant, Pinus nigra from four subspecies and deriving from eighteen provenances has undergone a significant change in its chemotypes. Plants of black pine seem to be adapted to local climate and soil conditions. This finding was supported by Amri et al. [12] who mentioned that there was a significant difference in the chemical composition of the essential oils from Pinus nigra subsp. laricio grown in Tunisia and those from other countries.

It has long been known that pedoclimatic conditions affect the volatile oil content as well as its chemical composition. Ormeño and Fernandez [18] mentioned that both biotic and abiotic conditions influence terpenoid production in plants, especially light and temperature. On the other hand, Staudt and Lhoutellier [19] determined the effect of these two environmental factors on monoterpene and sesquiterpene leaf emissions. In addition, water availability was known to be one of the most important environmental factors controlling volatile organic compounds from plants [20].

Under the same pedoclimatic conditions of the studied provenance trials, Pinus nigra plants showed a significant chemical variability. This could be explained by an eventual genetic variability among the eighteen provenances.

4. Conclusion

From the present study, we can conclude that the analyzed essential oils belonged to five different chemotypes. In addition, the obtained results showed differences in the quantitative and qualitative composition. Caryophyllene oxide was the main component of most of the studied oils. Therefore, this type of study will be useful for pharmacologists to promote pharmaceutical products and resolve several economic problems.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors’ Contributions

Sondes Fkiri and Faten Mezni performed some practical experiments, wrote and coordinated all the analysis, and calculated the results, statistics etc. Ghayth Rigane followed and checked the obtained results. Hanene Ghazghazi participated in some practical experiments. Ridha Ben Salem, M. Larbi Khouja, Zouheir Nasr, and Abdelhamid Khaldi supervised the scientific paper.

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