Influence of Geographic Position, Leaf Surface and Genetic Variability on Content of Total Essential Oils in 12 Distinct Populations of Bay Laurel (*Laurus nobilis L.*)

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Summary

Within research project "Taxonomy, Ecology and Utilization of Carob tree (*Ceratonia siliqua L.*) and Bay Laurel (*Laurus nobilis L.*) in Croatia" (HRZZ-IP-11-2013-3304), a total of 1200 plant samples were collected from 12 distinct populations of bay laurel in Croatian Adriatic area. After morphometric analyses of leaves, AFLP analyses of genetic variability, and variability of total essential oils content in leaves of bay laurel populations, significant correlation between leaf surface and essential oils content (Spearman's Rank Order Corr. Coeff. r_s = 0.15 ns) was not found, while the correlation between latitude and essential oils content was strongly negative (Spearman's Rank Order Corr. Coeff. r_s = - 0.78; $p<0.05$). However, the correlation between longitude and essential oils content was strongly positive (Spearman's Rank Order Corr. Coeff. r_s = 0.73; $p<0.05$). On the other hand, the results of Mantel test showed low, but positive and highly significant correlation between AFLP variability of populations and essential oils content (r = 0.39; $p<0.01$), while the significant correlation between AFLP variability of populations and leaf lamina surface (i.e. expectedly the most influential factor on accumulation of essential oils) was not obtained. Obtained results of these matrix correlations (i.e. Spearman's Rank Order Correlations and Mantel test) correspond with the results of Friedman's ANOVA and Kendall's Coeff. of Concordance for variability of total essential oils content between the populations (ANOVA Chi Square = 21.88; p = 0.025 and Kendall's Coeff. of Concordance = 0.99; Aver. rank r = 0.98). According to these results, it is possible to conclude that the populations of bay laurel from locations of south-east Croatian Adriatic islands and coastal area accumulate higher quantity of essential oils in the comparison with the populations of north-west islands and coastal area.

Key words

bay laurel, *Laurus nobilis*, essential oils, leaf lamina surface, biodiversity

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Introduction

Bay laurel (*Laurus nobilis L.*) is evergreen tree or large shrub. The genus *Laurus* belongs to the subfamily of Lauroideae, within the family of Lauraceae, to order Laurales, subclass of Magnoliidae, of the class Magnoliopsida (Takhtajan, 2009; Jørgensen et al., 2014). According to results of karyological studies of 16 species within the family Lauraceae, the basic chromosome number is the same ($x = 12$) for all 16 species. On the other hand, according to karyomorphological evidences these 16 species within the family of Lauraceae are divided into two subfamilies, Lauroideae and Cassythoideae (Okada and Tanaka, 1975). Within the genus of *Laurus* two species are included, *L. nobilis* and *L. azorica* (Seub.) Franco. *L. azorica* is characterized by densely tomentose to hirsute twig leaves, and it is distributed on the islands of Azores, Madeira and the Canary Islands. On the other hand, *L. nobilis* is characterized by glabrous twig leaves and it is distributed in the Mediterranean region, where it can be found as cultivated or naturalized species (Arroyo-García et al, 2001). Bay laurel is considered as one of the most important species of the late-Tertiary persisting in Mediterranean vegetation (Alessi et al., 2018). Moreover, according to some estimation, *L. nobilis* is a representative of Mediterranean bay laurel forests from the age of Pliocene (Kondraskov et. al., 2015).

The leaves of *L. nobilis* contain essential oils and the usage of its leaves is well known in Mediterranean cuisine and ethnobotany. Because of the inhibitory effects of essential oils of bay laurel on food borne bacteria and fungi the leaves, fruits and flowers of bay laurel has been used in food flavouring, as well as in solving digestive problems in ethnomedicine in some Mediterranean countries (Al-Hussaini and Mahasneh, 2009). Namely, the essential oils, extracted from the leaves of bay laurel, show significant antifungal and antimicrobial effects on *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19117, *Salmonella enterica* Enteritidis S64, *Aspergillus flavus*, etc. (Xie et al., 2004; Millezi et al., 2012; Nazzaro et al., 2017). However, the leaves of *L. nobilis* have components of essential oil, phenolic compounds, and sesquiterpenic lactones as the principal active substances and shows significant antioxidant activity (Politeo et al., 2007). The qualitative composition and quantitative content of these groups of essential oils compounds in bay laurel leaves depends of ecological conditions, geographical position, climatic factors and soil conditions (Nasukhova et al., 2017). Trees of bay laurel grown under cultivation produce the maximum content of essential oil in autumn and the lowest in spring and the young stems and old leaves have the highest content of essential oils. Approximately, one tree of bay laurel produces more than 5 kg of fresh matter or more than 50 ml of essential oil per tree per year, from the fifth year of growth (Putievsky et al., 1984). The essential oil of *L. nobilis* contains 39 different chemical compounds and the main components are 1,8 cineole, α-Terpinyl acetate, 4-Terpinenol, α-Terpineol (Bayar et al., 2018) and also linalool and limonene (Mansour and Ismail, 2018).

The aim of this research was to find possible differences in variability of total essential oils content in bay laurel leaves, collected in 12 distinct populations of Croatian Adriatic area, in relation with geographic position of locations, differences in leaf lamina surface and genetic variability between distinct bay laurel populations.

Materials and methods

Sampling of bay laurel plant material

The samples of plant material were collected from 12 distinct locations on Croatian Adriatic islands and coastal area (Table 1; Figure 1). Latitude and longitude of each location was detected using the Garmin (pocket) GPS locator. In order to provide statistical analysis the degrees and angular minutes of longitudes and latitudes of each location were transformed into decimal numbers of degrees

Table 1. Locations of sampled plant material from different bay laurel habitats in Croatian Adriatic area

Location	Latitude (transformed into decimal numbers of degrees)	Longitude (transformed into decimal numbers of degrees)
Lovran	45.48°	14.45°
Cres	45.41°	14.65°
Brač	43.05°	17.08°
Lošinj	44.88°	17.08°
Pelješac	43.43°	17.92°
Mljet	42.72°	17.85°
Korčula	43.53°	17.46°
Konavle	42.90°	18.55°
Lastovo	43.25°	17.45°
Šipan	43.20°	18.45°
Dugi Otok	44.60°	15.15°
Žirje	44.08°	16.08°

The samples of leaves with branches were collected in phyllotaxis of 360° per each plant, and young leaves were collected and stored into zip nylon bags with silica gel for further AFLP analyses. The leaves for morphometric analyses were collected from the medium insertions of each plant, per each location, i.e. from the middle insertions of plants and the upper and basic insertions were avoided during the sampling of plant material. For each plant sample a voucher number was assigned. A total of 10 900 samples of plant material were collected from 12 distinct populations, i.e. 10 000 of leaf samples for morphometric and chemical analyses and 900 of young leaf samples for AFLP analyses.

Morphometric analysis

Leaves of *L. nobilis* are typical dorsiventral of ellipsoidal shape. Because of typical ellipsoidal shape of bay laurel leaves length and width are measured as described in Figure 2. The surface of leaves was calculated using the following equation [1](Gusić et al., 2015), as described in Figure 2.

$$P_{a,b} = \pi ab \qquad [1]$$

Where: P means surface of leaf lamina, a means ½ of central nerve length measured from petiole to the end of leaf, and b means ½ leaf lamina width measured on the widest part of lamina.

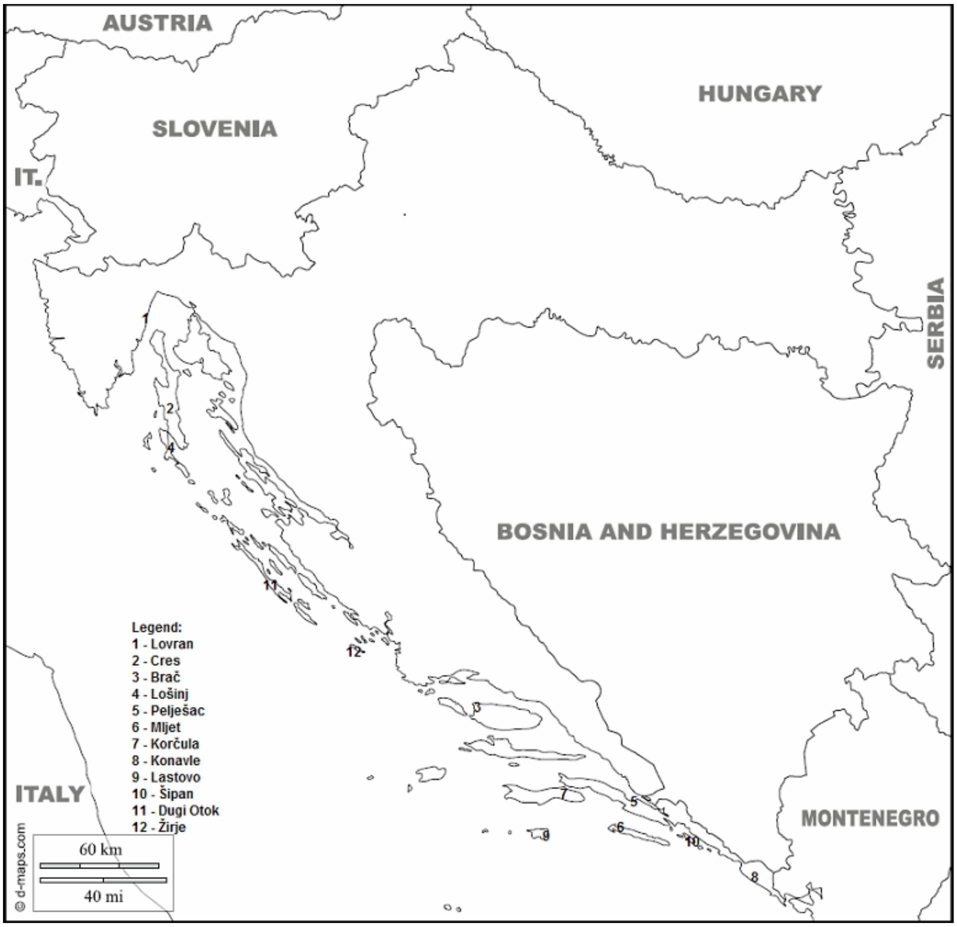

Figure 1. Locations of 12 distinct populations of *L. nobilis* in Croatian Adriatic area (source: d-maps.com free maps)

Per each of 12 locations, dimensions of 10 leaves, collected on different insertions from 10 plants were measured, i.e. the total number of measures and calculations of leaves surface were 1200 (i.e. N=1200; n=100). The rest of leaf material was used for isolation of total oils, as follows.

Isolation procedure of total oils

Dried plant material (100.0 g) was placed in a round-bottomed flask and 500 mL distilled water was added. Hydro distillation was performed for 3 h in Clevenger-type apparatus. The essential oil content in the plant material was determined as percentage yield of oil, which was calculated as the percent of the ratio of weight of oil to weight of plant material used for extraction. The obtained oils were dried over anhydrous sodium sulphate and stored at 4°C before the analysis (Dunkić et al., 2017). All the analyses were done in triplets.

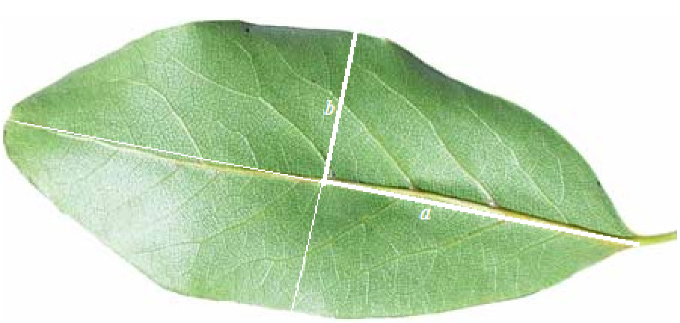

Figure 2. Illustration of leaf dimensions measuring for calculations of leaf surface

DNA isolation and AFLP analysis

Samples of fresh young bay laurel leaves collected *in situ* were dried in silica-gel within zip nylon small bags. Dried leaves were grinded for 60 sec at 25 Hz vibrational frequency into fine powder using mixer mill MM400 (Retsch GmbH, Germany), and used for DNA isolation. Genomic DNA was isolated using DNA plant isolation kit (DNeasy Plant Mini Kit; Qiagene, Netherlands) following the protocol provided by manufacturer. AFLP analyses were performed according to Vos et al. (1995). Genomic DNA was digested with *Eco*RI i *Mse*I restriction endonucleases. Adaptors *Eco*RI and *Mse*I were ligated at the end of digested DNA fragments and diluted in ratio 1:10 to generate a template DNA for preselective PCR amplification. In preselective amplification two primer combinations, each with one selective nucleotide (E01/M02 and E01/M01), were used. Obtained products were used as template DNA in selective PCR amplification where six primer combinations (E36/M62, E38/M48, E34/M60, E41/M48, E42/M54 and E31/M51) were used. Forward primers of selective primer combinations were labelled with fluorescent dyes (6FAM and VIC; Applied Biosystems, USA). Preselective and selective amplifications were performed in a VeritiTM 96 Well Thermal Cycler (Applied Biosystems, USA) in a total volume of 20 µl. Amplified AFLP fragments were separated in the four capillary electrophoresis (Genetic Analizer 3130, Applied Biosystems) using GeneScan™ 600 LIZ™ dye size standard (Applied Biosystems), and scored for presence (1) or absence (0) using the GeneMapper 4.0 software (Applied Biosystems). Scored fragments were entered into a binary matrix. Binary matrix was used for further statistical analysis.

Statistical analyses

After analyses of bay laurel leaves morphometry, total oils and AFLP, all analytical data were statistically analysed by parametric and nonparametric statistical methods. Variability of leaf surface and also essential oils between the 12 distinct populations were examined by Friedman's ANOVA and Kendall's Coefficient of Concordance. Correlations between bay laurel leaf surface and content of total essential oils, and also geographic position of each location and content of total essential oils, were examined by Spearman's Rank Order Correlation. Although, the multiple correlations were used in order to find the influence of latitude and longitude (as two independent variables) of each location on content of total essential oils (as dependent variable). Percentages of total essential oils were transformed by angular or *arcsin* transformation (Chanter, 1975). Also, the Cluster analyses by unweighted pair group method with arithmetic mean (UPGMA) were done for the traits of leaf lamina surface, as well as, for content of total essential oils of 12 distinct *L. nobilis* populations (Sokal and Michener, 1958). Described statistical analyses were done using the STATISTICA SixSigma software (Hill and Lewicki, 2007). The AFLP binary matrix was used to calculate squared Euclidean distances (Excoffier et al., 1992). The genetic distance between any two bay laurel populations is represented by its ΦST value and refers to as interpopulation distance. ΦST values were calculated using Arlequin ver. 3.1 (Excoffier et al., 2005) and used as input data for and Mantel test. Correlations between results of AFLP analyses of 12 distinct populations of bay laurel and total essential oils content within them and its geographical position were performed by Mantel test (Goslee and Urban, 2007). Mantel test was performed using the NTSYSpc 2.21L program (Rohlf, 2008).

Results and discussion

Opposite of our previous expectations, the average surface of bay laurel leaves has no influence on accumulation of essential oils in 12 distinct populations ($r_{s\ (\text{average surface of leaves vs. total oil content})} = 0.125$; $p>0.05$; Figure 7). Namely, it is obvious in Figure 3 that the highest rank of leaf surface is detected in *L. nobilis* population of Lovran, which is the northernmost population and then follows the population of Konavle, which is the southernmost population of *L. nobilis*. Than follows the population of south Adriatic island Korčula. Also, the population of middle Adriatic island Brač shares the same rank considering the leaf lamina surface with the population of north Adriatic island Lošinj (average ranks 8.75 and 8.25, respectively, with difference between average leaf surface of only 0.01 cm^2; Figure 3), which is very surprising considering the geographical distance between these two populations (Table 1, Figure 1). The smallest leaf lamina surface was noticed on the population of north Adriatic island Cres, which is the nearest location of the population of Lovran. The results of Friedman's ANOVA, for trait of leaf lamina surface, correspond with the results of UPGMA analysis for the same trait (Figure 5). Consequently, the correlations between leaf lamina surface and latitude and longitude of the location of investigated population were not obtained, i.e. the Spearman's Rank Order Correlations between leaf lamina surfaces were very low and not significant ($r_{s\ (\text{latitude vs. leaf lamina surface})} = -0.083$; $r_{s\ (\text{longitude vs. leaf lamina surface})} = 0.055$; $p>0.05$, respectively).

On the other hand, the content of accumulated total essential oils within the populations of north Adriatic islands and coastal area is generally lower in the comparison with the populations

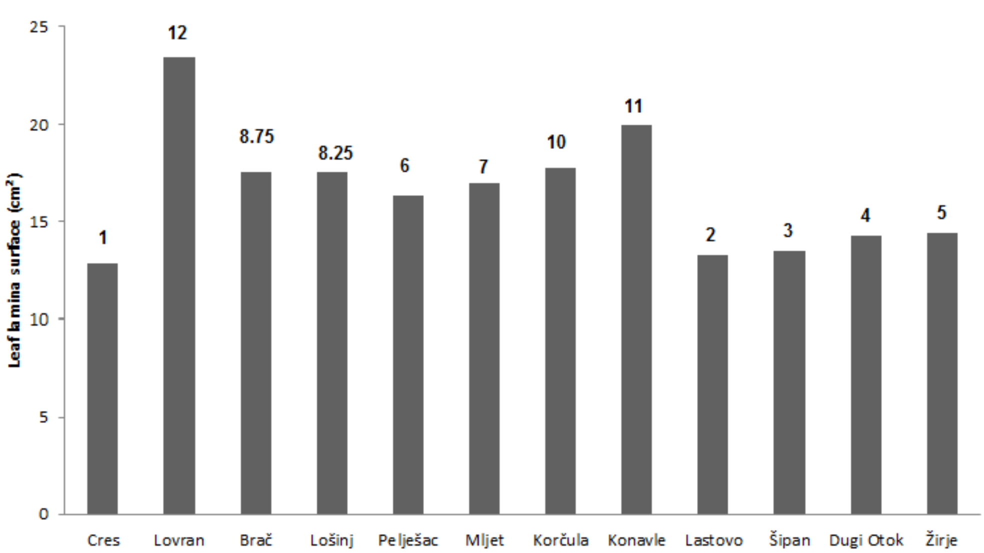

Figure 3. Variability of average leaf lamina surface of *L. nobilis* of 12 distinct populations (average ranks assigned above each bar; Friedman's ANOVA $\chi^2 = 21.98074$; $p = 0.0245$; Kendall's Coefficient of Concordance = 0.99912 Aver. rank r = 0.99825)

Figure 4. Variability of total essential oils in leaves of *L. nobilis* of 12 distinct populations (average ranks assigned above each bar; Friedman's ANOVA χ^2 = 21.88298; p = 0.0253; Kendall's Coefficient of Concordance = 0.9946 Aver. rank r = 0.9893)

of south Adriatic islands and coastal area (Figures 4 and 1). The highest content of accumulated essential oils in leaves of *L. nobilis* was noticed in the southernmost population of Konavle, than followed by the populations of south Adriatic islands Lastovo, Mljet and peninsula Pelješac, etc. But the lowest content of total essential oils in *L. nobilis* leaves were obtained from populations of middle Adriatic islands Dugi Otok and Žirje, followed by the northernmost populations of Lovran and northern Adriatic island Cres, etc.

However, the results of Spearman's Rank Order Correlations between latitude and total essential oils content in bay laurel leaves showed strongly negative, but highly significant correlation (r_s = -0.76; $p<0.01$, Figure 8). At the same time, Spearman's Rank Order Correlation between longitude and total essential oils content in bay laurel leaves of the same populations were strongly positive and also highly significant (r_s = 0.77; $p<0.01$, Figure 9). The results of Spearman's Correlations are also confirmed by the Multiple Correlation Analysis. Determined multiple correlation coefficient between longitude and latitude, treated as two independent variables, and total content of essential oils was very high and significant ($R_{(multiple)}$ = 0.78; $p<0.05$, Figure 10).

The Mantel test between results of AFLP analysis and content of total oils in leaves of *L. nobilis* of these 12 distinct populations showed low positive correlation (r = 0.39; $p<0.05$), yet no correlation was obtained by Mantel test between results of AFLP analysis and leaf lamina surface, latitude and longitude of locations (r = -0.06; 0.09; 0.17; $p>0.05$, respectively).

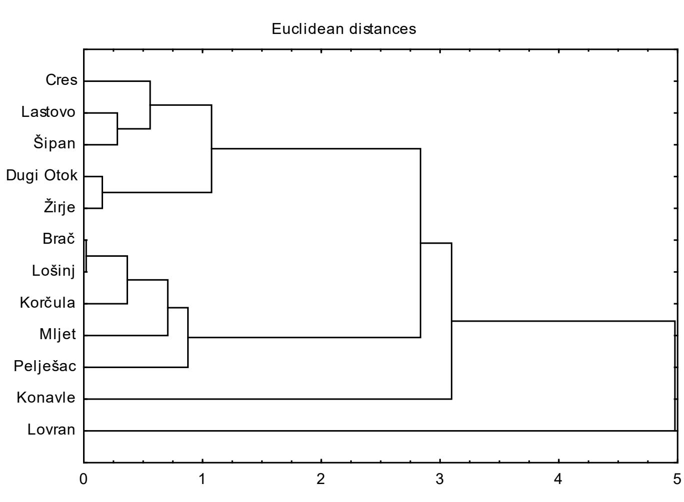

Figure 5. Cluster analysis of 12 distinct populations of *L. nobilis* for trait of leaf lamina surface

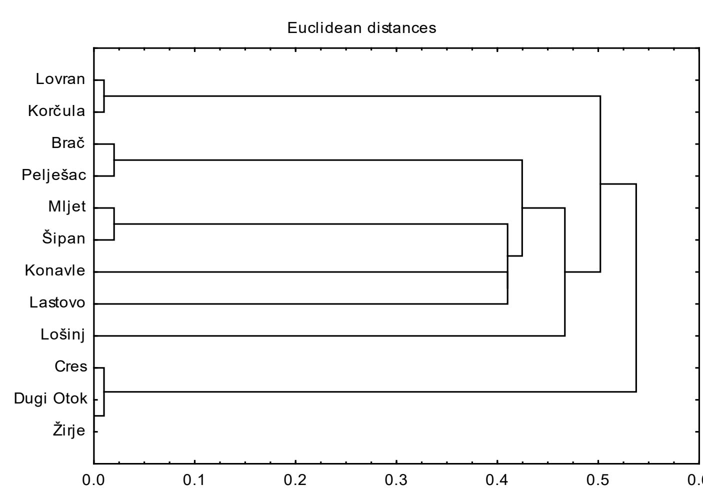

Figure 6. Cluster analysis of 12 distinct populations of *L. nobilis* for trait of total essential oils content

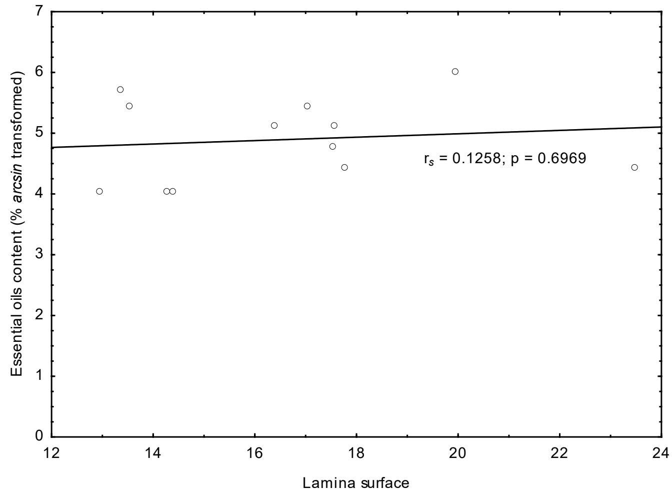

Figure 7. Spearman's Rank Order Correlation between average leaf lamina surface and average content of essential oils in *L. nobilis* leaves

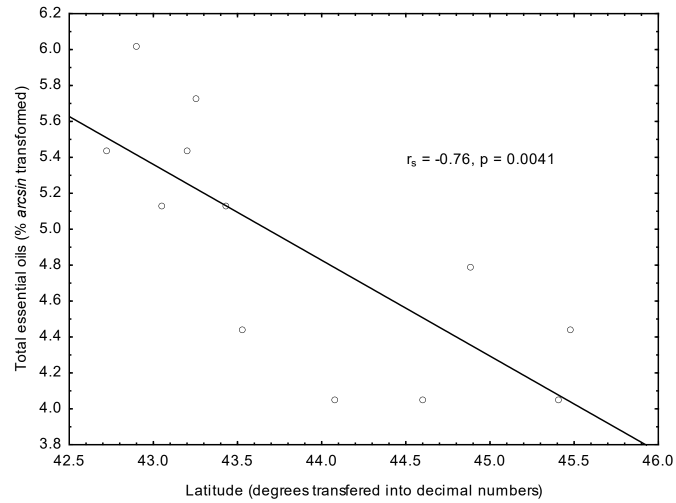

Figure 8. Spearman's Rank Order Correlation between latitude of *L. nobilis* populations and content of total essential oils in its leaves

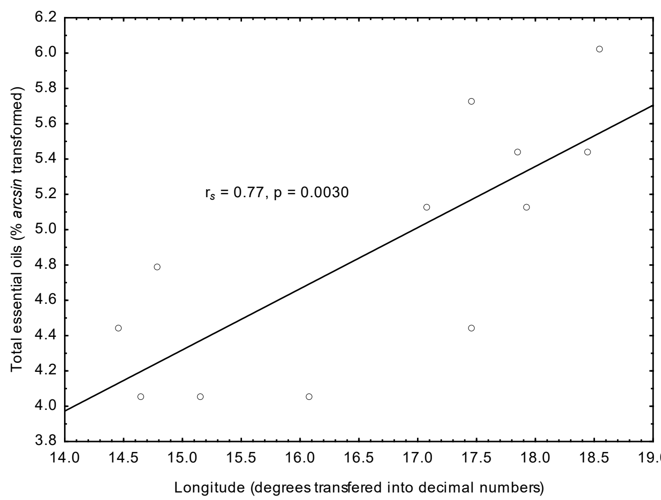

Figure 9. Spearman's Rank Order Correlation between longitude of *L. nobilis* populations and content of total essential oils in its leaves

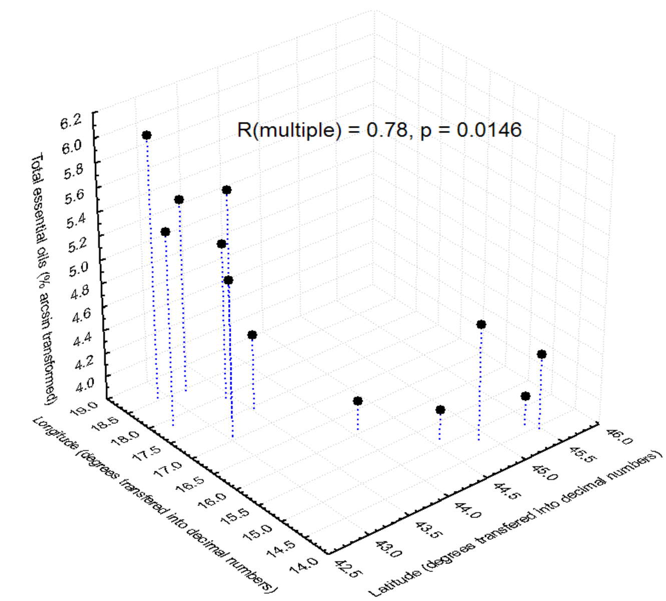

Figure 10. Multiple correlations between latitude and longitude of 12 distinct locations and content of total essential oils in *L. nobilis* leaves

Conclusion

According to results of this study, geographical position of the locations of 12 distinct populations has a stronger influence on accumulation of total essential oils in leaves of *L. nobilis*, rather than its leaf lamina surface. Namely, achieved results showed that content of total essential oils generally increases from the northern to southern populations and also from west to the east. However, considering the low positive correlation between results of AFLP analysis and content of total oils in leafs of *L. nobilis* of these 12 distinct populations ($r = 0.39$; $p<0.05$), it is not possible to exclude the influence of genetic factors and variability between populations on differences in accumulation of essential oils between them.

Acknowledgment

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