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Variability of Cortex Terpene Composition in *Cupressus sempervirens* L. provenances grown in Crete, Greece

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Abstract

The terpene composition of twigs in 106 trees from 6 provenances of *Cupressus sempervirens* L. grown in the island Crete of Greece was determined by GLC-MS analysis. The aim was to investigate the utility of cortex terpene composition to study the genetic variation between cypress provenances. Twenty nine compounds were detected in cortex resin of all trees, twenty one of

which identified. The major constituents were cedrol, α -pinene, 3- δ -carene and α -terpinyl acetate. A cluster analysis based on the amounts of the four evaluated compounds classified all the trees in five chemotypes. The chemotype pattern for every provenance was determined. Based on distribution of chemotypes two main groups of provenances can be suggested.

Key words: *Cupressus sempervirens* L., *Cupressaceae*, cortex terpene composition, provenance variability, GLC-MS, Crete, Greece.

Introduction

In the Mediterranean region, *Cupressus sempervirens* L. is a very important forest tree species for multiple purposes in forestry because of its ability to grow in adverse environments such as calcareous, clayish, dry and poor soils (XENOPOULOS et al., 1990). Through the geographical distribution the Mediterranean or Italian or common cypress comprises two main morphs: a)

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Cupressus sempervirens L var. *horizontalis* which has a broad crown form with wide angles between branches and stem and b) *Cupressus sempervirens* L var. *pyramidalis* with a conical crown form and small angles between branches and stem. The two morphs can hybridizes naturally – intraspecific hybridization- and produce progenies exhibiting a crown form which varies between the former and the latter morph (PANETSOS, 1967).

Terpene composition has been shown to be a very useful tool in studying provenance variation, hybridization etc. for a wide variety of conifer species. Terpene composition is found to be genetically determined and little influenced by environmental conditions. Additionally each component can be identified with high accuracy by GLC analysis and more recently by GLC coupled with MS (VON RUDLOFF, 1967, 1969; SQUILLACE, 1976; ADAMS et al., 1979, ADAMS, 1995). However, several variation patterns derived by terpene analysis showed little or no relevance with patterns observed at DNA markers, such as RAPDs, ITS sequences and iSSR (ADAMS et al., 2003), SSRs at chloroplast DNA (NAYDENOV et al., 2006) and DNA sequences (LEVIN et al., 2003), indicating that probably the evolutionary forces shaping the terpene variation may be different than the ones being responsible for variation at the DNA level. Terpene composition was also found to be related to disease and insect resistance (SQUILLACE, 1976; HANOVER, 1990, 1992). Therefore terpene composition was selected as an investigational approach in our research.

The objectives in the present study were: a) to determine the cortex terpene composition of *Cupressus sempervirens* provenances grown in Crete and b) to evaluate if terpene composition could be used to describe genetic variation within cypress provenances

Materials and Methods

All samples were collected from a provenance trial plantation in the location Kathiana in western Crete established in 1990. Samples included six provenances of *Cupressus sempervirens* from the western part of the island of Crete representing natural stands of different altitude, latitude and longitude (Table 1 and Figure 1). Low elevation stands are natural, but they probably have been in mating contact with introduced material. High elevation stands are considered to be pure natural (PAPAGEORGIOU, 1995). Each provenance was represented from 15–20 trees, belonging to 20 different families. In February 1997, a total of 106 trees were sampled (Table 1). All trees studied were 7 years old, healthy, without any sign of disease or insect attack.

Samples were obtained from lateral shoots of the tree. Twigs were then placed in hermetically sealed plastic bags in ice, transferred to the laboratory and stored in a refrigerator at -20°C until analyzed. After removing needles and wood, 0.5 gr. of a very well chopped cortex (less 1 mm) was extracted from each twig using a 100% hexane solution for 24h at room temperature. The solution was then transferred to auto-injector vials. For chromatographic analysis of all samples a GC-MS system was used according to ADAMS (1995) i.e. a H.P 5890 A II gas chromatograph equipped with a 7673 injector and a F.I.D. coupled with a VG-2000 spectrometer. Individual terpenes were separated on a capillary column of HP IV fused silica 50 m x 0.25 mm i.d coating FFAP. The injector temperature was set at 250°C and the F.I.D.'s at 280°C . Helium was used as carrier gas at a flow rate of 1 ml/min. Components were identified by comparison with the MASS LYNX software. Terpenes were quantified as percentage contribution of each peak to total terpenes present. For statistical evaluation of data, a

Table 1. – ANOVA analysis, mean (%), std. deviation, number of trees included in each one *Cypress* provenance and sites characteristics.

Provenances	Altitude (m)	Latitude	Longitude	N	statistics	Components			
						α -pinene	3- δ -carene	α -terpinyl acetate	cedrol
Fress (FR)	175	35°22.5'	24°09'	17	mean std.dev.	57.4 % 11.4	23.9% 8.5	10.6% 5.8	7.9% 6.6
Anopolis (AN)	1650	35°17'	24°08'	15	mean std.dev.	48.2% 10.0	26.0% 7.4	11.7% 5.5	14.0% 6.8
Askifou (AS)	850	35°15'	24°10'	19	mean std.dev.	58.3% 10.3	23.7% 7.8	8.9% 5.5	9.0% 4.3
Omalos (OM)	1050	35°19.5'	23°52.5'	18	mean std.dev.	54.9% 7.8	24.2% 6.7	7.5% 2.9	13.2% 6.8
Prasses (PR)	550	35°22.5'	23°50.8'	17	mean std.dev.	52.3% 9.6	22.5% 6.3	10.9% 4.2	14.1% 11.4
Zourva (ZO)	650	35°23'	23°57.5'	20	mean std.dev.	49.4% 9.3	26.0% 6.2	11.1% 6.3	13.3% 5.7
ANOVA significance						0.019 (*)	0.665 (ns)	0.161 (ns)	0.046 (*)

* = Significant differences at the 95% confidence level, ns = not significant.

SPSS/PC+ package was used. According to BIRKS and KANOWSKI (1988, 1993) the resin data evaluation should be carried out with a variety of standard techniques, such as cluster analysis, principal components e.t.c. In our study one way analysis of variance (ANOVA) followed by a cluster analysis with the WARD method and Squared Euclidian distance (seuclid) was used to determine the number of chemotypes based on the relative amounts (%) of the four major terpenes i.e. α -pinene, 3- δ -carene, α -terpinyl acetate and cedrol after Z- standardization. The relative (%) amounts of the 4 selected characters have been calculated again based on the same 4 terpenes (Table 1). Cluster analysis for the determination of chemotypes has been applied in many conifer species (SCHILLER and GRUNWALD, 1986, 1987; VON RUDLOFF and LAPP, 1987; ZAVARIN et al., 1990; SCHILLER, 1990; CHANG and HANOVER, 1991; SCHILLER and GENIZI, 1993, LANG, 1994; GALLIS and PANETSOS 1997; GALLIS et al., 1998). For every provenance the frequency percentage of each one chemotype was calculated and as well the chemotype pattern determined (Figure 1).

Results

Cortex terpene analysis by GLC-MS of all samples revealed that twenty nine components are present twenty one of which could be identified. The identified terpenes were: thujene, α -pinene, fenchene, camphene, sabinene, β -pinene, myrcene, 3- δ -carene, p-cymene, limonene, β -ocimene, terpinolene, linalool, camphoral, terpinen-4-ol, terpinen-4-ol acetate, α -terpinyl acetate, cedrene, caryophyllene, g-cadinene, cedrol. The 4 terpenes: cedrol, α -pinene, 3- δ -carene and α -terpinyl acetate were found to be the major components since they occurred in all samples in relatively large amounts. The remaining components are minor since they occurred either in traces or in relatively small amounts and were not evaluated. The amounts of the four major terpenes evaluated varied between the provenances studied. The analysis of variance showed significant differences among the provenances only for the characters α -pinene and cedrol at the 95% confidence level (Table 1).

The resin of provenance Fress contains high amount of α -pinene (57.4%) and the lowest amount of cedrol (7.9%). The lowest amount of α -pinene (48.2%) charac-

terises the resin of provenance Anopolis. It also contains the highest amounts of the characters 3- δ -carene and α -terpinyl acetate (26.0% and 11.7% respectively) as well high amount of cedrol (14.0%). The resin of provenance Askifou characterised by the highest amount of α -pinene (58.3%) and low amount of α -terpinyl acetate (8.9%). The resin of provenance Omalos contains a high amount of the character α -pinene (54.9%) and lowest amount of α -terpinyl acetate (7.5%) among all provenances investigated. The highest amount (14.1%) of cedrol and the lowest amount (22.5%) of 3- δ -carene characterize the resin composition of provenance Prasses. On the other hand, the resin of provenance Zourva contains the highest amount (26.0%) of 3- δ -carene and low amount (49.4%) of α -pinene.

A numerical analysis by WARD cluster analysis (SPSS/PC) of the 106 trees under investigation based on (%) amount of the four selected terpenes was carried out. The result of cluster analysis was the classification of all trees in five chemotypes which occur at different frequencies. Both chemotypes A and E occur with the same frequency 23.5% (25 trees), chemotype B occurs with frequency 14.1% (15 trees). The lowest total number of 10 trees (9.4%) comprises chemotype D, while chemotype C comprises the highest total number of 31 trees (29.2%). Each of the cypress provenances is composed by an ensemble of chemotypes occurring at various frequencies (Figure 1).

By means of the terpene composition of every individual tree included in each chemotype, the mean terpene (%) composition for each chemotype was calculated. Chemotype A contains high amounts of α -pinene (59.9%) and 3- δ -carene (24.7%) and low amounts of the other terpenes. Chemotype B is characterized by the highest amount of α -pinene (64.9%) and lowest amounts of 3- δ -carene (17.3%) and α -terpinyl acetate (6.8%) compared with the other chemotypes. Furthermore, the highest amount of 3- δ -carene (33.2%) characterizes the terpene composition of chemotype D. Chemotypes C and E contain similar amounts of α -pinene (45.4% and 46.4% respectively) but they differ in the amounts of the rest characters. Indeed, chemotype C characterized by the extremely high amount (24.2%) of the character cedrol compared to other chemotypes. Finally, chemotype E contains the highest amount (15.8%) of the character α -terpinyl acetate among all chemotypes.

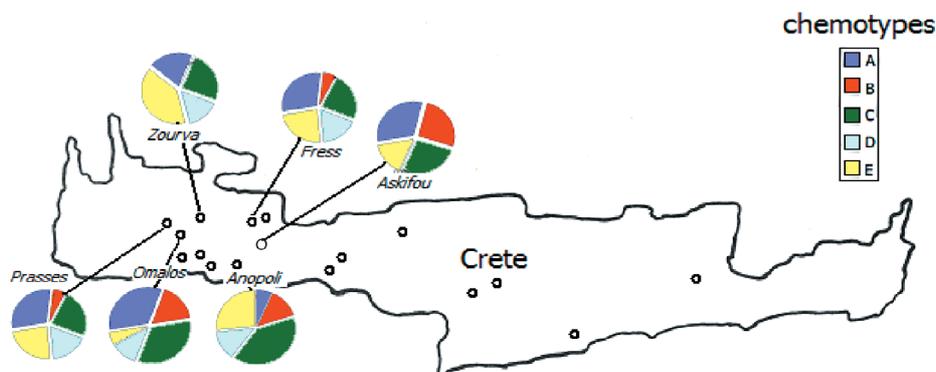


Figure 1. – Provenance chemotype pattern and distribution of *Cupressus sempervirens* L. in the Crete island. A, B, C, D, E: Chemotypes. ○: Cypress natural populations.

Discussion and Conclusion

Several studies dealing with the terpene composition of *Cypressus sempervirens* were conducted in the past. The resin composition in different tissues of cypress was determined by GARNERO et al. (1977), PIOVETTI et al. (1981), ANONYMOUS (1982), LOUKIS et al. (1991) and KASSEM et al. (1991). They all report also four major compounds (α -pinene, 3- δ -carene, α -terpinyl acetate, cedrol) in high quantities to be present in resin composition. SCHILLER (1990, 1993), SUFRA et al. (1990) and YANI et al. (1990) have used the terpene composition of cypress in order to describe its variation, to develop chemotaxonomic relationships and to identify clones. Differences in terpene composition in our research with the above mentioned studies should be the result of factors such as different kind of tissue, age of the trees analyzed, conditions of chromatographic analysis, etc. Recently, the qualitative and quantitative terpene composition of essential oils in seven *Cupressus* species, except *Cypressus sempervirens*, was determined by LEANDRI et al. (2003). These authors identified many monoterpenes as well as several sesquiterpenes and diterpenes in the essential oils of the *Cupressus* species analyzed. They also reported qualitative and quantitative differences in terpene composition proving that terpenes can offer a valuable tool for *Cupressus* species classification.

For the evaluation of our resin data and the determination of chemotypes a cluster analysis was applied. The results of the analysis revealed the classification of all the trees investigated in five chemotypes that occurred at different frequencies within cypress provenances. Three chemotypes (A, C and E) are common and occurred in all provenances. The chemotypes B and D are not present in all provenances and their frequencies range from zero to 26.3% and 17.6% respectively.

Based on chemotype distribution two main groups of provenances can be consisted. Group I includes the provenances Askifou, Fress and Zourva characterized by the complete absence of one chemotype. Group II includes provenances Omalos, Anopolis and Prasses characterized by the presence of all five chemotypes identified. From a general point of view, a smaller number of chemotypes implies lower genetic variability. So, the provenances belonging to group I appear to be with lesser genetic variability if compared to the provenances of group II.

The two provenances Askifou and Fress are characterized by the complete absence of the chemotype D. It should be noted here that provenance Fress includes trees of both cypress morphs (*pyramidalis* and *horizontalis*). The occurrence of the chemotypes C and E with the same frequency in combination with the complete absence of chemotype D suggests low genetic variability in the Fress provenance. Genetic studies based on isozyme analysis of the same provenances that are studied in our research have shown that provenance Fress contains less variability than the rest of the provenances (PAPAGEORGIU et al., 1994; PAPAGEORGIU, 1995). Differences in resin composition between the two morphs, *horizontalis* and *pyramidalis* were reported

also by SCHILLER (1990) in *Cypress* provenances grown in Israel. Isozyme based genetic studies indicate gene flow of introduced cypresses, growing in settlements nearby, into the natural stand of provenance Fress (PAPAGEORGIU, 1995). The results of the present study support this indication. We assume that a possible gene flow from var. *pyramidalis* in var. *horizontalis* was happened in the past. As mentioned above, provenance Askifou is characterized by the absence of chemotype D. Besides that, the chemotypes B and C occur with the same (26.3%) frequency. If compared to provenance Fress differences exist in the frequencies of chemotypes A and E. Based on chemotype pattern, it could be proposed that provenance Askifou has also low genetic variability. The two provenances Askifou and Fress are the most eastern provenances, growing on completely different sites and exposure. However, they both belong to a North-South wind passage, which defines the region in Crete with the highest precipitation. Especially the origin of provenance Askifou is found to have the highest precipitation all over Greece (PAPAGEORGIU, 1995). GHOSN et al. (2000) have analysed the same provenances using random primers (RAPDs) and have also grouped provenances Askifou and Fress close together.

Provenance Zourva is characterized by the absence of chemotype B as well by high frequency (40.0%) of chemotype E. This particular chemotype distribution differentiates the provenance Zourva from the rest of the same group. It could be proposed here to divide the first group into two subgroups. Subgroup I include the provenances Askifou and Fress that are characterized by the absence of chemotype D and the presence of chemotype B. Subgroup II contains only the provenance Zourva that is characterised by the presence of chemotype D, the absence of chemotype B and high frequency of chemotype E. The small number of chemotypes found in the Zourva provenance as well their distribution among its individuals, points to a low genetic variability in this provenance, too.

Concerning the second group, the provenances Anopolis, Omalos and Prasses are characterized by the occurrence of all five chemotypes. Chemotype D characterizes only a small number of trees, (10 trees or 9.4%, in total). This fact implies a rare genetic composition (SCHILLER et al., 1986) for the provenance Prasses. Provenance Anopolis possesses the highest frequency (40.0%) of chemotype C and the lowest frequency (6.6%) of chemotype A which actually means just one individual tree. The frequency of this chemotype among provenances ranges from 17.6% to 33.3%. The very rare occurrence of chemotype A in combination with the highest frequency of chemotype C represents a very specific pattern which characterizes this provenance and suggests a particular genetic makeup. The provenance Anopolis is growing at high elevation (*Table 1*) sites with relatively cold winters and dry summers as well poor soils. So, the environmental factors seem to be more or less responsible for this genetic composition. A relation between terpene composition and environmental factors of the provenance original sites has been also reported by TOBOLSKI and HANOVER (1971), RUBY and WRIGHT (1976), FORREST (1987) and HANOVER (1992). Provenance Omalos is char-

acterized by the very low frequency (5.5%) of chemotype E as well by the same highly frequent occurrence (33.3%) of both chemotypes A and C, which together occur in 66.6% of the Omalos trees. This specific chemotype pattern may possibly be a consequence of the particular genetic composition characterizing the provenance Omalos.

From our results it could be suggested that the genetic variability is less in the provenances growing in low elevation stands than the provenances growing in high elevation stands. As mentioned above, all provenances investigated represented natural stands but low elevation stands probably have undergone mismanagement by human populations. Domesticated cypress populations have been found to possess much lower genetic diversity than natural populations (PAPAGEORGIOU, 1995).

In conclusion, quantitative differences in cortex terpene composition seem to be a very useful tool to define the genetic variability between cypress provenances grown in Crete. The patterns of variation and differentiation observed in this study indicate that cypress in Crete is well adapted and – despite the fragmentation of its populations – maintains high diversity levels. In situ conservation measures, including mainly the reduction of anthropogenic pressure, are necessary, in order to secure the existence of the existing populations and keep valuable genetic resources for reforestations in sites with adverse ecological conditions.

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