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Pinus halepensis x *Pinus brutia* subsp. *brutia* Hybrids? Identification Using Morphological and Biochemical Traits[1])

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Summary

The aim of this study was to verify whether trees which exhibit very vigorous growth are F_1-hybrids of *Pinus halepensis* X *Pinus brutia,* and to what extent they differ from typical *P. brutia* subsp. *brutia* trees growing in the same plantations and from typical native *P. halepensis* MILL. trees.

Comparison of data regarding morphological traits and isoenzyme analysis of biological material taken from *P. halepensis, P. brutia* and the very vigorously growing trees, provided evidence that the trees targeted are F_1-hybrids. The results indicate that the seed used to establish these *P. brutia* forest plantations was probably imported from sites in Greece where *P. halepensis* and *P. brutia* grow in geographic proximity.

Key words: Isozymes electrophoresis, morphological traits.

FDC: 165.3; 165.51; 165.7; 174.7 *Pinus halepensis* x *Pinus brutia* subsp. brutia.

Introduction

Recently, attention was drawn to few trees in 2 plantations due to their morphological traits and vigorous growth which separate them from the other trees in these plantations. These plantations were planted in Israel 19 years ago with *Pinus brutia* subsp. *brutia* a introduced species. One plantation was planted on the Mt. Carmel range near the Muchraka peak (32° 42' lat. N., 35° 04' long E., alt. of 425 m a. s. l.), the second in the Judean foothills near Beqoa (31° 51' lat. N., 35° 04' long. E., alt. 185 m a.s.l.). The seed used for planting was registered as imported from Greece in 1975. HETH (1990) suggested that these trees might be hybrids, probably F_1-hybrids of *P. halepensis* X *P. brutia* which exhibit heterosis as their growth rate is 160% of the largest *P. brutia* tree in these plantations. These trees are also less susceptible to the pine bast scale, *Matsucoccus josephi* BODENH. et HARPAZ, the most noxious insect of planted Aleppo pine (*P. halepensis* MILL.) in Israel (MENDEL, 1984).

The aim of this study was to verify whether these trees are F_1-hybrids, and to what extent they differ from *P. brutia* subsp. *brutia* trees growing in the same plantations and from *P. halepensis* MILL. trees.

Material and Methods

Two different methods were used to analyze the trees thought to be hybrids in comparison with *P. brutia* subsp. *brutia* trees growing in the 2 plantations and native Israeli *P. halepensis* MILL. trees growing on the Mt. Carmel range.

1. Morphological-anatomical method and traits measured

Morphological traits were analyzed using methods described by CALAMASSI *et al.* (1988), CALAMASSI (1986), DEBAZAC and TOMASSONE (1965), PANETSOS (1975) and RIVA and VENDRAMIN (1983).

a. Two-year-old healthy needles were taken at random from 10 branches at the upper part of the canopy. Twenty needles selected at random from these branches were used to measure the length, width and number of stomata rows per needle. Fresh and dry weights were determined on 50 needles selected at random. Dry weight was measured after 36 hours at 70 °C.

b. Cone length and the largest diameter, petiole length and angle between cone length axis and the branch bearing it were measured on 10 cones selected at random from the last year crop.

c. Seed weight, and seed and wing length were measured on 20 seeds selected at random from approximately 800 seeds, the seed yield of 10 cones per tree.

d. Anatomical slides of radial, tangential longitudinal and cross-sections of xylem wood were prepared from each of the 28 hybrid trees and of some of the 22 *P. brutia* and 10 *P. halepensis* trees to evaluate differences in number of resin ducts and their dispersal within the year-ring, number and shape of cross-field pits.

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2. Biochemical methods

Using the methods described in the laboratory manual by CONKLE *et al.* (1982), with several adjustments, starch gel electrophoresis of isoenzymes was performed to analyze allele frequencies in several enzyme systems in which, on the basis of previous knowledge, *P. halepensis* differs significantly from *P. brutia* (CONKLE *et al.*, 1988; GRUNWALD *et al.*, 1986; SCHILLER *et al.*, 1986). Use was made of six endosperms (maternal haploid tissue) from each single tree. Seeds of 21 hybrid trees, 17 *P. brutia* subsp. *brutia,* and 10 native Israeli *P. halepensis* trees were analyzed.

The maternal tissue was homogenized in a grinding plate together with 35 µl of 0.2 M phosphate buffer pH 7.5 (CONKLE *et al.*, 1982), 0.1% Triton x-100, 1% BSA, 0.1% DTT for all enzyme systems. Four different electrophoresis buffer systems were used:

System I, Gel buffer: 0.02M Tris, 0.02M boric acid, 0.002M EDTA, pH 8.4.
- Electrode buffer: 0.2M Tris, 0.2M boric acid, 0.002M EDTA, pH 8.4.

System II, Gel Buffer: 0.01M Tris, 0.005M citric acid, pH 8.8.
- Electrode buffer: 0.05M NaOH, 0.3M boric acid pH 8.0.

System III, Gel buffer: 0.002M citric acid, adjusted with morpholine [N-(3-aminopropyl)] to pH 6.1.
- Electrode buffer: 0.04M citric acid, adjusted with morpholine [N-(3-aminopropyl)] to pH 6.1.

System IV, Gel buffer: 0.002M citric acid, adjusted with morpholine [N-(3-aminopropyl)] to pH 8.3.
- Electrode buffer: 0.04M citric acid, adjusted with morpholine [N-(3-aminopropyl)] to pH 8.3.

Following electrophoresis, gels were sliced and stained for each enzyme system according to CONKLE *et al.* (1982).

Statistical analysis of the data was done using the BIOSYS-1 computer program for the analysis of alleleic variation in genetics (SWOFFORD and SELANDER, 1981).

Results

1. Morphological-anatomical method

Means and coefficient of variation of the morphological traits measured, and the results of DUNCAN's Multiple Range Test, are given in *table 1.* Significant interspecific differences were found in several morphological traits, as follows:

a. Needle characteristics: (i) Hybrid needles were thicker than those of *P. halepensis* but thinner than those of *P. brutia.* (ii) Length of needles of *P. brutia* and hybrid trees was similar, and differed significantly from that of *P. halepensis,* which was much shorter. (iii) Number of stomata rows per needle was lowest in *P. halepensis* and largest in *P. brutia,* whereas hybrids had an intermediate number of rows. (iv) *P. halepensis* needles had the lowest fresh and dry weights, *P. brutia* needles the heaviest, and needles of hybrid trees had an intermediate weight.

b. Cone characteristics: (i) Cones of hybrid trees were significantly longer than those of *P. halepensis* and *P. brutia,* but differences in cone diameter were of mixed nature and minor. (ii) Petiole length of *P. brutia* cones was very short in comparison with the petioles of *P. halepensis* cones; that of hybrids was intermediate. (iii) The angle between the cone axis and the branch was widest in *P. brutia* (>100°), intermediate in the hybrid (50° to 61°) and smallest in *P. halepensis* (49°). The intraspecific differences in *P. brutia* and the hybrid trees can be attributed to site characteristics. These results are similar to those presented by PANETSOS (1975).

c. Seed characteristics: No significant differences were found in seed characteristics because of the very large variation in seed weight and in seed and wing length. These results are in contradiction to earlier findings by DEBAZAC and TOMASSONE (1965) and PANETSOS (1975) who found very significant differences between *P. halepensis* and *P. brutia* in their seed characteristics.

Except for the results concerning the seed characters, those regarding needle and cone characters are in agreement with

Table 1. – Means and coefficient of variation (c.v.) of the measured parameters of the different species and sites.

	Mt. Carmel				Beqoa					
	Hybrid		P. brutia		Hybrid		P. brutia		P. halepensis	
Parameters measured	mean	c.v.	mean	c.v.	mean	c.v.	mean	c.v.	mean	c.v.
Needle length (cm)	11.30	13.50 a	12.90	11.20 a	11.60	15.60 a	12.20	12.50 a	7.00	8.10 b
Needle width (mm)	1.17	0.70 b	1.41	0.50 a	1.10	0.84 b	1.34	1.37 a	0.98	0.56 c
Number of stomata rows	10.60	9.30 c	12.80	4.30 a	9.60	10.40 d	11.70	11.90 b	8.80	8.30 d
Needle wet weight (g)	7.28	26.80 c	12.60	17.30 a	7.11	27.50 c	10.49	18.70 b	3.50	13.70 d
Needle dry weight (g)	3.87	24.50 c	6.36	15.10 a	3.64	26.50 c	5.32	20.30 b	1.69	16.20 d
Cone length (cm)	9.60	7.70 a	7.30	5.90 c	8.30	13.40 b	7.10	13.40 c	7.60	15.00 c
Cone width at half length (cm)	4.20	8.20 a	4.00	8.00 ab	3.70	13.50 cb	4.00	12.40 ab	3.40	8.20 c
Cone's stem length (cm)	1.15	23.60 b	0.34	37.00 c	1.15	33.20 b	0.26	66.20 c	1.98	14.80 a
Angle between cone length axis and the branch (deg.)	60.80	18.20 b	113.30	10.40 a	53.80	20.40 b	106.30	14.00 a	48.50	35.40 b
Length of seed and wing (cm)	3.21	9.10 a	2.55	6.40 a	2.93	9.90 a	3.22	46.90 a	2.60	12.50 a

*) within rows, data not followed by a common letter differ significantly at P = 0.05, according to DUNCAN's Multiple Range Test.

Table 2. – Allele frequencies, observed and expected heterozygosity.

		Allele frequencies			Heterozygosity					
					observed			expected		
Enzyme/ Locus	**Allele**	**halep.**	**brutia**	**hybrids**	**halep.**	**brutia**	**hybrids**	**halep.**	**brutia**	**hybrids**
Aco	1	0.850	0.190	0.524	0.100	0.240	0.875	0.255	0.360	0.500
	2	0.150	0.810	0.476						
Acp	1	1.000	0.770	0.524	0.000	0.470	0.524	0.000	0.360	0.500
	2	0.000	0.240	0.476						
Adh-1	1	0.000	0.440	0.095	0.000	0.290	0.095	0.000	0.490	0.170
	2	1.000	0.560	0.905						
Adh-2	1	0.700	0.560	0.595	0.857	0.880	0.810	0.490	0.490	0.480
	2	0.300	0.440	0.405						
Gdh	1	1.000	0.000	0.500	0.000	0.000	0.900	0.000	0.000	0.500
	2	0.000	1.000	0.500						
Idh	1	1.000	0.000	0.500	0.000	0.000	0.900	0.000	0.000	0.500
	2	0.000	1.000	0.500						
Mdh-1	1	1.000	0.250	0.714	0.000	0.120	0.475	0.000	0.360	0.410
	2	0.000	0.750	0.288						
Mdh-4	1	0.300	0.000	0.595	0.000	0.000	0.714	0.420	0.000	0.480
	2	0.700	0.000	0.406						
6Pgd-1	1	0.000	0.650	0.476	0.000	0.000	0.286	0.000	0.490	0.630
	2	0.000	0.060	0.238						
	3	1.000	0.290	0.286						
6Pgd-2	1	0.000	0.120	0.818	0.000	0.000	0.000	0.000	0.520	0.540
	2	0.000	0.650	0.091						
	3	0.000	0.240	0.091						
Mean					0.096	0.200	0.558	0.116	0.310	0.470
S.D.					0.269	0.289	0.338	0.196	0.220	0.120

*) halep. = *Pinus halepensis* MILL.; brutia = *Pinus brutia* subsp. *brutia*

the findings of PANETSOS (1975, 1981) and MOULOPOULOS and BASSIOTIS (1961), who showed that hybrid morphological characters are intermediate in their measures between the 2 parent species.

In contrast to the differences found in morphological traits between the 2 species and the hybrid trees no differences were found in anatomical traits investigated, *viz.,* number and shape of cross-field pits, and number and distribution of resin ducts within a year-ring. These findings are in contrast to the results reported by LIPHSCHITZ and BIGER (1991) who found differences in this traits between *P. brutia* and *P. halepensis.*

2. Biochemical methods

The eight enzyme systems analyzed, using starch gel electrophoresis, allele frequenciec and results of statistical analysis are presented in *table 2.*

Gels stained for ACO had 1 zone of activity. Two alleles were found, which were exhibited by 2 one-band variants as homozygous, and by two-band variants as heterozygous. This locus is polymorphic in *P. halepensis* and *P. brutia.* In *P. halepensis* allele 1 and in *P. brutia* allele 2 were more frequent, whereas in hybrids allele 1 and 2 had nearly the same frequency.

Gels stained for ACP showed 1 zone of activity in *P. halepensis* and 2 zones of activity in *P. brutia* and the hybrids, i. e., ACP-1 and ACP-2. ACP-1 always stains very intensively and shows a single band, whereas ACP-2 stains more faintly. This might explain the fact that ACP-2 was not detected in *P. halepensis.* This locus was polymorphic in *P. brutia* but monomorphic and presumedly fixed in *P. halepensis* for one of the alleles present in *P. brutia,* whereas in hybrids, allele 1 and 2 had nearly the same frequency.

Gels stained for ADH showed 2 zones of activity, ADH-1 and ADH-2. The faster migrating zone ADH-1 stained faintly. In *P. halepensis.* ADH-1 locus was monomorphic and fixed for allele 2; in *P. brutia* and hybrid trees,- it was polymorphic with high frequency of allele 2. The ADH-2 locus was polymorphic in all species.

Gels stained for GDH showed one zone of activity that was controlled by a single locus. This locus was fixed for alternative alleles in *P. brutia* and *P. halepensis;* hybrids showed the 2 alleles in equal frequencies.

Gels stained for IDH showed 2 loci in 2 zones of activity; the faster moving zone always stained more intensively, it was monomorphic and fixed for the same allele in all trees analyzed. The slower moving zone IDH-2 appeared lightly stained and fixed for alternating alleles in *P. halepensis* and *P. brutia.* In hybrids, the 2 alleles had similar frequencies.

Gels stained for GDH, likewise gels stained for IDH, the locus IDH-2 was fixed for alternative alleles in *P. brutia* and *P. halepensis;* hybrids showed both alleles in equal frequencies.

Gels stained for MDH usually show 4 zones of activity, *viz.,* 4 loci. In our analysis, only 3 loci and a heterodimer could be easily read. MDH-1 was monomorphic and fixed for allele 1 in *P. halepensis;* in *P. brutia* allele 2 had a 3 times higher frequency than allele 1; in hybrid trees, allele 1 had a 3 times higher frequency than allele 2. Very weakly detectable bands with the same mobility occurred at the same site which we have

associated with MDH-3. In MDH-4, which had very slow mobility, 2 single band variants and 1 two-band variant which was assumed to be heterozygous were detected. This locus was polymolphic in *P. halepensis* and monomorphic and fixed for allele 2 in *P. brutia* (CONKLE *et al.*, 1988). In the present study, probably due to changes in the pH of the system used, MDH-4 allele 2 in *P. brutia* was not detected; in hybrid trees the 2 alleles had equal frequencies. In the present study allelic variation was noted only in MDH-1 and MDH-4, although in other conifer species variations in loci 2 and 3 have been reported (SCALTSOYIANNES *et al.*, 1994; THORMANN and STEPHAN, 1993).

Gels stained for 6PGD showed 3 zones of activity (SCHILLER *et al.*, 1986). This enzyme is usually dimeric (HERRIS and HOPKINSON, 1976), and therefore heterozygotes are exhibited by triple-band variants. Difficulties appeared in the interpretation of allele 1 of 6PGD-1 and allele 3 of 6PGD-2 due to a very small difference in migration rates. 6PGD-3 was always monomorphic in the 2 species and fixed for allele 1. In *P. halepensis*, 6PGD-1 was fixed for allele 3 and in *P. brutia* and hybrid trees allele 1 had the highest frequency. 6PGD-2 was not detected in *P. halepensis;* in *P. brutia,* allele 2 had the highest frequency, whereas allele 1 had the highest frequency in hybrid trees.

Comparison between results presented in *table 2* with those published in earlier studies (CONKLE *et al.*, 1988; GRUNWALD *et al.*, 1986; SCHILLER *et al.*, 1986) show several differences. In *P. halepensis,* differences occurred in the following enzyme systems: ADH-1 was not observed in earlier studies; ADH-2 was monomorphic and fixed for allele 1. MDH-4 allele 1 and 2 frequencies were in contrast to these found in our study. Large differences were found in allele frequencies in the 6PGD-1 and 6PGD-2 enzyme systems. In earlier studies 6PGD-1 and 6PGD-2 were nearly monomorphic and fixed for allele 1; whereas in our study, 6PGD-1 was fixed for allele 3 and 6PGD-2 could not be identified on the gels. In *P. brutia* several differences from an earlier study (CONKLE *et al.*, 1988) were also observed as follows: ACP has been reported to have 2 alleles with equal frequencies, whereas in our study allele 1 had a 3 times higher frequency than allele 2. ADH-1 was not observed in earlier studies, and ADH-2 was nearly fixed for allele 2. In our study the 2 alleles of ADH-2 had nearly equal frequencies. MDH-4 was not observed in our study whereas in earlier studies it was monomorphic and fixed for allele 2. There are large differences in allele frequencies in 6PGD-1 and 6PGD-2. Whereas in an earlier study (CONKLE *et al.*, 1988) 6PGD-1 had 2 alleles, the first one with a mean frequency of 0.90, we detected 3 alleles.

Table 3. – Observed single locus segregation of allozymes in hybrid trees.

Enzyme/ Locus	Allele-1	Allele-2	Allele-3	chi-square	P
Aco	**22**	**20**		**0.100**	**0.760**
Acp	**22**	**20**		**0.100**	**0.760**
Adh-2	**25**	**17**		**1.520**	**0.220**
Gdh	**21**	**21**		**0.000**	**1.000**
Idh	**21**	**21**		**0.000**	**1.000**
Mdh-1	**30**	**12**		**7.710**	**0.010**
Mdh-4	**25**	**17**		**1.520**	**0.220**
6Pgd-1	**17**		**9**	**2.460**	**0.120**

Very different allele frequencies were detected also in 6PGD-2; in the earlier study only 2 alleles where detected, allele one frequency was 0.690; in the present study 3 alleles where detected.

Observed and Expected heterozygosity for each of the loci analyzed in the 3 species are also shown in *table 2.* In *P. halepensis,* expected heterozygosity was 0.116, in *P. brutia* 0.310 and hybrids 0.470. Comparison of results obtained with those from earlier studies show that heterozygosity was lower in *P. halepensis* and higher in *P. brutia* in the present study than in earlier studies (CONKLE *et al.*, 1988; GRUNWALD *et al.*, 1986); the heterozygosity of hybrid trees was more than twice that found in an earlier study (unpublished).

Results of a chi-square test to determine the goodness of fit of segregating allozymes to the expected 1:1 ratio for megagametophytes from the hybrid mother trees, are shown in *table 3.* The data show that severe distortion from the 1:1 ratio was detected only in the MDH-1 locus.

Discussion and Conclusions

The occurrence of natural hybrids of *P. halepensis* X *P. brutia* in Greece, at sites were *P. brutia* was planted within the range of *P. halepensis,* was reported by PAPAIOANNOU (1936) and PANETSOS (1975), who investigated this phenomenon using morphological traits only. Results obtained in our study using these morphological markers verify earlier assumptions that trees which where the subject of this study are indeed hybrids of *P. halepensis* X *P. brutia.* In our case, the morphological traits analyzed have a greater similarity with *P. halepensis* than with *P. brutia.* Using biochemical markers, the present results which show the occurrences of the 2 alleles in equal frequencies in GDH and IDH enzyme systems, the nearly equal frequencies of alleles in other enzyme systems analyzed, establishes that the trees under consideration are F1-hybrids of *P. halepensis* X *P. brutia* subsp. *brutia.*

Differences between our results and earlier ones, quoted above, in loci identifications and allele frequencies might be the result of several factores: (i) changes and adjustments of the buffere systems and the pH used in comparison to the systems used in earlier studies (CONKLE *et al.*, 1982). (ii) limited number of trees analyzed in comparison with the numbers analyzed in the earlier studies cited. (iii) the fact that *P. brutia* subsp. *brutia* is not native to mainland Greece but planted (MENDEL and SCHILLER, 1993) and the seed material imported was probably collected in a plantation and; (iv) hybrids where the result of pollination of planted *Pinus brutia* by Greek *Pinus halepensis.*

There is an ever-growing desire to use hybrids of *Pinus halepensis* X *Pinus brutia* subsp. *brutia* that combine on the one hand the (a) high drought resistance, (b) capability to grow on highly calcareous bedrock formations and soils, (c) high quality and quantity of resin production and, (d) relatively rapid growth rate in early years of *P. halepensis,* with on the other hand the higher timber quality of *P. brutia.* Artificial hybridization between these two species has proved very successful when *P. halepensis* was the pollen donor, whereas reciprocal pollination proved unsuccessful (BASSIOTIS, 1972; DUFFIELD, 1952; MOULOPOULOS and BASSIOTIS, 1961; MOULALIS *et al.*, 1976; RIVA and VENDRAMIN, 1983). *P. halepensis* X *P. brutia* hybrids have shown heterosis (MOULOPOULOS and BASIOTIS, 1961; PANETSOS, 1981) probably due to their higher heterozygosity than that of the parent trees, which is at the most only about 0.056 in *P. halepensis* (SCHILLER *et al.*, 1986) and 0.118 in *P. brutia* (CONKLE *et al.*, 1988). Positive relationships between allozyme heterozygosity and tree growth rate, and higher

resistance to environmental stresses have been reported by KNOWLES and MITTON (1980), KNOWLES and GRANT (1980), LEDIG *et al.* (1983), LARSEN (1986).

The vigorous growth, of the few imported hybrid trees, under contrasting environmental conditions in Israel points to success of afforestations with: (i) local artificially developed hybrids between plus trees of Israeli *P. halepensis* X *P. brutia* and/or (ii) selection of plus trees among the identified hybrid trees and their vegetative propagation for the establishment of seed orchards.

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In Vivo Grafting and *In Vitro* Micrografting of *Acacia mangium*: Impact of Ortet Age

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Abstract

The possibilities of vegetatively propagating juvenile – 6-month-old – and mature – 3 to 5 year-old – *Acacia mangium* ortets by grafting were investigated using *in vivo* and *in vitro* techniques. The average success rates obtained for *in vivo* top-cleft grafting were 49% for scions coming from juvenile plant material and 0% when collected from mature ortets. *In vitro* micrografted apices gave rise to 52% and 46% of successfully established micrografts for the juvenile and the mature plant material respectively. No significant difference between juvenile and mature origins in terms of grafting success was observed for *in vitro* micrografting of shoot apices. However, the ones coming from the juvenile ortets elongated more readily than those from the mature origin which were more prone to rest. Overall, the *in vitro* micrografting technique used appeared to be an helpful tool for vegetative non-destructive propagation of mature selected *Acacia mangium* ortets, apparently recalcitrant to more conventional *in vivo* grafting techniques.

These results are discussed in terms of scion size and the related potential for grafting in relation to the age of the ortet.

Key words: Acacia mangium, age, grafting, *in vitro*, micrografting, ortet, shoot apex, vegetative propagation.

FDC: 165.442; 176.1 *Acacia mangium*.

Introduction

Grafting has been extensively used for centuries for asexually propagating tree species, mainly for fruit production. This vegetative propagation technique is still broadly utilized in