# LACK OF ADH POLYMORPHISM DETECTED BY STARCH GEL ELECTROPHORESIS WITHIN AND AMONG THREE POPULATIONS OF ALEPPO PINE IN ALGERIA

A. HARFOUCHE[1,2], S. GUENDOUZE[1] AND O. KEBAB[1]
1 - INRF, B.P 37 Chéraga, Alger, Algérie.
2 - Author for correspondence.

***ABSTRACT***

A horizontal starch gel electrophoresis has been carried out to determine allele frequencies at locus $Adh_2$ encoding for an alcohol dehydrogenase variant in Aleppo pine. Three populations, Zeralda, Setif and Tebessa, were studied. The locus revealed to be diallelic and characterized by a high frequency of the $Adh_{22}$ allele which codes for the slow allozyme. This allele was fixed in Zeralda population. Estimated genetic parameters showed a deficiency of heterozygotes and a weak genetic diversity at whole and within-population levels. The results are discussed in the light of available information on genetic diversity and control of ADH in Aleppo pine and about their consequences on the ability of the populations of the species to withstand environmental changes.

***Key Words*** **:** Aleppo pine, Starch gel electrophoresis, Genetic Diversity.

***RÉSUMÉ***

Une électrophorèse sur gel d'amidon a été mise en œuvre dans le but de déterminer les fréquenses alléliques au locus $Adh_2$ qui code pour un variant de l'alcool déshydrogénase chez le pin d'Alep. Trois populations, Zéralda, Sétif et Tébessa ont été étudiées. Le locus en question se révèle être biallélique dans l'échantillon de populations considéré et caractérisé par une haute fréquence de l'allèle $Adh_{22}$, qui code pour l'allozyme lent ; cet allèle est fixé dans la population de Zéralda. Les paramètres génétiques estimées à partir des fréquences alléliques observées et théoriques montrent un déficit d'hétérozygotes et une faible diversité génétique aux deux niveaux intra- et inter-populations. Les résultats sont discutés à la lumière de l'information disponible sur la diversité génétique et le contrôle de l'alcool déshydrogénase chez le pin d'Alep et relativement à la capacité des populations de l'espèce de faire face aux éventuels changements environnementaux.

***Mots Clés*** **:** Pin d'Alep, électrophorèse sur gel d'amidon, diversité génétique.

## INTRODUCTION

Aleppo pine (*Pinus halepensis* Milkl.) is a member of the *Halepensis* group which also includes Brutia pine (*Pinus brutia Tenore*) with its various forms (*Pinus eldarica, P. stanckewiczii, P. pithyusa*). Pines of this taxon are well adapted to severe dryness, which leads some authors to qualify them as "Dry country pines" (Spencer, 1985).

It is, nowadays, largely accepted that Aleppo pine is a distinct species from its middle-eastern vicariate brutia pine (Mirov, 1955 ; Mirov *et al.,* 1966 ; Nahal, 1962 ; Allegri, 1974). Morphological and physiological traits and/or biochemical and molecular markers have been used in order to refine the taxonomy and the phylogeny of the species and to evaluate its geographical and genetic variations (Iconomou *et al.,* 1964 ; Palmberg, 1975; Bellefontaine, 1979 ; Pelizzo & Tocci, 1978 ; Calamassi, 1986 ; Fisher *et al.,* 1986 ; Schiller *et al.,* 1986 ; Schiller & Grunwald, 1987 ; Conkle *et al.,* 1988 ; Schiller & Waisel, 1989 ; Gallis & Panetsos, 1997 ; Gallis *et al.,* 1998 ; Agúndez *et al.,* 1999 ; Matziris, 2000 ; Gómez *et al.,* 2001 ; Fady, 2005).

Aleppo pine is a circum-Mediterranean species whose range stretches over 28 degrees of longitude and 15 degrees of latitude, from Morocco and South-western Europe to the Near East, with, however, more numerous and largest populations in the western part of the range (Mirov, 1967 ; Panetsos, 1981 ; Thanos & Skordilis, 1987). In the eastern part of its range, Aleppo pine more often than not occurs as scattered and relict stands (Nahal, 1962 ; Weinstein, 1989).

In Algeria, This species occupies some 850,000.00 ha colonizing territories from the coastal regions, north, to the Saharan Atlas range, south, where populations are submitted to various natural pressures.

Kadik (1986), in a study carried out on several natural stands of the Algerian range of the species, highlighted a significant geographical variation for adaptive and morphological traits suggesting geographical et ecotypic differentiation. However, experiments in common environments and statistical replication are required for determining the part of heredity in the phenotypic expression of the characters. At the local scale (Western region of Algiers), more recent studies in common environments with statistical replication revealed that the genetic variation of cone, seed and plantlet traits mostly resides within stands with, however, a multi-trait differentiation beginning to take shape, roughly dividing western and eastern local stands (Harfouche *et al.,* 2003).

An important effort is to be made to have a deep understanding of the quantitative genetics (adaptive and morphological characters, including growth and resistance to drought) as well as the population genetics (biochemical and molecular markers) of the species in its Algerian range. It is within this framework the work here presented was carried out which dealt with the electrophoretic polymorphism of ADH (Alcohol Dehydrogenase, E.C. 1.1.1.1) in some Algerian populations of Aleppo pine.

## MATERIALS AND METHODS

### *Plant material*

Three Algerian natural, or assumed as such, populations of Aleppo pine have been used for this work (Figure 1). These populations are separated by hundreds of kilometers.

In each population, seeds were collected from at least 30 individuals taking care that they were enough far apart (50 m, at the minimum) for reducing risks of collecting related trees. The cones (10/tree) of an individual were collected from branches of various aspects (westerly, easterly, northerly, southerly).

Bulk seed lots were constituted per population by mixing all the cones of all the individuals of a population. The seeds were gently dried and then stored in a refrigerator (4 °C).

### *Extraction of enzymes and procedure of electrophoresis*

Enzyme extraction, gel and electrode buffers were prepared following the procedures described in Conkle *et al.,* (1982) with slight modifications.

Seeds were germinated on a piece of filter paper regularly moistened in a Petri dish.

As soon as the radicle emerged 2-3 cm over the seed coat, the seed is dissected and the endosperm (haploid tissue reserve) and the embryo (diploid tissue) separated; the seed coat and the thin underlying layer, which is the remnant of the diploid nucelle, are removed.

The embryo and its endosperm are grinded separately in Eppendorf plastic tubes containing 50 µl of a Phosphate buffer (0.2 M ; pH 7.5) for the embryo and 75 µl for the endosperm. The Phosphate buffer of

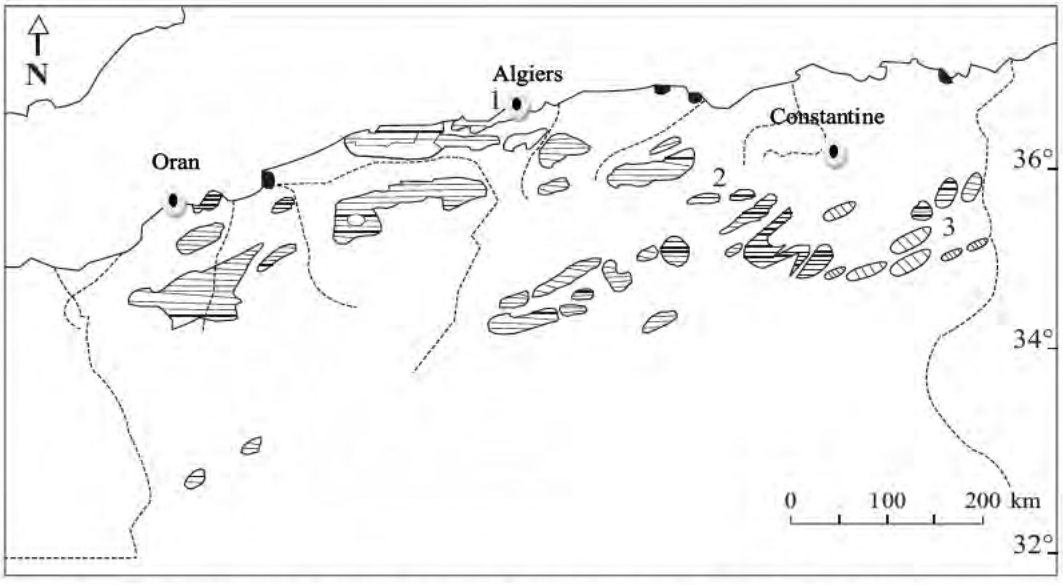


**Figure 1 :** The Algerian range of Aleppo pine and studied populations (1: Zeralda ; 2: Setif ; 3: Tebessa).

extraction is made of a mixture of 4.42 g Sodium phosphate monobasic monohydrate Sigma 3522 ($NaH_2PO_4$, $H_2O$) and 23.86 g Sodium phosphate dibasic anhydrous Sigma S3397 ($Na_2HPO_4$) dissolved in 1 l of distilled water.

The starch gel (12.5% of starch) is made of 47 g of Sigma starch S15651 cooked in a pH-8.3 buffer composed of 9 volumes of Tris-citrate pH 8.3 (6.2 g Trizma base Sigma T1503+1.46 g citric acid Sigma C7129+1000 ml of distilled water) and 1 volume of Lithium borate pH 8.3 (1.2 g Lithium hydroxide monohydrate Sigma L4256+11.89 g boric acid Sigma 0252+1000 ml distilled water).

The Lithium borate pH 8.3 described above is used also as electrode buffer.

The electrophoresis is carried out in a Maxi Plus tray connected to a Consort power supply unit working with a tension of 350 V and a current of 65 mA. While running, the tray is placed inside a refrigerator to prevent excessive heating.

***Staining enzymes***

For visualizing enzymes, we use 75 ml of a Tris-HCl buffer (0.05 M pH 8.0) containing a staining solution 1 ml absolute ethanol Riedel-de Haën 32205, 10 mg β-NAD Sigma N7004, 10 mg NBT (Nitro Tetrazolium Blue chloride) Sigma N6876 and 5 mg PMS (Phenazine methosulfate) Sigma P9625. The thin slices of gel are placed in containers containing the staining solution and put in an incubator at 37°C. After 30 to 60 minutes of incubation, zones of activity appear as spots on the slices.

***Interpretation of zymograms***

The count of the loci and alleles was done on the basis of the quaternary structure of ADH, which is a dimer, and the available knowledge and results on the alcohol dehydrogenase of Aleppo pine and other pines.

The enzyme is here transcribed in capitals followed by a first italicized Arabic numeral to refer to the involved locus and by a second italicized Arabic numeral which indicates the coding allele. For example, the transcription ADH*22* refers to alcohol dehydrogenase encoded by allele 2 at locus 2.

The encoding gene is transcribed in italicized lower-case letters preceded by a capital, also in italics. Loci are distinguished from each other by their electrophoretic mobility : the fastest (i.e. the closest to the anode) is pointed out by the numeral 1, the next by the numeral 2 and so on; the same for the alleles at a locus. For example, the transcription *Adh2* represents the locus 2 encoding alcohol dehydrogenase. Genotypes are transcribed as follows: *Adh2* (11) indicates homozygotes for the allele 1 at the locus 2, *Adh2* (12) indicates heterozygotes at the locus 2 and *Adh2* (22) indicates homozygotes for the allele 2 at the locus 2.

***Statistical analyses and genetic parameters estimate***

The probability to detect in a given population an allele X with a frequency f(X) is equal to :

$$\pi = 1 - (1 - f(X))^n \qquad [1]$$

Where n is the size of the sample representing this population (n=30 for Zeralda, n=10 for Setif and Tebessa).

Genotypic and allelic frequencies where deduced from the counts carried out on the zymograms.

At the within-population level, these frequencies allowed to calculate the following parameters : *ho*, the observed heterozygosity, *he*, the expected heterozygosity, *F*, the fixation index (Wright, 1965). In the case of a diallelic locus (2 alleles *i* and *j*), the allelic frequencies are calculated from the counts as follows :

$$Pi = \frac{Nii + \frac{1}{2} Nij}{Nii + Nij + Njj} \quad [2]$$

And,

$$Pj = \frac{\frac{1}{2} Nij + Njj}{Nii + Nij + Njj} \quad [3]$$

In addition,

$$p_j = 1 - p_i \quad [4]$$

And,

$$p_i + p_j = 1 \quad [5]$$

The observed heterozygosity is calculated as

$$ho = \frac{Nij}{Nii + Nij + Njj} \quad [6]$$

Where, *Nij* is the number of heterozygotes, *Nii*, the number of homozygotes bearing the allele *i*, *Njj*, the number of homozygotes bearing the allele *j* $(i \neq j)$.

The observed heterozygosity, under the hypothesis of Hardy-Weinberg equilibrium, is

$$he = 1 - \Sigma p_i^2 = 2p_i p_j = 2p_i (1 - p_i) \quad [7]$$

The local inbreeding coefficient of each population F is calculated as follows

$$F = 1 - \frac{ho}{he} = 1 - \frac{Nij /(Nii + Nij + Njj)}{2p_i (1 - p_i)} \quad [8]$$

At the whole level, i.e. the three populations, we calculated the following global F-statistics (Wright, 1965) : $F_{IS}$, which is the individual index of fixation, $F_{ST}$, which is considered also as a coefficient of differentiation among populations, and $F_{IT}$, which is the global fixation index based on global frequencies of the pooled populations.

$$F_{IS} = 1 - \frac{H_I}{H_S} \quad [9$$

With, $n_i$ being the size of the sample of the $i^{th}$ population

$$H_I = \sum_i n_i ho_i \Big/ \sum_i n_i \quad [10]$$

And,

$$H_S = \sum_i n_i he_i \Big/ \sum_i n_i \quad [11]$$

$$F_{ST} = 1 - \frac{H_S}{H_T} \quad [12]$$

With

$$H_T = 1 - \sum \bar{p}_i^2 = 2 \bar{p}_i \bar{p}_j = 2 \bar{p}_i (1 - \bar{p}_i) \quad [13]$$

Where $\bar{p}_i$ an $\bar{p}_j$ are the frequencies of alleles ($Adh_{21}$ and $Adh_{22}$) in the pooled population.

Because only a single enzyme system is available and the number of populations studied limited in this first work, more statistics cannot be estimated. Then, results

here reported must be regarded as preamble to subsequent investigations.

## RESULTS

### *Probability of detection of an allele of a given frequency*

The probabilities of detection of an allele of frequency 0.05, 0.10, 0.20, 0.30, 0.50, 0.70, 0.90 or 1 (fixation) in the three studied populations represented by finite samples are reported in Table 1 and illustrated in Figure 2. Of course, alleles with low frequency are less likely to be detected by sampling. For alleles with frequency below 0.20, the probability of detection falls importantly and it is necessary to take larger sizes of samples.

For the population of Zeralda, the size of the sample (n=30) allows to detect an allele with a frequency of 0.05 with a probability greater than 78%, whereas the probability to detect such an allele falls to 40% for the populations of Setif and Tebessa (n=10).

The probability of detection is high from the very moment when the allele frequency reaches 0.30 even if sample size is as small as 10. One can consider, on the base of these results, that such small samples of individuals allow to detect only alleles whose frequency is equal 0.20 or beyond. Thus, the risk is enough serious to miss alleles with low frequencies, all the more so the rare alleles. Therefore, greater samples are required to explore adequately the enzyme polymorphism in forest tree populations; for example, with a sample of 20 individuals, the probability of detection of an allele with a frequency of 0.05 moves up to 64%.

**Table 1 :** Probabilities of detection of an allele with different frequencies in the 3 studied populations (*n*=30 for Zeralda ; *n*=10 for Setif and Tebessa), where n is the size of the seed sample.

| | Frequency | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Population | *,05* | *,10* | *,20* | *,30* | *,50* | *,70* | *,90* | *1* |
| Zeralda | 0,7 85 | 0,9 57 | 0,9 98 | 0,9 99 | 0,99 99 | 0,9999 9 | 0,9999 99 | 1 |
| Sétif | 0,4 01 | 0,6 51 | 0,8 92 | 0,9 72 | 0,99 90 | 0,9999 94 | 0,9999 999 | 1 |
| Tebessa | 0,4 01 | 0,6 51 | 0,8 92 | 0,9 72 | 0,99 90 | 0,9999 94 | 0,9999 999 | 1 |

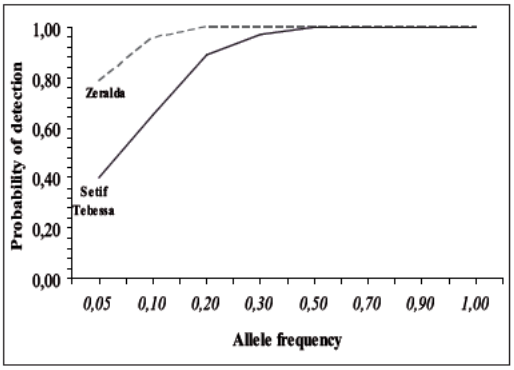


**Figure 2 :** Curves of detection probabilities of an allele with different frequencies of occurrence for the 3 populations studied.

### *Genotypic and allelic frequencies*

Zymograms and their interpretation are given in Figures 3 and 4, genotypic and allelic frequencies are reported in Table 2.

Homozygous genotypes *Adh2(22)* are clearly the more frequent ones. For the population of Zeralda, all of individuals in the analyzed sample present this genotype (Frequency equals 1). The allele 1 of Adh2 seems to be rare.

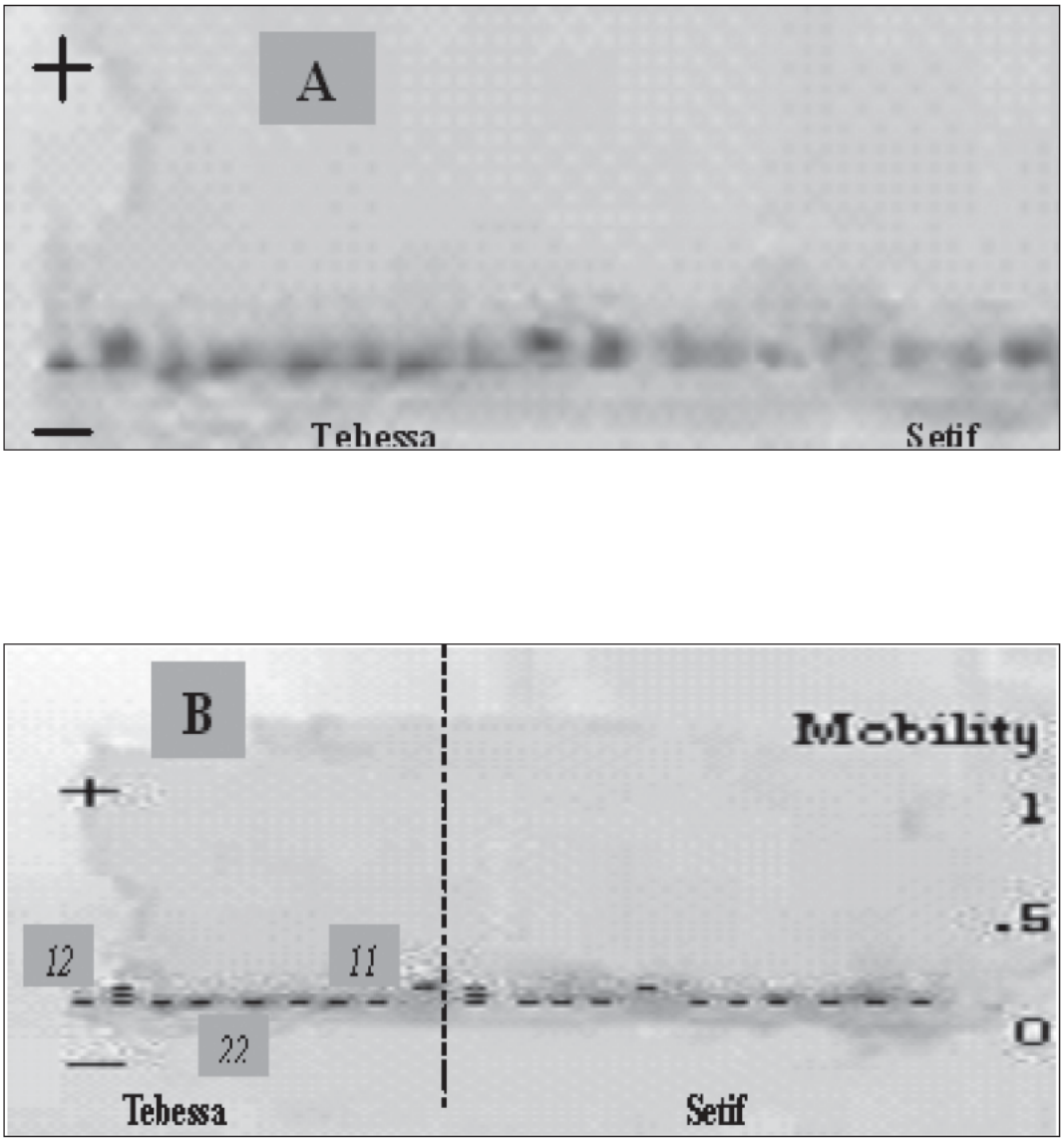


**Figure 3 :** Zymograms (A) of ADH*2* in the Aleppo pine Algerian populations of Tebessa and Setif and their interpretation (B). The indices *11, 12* and *22* respectively point out to the fast (ADH*11*), the intermediate (ADH*12*) and the slow (ADH*22*) allozymes corresponding respectively to the homozygous genotype *Adh2(11)*, the heterozygous genotype *Adh2(12)* and the homozygous genotype *Adh2(22)*.

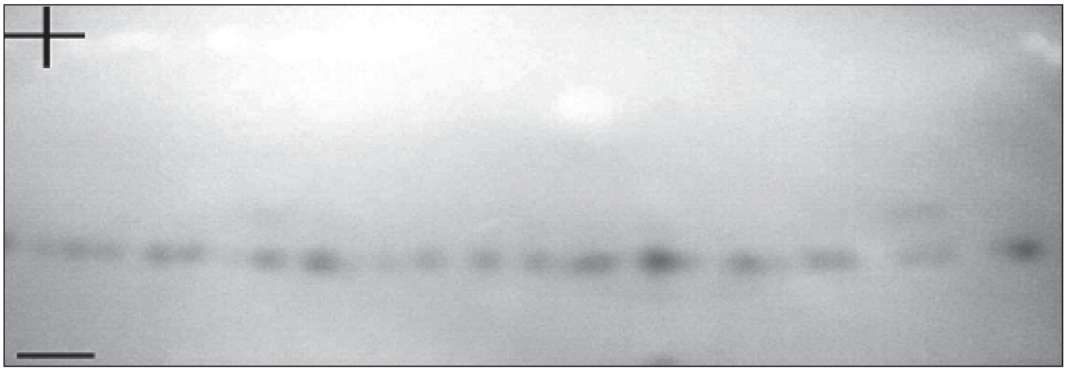

**Figure 4 :** Zymogram of ADH*2* in Aleppo pine population of Zeralda.
All individuals of the sample representing this population synthesize an identical allozyme ADH*22* which suggests that the locus for this enzyme is monomorphic in this population.

**Table 2 :** *Genotypes count and within-population allele frequencies*

| | Genotype | | | *Allele frequencies* | | |
|---|---|---|---|---|---|---|
| **Population** | *Adh2*(11) | *Adh2*(12) | *Adh2*(22) | *HWE Test** | *Adh21* | *Adh22* |
| Tebessa | 1<br>*0,4* | 2<br>*3,2* | 7<br>*6,4* | *1,40*<br>(0,495) | *0,20* | *0,80* |
| Setif | 1<br>*0,1* | 0<br>*1,8* | 9<br>*8,1* | *10,00*<br>(0,007) | *0,10* | *0,90* |
| *Zeralda* | 0<br>*0* | 0<br>*0* | 30<br>*30* | *0,00*<br>(1,00) | *0,00* | *1,00* |
| *Global* | 2<br>*0,18* | 2<br>*5,64* | 46<br>*44,18* | *4,22*<br>(0,121) | *0,06* | *0,94* |

** Hardy-Weinberg equilibrium: Adh21* is the allele (at the locus 2) encoding for the fast allozyme, *Adh22* the allele encoding for the slow allozyme.

The locus *Adh2* is polymorphic in the two populations of Tebessa and Setif, two alleles being identified (*Adh21* encoding for the fastest enzyme and *Adh22* encoding for the slowest enzyme). The fastest-enzyme encoding allele is however much more frequent than the slowest-enzyme encoding one. This allele is present in these two populations with frequencies of 0.80 and 0.90, respectively. The locus *Adh2* is monomorphic in the population of Zeralda, which is a stand westerly located relative to the two others. The allele *Adh22* would be fixed in this population.

Globally, the frequency of the allele *Adh22* is 0.94 (respectively 0.06 for the

allele *Adh21*). And, the frequencies of the homozygotes *Adh2*(11) and the heterozygotes *Adh2*(12) are very low ; homozygotes *Adh2*(22) represent the most part of the samples in the populations of Tebessa and Setif and the totality of the sample of Zeralda (Table 2). The deviation from the Hardy-Weinberg equilibrium is significant in the case of Setif (($\chi^2$=10,0 ; p=0,007) but not in the case of Tebessa ($\chi^2$=1,4 ; p=0,495). One cannot put forward such a conclusion for Zeralda given the complete deficiency of heterozygotes

***Genetic parameters***

Within - and global population genetic parameters are presented in Table 3. Within populations, positive values of F suggest that the studied populations were inbred and deficient of heterozygotes, particularly populations of Seif and Zeralda where F=1.

Globally, the high value of $F_{IT}$ ($F_{IT}$ =0,645) indicate a deficiency of heterozygotes over the total population, mainly due a deficiency of such individuals at the within-population level ($H_I$=0,04 ; $H_S$= 0,10 ; $F_{IS}$=0,600).

The value of the coefficient of population differentiation was moderate ($F_{ST}$=0.1134), essentially reflecting differences in allele frequencies between Zeralda, in one side, and Tebessa and Setif, in the other side, the fast allele *Adh21* being completely absent in the first population, whereas it was present at low frequency in the two others.

**Table 3 :** Within - and whole population Genetic parameters.

| Population | *ho* | *he* | *F* | $F_{IS}$ | $F_{ST}$ | $F_{IT}$ |
|---|---|---|---|---|---|---|
| Tebessa | *0,20* | *0,32* | *0,375* | / | / | / |
| Setif | *0* | *0,18* | *1* | / | / | / |
| *Zeralda* | *0* | *0* | *1* | / | / | / |
| ***Global*** | $H_I$=0,040 | $H_S$=0,1000 $H_T$=0,1128 | / | 0,600 | 0,1134 | 0,645 |

## DISCUSSION

In higher plants, electrophoresis revealed that enzyme alcohol dehydrogenase is encoded by two loci, *Adh1* (the fastest, i.e. the closest to the anode) and *Adh2* (the slowest, i.e. the closest to the cathode).

The locus *Adh1* seems to be monomorphic, and enzyme ADH1which it encodes is undetectable when investigated by means of standard starch gel electrophoresis ; this enzyme is, however, observable when electrophoresis is carried out under anaerobic conditions (Lefranc-Riandey, 1984).

In conifers, more particularly pines, the locus *Adh2* is polymorphic with, generally, two alleles (*Adh21* and *Adh22*) respectively encoding for two allozymes, one electrophoretically fast, the other slow (Schiller *et al.,* 1986 ; Grunwald *et al.,* 1986).

Despite limitations due to sampling, results obtained in this work are very consistent with those found by several authors having studied the variability of ADH system in Aleppo, on a local or at the Mediterranean region levels. This system revealed to be polymorphic only at he locus *Adh2* where the allele encoding for the electrophoretically fast allozyme can be considered as rare, several populations, especially in the western part of the range of the species, having fixed the allele encoding for the slow allozyme (Schiller *et al.,* 1986 ; Grunwald *et al.,* 1986 ; Conkle *et al.,* 1988 ; Teisseire *et al.,* 1995 ; Korol & Schiller, 1996).

Working on a circum-Mediterranean sample of 19 populations, including three populations from the western and central parts of the Algerian range of the species, Schiller *et al.,* (1986) found very low frequencies of the allele *Adh21* at the global level. The frequencies of this allele were respectively 0.00, 0.01, 0.02, in the three Algerian populations. It is also notable that the allele lacks completely in the Moroccan populations studied. According to these authors, it would exist a longitudinal cline of variation of frequencies of allele *Adh21* in Aleppo pine; this allele being more frequent eastward a meridian along Italy and Libya. The frequency of the allele increases moving away from this meridian, likely due to introgression of genes from Brutia pine, a close species of Aleppo pine in which the allele is frequent.

In North Africa, the frequency of allele *Adh21* in Aleppo pine reaches a maximum (referring to the works made until now on the topic) of 0.11 in the population of Wadi Latrum in the region of Syrte, Libya. We seen, in the work here presented, that this allele was present in the Algerian eastern populations of Tebessa and Setif but not in the western one, Zeralda. A recent study on 14 moroccan populations (Wahid *et al.,* 2010), also showed low allelic richness and heterozygosity using five enzyme systems (not including ADH). Results obtained from this study reinforce the knowledge that Aleppo pine in North Africa lacks genetic polymorphism for isoenzyme markers.

Other results on Aleppo pine enzymes made elsewhere showed the same trend of variation of allelic frequencies at *Adh2*, and a low genetic variation at several enzyme systems in the species (Grunwald *et al.,* 1986 ; Teisseire *et al.,* 1995 ; Korol & Schiller, 1996; Agúndez *et al.,* 1997, 1999; Puglisi *et al.,* 1999). Enzyme allelic fixa-

tion turned out to be frequent in Aleppo pine at the scale of its natural range. Such allelic fixation at enzyme loci is typical of coniferous species following from their evolutionary history linked to geological and climatic events at the end of the Tertiary and in the Quaternary, and Aleppo pine, as most pines, was genetically shaped by such events which largely determined the current structure of its populations, most of which descending from original small cores of founders in which genetic drift likely occurred.

And, similar patterns of polymorphism for several enzyme systems suggest adaptive processes affecting these markers (which would mean that enzyme markers are not completely neutral) and/or evolutionary problems such as bottle neck effects and linkage disequilibrium. Fires, which often get disastrous aspects in Aleppo pine forests, probably have accentuated and/or maintained such effects, because, and often, current populations regenerated from small groups of survivors, where genetic diversity is drastically reduced. Such populations could be highly vulnerable to environmental changes and/or pest threats, as it is regularly observed with attacks of the pine processionary caterpillar (*Thaumetopæa pityocampa* Schiff.).

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