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Theme

Establishment of *Cedrus atlantica* Manetti karyotype

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Abstract

A cytogenetic study was performed on two populations of an Algerianendemic species *Cedrus atlantica* Manetti belonging to two different bioclimatic zones i.e., Tala-Guilef (humid region) and Bellezma (dry region) in the aim purpose of establishing their karyotypes. Seed samples from both populations were studied for their mitosis by the classic staining technique based on the useof orcein. Using the morphometric measurements and the Levan classification (1964) the karyotype has been successfully established.The chromosome number and the ploidy level were determined demonstrating that both populations of this species are diploid with 2n=2x=24 chromosomes.

Keywords:

Cytogenetic; *Cedrus atlantica* Manetti ; karyotype ; mitosis ; chromosome ; ploidy level.

Résumé

Une étude cytogénétique a été réalisée sur deux populations d'une espèce endémique algérienne *Cedrus atlantica* Manetti appartenant à deux zones bioclimatiques différentes à savoir, Tala-Guilef (région humide) et Bellezma (région sèche) dans le but d'établir leurs caryotypes.

Des échantillons de graines des deux populations ont été étudiés pour leur mitose par la technique de coloration classique basée sur l'utilisation de l'orcéine. En utilisant les mesures morphométriques et la classification de Levan (1964), le caryotype a été établi avec succès. Le nombre de chromosomes et le niveau de ploïdie ont été déterminés démontrant que les deux populations de cette espèce sont diploïdes avec 2n = 2x = 24 chromosomes.

Mots clés :

Cytogénétique ;*Cedrus atlantica* Manetti ; caryotype ; mitose ; chromosome ;polyploidie.

ملخص

تم إجراء دراسة وراثية خلوية على مجموعتين من احد الأنواع النباتية الجزائرية ، الأرز الأطلسي، تنتمي إلى منطقتين بيولوجيتين مختلفتين، وهما تالا جيليف (منطقة رطبة) وبيليزما (منطقة جافة) بهدف تحديد أنماطها النووية.

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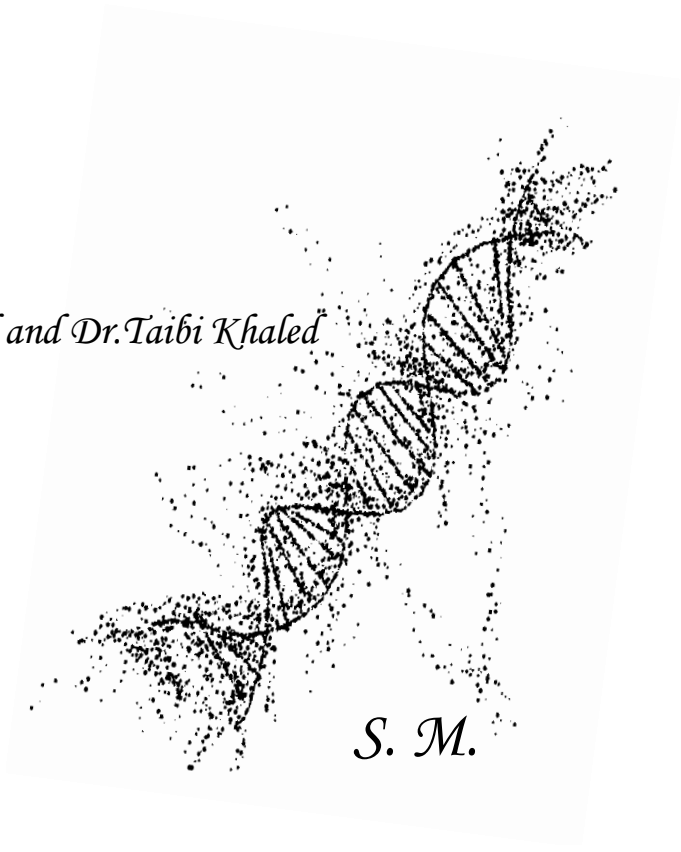
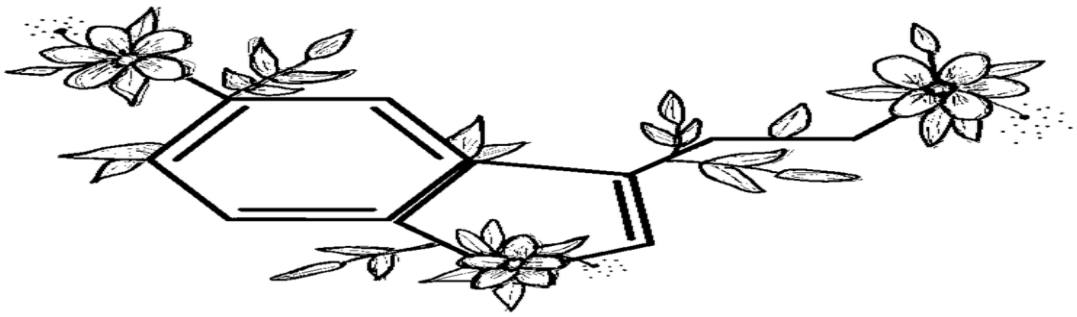


S. M.

To the soul of my father "peace to his soul" who left us before finishing this work.

B. SouAd

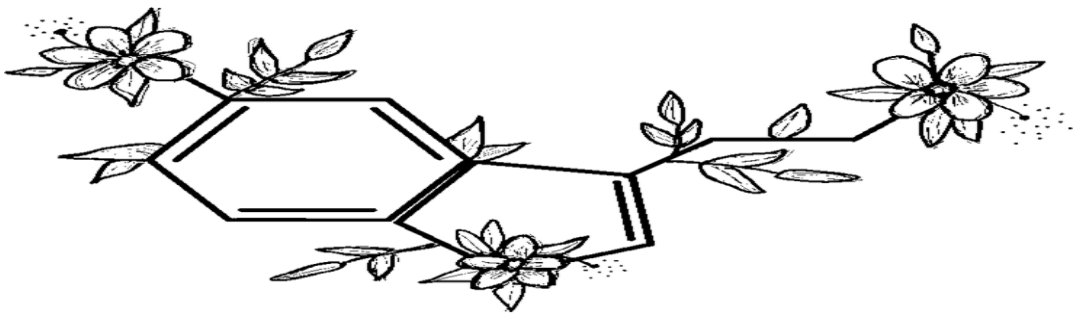


With great pleaser, I dedicate this modest work to:

- *The dearest person in my life…my mother and my father.*
- *To my brothers: Salek, Mohamed, Mansour, Arbi, Fathi, Ben Ouda, and my sisters.*
- *To my friends: Malika, Zinouba, SouAd and Meriem.*
- *To all my colleagues from the Molecular biology class.*
- *To all my family and any one with the name "ABID".*

I dedicate this work to all those who participated in my success.

A. Messouada



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List of abbreviations:

A: Number of Alleles

Ae: Effective number of frequency

ºC: Degree Celsius

C-bands: Constitutive heterochromatin or centromere banding

cm: Centimeter

CMF: The flow cytometry

DNA : Deoxyribonucleic acid

G-bands: Giemsa-bands

h: Hour

ha: Hectare

HCl: Chlorohedric acid

m: Miter

min: Minute

p: Short arm

Q- bands: Quinacrine-bands

q: Long arm

R-bands: Revers-bands

RFLP: Restriction Fragment Length Polymorphism

USSR: Union of Soviet Socialist Republic

Summary

Part I: Methodology

Introduction

Introduction

The genus *Cedrus* includes several species distributed in four distinct geographical areas: **a)** Algeria and Morocco, **b)** Cyprus, **c)** Lebanon, Syria and Turkey, and **d)** Afghanistan and Himalayan region **(Arbez et al. 1978; Arbez 1987; M'hirit 1987).** The Atlas cedar, *Cedrus atlantica* Manetti, is essentially a mountainous species that occupies areas of unequal importance and spontaneously forms seven geographical blocks in North Africa, four are in the Moroccan mountains and three in the Algerian mountains.

The urgent necessary to conserve these resources and to make the best use of them in the context of restoration projects for the highly degraded forest environments would justify the development of research programmes on this species in the countries around the Mediterranean.

Endemic taxa occupy smaller geographical areas compared to the other species systematically close and, therefore, they are generally rare species. Safeguarding the genetic heritage of rare, relictual and endemic species has become a necessity in the face of the threats of an increasingly invasive civilization **(Favarger & Siljak-Yakovlev 1986).**

Cedrus atlantica Manetti has been the subject of several studies that have focused on its ecology, regeneration, morphological variability as well as on the phenomenon of its dieback (decline), the chromosomes number and data on karyotypes of *Cedrus* genus species are rare, no enough genetic or cytogenetic studies have been carried out.

Cytogenetic studies make the link between cytology and genetics. The first work established in plants began in the second half of the 19thcentury but it was especially from the 1920s that cytogenetic developed and its importance continued to grow thereafter. Cytogenetic is one of many disciplines on which plant breeding is based. It participates in the knowledge of the plant material (Number of chromosomes, polyploidy) and the exploitation of intra-specific variability **(Jahier et al. 1992).**Karyological studies play a major role in biosystematic research to understand phylogenetic relationships **(Stebbins 1971; Grant 1986).**

The objective of this study is to identify the chromosome number of this endemic species and to evaluate the intra-specific diversity of the karyotype between two natural stands or provenances occupying two different ecological conditions in Algeria.

Part I:

Literature Review

Cytogenetic

I. Cytogenetic

I.1. Preview

Jahier et al. (1992) have defined the cytogenetic as the discipline which connect cytology and genetic, first application on plants was started during the second half of 19th century, but it is from 1920 that plant cytogenetic has known a prodigious development, and its importance has continued to grow afterwards.

On his investigative part, cytogenetic has an active part in understanding heredity mechanisms as well as species taxonomy and phylogeny. It's one of sciences which contribute to improve plants.

As such as cytogenetic can bring valuables information on chromosome number, karyotype, ploidy level, and also on genome size **(Abdel Samad 2016).**

Chester et al. (2010) have already expected that cytogenetic methods used to Improving plants and biodiversity study will be necessary in the coming years **(Abdel Samad 2016).**

I.2. Plant cytogenetic interest

Plant cytogenetic; this discipline which relies on plants improving, has known major progress during the recent decades. It also aims to exploit the plants species diversity by:

- Knowing the plant material: chromosome number, ploidy level.
- Genetic maps establishment by production and study of aneuploid.
- Interspecies, intraspecies or induced variability exploitation.

According to Jahier (1992), the experiment shows that cytogenetic tools are indispensable for a rational exploitation of interspecies hybrids; moreover, it found a new domain of application in study and usage of products resulting from the in vitro culture (somatic hybrids, somoclonal variation…)**.**

He claims that the cytogenetic can be also used for varietal creation by participating in explanation and resolution of punctual problems which facing selectors (instability, sterility).

I.3. chromosomes

Chromosomes known since the 19th century, we still know little about its assembly mechanism and support **(Almagro 2003).** A chromosomal theory of heredity established in the early 1900 by T. Morgan, and an idea which stipulates those gens are parts of chromosomes was issued these times by two scientists: W. Sotton and T. Bover.

These small bodies with dense marking seem like worms which exist within the nucleus **(Griffith et al. 2013)**, composed of approximately 1/3 DNA, 1/3 proteins called histones, 1/3 non histones proteins. It organized in a stick shape made of chromatin; it appears clearly during the metaphase of every single cellular division. The two chromatids are linked with a primary construction called "centromere" **(Almagro 2003; Griffith et al. 2002).**

I.3.1. Chromosome composition

The chromatin depends to material which formed chromosomes, grouping DNA and proteins. It can be subdivided into two regions according to a chemical staining (condensed DNA) are heterochromatin, however, region with low staining (relaxed DNA) are euchromatin, this one contains most of active gens (encoding DNA) **(Vincent 2007).**

I.3.2. Chromosome identification criteria

Three criteria allow distancing chromosome to establish the karyotype:

- Each chromosome length.
- Primary construction position (centromere).
- Existence and location of secondary construction generally corresponding to nucleolian organizers. Its frequently distal position on a chromosomal arm determines the existence of satellite **(Saada 2009).**

1.3.3. Chromosomes types

Chromosomes are classified in four types according to their form which is determined by the position of the centromere:

I.3.3.1. Metacentric chromosomes: the centromere is in the central position (median position) which gives it arms of roughly equal lengths.

I.3.3.2. Sub-metacentric chromosomes: the centromere is almost in a central position; the chromatids of this chromosome have arms of unequal length (a small arm "p" and a long arm "q").

I.3.3.3. Acrocentric chromosomes: the centromere is closer to one of the two ends (telomeres), the small arm is very short.

I.3.3.4. Telocentric chromosome: has a centromere very close to its telomeres. In case of loss of the centromere (abnormality), the chromosome is said to be acentric. Other abnormalities can cause the appearance of a chromosome with two centromeres called a dicentric chromosome. It is unstable and can break (during meiosis) **(Lemonde and Clement, 1983).**

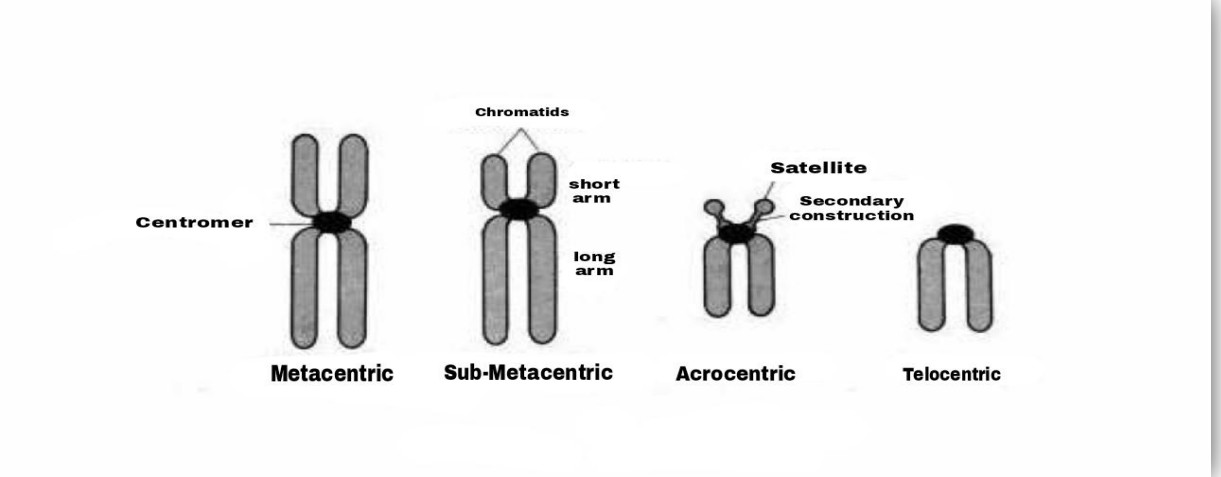


Fig 1. Schematic presentation of the different types of chromosomes (Andre et al. 1983).

1.3.4. Chromosomes marking techniques

In the last quarter of the twentieth century, and especially after 1970, chromosome banding techniques began to be applied to forest tree species.

Chromosomal bands techniques (banding) produce a series of coherent makers over the entire length of metaphasic chromosomes, which allow both recognition of individual chromosomes in a genome and identification of specific segments of individual chromosomes. These benchmarks facilitate the evaluation of chromosomal normality, identification of breaks and alterations sites, and localization of specific gens **(Schreck & Disteche 2001).**

Bernard & Bernard (1992) says that it's possible to make appear Q-bands (observables in fluorescence in the quinacrine mustard), G, C-bands (Constitutive heterochromatin or centromere banding) **(Robert & Barbieri 2009)** and N by Giemsa staining following a specific treatment, R-bands complementary to Q and G-bands by staining with orange acridine.

These works are developed almost simultaneously in plants and animals. These techniques therefore reveal on chromosomes a more or less important zoning, constituted the dark and

clear bands of each chromosome, which allows identifying each of their **(Bernard & Bernard 1992).**

I.5. Techniques for determining variability (polymorphism) in plant species

I.5.1. Morphological variability

The study of the intraspecies variability of forest species have often been approachedby the study of morphological and anatomical characteristics of vegetative and reproductive apparatuses **(Berka 1997).**

However, several researchers adopt the study of the influence of climatic conditions on morphological and anatomical structures in order to understand the phenomena of adaptation of the species with its environment.

Cedar's ability to develop under various climatic conditions induces interspecies variability in the Algerian area. A study published in this field by Berka (1997) according to the method of Ionescu 1969 (counting and measurements of stomata) showed that the cedars of the Saharan atlas are distinguished from that of the Tellian atlas by the number of rows of stomata.

I.5.2. Biochemical variability

Regarding biochemical variability, the use of enzymatic markers is desirable, this constitutes a direct expression of genome, it is clear of any uncertainties linked to the influence of the environment and its interaction with the genotype **(Aidrous-Larbi 2007).**

A study carried out by Aidrous-Larbi in (2007), aims to demonstrate the biochemical variability between the four cedar species, the technique consists in the study of the terpene components contained in the resiniferous pockets of the seeds (quantitative and qualitative analysis) terpene markers are often used to study intraspecies variations between provenances of the same species.

The results of this study showed that there are two cedar provenances (Ouled yaakoub "Aures" and Bab chiker "Riff") with the highest monoterpene content.

I.5.3. Molecular variability

The genetic variability of species is the subject of a precise classification and more based on the nature of the maintenance mechanisms **(Godelle et al. 1998).** Population geneticists and

quantitative geneticists use different parameters to estimate genetic variability within a population (variability between natural stands or provenances).These parameters are as follows: **(kremer 1994; Bariteau et al 1999)**.

I.5.3. 1. Genetic diversity of molecular markers

The diversity parameters at the allelic level are usually in number of three (3):

-The number of alleles (A)

- The genetic diversity of Nei (H)

-The effective number of frequency (Ae)

I.5.3.2. Genetic variability of phenotypic traits

According to Kremer (1994) the level of variability within population can be estimated from different components of genotypic variance (addictive, domain and genotypic variance).

Currently three (3) types of technology give access to the study of variability at the molecular (genetic) level: one-dimensional electrophoresis, which allows revealing the isomatic diversity or certain particular protein, the profiles of characteristic restriction of ADN segment (PLFR), and two-dimensional electrophoresis of denatured proteins **(LefortBuson et al. 1988).** Molecular markers also allowing a direct study of genome variability. It provides the necessary knowledge to quickly build a genetic typology of populations, In the case of cedar, this technique does not use until 1991 by Greek researchers. **(Briteau et al. 1999).**

I.5.4. Chromosomal variability

karyology, which is the study of the genome during the diploid phase of the individual, it makes it possible to distinguish the different taxa by the size, shape and number of their chromosomes, including the number and size of satellite **(Davis & Heywood 1973; Stace 1989).**As a rule, chromosome number is abiding among all individuals of the same species, and each chromosomal pair morphology characterized it. However, eco-geographical conditions can induce variations in genome size within the same species. Indeed, the intraspecies variability related to the phenomenon of polyploidy and correlated with ecogeogarphic conditions has been reported by some authors in several plants (**Grime 1983**; **Reeve et al. 1998**; **Lysák et al.2000**).

Cytogenetic

The karyology makes it possible to establish “chromosomal identity card"; karyotype or caryogram, which is a schematic representation of the haploid genome. It can be based on the classical technique of observing chromosomes, but also on more recent techniques of banding or in situ hybridization (use of specific DNA probes**) (Birkam & Kimber 1974; Teoh et al. 1983; Shang et al. 1988).**

Currently, a new rapid approach to determining the ploidy level of a species has been developed. it is the flow cytometry (CMF), based on fluorescence measurement, which allows the estimation of DNA content **(Arumuganathan & Earle, 1991)**, its distribution in the different phases of the cell cycle **(Jayat & Ratinaud 1993)** or simply the search for the degree of ploidy **(Belkadi 2003).**

The use of CMF demonstrated the existence of a natural level of polyploidy where the number of homologous chromosomes set (basic genome) is greater than two. **(Blakesly et al., 2002; Stebbins 1971; Mayer 1974; Jackson 1976; Bretagnolle et al. 1998**).
This last technique was used with the classical technique to establish the karyotype of the four species of *Cedrus atlantica* by Bou Dagher and his collaborators in 2001.

Atlas cedar

(Cedrus atlantica Manetti)

II. Atlas cedar

I.1. Preview on *Cedrus* genus

Cedrus genus; family of *Pinaceae*; sub-family of *Abietae,* is an old genus, considered as the oldest one after *Pinus* genus. It appears to have been widely distributed in the old wide in the upper tertiary **(Bou Dagher-kharrat et al. 2001).**It includes several species whose taxonomic value has long been controversial. First it integrated into a single species (*Cedrus libanotica* Link). After that, cedars evolved in distinction into four species meeting biogeographic criteria which its natural areas are well differentiated **(Benzyane & M'hirit 2006; Gaussen 1967).** There is an Atlas cedar (*Cedrus atlantica* Manetti) in North Africa (Algeria and Morocco), a Lebanon cedar (*Cedrus libani* Richard) in Minor Asia, Lebanon and Turkey, a Cyprus cedar (*Cedrus brivifolia* Henry) in the Cyprus island, and Himalaya's cedar (*Cedrus deodara* Loudon) in India and Afghanistan in Northwest of part of Himalaya **(Toth 1980).** The follow figure shows the distribution of the four species of cedars in the world.

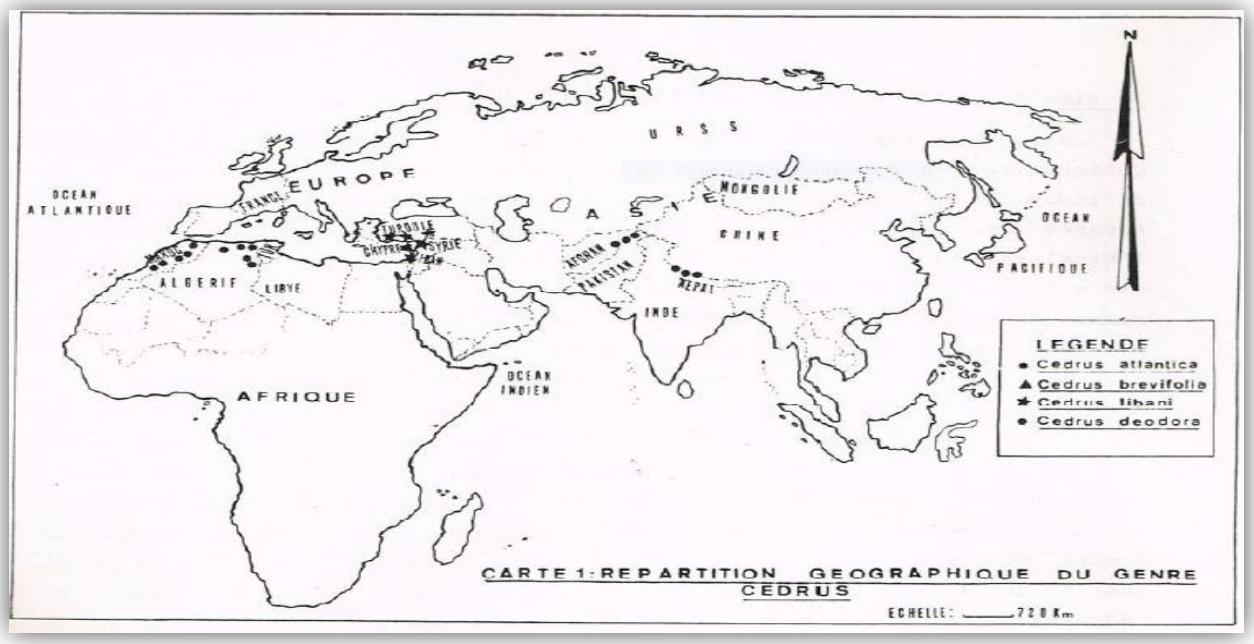


Fig 2. Geographic distribution of *Cedrus genus* in the world (Derrig, 1990).

I.2. *Cedrus atlantica* Manetti

The Atlas cedar (*Cedrus atlantica* Manetti) Arz El Atlas in Arabic, also Beguenoun **(Boukerker 2016)** or Idil in Berber. It is a monoic species, which represent an endemic forest of North Africa Mountain. It indisputably represents the noble essence of Algerian and Moroccan forest **(Benabid 1994; Benzyane & M'hirit 2006).**

Benzyane and M'hirit (2006) described cedar as the wonderful tree which can wait 40 m high, and 2 to 3 m in circumference. It has a very important longevity, where there is a Middle Atlas trees at 2000 m of altitude has more than 1200 years old.

I.2.1. Systematic

The *Cedrus atlantica* species has the following systematic: **(Aidrous-Larbi 2007; Emberger & chaufaud 1960).**

Kingdom: *Plantae*

Phylum: *Spermaphyte*

Sub-Phylum: *Gymnosperm*

Classe: *Vertices*

Ordre: *Conefirales*

Family: *Pinaceae*

Sub-family: *Abieteae*

Genus: *Cedrus*

Species: *Cedrus atlantica*

I.2.2. Botanical characters

I.2.2.1. Architectural units

Specific elementary architecture of *Cedrus atlantica* is characterized by 5 categories of vegetative axis Trans, branch, long twigs, small branch, short twigs **(Sabatier & Barthelemy 1994).**

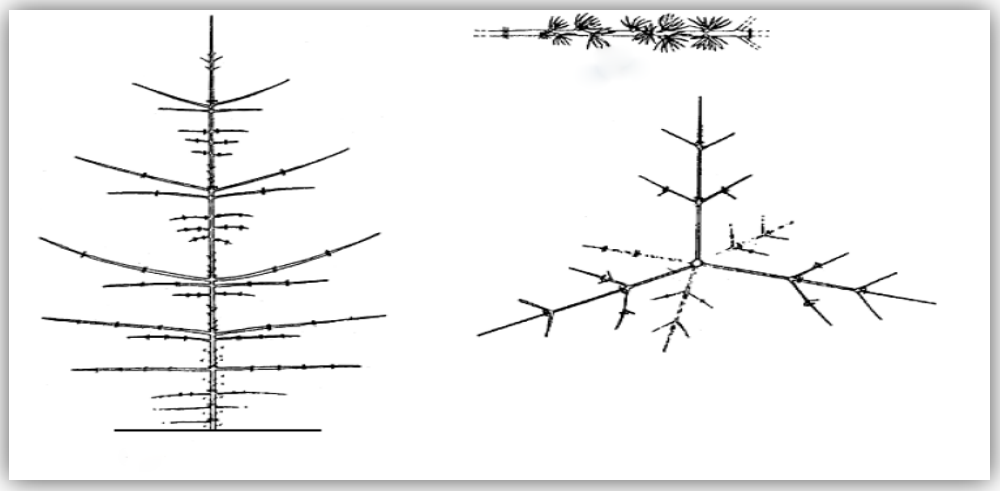


Fig 3. *Cedrus altantica* architecture (Sabatier &Barthelemy 1994).

I.2.2.2.Shape

A high size tree, which can wait until 50 m, and 40 m on average**.** However, old tubercular trees of 200 to 300 years reach only 20 m. The tree shape is pyramidal with straight bole **(Boudy 1952; Toth 1980)**.

I.2.2.3. Twigs

Cedrus genus characterized by two types of twigs:

- Short twigs (mesoblaste) bearing needles which inserted in rosettes.
- Long twigs (auxiblast) with isolated needles which are spiral inserted.

Twigs are light brown, densely pubescent in small rods, and low pubescent in old rods (**Aidrous-Larbi 2007).**

I.2.2.4. Crust

Cedar crust is thick and rough, with blackish color; contain deep furrows through it which lead water from rainfall to roots **(Benzyane & M'hirit 2006).**

I.2.2.5.Needles (leaves)

Needles are regrouped in small bouquets of 30 to 40 cm, located in short twigs summits. These needles measure from 1 to 2 cm length; are straight green glaucous. Usually live 3 years **(Benzyane & M'hirit 2006).**

I.2.2.6. Flowers

Male and female flowers are regrouped in an ovoid shape hornet, male hornets appears in mid-June, whereas females appear three months before, it is usually smaller with bluish-green color **(Boukerker 2016).**

I.2.2.7. Cone (Fruit)

A cylindrical cone with an egg size of 5 to 8 cm, usually with truncated summit, it smooth, velvety, very resinous formed of multitude scales. It developed immediately, but it will only be mature in the third-year autumn **(Benzyane & M'hirit 2006).**

I.2.2.8. Seeds

Cedar cone carry big triangular seeds, with a various length between 8 and 12 mm with a brown color, ending with wide triangular wings, wrapped in protective resinous film **(Boukerker 2016).**

I.2.2.9. Roots

Atlas cedar has an extremely strong root system, similar to a gigantic claw, which penetrate in soil in order to absorb water and mineral elements and give the tree its strength and its majestic shape which characterized it **(Benzyane & M'hirit 2006).**

Fig 4. Botanical characters of *Cedrus atlantica* (Agronomie.info/fr/).

I.2.3. Distribution area

I.2.3.1. Natural area

Over its entire natural area, cedars are observed between 1500 and 2600 m of altitude distributed between Algeria and Morocco in vegetation stages of the Mediterranean and supra-Mediterranean mountaineer **(Benabid 1994).**

In Algeria, it occupies discontinuously between 25000 and 29000 ha in unequal scatted islets **(Benabid 1994; Toth 1980).**It can be said that Atlas cedar is a transitional climate species; it can be grown in cold and humid climate, and also in hot and dry climate so it is possible to distinguish its natural area into two big groups: **(Boukerker 2016).**

-The northern group adapted to moisture, which distributed from east to west, includes the massif of Babour and Tababour (1300ha), and the Djourdjra massif (2000 ha) which include the populations of Tala guilef in the Northwest and Ait-ouabane in the Northeast, Tikijda on the southern slopes and that of the Oursanis (1500ha) and the Blidian atlas (1000ha).

-The southern group adapted to drought, represented by the massifs of Aures and Hodna, of which that of Aures covering 17450 ha includes the massif of Bellezma (8100 ha) to the west, the Chelia, Aidel, and Sgag to the east **(Derrig 1990; Krouchi 2010).**

The following map **(Fig 5)** represents the natural area of *Cedrus atlantica* Manetti in Algeria and Morocco.

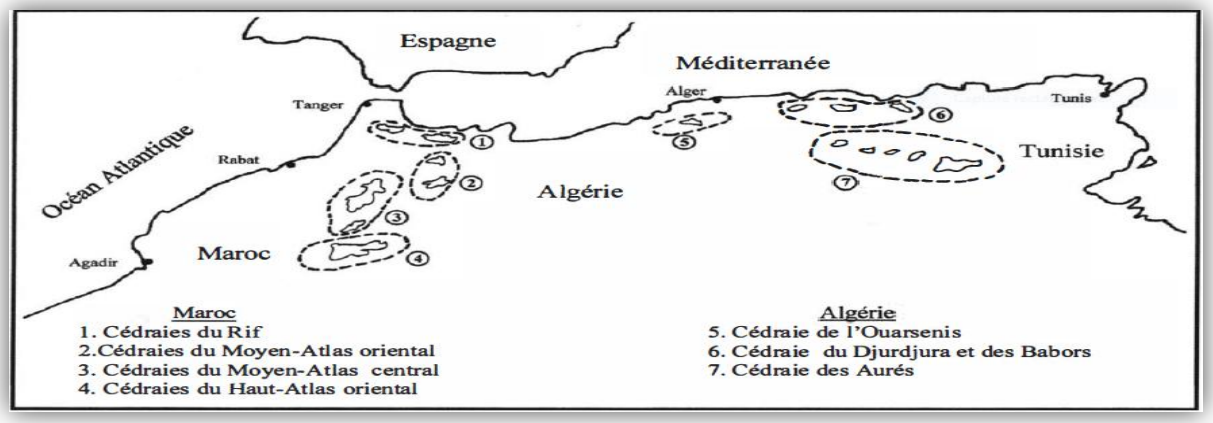


Fig 5. Atlas cedar distribution in Algeria (M'hirit 1999).

I.2.3.2. Introduction area

During the second half of the 19th century, several attempts to introduce the Atlas cedars in Europe have given unequal success according to ecological conditions of interested countries, and also invested efforts by the forest initiative. He cites cedar's introduction dates as follows: 1864 in Italy; 1886 in France; 1890 in Bulgaria, it also introduced in some American states: Pennsylvania; New York, the Pacific coast. Also introduced in URSS, Crimea and Caucasus in 1890.Firstly, it introduced as an ornamental species than as a reforestation species. **(Toth1980; M'hirit 1999).**

I.2.4. interest

I.2.4.1. Ecological

Cedar represents indisputably the noble essence of Algerian forest (**Benabid 1994).** This essence, which fully successful despite the difficult conditions is able to fill out several rules at the same time **(Toth 1980).**

- Protect and Improve soil.
- Eliminate the herbaceous vegetation, and especially in dense stands, which make it less flammable.
- Adaptation with difficult conditions as drought, because of its root system which penetrate soil (**Boudy 1995; Benzyane & M'hirit 2006; Grieu & Aussenac 1988).**
- Offer a remarkable aesthetic for the environment because of its ornamental aspect.

These features give to this fantastic species an important place from the ecological side, in particular, in reconstruction, creation, and upgrading project especially in Mediterranean region (**Yahi & Djelloli 2011; khanfouci 2005).**

I.2.4.2. Medicinal

Traditional medicine and treatment with plants is an interesting domain in Algeria (**Rebbas et al. 2012).** Whereas Atlas cedar has an important interest because of several forest quality (**Abdelhamid et al. 2017).** According to an enthobotanic survey was conducted by Rebbas and his collaborators (2012), Atlas cedar used a lot in herbal medicine, its resin used from antiquity as incense in cosmetics; embalming; leprosy and parasite treatment. Its wood's essential oil used for vermin hurt, the aromatherapy used it against chronic anxiety, cystitis, dermatological affection and bronchi.

I.2.4.3. Industrial

Cedar Product a valuable wood, whereas cedar wood is greater than that all of pines in Mediterranean region (**Toth 1980; Benzyane & M'hirit 2006)**, it is because of its mechanical, chemical and technological properties, as well as its paper propriety and natural durability. So, it is widely used in reforestation since several years. Its potential extension area is estimated at about 200000 ha in Mediterranean region. And perhaps the clearest example is Morocco, where cedars represent the main source of wood (80% of national production). (**El-Azzouzi & keller 1998).**

Cedar wood has also been intensively used over countries by Assyrians, Babylonians, Phoenicians and Egyptians in shipbuilding, temple decoration, mummification, and embalming and resin production **(Bou Dagher et al. 2001).**

Part II:

Methodology

Materiel and Methods

I. Objective:

The purpose of this study is to establish the karyotype of an Algerian endemic species *Cedrus atlantica* Manetti and the search for possible chromosomal polymorphism.

II. Materiel and Methods

II.1. Plant material used

The plant material collected from both populations consists of mature cones collected between the middle-October and the end of November from several stands of the Atlas cedar.

The two sampling sites are located on different bioclimatic zones, namely Bellezma cedars (Bellezma national park) and Talaguilef cedars (Djourdjra national park), the cones have been conserved at room temperature until February.

II.2. Samples sites

The table shows the geographical data as well as the sampling periods of plant material (mature cones).

Sites	Latitude	Longitude	Altitude	Period of sampling
Bellezma	35.3437.125	5.5901.087	1674	End of November 2020
Tala Guilef	36.2827.646	4.0126.741	1781	Middle-October 2020

II.3. The used seeds

The fertile cedar seeds used for germination are from the two-year ripe cones sorted manually in our laboratory. The extraction of the seeds is a manual operation that consists of soaking the ripe cones in water for at least 24 h. After drying it for a few hours in the open air, we disarticulated it; for this, it is necessary to hold the cone, in the hands with firmness, it moved in a rotational movement in the opposite direction, in order to separate the scales and seeds. This way of process allows us to easily extract the maximum of seeds without harming it variability.

Fig 6: *Cedrus atlantica* seeds (photography).

III. Methods

III.1. karyotype establishment

Note: methods of preparation of solutions used in this protocol are described in the annex below.

III.1.1. Disinfection and put in germination of seeds

a. Disinfection

Seeds taken from cones were sorted and stripped of their wings and then disinfected in a mixture of bleach (12 °) and distilled water (1:2) for 5 minutes followed by rinsing with distilled water three times 5 min each.

b. Germination

Seeds were germinated in Petri dishes covered with wet filter paper **(Fig 7)**, then put in the refrigerator at 4°C temperature during three weeks for a cold stratification that allows lifting the state of their dormancy in order to cause germination.

When the root apexes reach about 1.5 cm in length (it is at this stage that the cell division zone is more active), the samples were taken.

Fig 7. Germination of cedars seeds.

III.1.2. Pre-treatment

This is a step that separates the radical of the seed having started germinating after a period of germination. Pre-treatment is done by dipping of these radicals in a mitoclastical agent which has the main effect of:

- Block the mitotic divisions at the metaphase stage.
- Contract chromosomes.
- Obtain a large number of metaphasic plates.

Different mitoclassical agents are used namely:

- Colchicine, at 0.05% for 2 hours at room temperature.
-The 8- Hydroxyquinolein, solution of 0.02 Mol / L at 16 °C for several hours.
- Cold "icy" water (0°C) between 16 and 24 hours.
- α-bromonaphtalene, 3 to 4 hours (room temperature).

For our study, radicals are put in colchicine at 0.05% in 1.5ml Eppendorf tube with labelling and preserved during the pre-treatment period of 2hat room temperatureat the darkness **(Fig 8).**

Fig 8. Tubes Eppendorf containing radicals immersed in colchicine solution.

III.1.3. Fixation

The removed radicals of the metoclassical agent are fixed (Carnoy I); a mixture alcohol - Acetic acid (icy) in the following proportions: 3 volumes of absolute ethanol + 1 volume of acetic acid contained in an Eppendorf tube of 1.5 ml. The preparations are conserved at 4°C for 48 h.

III.1.4. Hydrolysis

This step is generally necessary to obtain a further spread of cells and chromosomes between slide and lamella. The radical extremities are removed from the fixer and rinsed to distilled water and then in a hydrochloric acid solution (1N-HCl) at 60 °C for 10 to 12 minutes in heating plate **(Fig 9)**. The hydrolysis allows dissolving the pectic cement of the middle lamella and also the clarification of cytoplasm. Furthermore, hydrochloric acid releases aldehydic groups on the DNA sugar molecules by destruction of bonds between purine bases and deoxyribose. Hydrolysis also allows the softening of the meristimatic tissues and thus facilitates its crushing.

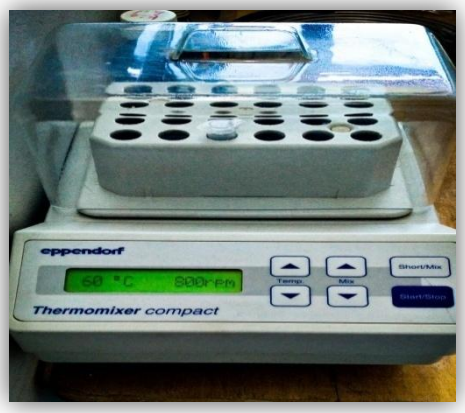


Fig 9. Hydrolysis of a radical in heating plate.

III.1.5. Staining

Radicals were rinsed again in distilled water for 5 min, and emerged in aceto-orcein at 2% for 1h-1h30 **(Fig 10)**. In comparison with other stain tested (SCHIFF reagent), the Orcein gave us a better color, it does not colors neither cytoplasm, nor the cytoplasmic membrane, it mainly colors chromosomes. The aldehydes groups released during hydrolysis give a red color to chromosomes in the presence of aceto-orcein 2%.

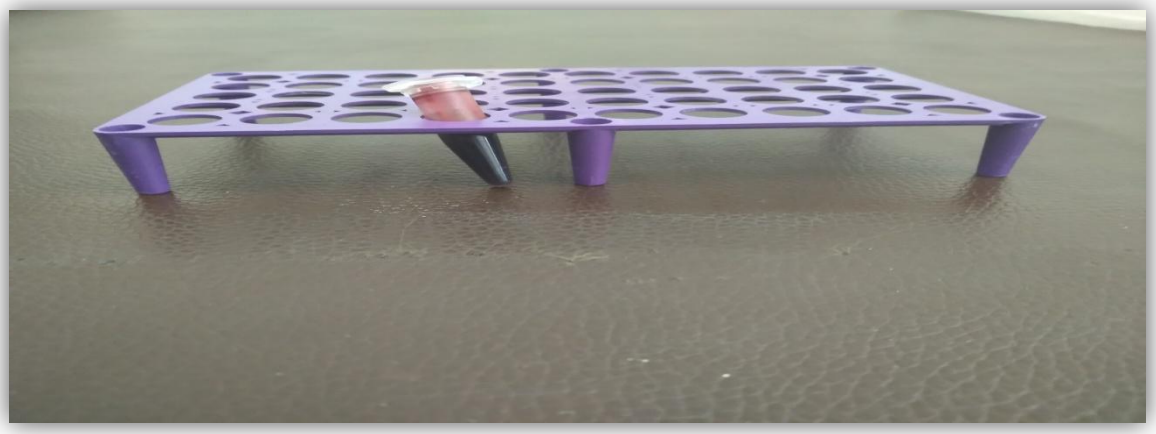


Fig 10. A radical stained with orcein.

III.1.6. Montage

The mounting can be done after one at one hour thirty minutes after staining. The hydrolyzed and stained meristematic zone is isolated with a scalper and mounted between slide and lamella in a drop of acetic acid 45% (to accentuate the contrast between chromosome and cytoplasm). A crushing perpendicular pressure of the finger is carried out on the preparation covered before a paper after a light heating of the slide, to ensure a good spread. Spread continues to gain dried blows on the slide using a small stick. The observation is carried out on a photonic microscope with a digital camera device, connected to a microcomputer **(Fig 13).**

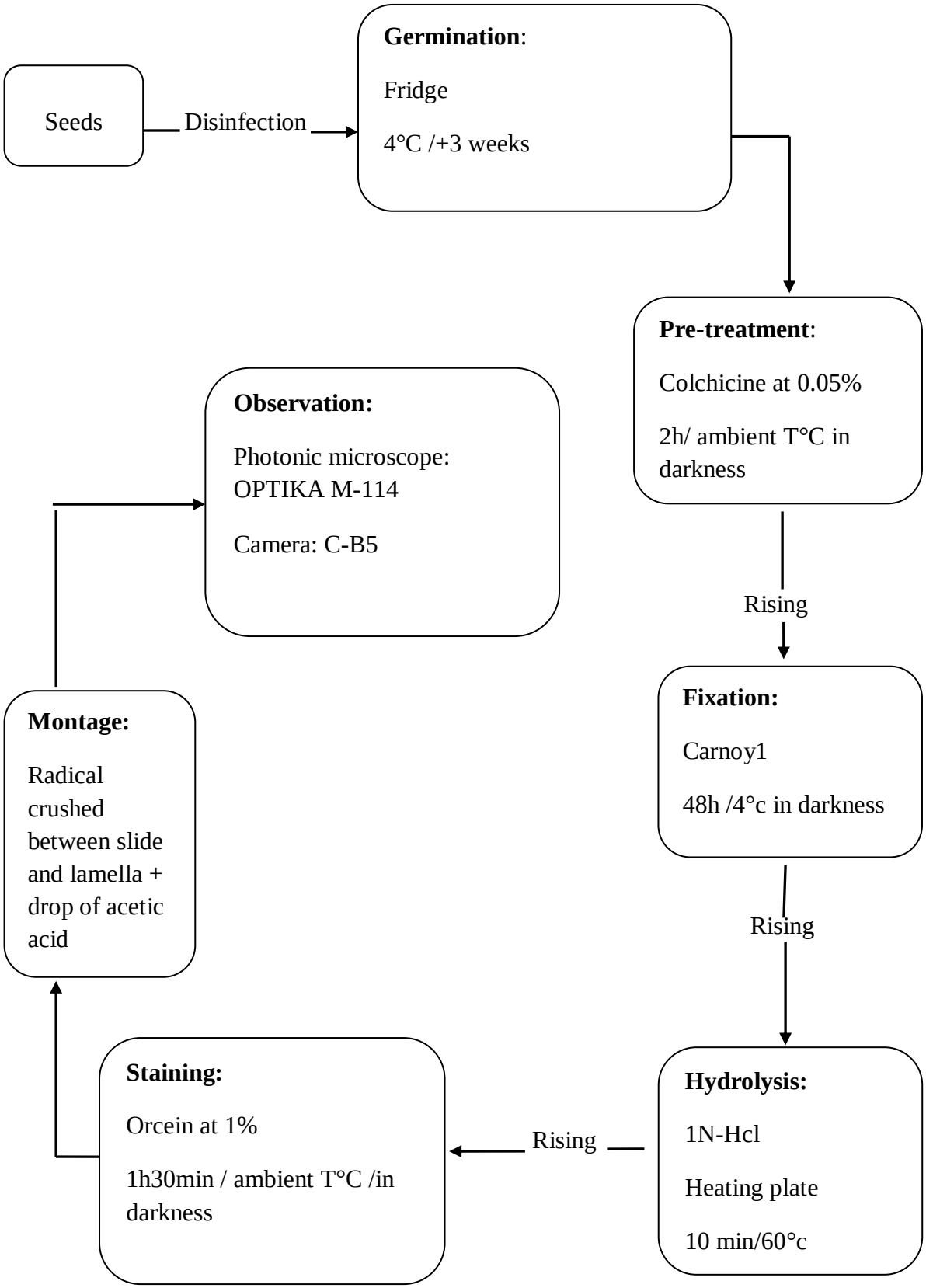
III.1.7. Observation

Slides are observed using an OPTIKA M-114 photonic microscope with a C-B5 digital camera. The first step is to identify the dividing cells, using the lens of low magnification (G= x 10), then G = x 40). Chromosome observation is made at a higher magnification (G = x 100) with immersion oil. The detected metaphasic plates are recorded directly on computer, each photo is recorded by indicating the date, time, minute and second of its capture **(Fig 11).**

Fig 11. Microscope and camera used in chromosomes observation.

Morphometric measures are carried out on all the chromosomes of the best metaphasic plates obtained, using the "ImageJ". The schema below resumes all steps of the used protocol **(Fig 12).**

Fig 12. Schema of the protocol used



Results and Discussion

I. Results and discussion

The analysis of mitosis focused on about thirty root meristems belonging to thirty seeds of *Cedrus atlantica* showed in most cases an insufficient separation of chromosomes at the metaphase, which made counting sometimes very difficult or impossible because of the low dispersion of chromosomes in some cases and because of its superposition in other cases.

The successful metaphasic plaques allowed us to count the chromosomes and determine the level of ploidy in the two sampled populations. All stages of mitosis were observed on the different successful preparations.

In all metaphasic plaques analyzed in the root tips of *C. atlantica* seeds from the two sampled populations, we observed a number of diploid chromosomes of 2n = 2x = 24 **(Fig 13)** and **(Fig 14).**

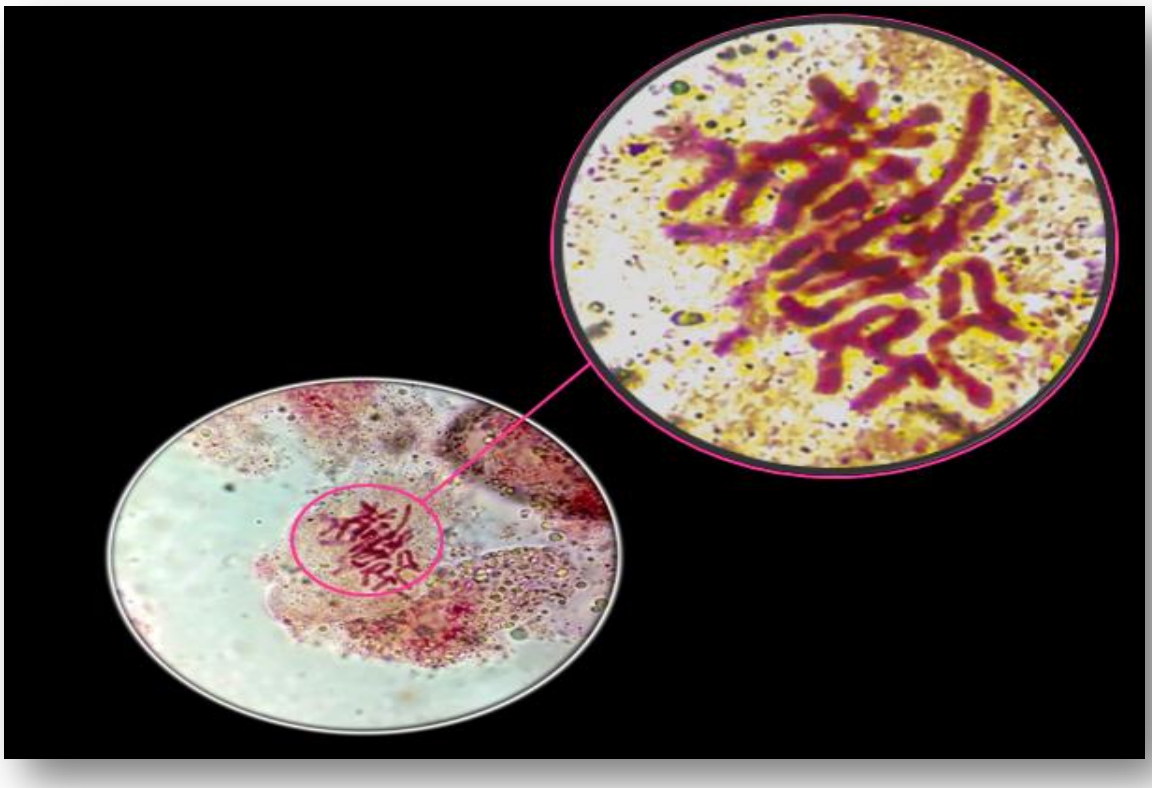


Fig **13. Metaphasic plate with 2n=24 (Tala-Guilef population).**

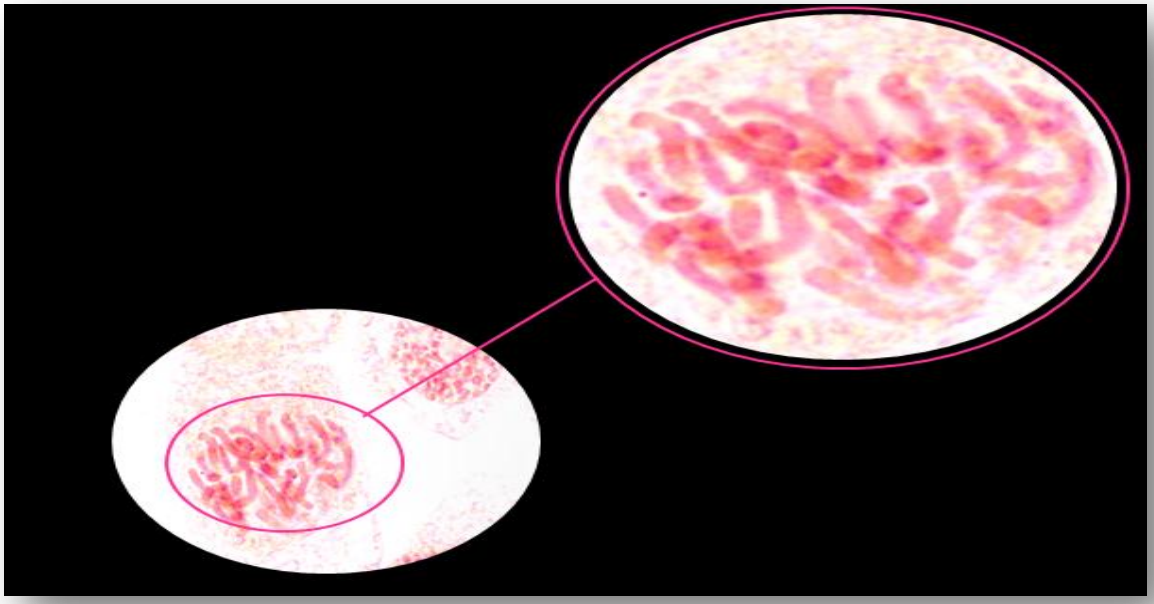


Fig 14. Metaphasic plate (Bellezma population).

The number of chromosomes that characterize the two studied populations corroborates with the obtained results of Bou-dagher and his collaborators (2001), on the four species of the genus *Cedrus* including one taken from Algerian and Moroccan cedars.

Chr	r	IC%	Type
1	1,49	40,16	M
2	1,384	41,94	M
3	4,399	18,52	St
4	1,5	39,98	M
5	1,558	39,09	M
6	1,291	43,66	M
7	1,298	43,51	M
8	1,519	39,7	M
9	1,486	40,23	M
10	1,618	38,19	M
11	2,146	31,78	Sm
12	2,345	29,89	Sm

Tab 1. Morphometric measurements of each pair of chromosome "Tala Guilef population" (Leavan classification).

We found light dissimilarities in the morphometric measurements between the two studied populations, perhaps due to the resolution of the microscope that did not allow the chromosomes to be clearly visible or because of the shifts between the stages of division that influence the degree of shortening and thickening of the chromosomes (the size).

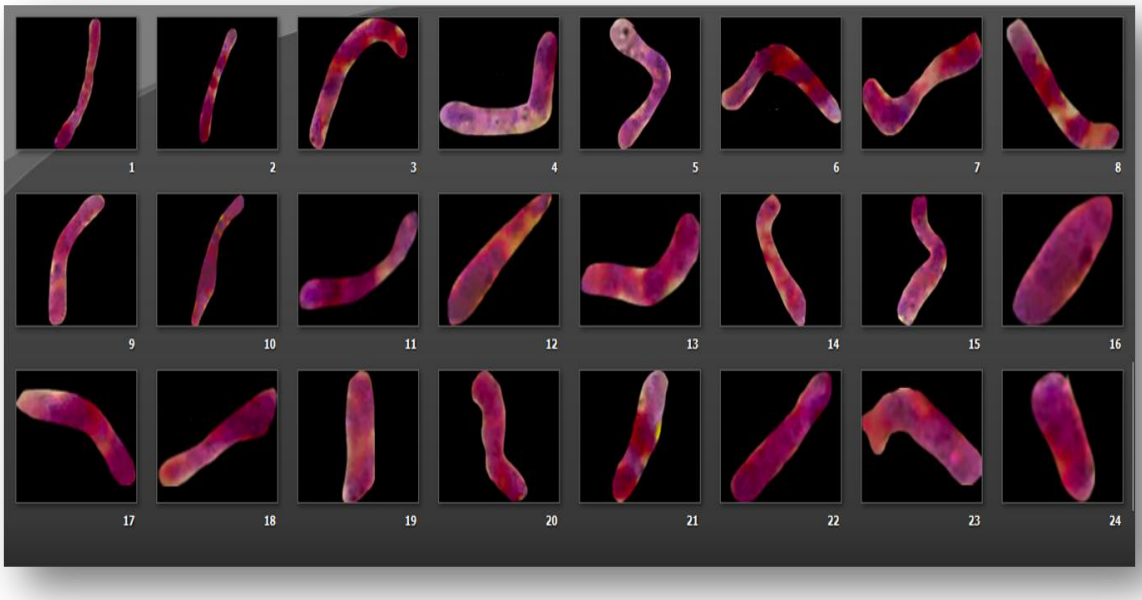


Fig 15. Separation of chromosomes of Tala-Guilef population.

Analysis of karyotype **(Fig 16)** reveals a great similarity in the shape and size of chromosomes. The latter are mostly metacentric, large in size; all pairs are substantially the same size. This trait is related to the symmetrical and primitive karyotypes that characterize the different species of the *Pinaceae* family.

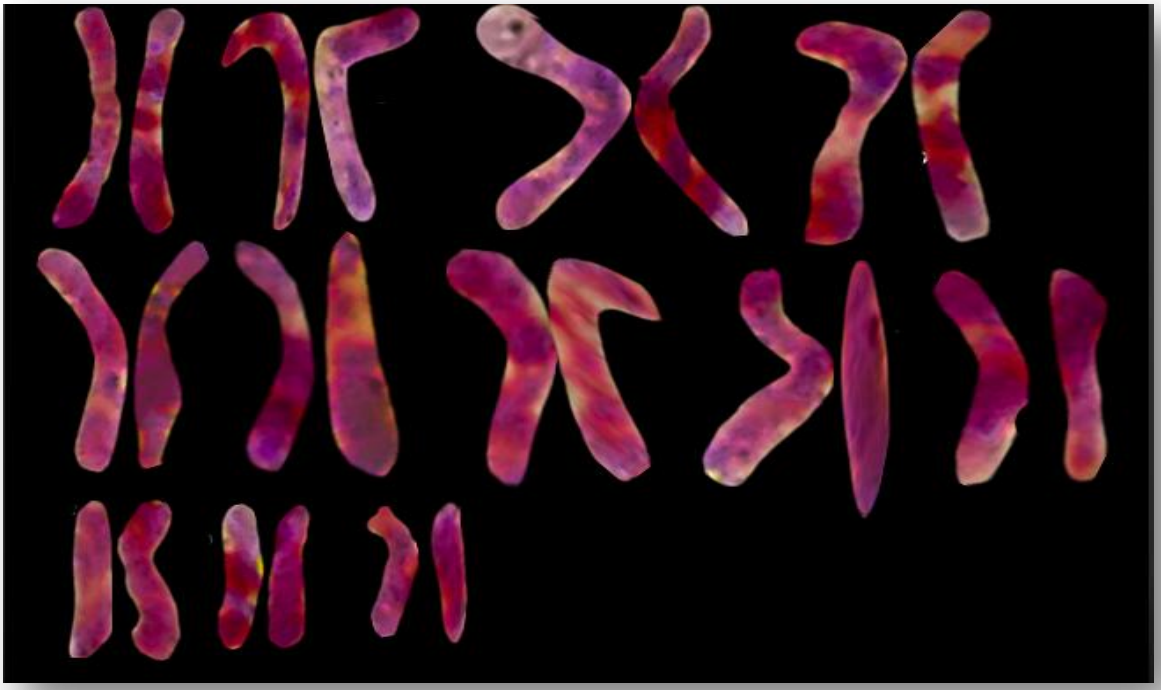


Fig 16. *Cedrus atlantica* karyotype.

The long metacentric chromosomes were so similar in shape and length that they were barely distinguishable. The individual identification of chromosomes depends on the location of secondary constriction, the position of the centromere and the length of the chromosomes. Because the differences between *Abies, Cedrus, Larix, Picea* and *Pinus* species in chromosome size, presence of secondary constrictions, and centromere position are small, the probability of chromosomal inversion is significantly high **(Nkongolo et al. 2012).**

The results obtained by Bou Dagher-Kharrat and his collaborators in 2001 show that the karyotypes of the four species of the genus *Cedrus* were similar in size and symmetry. Chromosome length ranged from 8.8 to 13.8 µm, with eight to nine pairs of metacentric chromosomes of similar size and three to four pairs of smaller submetacentric chromosomes. The total chromosome length and asymmetric index were 139 µm and 54.5 for *C.atlantica* respectively.

In the Turkish flora and in the "Med-Checklist"**(Bariteau et al. 1999),** all Mediterranean Cedars have been described as a single species, called *Cedrus libani* (*C. libanitica*), with 4 subspecies: *C. libani* ssp. *atlantica* in Morocco and Algeria, *C.*

*libani*ssp. *brevifolia* in Cyprus, *C. libani* ssp. *libani* in Lebanon and Syria, and *C. libani* ssp. *stenocoma* in Turkey.

The interspecies relationship between *Cedrus* spp. have recently been resolved using paternal chloroplast genomes and maternal mitochondria **(Nkongolo et al. 2012).** The two *Cedrus* spp., *Cedrus libani* and *Cedrus brevifolia* (from Cyprus), were found to be the most closely related. The Himalayan cedar (*Cedrus deodara*) diverged first and then the North African species *Cedrus atlantica* separated from the common ancestor of the two Mediterranean *Cedrus* spp **(Nkongolo et al. 2012).**

No abnormalities (euploidy or aneuploidy) were found on the tested plant material of the two populations that manifested a single cytotype with the same degree of ploidy.

According to Chagne (2004), the *Pinaceae* family is composed mainly of diploid species (2n = 24), which are characterized by a large genome, a constant number of chromosomes within the different families and a highly conserved karyotype between species. In addition, very few conifers are polyploid. Karyotypes are remarkably conserved among gymnosperms compared to angiosperms **(Sax and Sax 1933; Bou Dagher-kharrat et al. 2001).**

Conclusion and Perspectives

I.1. Conclusion

Two populations of *Cedrus Atlantica* located in two different ecological conditions were the subject of a cytogenetic analysis that concerned the establishment of its karyotypes through the mitotic metaphase of the root apexes issued from the germinated seeds.

The analysis of mitosis made on the root apexes allowed us to observe the main stages of mitosis. *Cedrus atlantica* showed in most cases insufficient separation of chromosomes at metaphase, but on some metaphasic plates, 24 chromosomes could be counted.

The obtained results reveal a single degree of ploidy that is to say a single cytotype (2n = 2x = 24) for the two provenances studied.

No abnormalities or presence of B chromosomes were found on the treated plant material. Karyotype analysis reveals a great similarity in the shape and size of the chromosomes between the two origins. The latter are mostly metacentric, large in size; all pairs are substantially having the same size, which indicates stable, symmetrical and primitive karyotypes.

The number of chromosomes 24, correspond to the chromosomal numbers found in some natural populations of Atlas cedar that develop in Algeria and Morocco and in other species of the genus *Cedrus*, it corroborates with the number of the majority of species belonging to the family of *Pinaceae*.

I.2. Perspectives

It would be interesting to:

- Extend the study to a greater number of cedar populations in Algeria.

-Improve the techniques used to analyze mitosis; and expand the study to meiosis.

-Apply modern molecular cytogenetic techniques.

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Annex

Preparation of solutions

A. Colchicine (mitoclassical agent)

The Colchicine at 0.05% is used as a mitoclassical agent. A quantity of 50mg of colchicine powder is added to the 100 ml of distilled water, preserved in the dark for 1 hour before using.

B. Carnoy 1 (fixer):

The fixer used is the Carnoy1 (3v:1v) which prepared with .....ml of ethanol and.....ml of acetic acid.

C. Ethanol at 70% (storage solution)

For obtaining a 70% ethanol solution, we used, as a reference method, the Gay-Lussac table (alcohol wetting table), the table indicates that we must add 45.98 ml of distilled water to 100 ml of 99° alcohol.

Table pour la dilution de l'alcool (Table de Gay-Lussac) appelée aussi Table de mouillage de l'alcool

		Concentration initiale													
		100	99	98	97	96	95	90	85	80	75	70	65	60	50
Concentration finale	95	6,5	5,15	3,83	2,53	1,25									
	90	13,25	11,83	10,43	9,07	7,73	6,41								
	85	20,54	19,05	17,58	16,15	14,73	13,33	6,56							
	80	28,59	27,01	25,47	23,95	22,45	20,95	13,79	6,83						
	75	37,58	35,9	34,28	32,67	31,08	29,52	21,89	14,48	7,2					
	70	47,75	45,98	44,25	42,54	40,85	39,18	31,05	23,14	15,35	7,64				
	65	59,37	57,49	55,63	53,81	52	50,22	41,53	33,03	24,6	16,37	8,15			
	60	72,82	70,80	68,8	65,85	64,92	63	53,65	44,48	35,44	26,47	17,58	8,76		
	55	88,6	86,42	84,28	82,16	80,06	77,99	67,87	57,9	48,07	38,32	28,63	19,02	9,47	
	50	107,44	105,08	102,75	100,44	98,15	95,89	84,71	73,90	63,04	52,43	41,73	31,25	20,47	
	45	130,26	127,67	125,11	122,57	120,06	117,57	105,34	93,30	81,38	69,54	57,78	46,09	34,46	11,41
	40	158,56	155,68	152,84	150,02	147,22	144,46	130,8	117,34	104,01	90,76	77,58	64,48	51,43	25,55
	35	194,63	191,39	188,19	185,01	181,85	178,71	163,28	148,01	132,88	117,82	102,84	87,93	73,08	43,59
	30	242,38	238,67	234,99	231,33	227,70	224,08	206,22	188,57	171,05	153,61	136,04	118,94	101,71	67,45
	25	308,9	304,52	300,18	295,86	291,56	287,28	266,12	245,15	224,3	203,61	182,83	162,21	141,65	100,73
	20	408,5	403,13	397,79	392,47	387,17	381,9	355,8	329,84	304,01	278,26	252,58	226,98	201,43	150,55
	15	574,75	567,43	560,53	553,55	546,59	539,66	505,27	471	436,85	402,81	368,83	334,91	301,07	233,64
	10	907,09	896,73	886,4	876,1	865,15	855,15	804,5	753,65	702,89	652,21	601,6	551,06	500,50	399,85

Fig . photographie de table de Gay-Lussac

The orcein eat 1% (stain)

0.5g of the orcein powder is dissolved in 22.5ml of acetic acid; the solution is stir until boil at 120 ° C. We stop the stirring 10 minutes after boiling.

The evaporated volume is completed by drops of acetic acid, after cooling of the solution (up to 30 min) the volume is completed by 27.5 ml of distilled water to obtain 100 ml of the stain solution. The vial is covered with an aluminum paper to prevent exposing to light rays. The vial stored at 0°c. After 7 to 8 hours, the solution is filtered with classical filtration (by filter paper).

Chlorohydric acid (1N-Hcl)

8.33 ml of Hcl is diluted with 91.21 ml of distilled water for obtaining 1N-Hclsolution (1 time normal).

Acetic acid at 45%

A volume of 22.5ml of acetic acid is diluted with 27.5ml of distilled water for obtaining 50ml acetic acid at 45%.