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# Analysis of Toxin Amnesiac by High Performance Chromatography Liquid in Bivalves of the Dakhla Region

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## ABSTRACT

In this study we performed an ASP analysis in bivalve molluscs of the Dakhla region, especially mussels and oysters. This work was performed between March and June 2015. The sample was conducted in four sites in the Dakhla region, namely Boutalha, Duna Blanca, Puertitto and Oum Labouir. After analysis by HPLC / UV it was shown that the greatest percentage of toxicity of domoic acid (DA) was recorded in June, followed by the months of April and May, while the month of Mars was marked by a low DA toxicity.

**Key word:** ASP, Domoic Acid, toxicity, HPLC/UV, *Pseudo-Nitzshia*, efflorescence, phytoplankton.

## I. INTRODUCTION

There are indications that toxic algal blooms are increasing because of pollution of coastal waters and worldwide shipping. This article deals with the marine biotoxin domoic acid, also known as amnesic shellfish poison (ASP), and its main producing pinnate diatom genus Pseudo-nitzschia (Bacillariophyceae) (Mos, 2000). The Domoic acid, belongs to a group of amino acids, called the kainoîds, which are classed as neuroexcitants or excitoxins that interfere with the neurotransmission mechanisms in the brain. The toxin can be accumulated in shellfish feeding on a number of toxic Pseudo-nitzschia species (Bates *et,al.,* 1998). Ingestion of seafood contaminated with domoic acid can lead to an intoxication which symptoms include (among others) abdominal cramps, vomiting, disorientation and memory loss (amnesia) and can become severe in certain cases (Wright *et al.,* 1989).

Consequently, DA assays in shellfish have become systematic since 2003 in Morocco, in the context of the phytoplankton monitoring network, as soon as the threshold for Pseudo-nitzschia spp. rises above $10^5$ cells/ litre (themean threshold for Europe) (Amzil, 2002).

Domoic acid is extracted from bivalve tissue with a mixture of methanol and water. The extract is filtered through a membrane filter and measured using HPLC equipment with isocratic elution and UV detection. The amount of domoic acid is calculated by the external standard method.

The main purpose of the present study is the assessment of the monitoring concerning amnesic toxins in Pseudo-nitzschia spp. blooms in Dakhla coast in terms of: domoïc acid accumulation in shellfish.

## II. METHODS AND MATERIAL

### Shellfish Samples

Tow shellfish species were sampled at different sites of dakhla coasts (fig.1): *Mytilus galloprovincialis and Crassostrea gigas.*

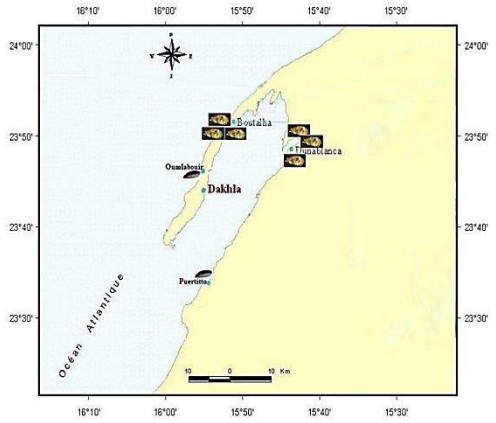


**Figure 1 :** Sampling locations of Dakhla coasts.

**Shellfish Extracts**

One hundred grams of total shellfish meat were ground in an Ultra-turrax. The assay amount (4 g) was precisely weighed in a graded centrifuge tube to which 16 ml MeOH/H O (1:1) were added. The sample was homogenized followed by centrifugation at 4,800 g for 10 min. The supernatant was filtered on 0.2 µm, and 20 µl were analysed by HPLC/UV.

**High performance Liquid chromatography with ultraviolet detection (HPLC/UV).**

High performance liquid chromatography with ultraviolet detection (HPLC-UV) was the first chemical analytical method for domoic acid and is still the most commonly used for monitoring shellfish. Domoic acid detection is facilitated by its strong absorbance at 242 nm (Quilliam *et al.*, 1995).

The DA assay by HPLC/UV was performed according to the method of (EU-Harmonised Standard Operating Procedure, version 1, 2008) using a $C_{18}$ reverse phase (Vydac : 5 µm, 250 mm x 4,6 mm) at 40° C. The elution solvent for single pump systems was a 10% acetonetrile; 0,1% TFA and dilute to 1L with water with, a flow rate of 1 ml/min. DA detection was performed at a wavelength of 242 nm. The DA in the sample was quantified in duplicate using a certified standard DA provided by NRC, Halifax, Canada ( SOP, 2008).

## III. RESULT AND DISCUSSION

Results analysis during the study period showed that at 4 sampling sites, Mussel samples were the most contaminated with DA compared to Oyster samples. The maximum concentrations found (7.6 mgDA/Kg meat) were not harmful for consumers as they were largely below the legal threshold (20 mg DA /Kg of meat) (fig.3).

Fig. 2 summarises the results of analyses of the shellfish samples in 4 the study sites. Domoic acid was detected in different bivalve species from the 4 sites on the Dakhla coast. With a percent of toxicity that augments in Boutalha to Oum labouir. The maximum % of txicity found (75% in Oum labouir )

However, at the study areas, it was noted that the percentage of toxicity was much more important in the area and Puertitto Oum Labouir in areas of Boutalha and Duna Blanca.

Knowing that the DA is responsible for the ASP (Amnesic shellfish poisoning) syndrome is synthesized directly by Pseudo-Nitzshia which is a phytoplanktonic micro-alga, it is assumed that this high rate of toxicity is due to a phytoplankton bloom of this micro-alga, which is stimulated by different environmental factors. According to studies carried out on the phytoplacton, the periods of annual blooms are recorded around the middle of the year between spring and early summer. (Andrew et al, 2012). This may explain the rate of toxicity between April and June 2015.

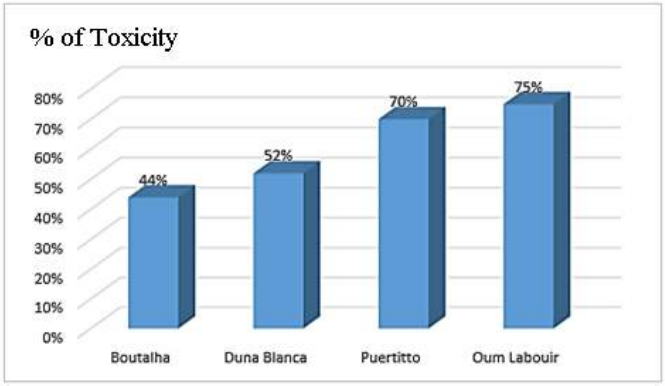


**Figure 2 :** Domoic acid concentration in shellfish at different sites of dakhla coasts.

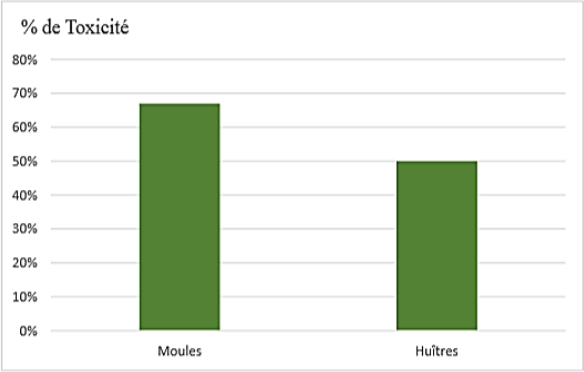


**Figure 3 :** Domoic acid concentration in shellfish extracts analysed by HPLC/UV.

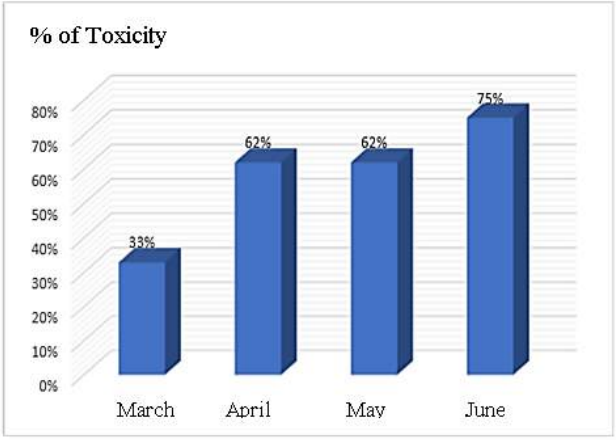


**Figure 4 :** Domoic acid concentration in shellfish extracts since March to June .

The occurrence of amnesic toxins in Moroco shellfish lends support to the idea that phycotoxins can be disseminated throughout the world via ballast waters and shellfish transfers. In fact, potentially toxic species of spp. have already been observed and implicated in shellfish toxicity events in neighbouring or nearby countries such as Spain and Portugal (Arévalo *et al.*,1998; Vale *et al.*, 1998).

## IV. CONCLUSION

During the monitoring programme of harmful algal blooms established along the dakhla coast, the weekly determination of phycotoxins analysis in Mussels and Oyster is carried out from marsh 2015 to June 2015. Results analysis during the study period showed that at 4 sampling sites, Mussel samples were the most contaminated with DA compared to Oyster samples. However, at the study areas, it was noted that the percentage of toxicity was much more important in the area and Puertitto Oum Labouir in areas of Boutalha and Duna Blanca. the greatest percentage of toxicity of domoic acid (DA) was recorded in June, followed by the months of April and May, while the month of Mars was marked by a low DA toxicity. According to studies carried out on the phytoplacton, periods of annual blooms are recorded around the middle of the year between spring and early summer. This may explain the rate of toxicity between April and June 2015.

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# Study of Toxic Profile of *Staphylococcus Aureus* Isolated From Raw Milk Samples of Mastitis SOR Consumption in Oran Area, Algeria

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## ABSTRACT

Mastitis *S. aureus* is considered one of the major diseases in dairy cattle. The presence of *S. aureus* causes damage to the mammary gland tissue that will impact milk production in both volume and quality. The many virulence factors such as toxins and proteins or surface polysaccharides are involved in the pathology. This study determines the toxic profile of *S. aureus* strains isolated from raw milk samples from bovine mastitis diagnosed in dairy farms in Oran.

After identification by MALDI-TOF, spa typing and MLST have identified the phylogenetic position of the strains. Search for virulence factors is performed by real-time PCR TaqMan type, to detect manipulation in one of the four genes of virulence factors of *S. aureus:* tst for *TSST-1*, *pvl* leucocidin for ED, *sea* for enterotoxin A, *seb* for enterotoxin B, in this study sought.

Toxins research shows that only a few strains have proven carriers of different virulence genes including the gene encoding the *pvl* Panton-Valentine leukocidin. Other strains were positive for the presence of tst gene encoding the toxin of toxic shock syndrome (TSST-1), 100% of the *Staphylococcus aureus* isolates identified were sensitive to methicillin.

In conclusion, the results obtained in this work have determined the phylogenetic profile, toxic and sensitivity profile of the méticiline strains studied. Stem found produisantes as the Panton-Valentine leukocidin are sensitive to méticilline. Cette study is the first molecular characterization study of animal strains of *S. aureus* isolated in the region of Oran.

**Keywords:** milk, mastitis, *Staphylococcus aureus*, Toxic profile, *pvl*, *TSST-1*, *sea*, *seb*.

## I. INTRODUCTION

*S. aureus* is an important human and animal pathogen responsible for skin and soft tissue infections, the bovine intramammary infections are de most infections caused by *S. aureus.* As an agent of intra-mammary infections, this pathogen can contaminate the bulk milk tank and thus may constitute a bacteriological hazard for raw milk dairy products consumed. In this context, molecular subtyping tools are of great interest for the comparison of genotypes in order to identify sources and transmission routes for control improvement.

The pathogenicity of *S. aureus* is connected to the expression of virulence genes carried by the chromosome. Scientific studies have proven that in the case of *S. aureus* that has structural factors responsible for the pathogenic activity. The virulence factors such as the ability to secrete toxins, invasiveness and adhesiveness and hydrolytic enzyme production are the factors that determine pathogenicity. The Molecular biology studies on *S. aureus* have revealed the presence

of several genes involved in the virulent power or Table Some *S. aureus* strains are able to produce some virulence factors causing infections. 15 list the 14 genes that encode components of the cell wall, responsible for adhesion, attachment, invasion and the invasion of the host with super antigens.

*Staphylococcus aureus* is the most predominant contagious pathogen responsible for clinical and subclinical infections in lactating cows (KerroDego et al., 2002).The purpose of this study was to investigate a research staphylococcal virulence factors of *S. aureus* strains sensitive to the methicilin; Panton Valentine leukocidin pvl, Enterotoxins sea, seb Enterotoxins and exotoxin, staphylococcal toxic shock syndrom: tst.

## II. METHODS AND MATERIAL

We selected 52 *Staphylococcus aureus* Methicillin susceptible strains isolated from both subclinical and clinical cases. The strains were identified firstly by the spectrometry de mass (MALDI-TOF) and diffrents *spa* types and *MLST* types.

Detection of virulence factors in *Staphylococcus aureus* by PCR time real. DNA extraction (the technique used InstaGene the kit).

Preparation of primers and probes : the reaction mixture contained 5µl of extract and 20 .mu.l of the mix. It is performed in the extraction part. It is achieved, for sample (DNA extract of *S. aureus* strains) and virulence factors, positive control and negative control.

**Table 1 :** Taqman primer sequences and probes for real-time PCR

| | |
|---|---|
| Amorce Forward (*pvl*) | 5' -TTC ACT TGT ATC TCC TGA GCC TTT T -3' |
| Amorce Reverse (*pvl*) | 5' -AGT ACA CAG TGG TTT CAA TCC TTC AT -3' |
| Sonde FAM-MGB (*pvl*) | 5' -CAT GAG AAA CAG TTG CAA TA-3' |
| Amorce Forward (*sea*) | 5' -CGA AAC GGT TAA AAC GAA TAA GAA AA-3' |
| Amorce Reverse (*sea*) | 5' -CCT GTA AAT AAC GTC TTG CTT GAA GA-3' |
| Sonde FAM-MGB (*sea*) | 5'-TGT AAC TGT TCA GGA GTT G-3' |
| Amorce Forward (*seb*) | 5' -CTG TTA GGG TAT TTG AAG ATG GTA AAA AT-3' |
| Amorce Reverse (*seb*) | 5'-TCT AAT TCT TGA GCA GTC ACT TTT TTC T -3' |
| Sonde FAM-MGB (*seb*) | 5 '-TCT TTT GAC GTA CAA ACT A-3' |
| Amorce Forward (*tsst*) | 5' -GCT TGC GAC AAT CGC TAC AG -3' |
| Amorce Reverse (*tsst*) | 5' -GAT GCT TTT GCA GTT TTG ATT ATT TG -3' |
| Sonde VIC-MGB (*tsst*) | 5' -TTT TAC CCC TGT TCC C -3' |

## III. RESULT AND DISCUSSION

Production of toxins: Two strains cards are producing test, two producing strains and the pvl. No strains produced the other two toxins (sea and seb). TST + (t007) (4 strains) (2 strains in winter, in summer one isolated strain 1 strain isolated in spring). pvl + (t267) (1 strains) (isolated in winter), presence of the gene encoding the toxin (TSST) by the detection of toxins through the real-time PCR StepOne. The results obtained are comparable to those shown in the study of Velusamy et al., (2006). The distribution of the types spa of Staphylococcus *aureus* strains revealed a diversity of strains of three to two types in each studied herd, as in farms F2, F3 presence of three types of strains against farms F4, F5 two types of strains of Staphylococcus *aureus* and one type of strain was isolated in the F6 farm.

*Staphylococcus aureus* is considered common agent of clinical and subclinical mastitis in dairy cows. The main reservoir of S. *aureus* is the mammary gland and transmission from one cow to another usually occurs during milking. *Staphylococcus aureus* produces a spectrum of extracellular protein toxins and virulence factors, which are thought to contribute to the pathogenicity of the microorganism. The enterotoxin (SE) and staphylococcal toxic shock syndrome (TSST-1) are recognized as food poisoning syndrome and officers may be involved in other types of infections in humans and animals (Akineden et al., 2001). The results obtained in this first study in the region of Oran, we determined four strains of S. *aureus* carriers of the gene

encoding the toxic shock syndrome (TSST-1) and two other strains carrying gene (pvl- luk) encoding the toxin 'Pantin-Valentine-leucocidin.

Search SE enterotoxins show an absence in all strains tested. We know that about 95% of outbreaks of staphylococcal food poisoning are caused by type SE SEA-SEE, and the remaining 5% epidemics can be combined with other new identified SE (Kokan and Bergdoll, 1987).


*tst*+ (t007) (4 souches) (2 souches en hiver,
1 souche isolée en été, 1 souche isolée en printemps).
*pvl*+ (t267) (1 souches) (isolée en hiver).

**Figure 1:** Detection of Staphylococcal Toxin Genes

## IV. CONCLUSION

This first study on bovine mastitis in the region of Oran allowed us to determine the molecular profile of toxic bacterial species *S. aureus* that is dominant among bacterial isolates in the study.

*S. aureus* strains isolated in these study antibiotic susceptible strains and some strains of *Staphylococcus aureus* have proved sensitive carrier and gene encoding toxins (*Luk-pvl*, *TSST-1*) for the toxin-Pantin Valentine - leucocidine and toxic shock syndrome. These results revealed for the first time an animal strain identified with this toxic molecular profile in the region of Oran.

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# Biological Control of *Botrytis cinerea* by *Bacillus* sp. Strain S7LiBe Under Abiotic Stress

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## ABSTRACT

In this investigation, the isolate S7LiBe isolated from trefoil rhizosphere in Barbacha, Bejaia (northern Algeria), was selected on the basis of plant growth promoting attributes. This isolate identified as Bacillus sp. based on 16SrDNA sequence analysis, exhibits the highest similarity of 99.1% with *Bacillus pichinotyi* AF519463 andAF519460 and *Bacillus* sp. JX152775. *Bacillus* sp. S7LiBe has been subjected to the study of its antifungal activity against *Botrytis cinerea in vitro* and *in vivo* on the detached lettuce leaves. The effect of some abiotic factors on the performance of this bacterial strain as biocontrol agent and the production of some antifungal metabolites (siderophores, ammonia, hydrogen cyanide and chitinase) were also carried out. The results showed that *Bacillus* sp. S7LiBe strain inhibit the mycelial growth in high level (70%±1,33) and the fungal spores germination (82,54%±3,58 ). The strain produce siderophores, ammonia, hydrogen cyanide and chitinase. Its antagonistic activity was found to be optimal on CZA modified medium at a pH of 6.5 and a temperature of 30 ° C. The antagonist activity on the detached lettuce leaves showed that the bacterial strain inhibit 57.0% of the lesion extension.

**Keywords:** *Bacillus* sp., *Botrytis cinerea*, PGPR, Biocontrol.

## I. INTRODUCTION

Pathogens affecting plant health are a major menace to food production and ecosystem stability worldwide [1]. It has been estimated that approximately one third of the food crop is destroyed every year due to attack by insects, pathogenic fungi, bacteria, and nematodes [2]. *Botrytis cinerea* is one of the most hazardous plant pathogen infecting a large number of vegetable plant. This phytopathogene infect leaves, stems, flowers and fruits of plants, either by direct penetration or through wounds caused by cultivation practices [3; 4; 5; 6].To control plant disease, producers had resort to the chemical products. However, many of the chemicals are hazardous and causes several negative effects on the environment and human health. For that reason, biocontrol has become an interesting alternative that are more "friendly" to the environment. Plant growth promoting rhizobacteria (PGPR) are the important group of microorganisms, which play a major role in the biocontrol of plant pathogens [7]. *Bacillus* species have been reported as plant promoting bacteria in a wide range of plants [8; 9; 10; 11], it´s one of the principals PGPR groups known for their application as biocontrol of several pathogenic fungi. *Bacillus* is the key bacterial genus that have shown greatest potential for *Botrytis* disease control [12], and that by several mechanisms which include; secretion of lytic enzymes, siderophores and antibiotics, competition for space and nutrient, defense plant stimulating and combination of mechanisms [7; 13]. In addition, *Bacillus* species produce spores that are resistant to heat and desiccation, which allows the preparation of more stable and durable formulations, this explains the greater availability of commercial products based on Bacillus [14; 15]. Commercial available biocontrol products include: Kodiak (*B. subtilis* strain GB03), Serenade (B. subtilis QST 713), YieldShield (*Bacillus pumilus* strain GB34), EcoGuard (*Bacillus licheniformis* strain SB3086) [16; 17]. and Subtilex (*B. subtilis* strain MB1600) which is active against *Botrytis* spp. infection of vines, strawberry, cucumber, powdery mildew of tomato, and brown rust of cereals [2; 17].

In the present investigation, Bacillus sp. S7LiBe strain isolated from trefoil rhizosphere in northern Algeria has been subjected to the study of its antifungal activity against *Botrytis cinerea in vitro and in vivo on the* detached lettuce leaves. Many abiotic factors, such as pH, temperature, moisture, inorganic and organic constituents, may influence the mechanisms of biocontrol, in this research we study the influence of some abiotic stress on the performance of *Bacillus* sp. S7LiBe against *B. cinerea. The* production of some antifungal metabolites (siderophores, ammonia, hydrogen cyanide and chitinase) was also carried out.

## II. METHODS AND MATERIAL

*A. Fungal strain and culture conditions*

A strain *Botrytis cinerea* BC1 used in this study was obtained from the laboratory of Mycology, Bejaia University, Algeria. It was originally isolated, in the Laboratory of Plant Protection(INRA, STPV, Avignon, France), from tomato plants with typical symptoms of grey mold. The fungal strain was grown in Petri plates containing potato dextrose agar medium (PDA, Difco), The plates was incubated at 21ºC for 3-10 days, Stock cultures of *B. cinerea* was maintained on PDA medium and stored at 4°C.

*B. Bacterial strain and inoculum*

*Bacillus* sp.S7LiBe was isolated from trefoil rhizosphere in Barbacha, Bejaia (northern Algeria). This strain was selected for its ability to produce several promoting growth traits (production of siderophores, indole acetic acid, inorganic phosphate solubilization and heavy metal tolerance), and was identified by sequencing their entire 16S rRNA. Bacterial suspension was prepared by inoculating a loop full of cells in LB medium and incubating at 30°C for 24h.The bacterial concentration was calculated by serial dilutions and checked by optical density ($OD_{600}$), and then adjusted to $10^8$c.f.u. /ml before use.

*C. Molecular identification*

The isolate S7LiBe was subjected to the molecular identifications, genomic DNA extraction from pure bacterial colonies was carried out using the FastDNA® SPIN kit in conjunction with the FastPrep FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The genomic DNA was further PCR amplified for 16S-rDNA gene sequencing using the flanking primer pair 616F (5´AGA GTT TGA TYM TGG CTCAG 3´) and 630R (5´ CAK AAA GGA GGT GAT CC 3´). For phylogenetic analyses the obtained 16S-sequences were aligned with the *Sina Aligner V1.2.11* on the Silva website *(www.arb-silva.de)* and phylogenetical allocated with the sofware package ARB [18]. Phylogenetic tree construction was performed by using the Maximum-Likelihood[19].

*D. Effect of the antagonist on the growth of B. cinerea mycelium*

The inhibitory effects of the strain on the growth of *B. cinerea* were tested by the agar diffusion method as described by [20]. $1cm^2$ fungal plug was inoculated in the center of PDA plate, each isolate was sown at a distance of 2.5 cm from the fungus. Plates without antagonist served as control. Three replications were performed for each confrontation experiment. The plates were then incubated at 25±2°C for 5 days and verified every day. The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula: PGI (%) = [(KR-R1)/KR] x100 where KR corresponds to the distance from the point of inoculation to the colony margin on the control dish (mm). R1 represents the distance (mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes.

*E. Effect of the antagonist on the germination of B. cinerea spores*

20µl of spore suspension adjusted to $10^6$ spores/ml and 20µl of 24h bacterial culture grown on LB medium adjusted to $10^8$c.f.u./ml, were pipetted into an Eppendorf tube containing 1 ml of sterile distilled water with 5% of glucose and then incubated at 21°C for 24 h. Control tubes were inoculated only with fungal spores. The experiment was realized in triplicate. The germination studies were performed on hemocytometer using a light microscope (40X).

*F. Effect of the antifungal volatiles on the growth of B. cinerea mycelium*

The strains *Bacillus* sp. S7LiBe et *Pseudomonas* sp.S5LiBewere streaked on a Petri dish containing LB agar medium. a second Petri dish containing PDA medium, was inoculated with a 5 mm plug of the fungus *B. cinerea* in the center of the plate, and inverted and placed over the bacterial culture. The two plates were sealed together with parafilm and incubated at 25°C. The percentage of growth inhibition (n) of the fungus, was recorded and calculated using the formula: N= [(A-B)/A] x 100, where A corresponds to the diameter of the mycelium in the control (cm) and B represents the diameter of the mycelium in the plates inoculated with bacteria (cm).

*G. In vivo antagonism experiments*

The evaluation of the antagonistic activities of S7LiBe against *B. cinerea* is realized on the detached lettuce leaves. The test was done according to the protocol of INRA Avignon France with modification. Three lettuce leaves were deposed on box lined beforehand with absorbent paper soaked in distilled water, after this, the bacterial suspension ($10^8$ CFU/ml.) was sprayed on the surface of the detached lettuce leaves. The leaves were allowed to air dry in a sterile cabinet for up to 30 min, followed by inoculation with a mycelium plug (5mm) at the center of each leaf. A control was done with lettuce leaves sprayed with sterile distilled water and then with *B. cinerea* plug in the center. The leaves were then stored in a growth chamber at 24°C for 72 h. The percentage of disease reduction of the gray mold on the lettuce leaves was calculated using the following formula:

Reduction rate (%)=[(A-B)/A] X 100, Where; A is the lesion diameter recorded in untreated control, and B is the lesion diameter in the infected lettuce fruit treated with the antagonists [21]. For each treatment, nine leaves were assayed (three leaves as one replicate).

*H. Production of antifungal compounds*

*1) HCN and NH3 production:* Bacillus sp. S7LiBe was tested for the production of hydrogen cyanide by adapting the method of[22]. Bacteria were streaked on nutrient agar medium amended with 4.4 g glycine/l, a Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28°Cfor 4 days. A change in color of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.The production of ammonia was tested in peptone water. Freshly grown cultures were inoculated into 5 ml peptone water, after incubation period 48hrs at 30°C, 0.25 ml of Nessler's reagent was added to each tube. Development of brown to yellow color indicate production of ammonia [23].

*2) Siderophores production:* The experiments were carried out in Chrome Azurol S agar according to the method of Schwyn and Neilands [24]. CAS agar was prepared from four solutions, which were sterilized separately before mixing. At 50°C, after autoclaving, nutrition solution and casamino acid solution were added to the buffer solution. Indicator solution was added last with sufficient stirring to mix the ingredients. The bacterial strain was streaked on the surface of the bleu agar plates and incubated at 30°C for 72h and examined for growth and production of orange halos surrounding the colonies.

*3) Chitinase Activity:* Chitinase activity was determined as described by Kopečný et *al*. [25], Bacterial strain was inoculated in minimal salt medium containing 0.8% of colloidal chitin as carbon and energy source and incubated at 30°C. Clear zone around the colony indicate chitinas activity.

*I. Effects of culture medium, pH and temperature on the antagonistic activity of S7LiBe*

The influence of the culture medium; pH; salinity and incubation temperature on the antagonistic activity of the bacterial strain was studied according to the method cited in the section (D). Four culture medium (Potato Dextrose Agar PDA; Sabouraud Dextrose Agar SDA; Czapek Dox Agar CZA; Malt Extract Agar MEA) were tested. The culture medium which gave a high PGI%, was prepared at deferent pH value (4.5, 6.5, 8.5, 10.5) to determine the optimum pH. The optimum temperature was studied at, 4°C; 25°C; 28°C; 30°C; 37°C and 44°C,

using the optimum medium and optimum pH. After incubation, the PGI% was calculated as described previously.

## III. RESULT AND DISCUSSION

### *A. Phylogenetic identification of S7LiBe*

Comparative analysis of the 16S rDNA sequence of the strain S7LiBewith already available database showed that the Gram-positive isolate was member of the genus Bacillus and the 16S rRNA gene sequence was 99.1% similar toBacillus pichinotyi AF519463 andAF519460 and Bacillus sp. JX152775.The taxonomic positions of S7LiBe is shown in the phylogenetic tree (Figure 1).

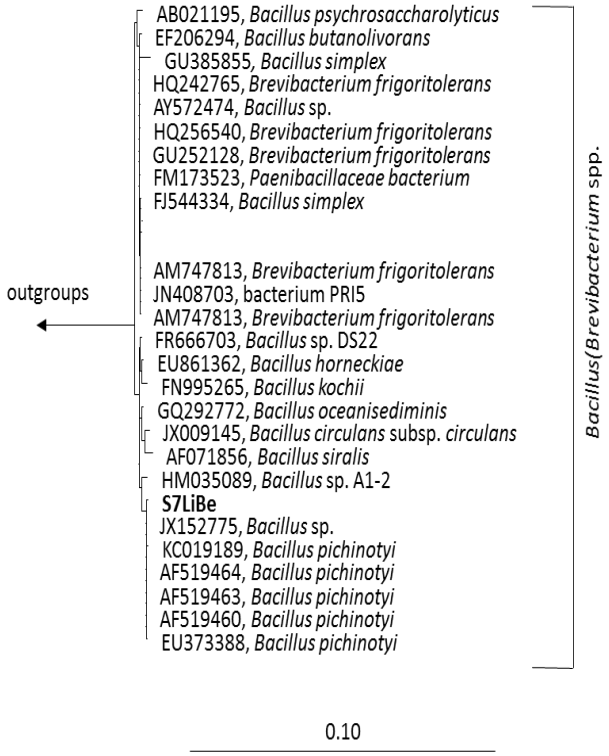


**Figure 1 :** Phylogenetic relationship of S7LiBe, Dendrogram based on maximum likelihood tree calculation.

### *B. Effect of the antagonist on the growth mycelium and the germination spores of B. cinera*

Six days after incubation, the inhibitory zones were observed in the dual culture dishes (Fig. 2a and b2). *Bacillus* sp. S7LiBe inhibited 70%± 1.33 mycelia growth of *B. cinerea* and it was effective in suppressing germination of *B. cinerea* spores (Fig. 2c and 2d). The percentage spore germination was 25.65% ±6.69 after 24 h of incubation. These results indicate that *Bacillus* sp. S7LiBe grown on PDA plate released an extracellular diffusible metabolite(s) that inhibited the hyphal growth of *B. cinerea*. Several mechanisms have been proposed to explain the antagonist effect of *Bacillus spp.*, including antibiotic production, hydrolytic enzymes synthesis, competition for nutrients, or a combination of these mechanisms in synergy [26, 27, 28]. Alabouvette et al. (2006)[29], suppose that the inhibition of spores in soil germination is due to the competition for nutrients, especially the carbon. Elad and Stewart (2007) [12] showed the sensibility of *B. cinerea* to several antifungal produced by *Bacilus* sp. *Bacillus brevis* and *Bacillus polymyxa* produce the gramicidin S and the polymyxin B which inhibit *B. cinerea* germination *in vitro* and *in vivo.*

### *C. Effect of the volatile compoundson the growth of B. cinera mycelium*

Although the absence of direct contact between bacterial strain and the fungi, a strain *Bacillus* sp. S7LiBe inhibited up to 65.81% ±4,06 of *B. cinerea* mycelium. These result approve that *Bacillus* sp. S7LiBe release a volatile compounds which affected fungi growth.Guetsky et al. (2002)[30] showed that the volatile compounds produced by *Bacillus pumilus,* inhibit the growth of *Botrytis cinerea*. Several studies have been demonstrated that the production of volatile compounds, e.g. HCN; NH3; 2,3-butanediol; acetoin, can influence growth of fungi[31, 32] and improve the plant growth [33, 34, 35, 36].

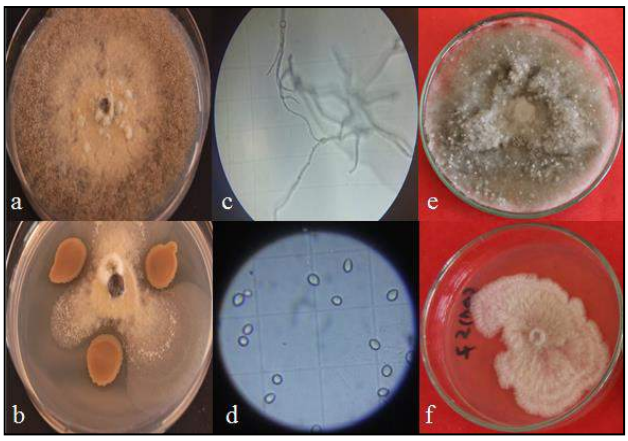


**Figure 2 :** Antifungal activity of *Bacillus* sp. S7LiBe against *B. cinerea.*(a, c and e) *B. cinerea*BC1 Control in

agar diffusion test, spores germination test and antifungal volatiles test respectively. (b, d and f) Strain *Bacillus* sp. S7LiBe in agar diffusion test, spores germination test and antifungal volatiles test respectively.

*D. In vivo antagonism experiments*

*In vivo* evaluation of *Bacillus* sp. S7LiBe as antagonists towards *B. cinerea* on the lettuce leaves showed a remarkable potential (Fig. 3).The antagonist inhibited up to 57% ± 1.65 of *B. cinerea*. These results confirm those obtained in the in vitro tests and those reported by Wang et al. (2009)[37], they showed the potential of *Bacillus subtilis* to inhibit up to 52.4% of *B. cinerea*. According to Elad and Stewart (2007)[12], *Bo. cinerea*, is sensible to the antibiotics produced by some *Bacillus* species in several host.


**Figure 3 :** Lesion extension in the lettuce leaves by *B. cinerea*. (S7) *Bacillus* sp. S7LiBe, (T) Control.

*E. Production of antifungal compounds*

*1) HCN and NH3 production:* In the present study, *Bacillus* sp. S7LiBe was a moderate producer (++) of cyanide and ammonia. Researchers have reported that many *Bacillus* species could secrete several antifungal metabolites such as CHN [33, 34, 38], and NH3 [34, 39]. According to Prashar et al. (2013) [38], the production of antifungal compounds diffusing in the culture medium or volatiles as cyanide and ammonia, affect negatively the fungi growth.

*2) Siderophores production*: Siderophores production is a major mechanism involved in bio-control by many PGPR groups, including *Bacillus* sp., which produces a large variety [40]. Siderophore production in the CAS agar is characterized by the production of orange halos surrounding the colonies (Fig. 4). In this investigation, *Bacillus* sp. S7LiBe produce an important zone around the colonies and the diameter was up to 4 cm ± 0.6.Several studies have demonstrated the production of siderophores by *Bacillus* sp.[41], the role of these molecules in the control of diseases has been well documented[42], they deprive pathogenic fungi of iron since the fungal siderophores have loweraffinity [43, 44]. These molecules may indirectly stimulate the biosynthesis of other antimicrobialcompounds by increasing the availability of these minerals to the bacteria [45].

*3) Chitinase Activity:* It has been reported that antifungal mechanism of antagonists has been attributed to the production of hydrolytic enzymes such as chitinase, [46, 47,]. In this investigation, *Bacillus*sp. S7LiBe produce a clear zone around the colonies, indicate the production of Chitinase enzyme. Chitinase-producing microorganisms have been reported as biocontrol agents for different kinds of fungal diseases of plants [48, 49, 50]. Some studies revealed a relationship between chitinases of *Bacillus Sp,* and its ability to inhibit *Fusarium oxysporum* and *Fusarium solani* mycelial growth [51].

*F. Effects of culture medium, pH and temperature on the antagonistic activity of S7LiBe*

Understanding which environmental factors are important and how these influence disease suppression is widely recognized as a key to improving the level and reliability of biocontrol [45]. However, little knowledge of how specific factors affect the interactions among soil borne plant pathogens and their antagonists[45, 52, 53].In this investigation, the PGI% obtained showed that *Bacillus* sp. S7LiBe inhibit the mycelial growth at different conditions tested (Fig. 4, 5 and 6), however, PDA, pH 4,5 and the temperature 4ºCwerethe least suitable condition for *Bacillus* sp. S7LiBe ($0{,}05 \geq p \geq 0{,}01$).Many factors have been discussed that may affect rhizosphere microbial communities and it is likely soil type, pH, temperature, salinity, organic carbon and inorganic nutrients, as well as the presence of other soil organisms will effect PGPR performance [54, 55, 56].

Carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy [45].

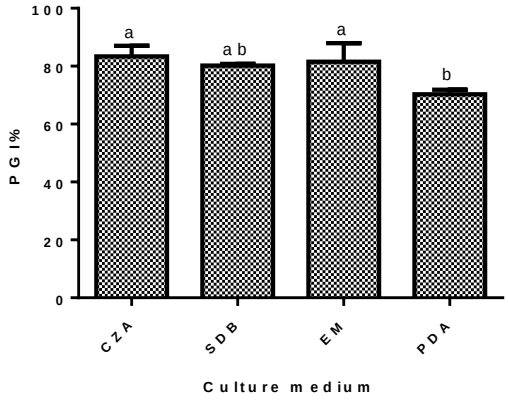


*Figure 4:* Effects of culture medium on the antagonistic activity of S7LiBe

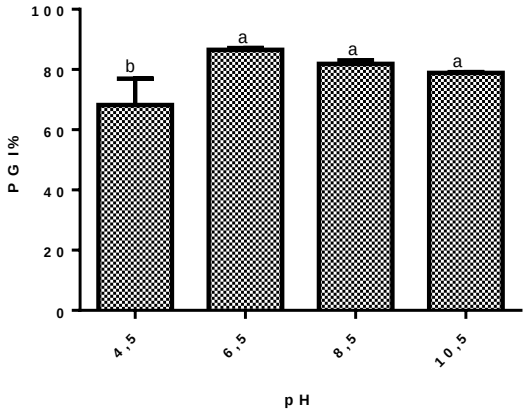


***Figure 5 :*** Effects of pH on the antagonistic activity of S7LiBe

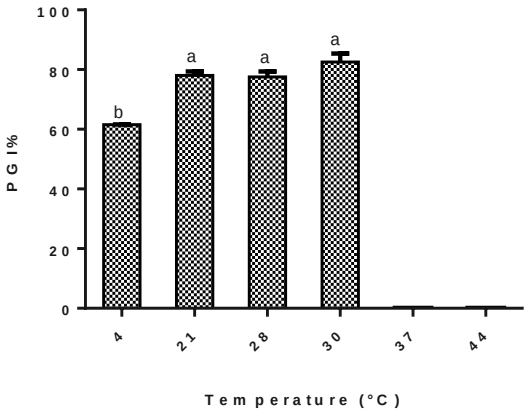


**Figure 6 :** *Effects of temperature on the antagonistic activity of S7LiBe*

## IV. CONCLUSION

Thus the strain S7LiBe selected previously for its ability to produce several promoting growth traits, could be applied in the same time as a PGPR and a biocontrol agent, to improve crop productivity and to prevent fungal attack in crop plant.

## V. ACKNOWLEDGMENT

Our special acknowledgments are addressed to Prof. Dr. Hartmann A. and Schmid M. from Helmholtz Zentrum München, Research Unit Microbe-Plant Interactions, Neuherberg, Germany for their molecular identification. We also want to consider this article as dedications to Prof. Dr. Hartmann for all his continuous and effective helps. We would be very grateful.

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# Accumulation of Two Metallic Elements (Zn, Pb) in the Mule (Flathead Grey Mullet Linnaeus 1758) Fishing in the Bay of Oran

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## ABSTRACT

Our present study focused on the evaluation of two concentrations of heavy metals (Pb, Zn) in the mullet (Mugil cephalus Linnaeus. 1758) caught in the Bay of Oran. This fish reflects very well the quality of its habitat, it is a very abundant species in Algerian coastal waters and much appreciated by the Algerian consumer. The monthly sampling took place over a period of six months from February to July 2010, three bodies have been considered: the liver detoxification organ, the gonads, reproductive organs and muscle representing the portion consumed by 'Man.The heavy metal concentrations were determined by the atomic absorption spectrophotometry flame in function of several parameters (gender, month, organs, and the sampling site).It appears from this study that the mullet (Mugil cephalus) contains the two wanted metal pollutants, the most important grades are those of Zinc is 80.55mg / kg and the lowest are those of the Lead 0.03 mg / kg. The results obtained treated showed no statistically significant difference between the heavy metal content of both sexes at the target organs and between the sampling area. The levels of trace metal concentrations reflect a certain pollution of target areas.

**Keywords**: Mulet, Flathead Grey Mullet, heavy metals, Lead, Zinc, contamination, pollution, Oran.

## I. INTRODUCTION

The marine environment is contaminated by many chemicals including metal elements discharged by industries, agriculture and urban communities. Estuarine and coastal areas under strong continental influence, are the most affected by this contamination. The latter can affect the health of the marine environment, since it does not undergo any biological or chemical degradation. It can therefore accumulate in food chains of different links to toxic concentrations in marine organisms (Neathery & Miller, 1975).

Bay of Oran is the site of a very high industrial concentration including eastwards ie Arzew which is the seat of incessant pollution. Note also the use of coastal waters for cooling thermal power stations in addition to discards a lot of waste and pollutant that can cause many strandings of certain marine fauna, observed on the Oran coast (Boutiba et al., 2003). For this reason it seemed so interesting to begin a study on the bioaccumulation of heavy metals and to detect the level of contamination in a target species, mullet (Mugil cephalus Linnaeus, 1758), fished in the bay Oran, since it forms an important link in the trophic chain.

## II. METHODS AND MATERIAL

Our study area is located on the Algerian west coast (Figure 1). The Oran coast is a set of landforms including shaping depends directly or indirectly shares in the sea. Bay of Oran occupies the central part of Oran coast and opens from west to east; it is bordered on 30 km of high ground and draw a half circumference roughly steady from Cape Falcon to Cape Aiguille. It is between the Andalusian Bay and the Gulf of Arzew. (Leclaire 1972).

The species Flathead Grey Mullet waschosen in this study because it plays an important role in ecological energy flow in marine communities. Through its regular abundance in the Mediterranean Sea, it is a characteristic link in the food chain and it also serves as prey (BESTER, 2004). Many studies have been devoted to his eating habits (SUZUKI, 1965; ODUM 1968; ZISMANN et al, 1975;. & MIGLARESE BISHOP, 1978). Finally, it is of great local importance because it is one of the most consumed fish and appreciated by the Algerian population.

Sampling took place over a period of six months from February to July 2010; 110 individuals were sampled at these two bays. After measurements, liver, muscle, and gonads were removed, weighed and frozen until the time of chemical analysis.

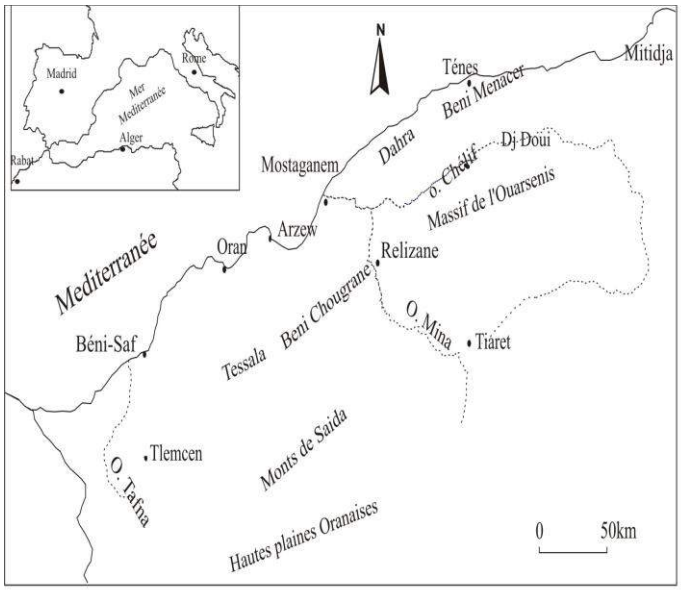


**Figure 1 :** Geographical location of bay of Oran(Perrodon, 1957).

**1-Chemical analysis**

The determination of trace elements in the fish commonly used for atomic absorption spectrometry method (SAA). Indeed, sample Mugil cephalus must first undergo mineralization.

Mineralization wet samples was performed according to the method of AMIARD et al. (1987): 1 ml of nitric acid is added to 1 g of sample and then adjusted to 4 ml of bidistilled water after one hour at 95 ° C.

This mineralization samples is accompanied on the one hand, by the white, consisting of solutions containing the mineralization reagent (nitric acid) and undergoing the same experimental conditions as the sample, and on the other hand, the series of intercalibration samples on a standard biological material tissue of Mytilus galloprovincialis (SRM 2976) from the International Agency for Atomic Energy of Monaco, allowing us to determine the coefficients of variation for each of the desired metals and control accuracy and the precision of the analytical protocol.

**2-Statistical treatment**

The statistical data processing was carried out using the Stastica software, and the results are shown as mean with standard error (m ± SD) mg / kg. Student's t test (T) was used to determine the significance of differences between the calculated average. The difference was considered significant at a confidence level (p) of less than 5% (p> 0.05).

## III. RESULT AND DISCUSSION

Both sought metal pollutants, zinc (Zn) and lead (Pb), are present in the targeted sub Flathead Grey Mullet samples from study sites. These concentrations are not entirely heterogeneous.

**A- Monthly change in average concentrations of heavy metals in Flathead Grey Mullet (Figures 2)**

In general, in the Bay of Oran, we see episodes of rising and falling concentrations of metallic elements. The average concentration of zinc in fish, higher during the month of April, reaching 36.5 mg / kg. Plombiques the concentrations are very low during all sampling months. Nevertheless, we found that lead concentrations recorded during all months are very low and almost homogeneous. The latter is a xenobiotic contained in the list of dangerous substances (CEE 1982), and is also considered highly toxic and polluting non-biodegradable (EEA 1997).

Note that in target sites during the month of April, the average concentrations are highest, while during the months of June and July, the average concentrations are minimal.

The sexual rest period is a gametogenesis phase characterized by increased accumulation of nutrient reserves with a summary and Storage of carbohydrate materials, lipid and protein (Webb, 1979). A spawning, nutrient reserves to draw automatically the heavy metal concentrations drop (release of metals that time) and reserve accumulation will slowly resume at the beginning of the period of sexual rest (WEBB, 1979 ).

According Landret, (1974), GREELY et al. (1987) and IBAÑEZ, (1994), reproductive Flathead Grey Mullet occurs from October to January. In the Mediterranean, M. cephalus different populations breed between June and October (FAOUZI 1938; ERMAN 1959; Morovic 1963 Farrugio 1975; BRUSLE & BRUSLE 1977; BRUSLE 1981).

The Student t test gave no significant difference at all concentrations of heavy metals (Pb, Zn) found in the study area (P> 0.05).

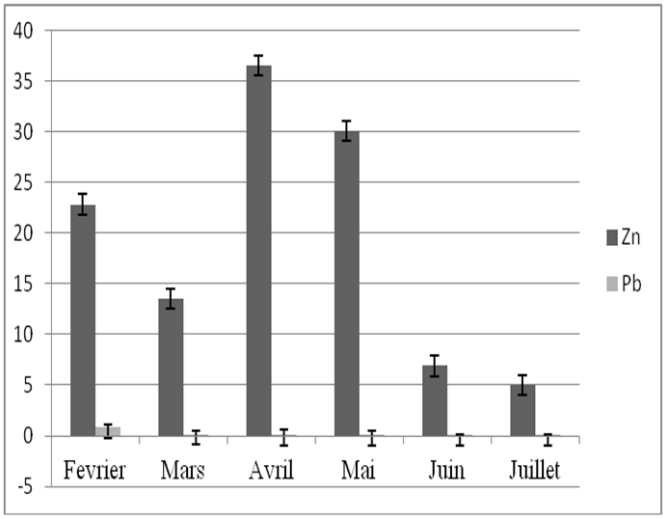


**Figure 2:** Monthly evaluation of the average concentrations in heavy metals (Pb, Zn) in mg/kg of P.F at *Mugil cephalus* in bay of Oran.

**B- Monthly change in average concentrations of heavy metals in sex functions (Pb, Zn) in Flathead Grey Mullet**

Based on the results in Figure 3, we can say that bioaccumulation of both inorganic pollutants is higher in females than in males individuals individuals. Comparing the average concentrations of sex indicates that zinc is accumulated by more females than males. Lead has a low concentration in both sexes and in all organs. Metals are more accumulated in the liver of females than males.

In fact, Powell et al. (1981) had already shown that heavy metals were concentrated in the organs of the teleost in descending order: Liver> Kidney> Muscle. The liver is considered the primary organ accumulation. Some authors (GUNS et al, 1984;. Nabawi EL et al, 1987;. & HORNUNG RAMELOV, 1987), muscle tissue, specifically the fish is barely involved in the metabolism. The preferred accumulation of these metals, in particular zinc, in females compared to males, this may be due to differences in concentrations of the gonads.

According SIDOUMOU et al. (1991), the female gonads focus more zinc than males. Females are more infected than males, this may be due to their migration to the coast polluted compared to spawning, which explains the high contamination with heavy metals via the various sources of pollution.

The results obtained by THIBAUD (1976) contents slightly higher trace metals were detected in fish caught near the coast.

For the entire population of the study area, the Student t test does not record any significant difference (P> 0.05) between the concentrations of separate sexes specimens.

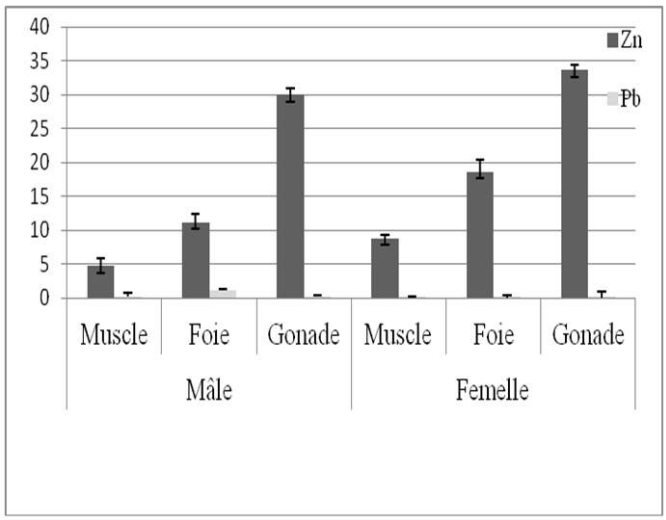


**Figure 3 :** Variation of the average concentrations in heavy metals according to sex (Pb, Zn) at *Mugil cephalus* fished in bay of Oran.

## IV. CONCLUSION

The study we have undertaken demonstrates that, given the results obtained and compared with the limit values found in the literature, we can conclude that the bay is not polluted, despite the presence of metal contaminants but with no significant difference. Both metals studied (Pb and Zn), bioaccumulation is preferentially in the liver and gonads relative to Mugil cephalus muscle. Moreover, the dominant metal, zinc stands out clearly in relation to the other member, and it has very high contents.

Regarding sexual maturity, a relationship was established between the laying period and high values of trace metals which corresponds to increased hepatic activity occurring Lord fattening of the case after the breeding season. Compared to sex, females are more heavily infected than males.

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# Bacteriological quality of Sea water bathing water for two stations of Eastern Oran (Dahliss, Sidi-Moussa) from Western Northern Algeria

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## ABSTRACT

The bathing water beaches of the coast of East Oran of Western Algeria are threatened by waste water discharges untreated or insufficiently purified bacteriological and deteriorating quality. This study is to evaluate bacterial contamination of two stations is Oran: Dahliss and Sidi-Moussa. Three biweekly samples were taken at different distances: 1m, 5m and 10m from June 1 to September 30 of 2012. For each of the two stations, the analysis focused on the estimation of the following parameters: fecal and total coliforms, fecal streptococci, *Salmonella*, *Staphylococcus*, sulfite-reducing *Clostridium* spores and the *Vibrio cholerea*. The statistical analysis of samples confirmed that they are in favour of a very significant correlation. The results showed that the Dahliss station is free from bacterial contamination; however the Sidi-Moussa station showed a level of pollution and subsequently it is unsuitable for swimming. Pathogens (*Salmonella*, *Staphylococcus* and *Vibrio*) are absent in all the samples taken. The East Oran is free from pathogen contamination.

**Keywords**: Bathing water, Fecal and Total coliforms, Fecal streptococcis, *Salmonella*, *Staphylococcus aureus*, *Vibrio cholerea*.

## I. INTRODUCTION

Demography, technology development and all anthropogenic factors constitute a danger to public health. Marine and coastal environments in particular are subject to constant changes of physical, chemical and bacteriological (**Kerfouf** and ***al.***, **2010**).

Water is an essential and irreplaceable element to ensure the continuity of life. However, it can be a source of disease (**El Haissoufi** and ***al.***, **2011**), playing role of vector of potentially dangerous agents, including pathogenic microorganisms (**Hassoun** and ***al.***, **2010**). The deterioration of water quality is a threat as great as that related to quantitative imbalance (**El Addouli** and ***al.***, **2009**).

Monitoring quality of sea water along Algerian west coast has been the subject of much research (**Hebbar**, **2005**; **Mouffok**, **2005**; **Djad** and ***al.***, **2015**).

During the summer, swimming is a much practiced recreational activity. On this occasion, the Algerian population wants to find a welcoming environment, protected from various forms of nuisance. This quality is a health factor but has also become an important part of tourism development. Improving the quality of bathing water may reduce health problems and improve recreation of the local population, making it as a more attractive region for tourism.

The choice of sampling sites was motivated by certain factors:
-proximity of beaches to the city;
-importance of attendance by beaches vacationers;
-presence of the discharge of domestic sewage;
-few researches at site (**Hebbar, 2005**).

This work reflects the overall results collected during bacteriological analysis of bathing water of east of Oran, targeting both sites Sidi-Moussa and Dahliss, and conducted during the summer of 2012.

## II. METHODS AND MATERIAL

### Study Area

Kristal is located in North West Gdyel on Western slopes of Jebel Bouhaichem an altitude of 426m and 497m at the Kristal. This isolation allows it to maintain very close relations with Oran. It is a tourism expansion zone defined by Decree n°88-232 of 05/11/1988. Its suburbs cover an area of 45,6 hectares, with a total population of 5498. The predominance of employment is in the agricultural and fisheries sector with 35,5% of total employment; this is explained by the presence of farmland that by pass central Kristal one hand and the existence of an artisanal fishing activity on the other (**POS**, **1998**). The best sand beaches very attended are located at the East of Oran (Kristel) "Fig. 1".

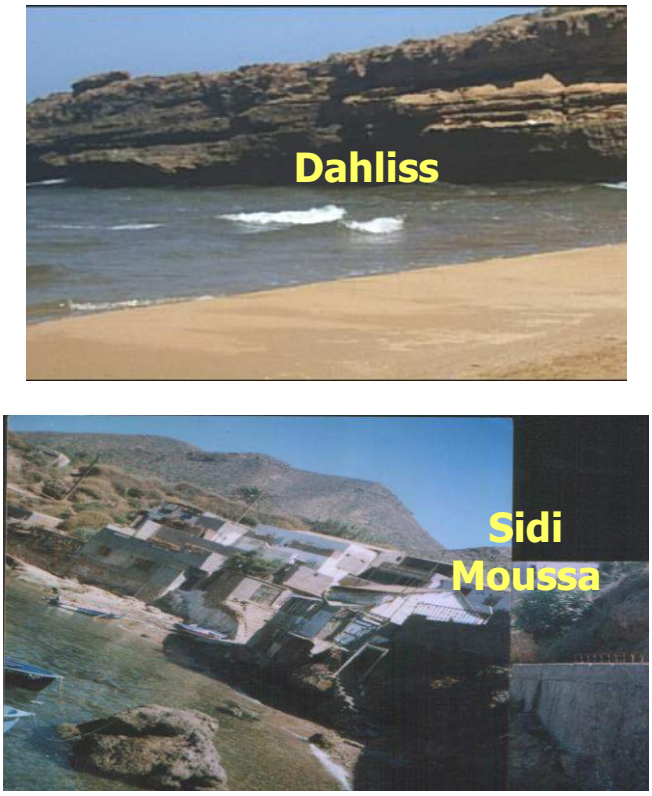


**Figure1:** Dahliss (on the left) and Sidi-Moussa (on the right) (Hebbar, 2005)

The coastal strip of Kristal site is formed by:

-Of shores accessible: Sidi-Moussa beach, Tamda beach, Dahliss beach and Ain-defla beach.
-Of inaccessible shores, generally corresponding to the slopes with slopes exceeding 25%: cliffs and headlands.

### Sampling

Method used for the detection of total and fecal coliforms and fecal streptococci is the series of dilutions in liquid medium (NPP), described by **WHO** in **1995**.

Bacteriological analyzes of this work consisted in search of following tests and pathogens germs: Total coliforms including fecal coliforms , streptococci fecal (or Intestinal Enterococci), *Escherichia coli*, *Vibrio cholerea* (**Pilet**, **1996**), *Staphylococcus aureus*, *Salmonella* (**Guiraud**, **1998**) and spores of sulphite-reducing *Clostridium* (**Rejsek**, **2002**) under aseptic conditions, hours and days or population of bathers is stronger and at a distance of 30cm below water surface at article 3 of Interministerial decree n° 8 of 17.01.1994 .

Total coliforms on BPCL environment, thermotolerant fecal coliforms in the middle Schubert at 44°C for 48 hours and fecal streptococci Rothe medium based on sodium azide at 37°C for 48 hours. Search spores of sulfite-reducing *Clostridium* fits on liver meat medium agar supplemented with iron alum and 5% solutions of sodium sulfite 10% crystallized.

*Salmonella* by two enrichment on SFB medium with SFB additives, isolated on two agars (Hektoen and SS) and biochemically identified on all suspect colonies.
*Vibrio cholera* on two enrichments middle EPA 10 times concentrated , followed by two isolations on two separate agar (TCBS and GNAB) and biochemical identification of any suspicious colony.

*Staphylococcus* enrichment broth by Giolitti and Cantoni followed by isolation on agar Baird Parker and confirmation of pathogenicity test by looking for staphylocoagulase.

Note: All samples must be accompanied by an information sheet on which we note: the source of the sample, date and time of collection, the temperature of the water, the sea state and speed and the wind direction.

To contribute to the study of evolution of bacteriological quality of seawater of East Oran, we chose three sampling points (1, 5 and 10m), which are located in two stations: Sidi-Moussa and Dahliss "Fig. 2". The closest

beaches to cities were controlled at the same time sensitive to water pollution.

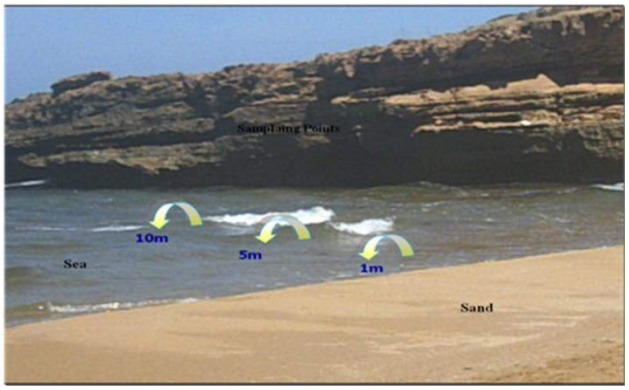


**Figure 2:** Sample points at the study area

## III. RESULT AND DISCUSSION

It is interesting to note the continued presence of fecal contamination indicators (total and fecal coliforms and fecal streptococci) and spores of sulphite- reducing bacteria and total absence in the bathing water beaches of bacterial genera: *Salmonella*, *Staphylococcus aureus*, *Vibrio cholerea*, although sea water is their natural habitat seen its high salt concentration and thus spreading the standards that specify their absence in the bathing water (cf. Executive decree n°: 93-164 ).

The indicators of fecal contamination have a high concentration in sea water of Sidi-Moussa station, explained by the proximity of this beach to discharges of domestic wastewater. **Essid** et ***al.*** (**2007**) explained the increase in sedimentary bacterial loads by the richness in organic matter. The survival of these bacteria would be under the influence of temperature (**Rozen** and **Belkin**, **2001**). In terms of *E. coli*, it is observed that its presence is dominant in most of the water samples analyzed in July and August, which indicates recent occurrence pollution. Fecal coliform (E. coli) and fecal streptococci are very well correlated with the health risk (**Fewtrell** and **Bartram**, **2001**).

Research for *Salmonella* leads to knowledge of dangerous pollution areas and assesses the value of the treatment of a wastewater treatment plant for sewage effluent which must be cleared of any pathogen before its release the natural environment (**Mouffok**, **2004**).

Microbial contamination levels have been the subject of several studies. A high bacterial pollution with an impact on public health was highlighted in the bay and the port of Algiers (**Delali** and ***al.***, **2000**; **Gueddah**, **2003**).

Industrial pollution of Kristal site is spread in the absence of industrial activities; by cons were domestic waste water that takes precedence. It is an agricultural village which delivers a relatively large volume of wastewater without treatment in that the village has no sewer system and is real permanent injector groundwater pollution flowing into the sea they even come out of the range used by the public during the summer season.

**Evolution of seeds depending on the time and distance**

Tests germs increase with the summer temperature and decrease gradually as one move away from the seaside. The germ rate is decreasing 1m =, 5m and 10m “Fig. 3”.

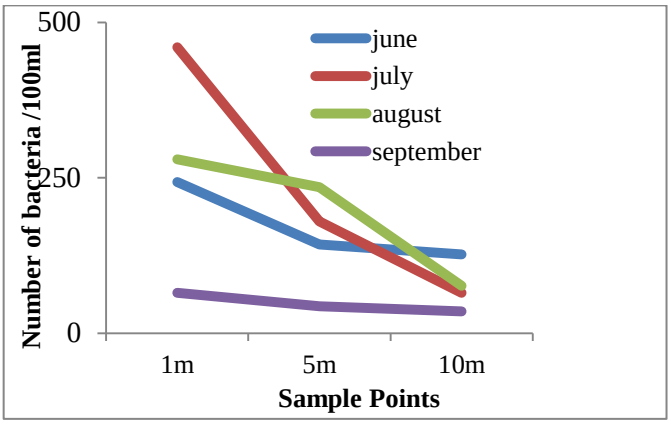


**Figure 3:** Sampling points of the study area

**Monthly evolution of bacterial contamination**

For total coliforms and fecal coliforms, it was found respectively:
- 460 cfu/100 ml and 110 cfu/100ml for Dahliss beach;
- 2700 cfu/100 ml and 1300 cfu/100ml for Sidi-Moussa beach.

Tests germs increase with the summer temperature and decrease gradually as one move away from the edge of the sea. The rate is decreasing germs 1m, 5m and 10m “Fig. 3”.

The minimum value of coliforms concentration (total and fecal) is visible at the beginning of June and by the end of September (Dahliss station); this is explained by the low existing population (only the employed staff is present). Rate of fecal coliforms in various samples of Sidi-Moussa station far beyond the standards. This massive presence reflects a strong fecal contamination and correspondingly risk of the presence of pathogens (**Bourgeois** and **Leveau**, **1991**); their origin may be either animal (intestines) or human (hand).

For fecal streptococci, their presence is visible in both beaches especially for Sidi-Moussa station where the concentration is 400 cfu/100ml during August. In contrast, normal concentrations appear to Dahliss station. Fecal streptococci are responsible for gastroenteritis and are specific indicators of ancient human fecal pollution (**WHO/UNEP**, **1995**); they are more resistant and have a long persistence in seawater (with the opposition of *E. coli*) Having regard to their resistance at a concentration of 6,5% NaCl and a temperature of 10 to 45°C (**Prescott** and ***al.***, **1995**).

The analysis of sulphite-reducing bacteria (specifically spores *Clostrdium*) revealed a maximum value of 20 (Sidi-Moussa station). Their presence indicates an old or intermittent fecal contamination and persistence in the environment (**Kunin**, **1993**). Presence of *Clostridium perfringens* in intestines of humans and many animal species is its presence in water and food due to fecal contamination (**Monteil** and ***al.***, **1992**).

Oran East is free of bacteriological pollution and remained almost in line with the regulations in force in comparison with the western Oran. Ain-Turck, Bousfer and Madagh beaches have high rates of total and fecal coliforms and fecal streptococci. The analysis of these waters has detected a presence of the same genus *Salmonella* during August (**Mouffok**, **2005**). The absence of pathogenic bacteria: *Staphylococcus aureus* and Salmonella and presence of fecal bacteria (total and fecal coliforms and fecal streptococci) in seawater Ain-defla Eastern of Oran have been authenticated. The same study confirms presence of *Staphylococcus aureus* on sampling seven months (December to June) and that of *Salmonella* three months (April to June) on Genets station west of Oran (**Seddik**, **2008**; **Souidi**, **2008**; **Maatallah** and ***al.***, **2011**). The study of **Messaoui** (**2011**) showed that the dry sand contains fecal contamination germs rates much higher than the wet sand and seawater at beaches of BeauSejour, Eden, Andalusians and Maddagh of Oran west.

**Spatial Evolution of bacterial pollution**

For Dahliss beach (commonly called French beach), the quality of its bathing water is good. This is due to several characteristics: its location far from any urban center, the absence of industrial facilities, low population absent throughout the year except for weekends and summer season. These results are lower than the standards or guides peremptory norms; but that does not exempt subject to continuous monitoring throughout the period of attendance at the beach by the various summer visitors (resident or foreign).

Sidi-Moussa station is polluted throughout summer period when the maximum value is very high in August. This is due to the different mansions built, excessive population density, number population varying from 500 to 1000 (**POS**, **1998**), heavy rainfall recorded in April and May, values are respectively 47,4 and 68,6 mm causing uncontrollable flows inexorably spilling pollutants flow (**ONM**, **2010**) and discharge of domestic sewage directly into the sea without any treatment. All homes feature pits, lacking the sewage system without forgetting the presence of a small port at this station, frequented by many trawlers and pleasure boats.

The results of analyzes and commentaries on the state of the beaches, and interpretation of results, are regularly transmitted in the form of weekly and monthly reports to the Wilayas and agencies, to keep them informed on the quality of bathing water. Such information shall be made through the website of the agency (www.APPL.dz), to public knowledge, as stipulated in Law n°03-02 of 17.02.2003 laying down general rules for use beaches and tourist exploitation.

**Statistical Analysis**

**Standard method ranks**

The order numbers associated with these percentages are: $n_{50} = 24 * 0{,}50 = 12$ and $n_{90} = 24 * 0{,}90 = 21{,}6 \approx 22$

The concentrations of total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) can be read as being associated with order numbers $n_{50}$ and $n_{90}$ = 12 = 22. Bacterial concentrations estimated in 50 and 90% are stated in Table1.

**Table1.** Bacterial concentration of two stations

| Parameters of quality of bathing water | Microbial concentrations | | |
|---|---|---|---|
| | Criteria | Sidi-Moussa | Dahliss |
| $CT_{50}$ | 500 | 950 | 90 |
| $CT_{90}$ | 5000 | 2200 | 460 |
| $CF_{50}$ | 100 | 93 | 17 |
| $CF_{90}$ | 1000 | 950 | 70 |
| $SF_{50}$ | 100 | 40 | 08 |
| $SF_{90}$ | 1000 | 150 | 40 |

Results of table1 confirm that bacteriological quality of water of beach Dahliss is good for swimming and poses no health risk to bathers, for against that of Sidi-Moussa is polluted and requires a ban permanent bathing saw the dramatic depletion of sand and presence of a resident population on these sites and absence sewerage of domestic waste water.

The correlation coefficients between TC/FC and FC/FS recorded for sea water taken from both sites are respectively r = 0,928 and r = 0,944. These results are in favor of a very significant correlation.

## IV. CONCLUSION

Good quality of bathing water is an undeniable tourist attraction for coastal communities; it is synonymous with a good management of Environment and of coastal ecosystems.

The bacteriological analysis of seawater is important for the chemist where it is interesting to know the exact chemical composition of the water and for the hygienist to know its particular quality bathing water that must obey defined criteria (**Mouffok**, **2004**).

In our study, although short (spread over four months of the summer season 2012), we deduce that the waters of Sidi-Moussa station are more contaminated than beach Dahliss. We counted fairly high levels of bacteria (total and fecal coliforms, fecal streptococci), which reflect the risks to people attending these environments but we have not isolated *Salmonella*, *Staphylococcus* and *Vibrio cholerae*. The installation of waste water treatment plant gray water is needed to avoid any risk of contamination and pollution, the consequences are often irreversible.

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# Assessment of Contamination by Xenobiotics (Lead and Cadmium) in the Muscle Tissue of two Teleost Spotted Weever (*Trachinus Araneus,*Cuvier, 1829) and the Axillary Seabream, (*Pagellus Acarne*, Risso, 1826) in the Algerian West Coast

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## ABSTRACT

Metals pollutants differ from other chemical pollutants by a low biodegradability and a high capacity to bioaccumulate along the food chain, which could be detrimental to the population as well as the flora and fauna. Our study focused on the assessment of contamination by the toxic metals: lead and cadmium in muscle and liver from two bioindicators in the western Mediterranean coast of Algeria, the axillary seabream (*Pagellusacarne*,Risso, 1826) and bright spider (*Trachinusaraneus* Cuvier, 1829). The concentrations of the metal elements were determined by Atomic Absorption Spectrophotometry flame, depending on two parameters (size, organs and seasonal). And levels of metal accumulation were determined. The present study showed the ubiquity of two toxic trace elements (Cd and Pb) in both organs of both species. The results show differences more or less marked between the two species and organs as well as significant in the two metals analyzed variations. Although average metal concentrations measured in fish muscle are low, high levels of Pb and Cd were observed in the liver of two species of fish. This is due to biomagnification of these elements in both biological bioindicators who occupy high trophic level of the marine food chain. The treatment has stressed no statistically significant difference between the heavy metal content of the two species in the muscle against by significant values are noted in the liver. Similarly, it is clear that the chemical pressure is well marked among younger aquatic individuals. The concentrations of heavy metals recorded in the edible parts of poisons were within the Maximum Dose Permissible (MPD). They are safe for human consumption, but can lead to serious dysfunction in these fish.

**Keywords:**
Axillary seabream,*Pagellusacarne*, Spottedweever,*Trachinusaraneus*, lead, cadmium,marine pollution, MPD, Bay of Oran, Algeria.

## I. INTRODUCTION

Metal contaminants released from current and past human activities are ubiquitous in the environment, especially in the sediments of rivers, these compounds tend to accumulate, which pose a potential threat to aquatic organisms.

It is in this context that our work is. This is to assess the state of the quality of the coastal environment by studying contaminants (Cd, Pb) in muscle and organs and liver muscle of the living spider caught in the Bay of Oran since it forms an important link in the trophic chain.

## II. METHODS AND MATERIAL

Oran Bay (Fig. 1) is located north west of Algeria and South West of the Mediterranean, it belongs to the Coast Mountains Tel Septentrional (Jebel Murdjadjo and Khar) (Leclaire, 1972).

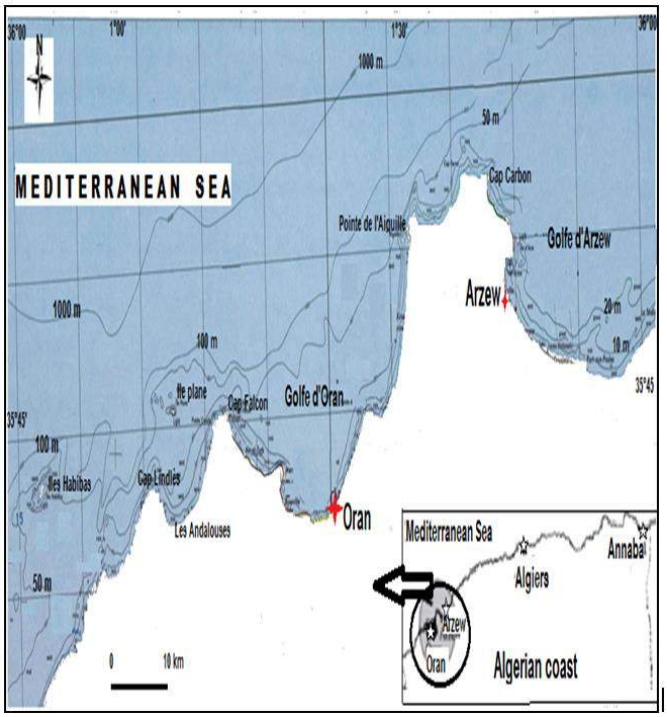


**Figure 1:** Geographical position of the study area: the Bay of Oran

Bright (Fig. 2) are a family of marine fish perciformes. Vivid found in the eastern Atlantic and the Mediterranean to the Black Sea. They stand near the coast in summer and offshore in winter. Bright spider Trachinusaraneus(Cuvier, 1829) (Fig. 2) is a coastal benthic species that lives buried in the substrate on coarse detrital sandy and sandy mud, up to about 100 m depth are revealing that their spines erected and the top of their head (Geistdoerfer 1983; Skeie, 1966).

The axillary seabream (Fig. 3) is found in the Mediterranean, Atlantic, in the Canary Islands, Cape Verde ... It usually lives at a depth of between 40 and 100 m (max 500 m).

**Figure 2 :** The random spider Trachinus araneus Cuvier, 1829.

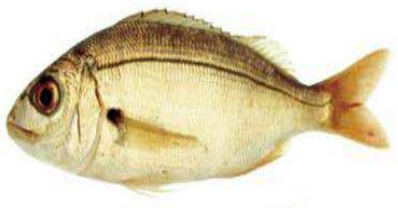

**Figure 3 :** The axillary seabream Pagellus acarne Risso, 1826

Sampling took place over one year from July 2013 to June 2014, both organs were removed: liver, and muscle.

The first step in our technique is to group individuals into batches of size classes. In a second step, one proceeds with mineralization (wet flue), depositing 1 g fresh weight of each sample (muscle, liver) in a flask to which was added 1 ml of nitric acid (HNO3) and 65% purity the temperature at 95 °C is heated for one hour, after cooling, the complete contents to 4 ml of double-distilled water, the solution is ready to mix with the atomic absorption spectrophotometry flame (SAA af) (Amiard et *al*, 1987).

## III. RESULT AND DISCUSSION

Each series of samples of our mineralization is automatically accompanied by a hand, of a mineralization white, comprised of reactant-containing solutions of mineralization (nitric acid) and suffer from the same experimental conditions as the sample, and secondly, by a series of samples of inter-calibration of a standard biological material Fucus sp 140/TM coded, provided by the International Atomic Energy Agency, Monaco (IAEA, 1995), allowing, thus defining the coefficients variation for each of the desired metals: lead (Pb) and cadmium (Cd) and check the correctness and accuracy of the protocol analytique.les results are summarized in Table 1.

Table 1: Results obtained from the inter calibration exercises expressed in ppm dry weight P. acarne and T. araneus

| **Element** | **Reference Value (A.I.E.A, 1995, Monaco)** | | **Value Found** | |
|---|---|---|---|---|
| | **Min - Max** | **Medium** | ***P. acarne*** | ***T. araneus*** |
| **Cadmium** | (0,50 - 0,57) | 0,53 | 0,54 | 0,55 |
| **Lead** | (1,91-2,47) | 2,19 | 2,32 | 2,45 |

These intercalibration exercises have shown that our analyzes were carried out in satisfactory conditions, the analytical technique used was reliable and accurate.

The average water content in the muscle of the axillary seabream is 72.51% andthat of the living spider is 76.06%. We adapted this mode of expression, because it allows a good comparison with different values from the literature.

Changes in levels of heavy metals (Pb, Cd)

Figures 4 and 5 leaves appear that individuals of both species display higher average concentrations of lead and cadmium. The rate of cadmium is noticeably lower for two fiches

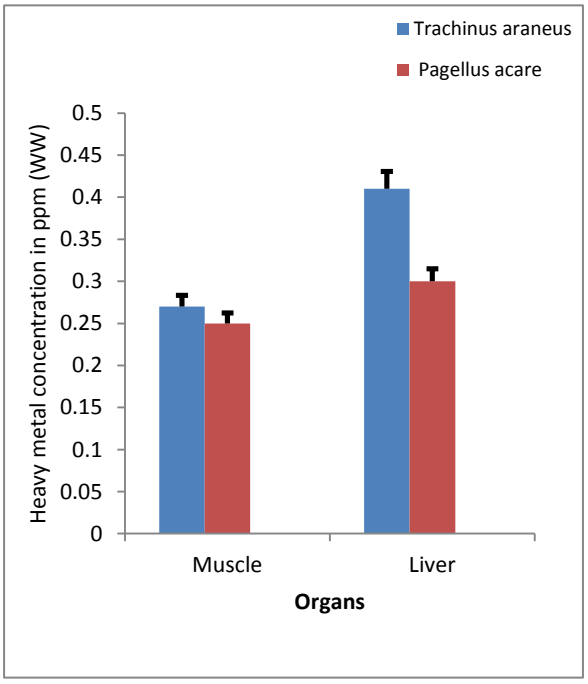


**Figure 4 :** Change in mean levels Lead (mean ± SD ppm WW) muscle and liver of Pagellu sacarne and Trachinus araneusand fished in the bay of Oran.

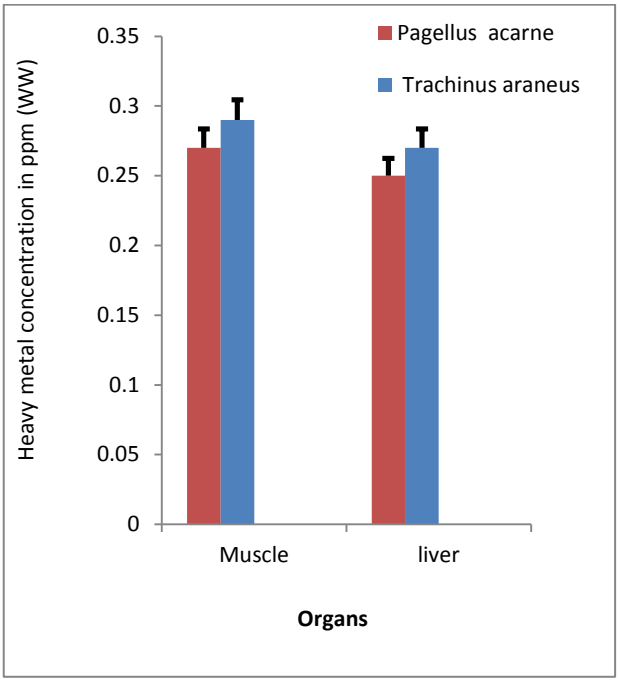


Figure 5: Change in mean levels cadmium (mean ± SD ppm WW) muscle and liver of Pagellus acarne and Trachinus araneus and fished in the bay of Oran.

The mean levels of Pb found in the different organs of the living spider and the axillary seabream show that the highest value is in the range of 0.33 mg /kg and 0,26 mg /kg successivelyobtained in the Liver. While the content is zero in the muscles. According to the contents, we can establish an order of accumulation of Pb in different organs of the two species: Liver> Muscle

The mean levels of Cd found in the different organs of the living spider and the axillary seabream show that the highest level is in the range of 0,29 mg / kg and 0,25 mg/kg obtained in the liver. While the lowest level is in the range of 0.016 mg / kg obtained in muscles. According levels can establish an order of Cd accumulation in different organs of the two species: Liver > Muscle.

We based on the results shown in Figure 3, we see that cadmium levels bioaccumulate in the liver of the living spider are (0,27±0,02ppm WW (Wet Weight)) and the *Pagellus acarne* of are (0,25±0,04 ppm WW). However, no significant difference was observed in the levels of Cd in the muscle compared to that observed in the liver in two species (Fig. 5).

Comparison of lead levels showed no significant difference between the liver and muscle samples from two fiches, they are generally of the same order of magnitude (Fig. 4).the levels of metal pollutants livers of bioindicator organism are greater than those observed in the muscles, expressed as total concentration, or individual for Pb and Cd One possible explanation is the presence in the physiological state of these elements in liver tissue as enzyme co-factors, but also the fact that they are subject to more rapid elimination from the muscle, as described by several authors for cadmium (Marcovecchio & Moreno, 1993; Cinier et *al*., 1999; Belhoucine et *al*., 2014).

According Ramade (1979), teleost fish, metallic elements are particularly concentrated in the liver but also in the kidneys and more modestly in the muscles. Thus, Powell et *al* (1981) had already shown that heavy metals were concentrated in the organs of teleost decreasingly: Liver> Kidney> Muscle.

Many studies emphasize the affinity of heavy metals such as Cu, Zn and Cd in the liver with storage capacity and regulation of these metals have been widely described in the literature in fish (Kalay & Canli, 2000; Usero et *al*, 2003. De Boeck et *al*, 2004. Oliveira Ribeiro et *al*, 2005).

Seasonal variations seem to govern the distribution of heavy metals. Indeed, we found that these fluctuate significantly in the liver and muscle of two fishes. This bioaccumulation of trace metals in these specimens of western Algeria experienced a sharp seasonal variation (Fig 6 and 7). It is possible that the high concentrations of heavy metals encountered during the summer, would be attributed to the importance of boating activity during this period. Moreover, Sadiq (1992) argues that an increase in the temperature and salinity of sea water increased bioaccumulation of xenobiotics. Indeed, these two factors are highest during the summer season in Algerian waters (Boutiba, 1992), thus promoting the bioaccumulation of micropollutants in summer compared to other seasons. The seasonal factor is important and many studies have also shown that metal concentrations in marine species vary seasonally (Majori et al, 1978;.Cossa et al., 1990; Augier et al., 1992; and Bei al., 1998; Wright and Mason, 1999; Kaimoussi et al., 2000; Orban et al, 2002, Belhoucine et *al*., 2014).

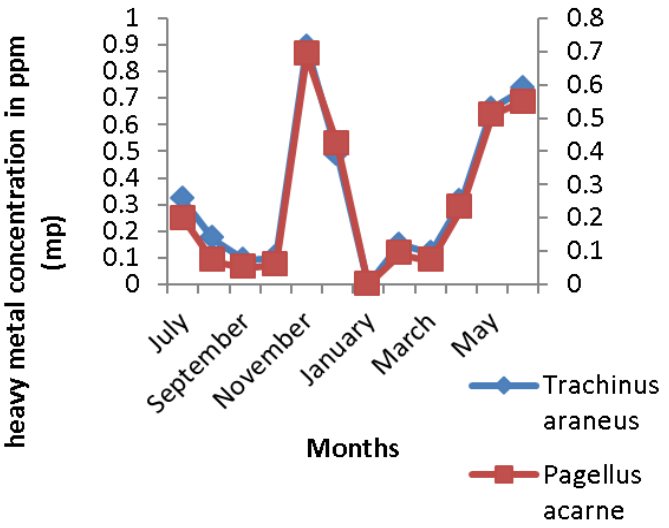


**Figure 6 :** Changes in concentrations of Cadmium (mean ± SDWW ppm) based on the months in Pagellus acarne and Trachinus araneus caught in the Bay of Oran.

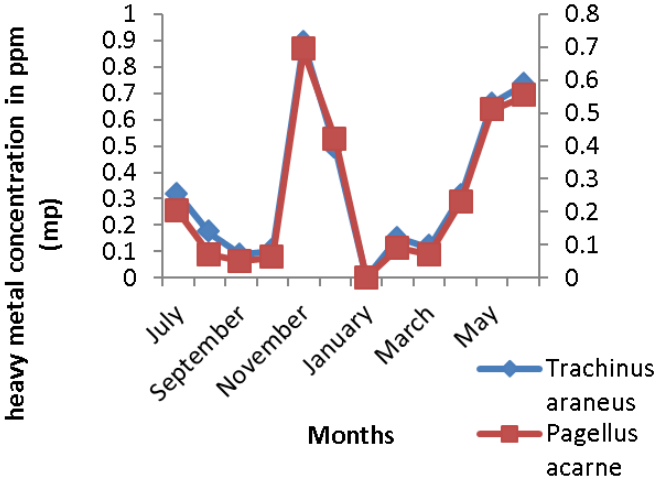


**Figure 7:** Changes in concentrations of Lead (mean ± SD ppm WW) based on the months in Pagellus acarne and Trachinus araneus caught in the Bay of Oran.

The obtained results point out that the average concentrations of heavy metals in the flesh and liver of the living spider are larger than those obtained from *Pagellus acarne*. This is probably due to the fact that the living spider is a coastal benthic species that is common on sandy bottoms and sandy-muddy coasts. It is not very active and stands buried in sediment which represents a reservoir traces elements and consequently the fish to living in this environment will be contaminated by this metal pollution.

**Location level of metal contamination *Trachinus araneus* and *Pagellus acarne* against the maximum permissible dose (MPD).**

**Table 2:** Comparison of heavy metal concentrations (ppm WW) in *Trachinus araneus* and *Pagellus acarne* against the maximum permissible dose (MPD)
(a) Augier et *al*, (1988) – (b) G.I.P.P.M (1973) – (c) C.N.R.S (1971) (d) O.M.S (1971) – (e) F.A.O (1971) – (f) CSHPF (1990) - (g) CNRMS d'Australie (1992)– (h) I.O.P.R (1996)

| **Metal** / **Species** | **Cadmium** | **Lead** |
|---|---|---|
| Presentstudy ***Trachinusaraneus*** | 1,689 ppm **W.W** 0,412 ppm **D.W** | 5,803 ppm **W.W** 1,416 ppm **D.W** |
| Present**study** ***P. acarne*** | **0,24±0,11** ppm **D.W** | **0,27±0,16** ppm **D.W** |
| **Fishes** | 1 ppm **D.W** (a) 0.15-3 ppm **D.W** (h) 0.1 ppm **W.W** (f) | 0.3 à 6 mg/Kg **D.W** (b) 0.5mg/Kg **W.W** (f) |

Location level metal contamination of ***P. acarne and the T. araneus***from the maximum permissible dose (MPD) estimating quantities of heavy metals in ecosystems and organisms is an important part of the work and research carried out in ecotoxicology (Huang et *al*., 2007). These xenobiotics are problematic because of their persistent nature and toxicity. This is because the regulations impose on the low thresholds.

By comparing our results of heavy metals in the*P. Acarne and the T. araneus*from the tolerated threshold (Tab. 2) muscle, we could deduce that the values recorded in the Bay of Oran remain below the critical values of contamination.

We see only the average doses of heavy metals found in the compared with those from the literature relating to MPDaxillary seabream and the Spotted weever are not worrisome.
There is currently, no indication that the levels are high enough to cause morbidity or mortality among the fish themselves or ask threats to human health from the consumption of these fish.

This fact does not diminish the potential risk to humans in the medium and long term if urgent measures are not put in place to monitor the safety of seafood, vectors of toxic agents, particularly lead and secondary cadmium, since these metals recorded alarming levels for public health. The reliable assessment of the risks posed by these pollutants on both human health and the environment is a major challenge (Maroni et *al.*, 2000; Eason and O'Halloran, 2002; Alavanja et *al.*, 2004).

**Changes in average concentrations of trace heavy metals (mean ± SD PF ppm) in different marine organisms**

Pollutant concentrations of animal species reflect a more representative average situation of the state of a medium.

The concentrations of trace metals observed in the muscles of both targeted species sin in the Bay of Oran are comparable to those measured in other fish species caught in areas of the Algerian west coast (Table 3).

**Table 3:** Changes in mean levels of heavy metal traces (mean ± SD WW ppm) in different marine organisms caught along the coast of Oran.

| Metals / Species | **Cd** | **Pb** | **Authors** |
|---|---|---|---|
| *Boops boops* | 0,02±0,01 | 0,40±0,18 | Aoudjit (2000) |
| *Mullus barbatus* | 0,08±0,02 | 1,19±0,04 | Bensahla (2001) |
| *Mullus surmuletus* | 0,15±0,01 | 0,23± 0,98 | Borsali (2006) |
| *Sardinella aurita* | 0,01±0.08 | 0,29±0,01 | Benamar (2006) |
| *Trachurus trachurus* | 0,01±0,003 | 0,06±0,04 | Benadda (2009) |
| *Diplodus argus* | 0,11±0,12 | 0,32±1,85 | Ayad (2010) |
| *Mullus cephalus* | 0.3±0.02 | 0.4±0.021 | Bouhadiba (2011) |
| *Pagellus acarne* | 3,15± 0,17 | 2,16±1,19 | **Present study** |
| *Trachinus araneus* | 0.01±0.07 | 0.01±0.08 | |

## IV. CONCLUSION

Because of the large volume of water in the Mediterranean has a great capacity to absorb pollution, but the large amounts of waste discharged cannot be assimilated in coastal areas. This pollution is such a serious threat, and anxiety is so high in public opinion that states seek individually and jointly, all the necessary tools to stop, reduce or stop this marine pollution.

Man, the end consumer marine products and occupying the final link in the food chain, may at any time be vulnerable. The use of marine organisms for evaluating and determining the level of contamination has been facing in the light of this objective.

The results of this study revealed that : The two metal elements bioaccumulate in the liver tissue better than in the trace elements among the muscle tissue lead concentration still much higher than the cadmium.

Compared to the size of individuals, juveniles are more contaminated than adults because as and as they age, fish salt out some of the contaminants from their bodies lay. Their size is often based on their age, larger fish contain less contaminants, this factor is closely linked to growth imposes on individuals a diet rich. The latter being carnivorous type, it potentially increases the risk of bioaccumulation.

The average concentrations of metals seems well below the maximum permissible dose (MPD), it seems not presented a real danger to the consumer, but it should be remembered that these micro have a cumulative effect through the food chain, and they also have a detrimental long-term effect on public health.

For the sound management and control of water pollution, we must come to study everything related inputs (expenses), the distribution and fate of contaminants, including heavy metals from land that drain into aquatic ecosystems. It is particularly important to study the quantity and quality characteristics, and the routes they travel when they disperse, their destiny and their effects on biota.

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# Evaluation of levels of plumbic pollution near Highway using phanerogamic and cryptogamic species in the city of Annaba (Algeria)

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## ABSTRACT

Nowadays, a serious problem emerged in the natural environments of many countries: they are increasingly subjected to a large number of air pollution: industrial pollution, pollution related to agriculture, but also pollution related to transport. The pollution of lead has constantly evolved over time, due to the considerable increase in the number of vehicles on the market. Our work is based primarily on the use of plants as bio indicator of air pollution in the region of Annaba. The evaluation of the pollution levels near the main roads is a complex exercise, given the many factors to consider at this scale. To do this, we chose three locations on three main roads Greater Annaba: ((RN 44): Annaba - El Kala; (RN 16): Annaba - El Hadjar; (RN 44): Annaba - Skikda), plants used are: phanerogamic namely species (*Fraxinus angustifolia*, *Eucalyptus camaldulensis*, and *Eriobotrya japonica*) and a cryptogamic species (a lichen (*Ramalina farinacea*)). An appropriate sampling strategy, a spatio-temporal monitoring, a counting vehicle at our study sites and measurements of physiological parameters combined with the determination of lead allowed us to assess not only the state of the air quality but also the impact of this pollution on the environment caused by a heavy traffic in the area.

**Keyword:** Pollution, lead, bio indication, bioaccumulation, road traffic, Annaba, Algeria.

## I. INTRODUCTION

During the last century, the industrialization and the development of transport played a major role in the evolution of society. These activities were synonymic of progress, modernity and enrichment. But since, an increasing awareness is felt as for the engendered environmental consequences. Indeed, big quantities of chemical substances are loosened in the environment, of which most of them being considered as dangerous. The introduction of these compounds implies serious risks for the environment and the alive bodies, in particular the human health **[1]**. Among different pollutants poured regularly in the atmosphere, the lead of motor origin occupies a dominating place; its toxicity for the biocenosis is evident and deteriorates more and more through the food chain to become dramatic by affecting the man **[2]**.

In Algeria, our capital was classified number one in the report of the World Bank, due to the 180 tons of lead which glide permanently in the atmosphere **[3].** For that purpose, at the level of the region is from Algeria, and more particularly in the region of Annaba; there is for several years a progressive problem of atmospheric pollution bound to an important road network **[4,5,6];** On one hand because of the existence of a very important motor vehicle population with regard to the traveled distances, and on the other hand of certain topographic characteristics (the closeness of the sea, the existence of plans of water, the presence of the heights and their orientations, these topographic devices in basin and in corridor favors the phenomenon of temperature inversion and its obstinacy) and climatic (the relative humidity always very high all year long and the direction of winds pulling alternative movements of air land breeze, sea breeze, contributing to maintain pollutants above the zone of broadcast, as well as a

naturally frequent fog to Annaba) which create a climate convenient to the development of the pollution **[5,6].**

Since the seventies of numerous researches were led on the use of vegetables as bio indicators and bio accumulators of the pollution, particularly the lichens which reveal excellent results concerning the bio accumulation in particular that some heavy metals. **[7-15 ; 5,6].** The various components of the environment react to the pollution differently, the lower vegetables especially lichens often present physiological, morphological and structural changes before even the appearance the slightest symptoms of poisoning at the man **[16].**

Our research on the study of the pollution plumbic of automobile origin in the region of Annaba by using in a relevant way the bio indicators, in particular lichens and some vascular plants to the objective to characterize the environmental state of the middle studied by highlighting a plumbic pollution bound to the road traffic, to study the impact of the latter on the morphology and the physiology of the used vegetables and to propose relevant bio indicators of this pollution.

## II. METHODS AND MATERIAL

Annaba, coastal city, renowned for its wet ecosystems, bathed in a Mediterranean climate with character sub wet, leaned in the mountain range of Edough. It is considered as being one of the cities the most polluted on the national territory and in the North of Africa; the main broad casting source of the lead is the road traffic which evolves in a disturbing rhythm.

The analysis of the built-up area of Annaba allows to distinguish in the global scale three expanding main trunk roads of growth and development and which converge on the city center of Annaba:

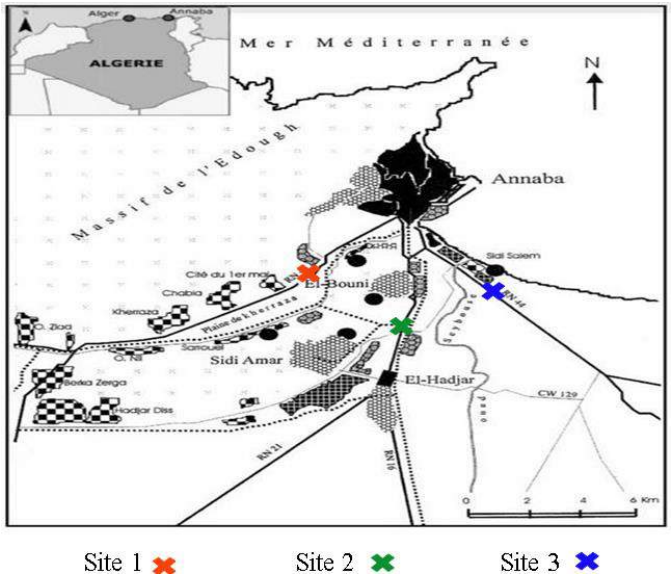
- The axis RN 16 which connects the big and old two poles Annaba - El Hadjar,
- The axis RN 44 East- is connecting Annaba - El Tarf,
- The axis RN 44 West – connecting Annaba to Constantine.

The population of the wilaya of Annaba did not stop increasing during these last years to reach609 499 inhabitants where we register more over an annual average of growth closely 1,01 % and an irregular distribution of the population with a variation of the density from a municipality to another one. The axis Annaba - Sidi Amar and El Bouni represents the sites where the majority of the population are concentrated (44,65% to Annaba, 20,04% to El Bouni and 12,80% to Sidi Amar). The socioeconomic characteristics (commercial, industrial, university pole and the quality of the services) are factors limiting some distribution of the population **[17].**

Nowadays we find an important motor vehicle population by which the annual growth rate of car registration documents is only increasing year by year. From 2002 till 2003, the rate considerably increased from 0,92 % to 3,57 % **[18]**. In 2005, the realized analysis reveals that the vehicle of tourism represents a 68 % rate with regard to the other ways of transportation. Compared with the other Algerian wilayas, Annaba is ranked second after the capital with a park automobile reaching100 000 vehicles, with 94 passenger cars for 1000 inhabitants and overtake widely Constantine and Oran which are respectively 79 and 81 cars for 1000 inhabitants **[19].**

Since 2003, we registered an acceleration of motorization of more than 9 %, the latter rose during these last year's respectively with a rate of increase of more of 14 % in 2004 and more than 42 % between 2004 and 2008.

### A. Presentation of the Sites of Surveillance and Measure of the Automobile Pollution:




Site 1 ✖ Site 2 ✖ Site 3 ✖

**Figure 1 :** Geographical location of the sites of study 1, 2 and 3 on three main highways harming the city of Annaba (Source : P.D.A.U, 2004).

The evaluation of the levels of pollution near the axes of circulation is a complex exercise, considering the numerous factors to be considered in this scale. The concentrations in pollutants registered in border of way indeed depend on local broad casts generated by the car traffic (depending themselves on conditions of traffic and on the composition of the motor vehicle population), parameters influencing the dispersal of pollutants (local meteorology and configuration of public road network) and levels of thorough concentration of the surrounding zones.

To do it, we chose three sites located on three main highways serving the urban area of Annaba (Fig. 23):

**Site 1:** (R.N. 44): Annaba-El Kala, it is approximately 4 km in the Southeast of Annaba.
**Site 2:** (R.N. 16): Annaba-EL Hadjar. It was chosen in 5 km in the South of Annaba.
**Site 3:** (R.N. 44): Annaba-Skikda: it was realized in 3 km in the Southwest of Annaba.

B . **The Climatic Parameters**

Certain climatic parameters are considered in our study because they have a role particularly mattering in the distribution and the dilution of the impurities:

· The city of Annaba presents in general lines of Mediterranean type with floors bio - climatic sub-wet and wet;

· The climate is characterized by sweet temperatures in winter, warm in summer and plentiful precipitation;

· The rose of winds allows to put in evidence a dominant direction of the wind of Northeast the western South **(Fig. 2).**

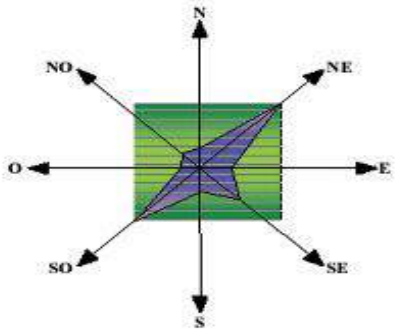



**Figure 2 :** Wind rose in Annaba built upon 10 year history (1999-2008).

## B. Choice of the Vegetal Species

In the current studies of bioaccumulation of elements atmospheric metal tracks, three big types of bodies are used: lichens, mosses and superior vegetables. We distinguish two approaches: the first one consists in harvesting the naturally present individuals on the zone of study, the second to be exposedon sites chosen by the individuals beforehand cultivated in standardized conditions or harvested or in not contaminated circles.In this article, our choice concerned as well cryptogams as phanerogams:

### 1) Phanerogamic species "in situ":

We chose the most representative vegetables of the region: species leaves of which were taken are: *Fraxinus angustifolia*, *Eucalyptus camaldulensis* and *Eriobotrya japonica.*

➢ ***Fraxinus Angustifolia***

**Figure 3 :** *Fraxinus angustifolia*

**Classic classification**

| | |
|---|---|
| **Reign:** | *Plantae* |
| **Division :** | *Magnoliophyta* |
| **Class:** | *Magnoliopsida* |
| **Order:** | *Scrophulariales* |
| **Family:** | *Oleaceae* |
| **Genre:** | *Fraxinus* |

- ***Eucalyptus Camaldulensis***

**Figure 4:** *Eucalyptus camaldulensis*

**Classic classification**

| | |
|---|---|
| **Reign :** | *Plantae* |
| **Division :** | *Magnoliophyta* |
| **Class :** | *Magnoliopsida* |
| **Under class :** | *Rosidae* |
| **Order :** | *Myrtales* |
| **Family :** | *Myrtaceae* |
| **Genre :** | *Eucalyptus* |

- ***Eriobotrya Japonica***

**Figure 5 :** Fruits of *Eriobotrya japonica*

**Classic classification**

| | |
|---|---|
| **Reign:** | *Plantae* |
| **Under reign:** | *Tracheobionta* |
| **Division:** | *Magnoliophyta* |
| **Class:** | *Magnoliopsida* |
| **Order:** | *Rosales* |
| **Family:** | *Rosaceae* |
| **Genre:** | *Eriobotrya* |

**2) Cryptogamic Species**

The lichen is a complex vegetable trained by the association of a mushroom the mycosymbiote, Lichens are included in Thallophytes, vast group vegetables devoid of stalks, leaves and roots and which are not thus vascularized. Their thallus or vegetative device which appears in more or less regular heap of cells, in more or less cut blades and offers an original morphology with regard to that of the seaweeds and the mushrooms which make up it. The capacity of bioaccumulation of lichens **[20],** appears to us a sensible approach to estimate the impact of the road traffic on the environment. These vegetables are capable of accumulating pollutants whatever are their conditions of broadcast, distribution and dispersal, when the physic-chemical measures can turn out to be difficult to realize. Our choice concerned a fruticose species, *Ramalina farinacea*, on one hand because of its sensibility and on the other hand because of its abundance at the level of the site of origin. In Algeria, the works of Semadi **[16]** in the zone of Annaba and those of Alioua **[15]** in the region of Skikda demonstrated the sensibility of this species to rates mattering of pollutants.

We took branches covered with Thallus of *Ramalina farinacea* in their original environment, El Kala, situated east of Annaba (national park). This site of taking is situated except the polluted zones; the qualitative methods used on numerous occasions in Europe allow to determine the degree of pollution directly from the observation of the lichen populating **[8, 21, 22].** We transferred these samples to the levels of the various chosen sites of transplantation. The transplantation took place on January 15th, at the level of three chosen sites. Vegetables chosen at the level of sites located on main highways are as follows:

- Site 1: (R.N.44): Annaba-El Kala, the twigs of branches of *Olea europaea* covered with *Ramalina farinacea* were fixed to *Fraxinus angustifolia* perpendicularly in the main highway in 2 m of the road.
- Site 2: (R.N.16): Annaba-EL Hadjar. The transplants of *Ramalina farinacea* was fixed to *Eucalyptus camaldulensis* between both senses of the highway in 2 m; besides, the leaves of *japonica Eriobotrya* situated in 5m were also used.
- Site 3: (R.N.44): Annaba-Skikda: the transplantations of the fragments of branches of *Olea europaea* covered with *Ramalina farinacea* were fixed to *Eucalyptus camaldulensis* between both senses of the highway in 2 m; we also used the leaves of *Fraxinus angustifolia* in 2m.

A taking is made at the beginning of every month, by removing a part of the thallus of lichens on the phorophyte. The study lasted seven months and the treatment of samples was made that very day by the taking or the next day with three repetitions for every site and every measure.

### D. Technique of Takings of Samples

To realize our sampling, we operated on a height varying 1, 50 m and 2 m of the ground. The takings took place according to the specific nature of the vegetable. For the treelike species, we took every time 10 in 20 sepals around of the tree at the level of man to have a homogeneous average sample.

For lichens, we removed a part of thallus on the phorophyte by means of a knife for every sampling. The taken samples are placed in plastic labelled bags carrying all the indications (in particular date and place of taking), closed by means of an elastic to limit the losses of water by evapotranspiration until the arrival to the laboratory.

### E. Analytical Techniques

After drying of samples in the steam room in 105°C, they are carefully crushed, put in pill box where they are handled by the peroxide of hydrogen until complete mineralization. The recent dosages of the lead were made by using the technique of spectrophotometer of atomic absorption (S.A.A.). The measures were made from the solutions of 20ml of nitric acid for 2 %. For the same solution, three measures (repetitions) are made, the average being considered.

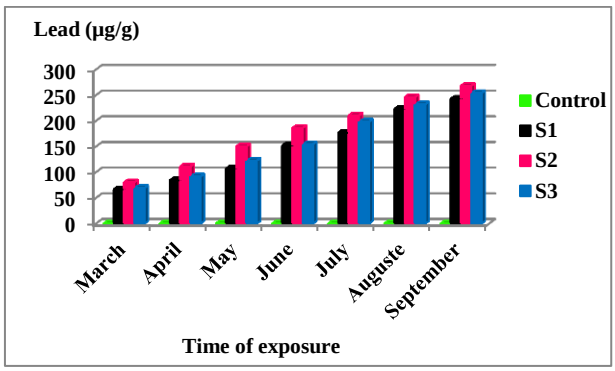
Before proceeding to the dosage of the lead in samples, it is necessary to establish at first a curve of calibration from the solutions of lead known concentrations. The results are directly read on the device if it is

## III. RESULT AND DISCUSSION

### A. Variation of the average monthly traffic in the three axes serving the urban area of Annaba during rush hours

On average, we see that road traffic increases during the summer especially at the axis 2. This is related to the environment of this axis and its features (surrounded by several important facilities: commercial, industrial, settlements etc...), so it seems to be the busiest.
Analysis of variance with two criteria for classification on the patio-temporal variations of traffic in the three axes serving the metropolitan area of Annaba during peak hours shows that it is highly significant in space (**p = 0,000*****) and time **(p = 0,000***).**

### B. Bioaccumulation of the lead by vegetables




The **Fig. 7** reveals a fluctuation in the spatiotemporal accumulation of the lead by *Ramalina farinacea* transplanted at the level of all the sites with an ascendancy at the level of the site 2, this can be explained by the intense road traffic registered at the level of the latter. The comparison of the spatiotemporal variation of the lead accumulated by *Ramalina farinacea* show that this variation is very highly significant in the space **(p = 0,000 ***)** and in the time **(p = 0,000 ***)**,

that is the more transplants is exposed and the more the accumulation of the lead is important.

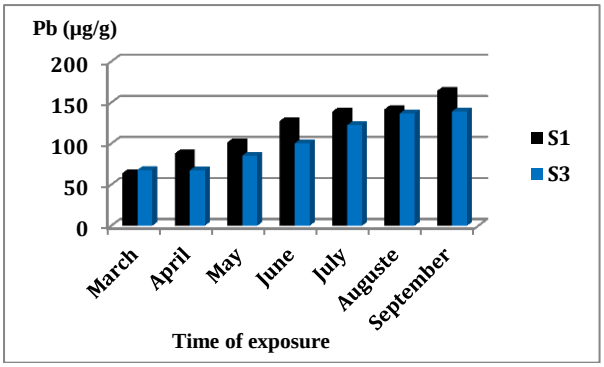



**Fig. 8:** Variations spatiotemporal of lead accumulated by *Fraxinus angustifolia*

According to **(Fig. 8)** relative to the spatiotemporal variation of the accumulation of the lead by Fraxinus angustifolia, we notice that this accumulation is more and more important in summer, what is perfectly understandable by the impact of the climatic factors, in particular the absence of the precipitation which tend to wash the various particles fixed to the foliage in winter. At *Fraxinus angustifolia*, the analysis of the variance in two criteria of classifications relative to the spatiotemporal variation of the lead shows that the latter is very highly significant in time**(p = 0,000***),** and highly significant in the space **(p = 0,007**).**

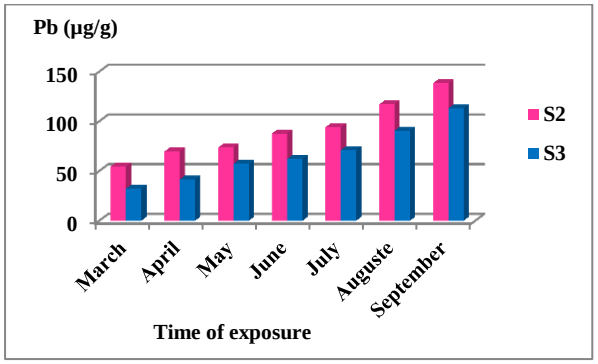



**Fig. 9:** Variations spatiotemporal of lead accumulated by *Eucalyptus camaldulensis*

Eucalyptus camaldulensis answers in the same way as the previous species, always with an ascendancy at the level of the site 2 which presents a strong volume of road traffic what explains the lead contents accumulated by the vegetable.

As well as for the comparison of the variation of the lead average accumulation to Eucalyptus camaldulensis who shows that it is very highly significant in the space **(p = 0,002**)** and in the time **(p = 0,000***)**, this is demonstrated by the accumulation of the lead to the Eucalyptus according to the time of exposure as well as the site of transplantation.

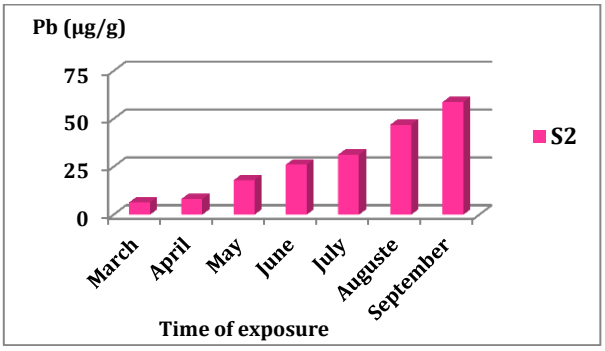



**Fig. 10:** Variations spatiotemporal of lead accumulated by *Eriobotrya japonica*

In the same way the spatiotemporal variation of the accumulation of the lead follows the same trend as that of the previous species with a clear progress from the first taking to affect a maximal value in September of **58,8 µg / g**, however this value remains lower than that registered to the previous species.

## C. Variation of the Content in Chlorophyll

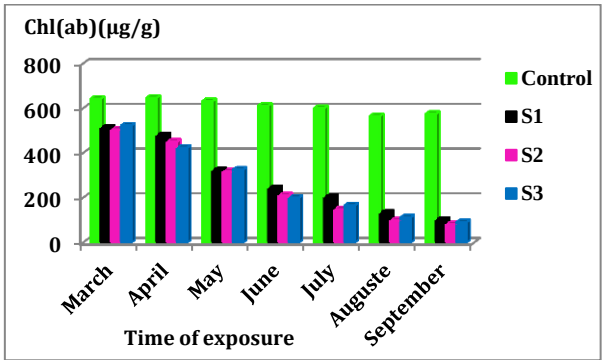



**Fig. 11:** Variations spatiotemporal of chlorophyll by *Ramalina farinacea*

We recover that the rate of the chlorophyll tends to decrease in time in exposure, in other words as the lichen weakens considering its exhibition in the pollution; this is going to influence the photosynthetic process. The rate of the chlorophyll varies well according to the site of exposure.

The comparison of the content average some chlorophyll (ab) at *Ramalina farinacea* shows that it is very highly significant in the time **(p = 0,000***)** while it is only significant in the space **(p = 0,010*)**.

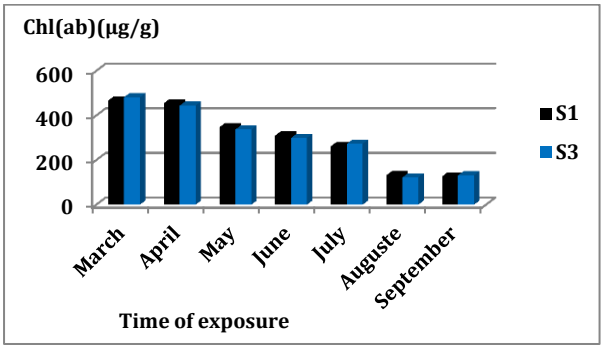



**Fig. 12:** Variations spatiotemporal of chlorophyll by *Fraxinus angustifolia*

The fluctuations in the time in the chlorophyll (ab) at Fraxinus angustifolia vary between 482.15 and 1.34 µg/g at the level of the site 3 and between 466.81 and 17.23 µg/g for the site 1.

The comparison of the content average in chlorophyll (ab) at *Fraxinus angustifolia* shows that the variation of the chlorophyll (ab) is very highly significant in the time **(p = 0,000***)** but it is not it in the space **(p= 0,722).**

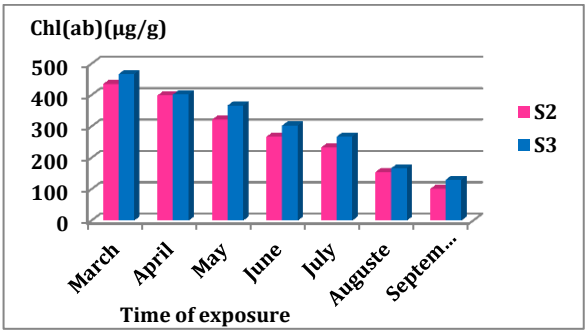



**Fig. 13:** Variations spatiotemporal of chlorophyll by *Eucalyptus camaldulensis*

According to **fig. 13**, we notice that the chlorophyll is more important for spring. The tree in question is subjected to a single pollution the spring vegetative push allows a good photosynthesis which generally will eventually perturbed after a period which will stay function of the environmental factors. In other words, for the rest of the time we register a decrease of the chlorophyll. The comparison of the content average in chlorophyll (ab) at *Eucalyptus camaldulensis* shows that the variation of the chlorophyll (ab) is very highly significant in the time **(p = 0,000***)** and in the space **(p= 0,003***).**

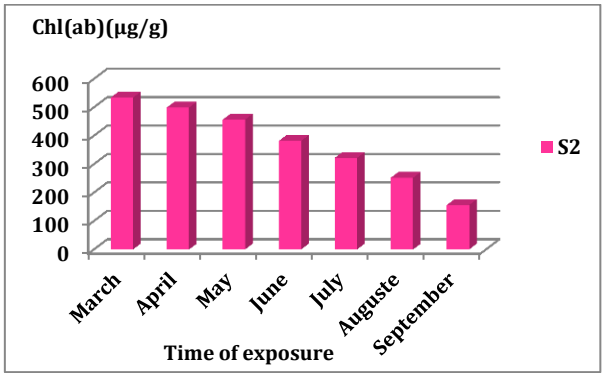



**Fig. 14:** Variations spatiotemporal of chlorophyll by *Eriobotrya japonica*

According **fig. 14**, the chlorophyll seems to fluctuate also as well in the time as in the space.

## D. Variation of the Content in Proline

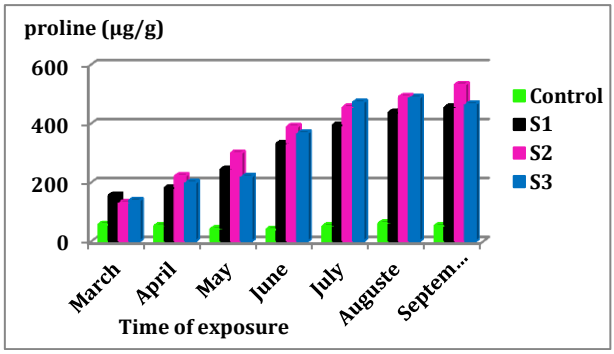



**Fig. 15:** Variations spatiotemporal of proline at *Ramalina farinacea*

**Fig. 15** show that the contents it proline at *Ramalina farinacea* are important at the level of three main highways during the months of exposure.

Concerning the comparison of the spatiotemporal variation of the proline at *Ramalina farinacea*, it shows that the latter is very highly significant in the time **(p = 0,000***)** while it is only significant in the space **(p = 0,017*)**.

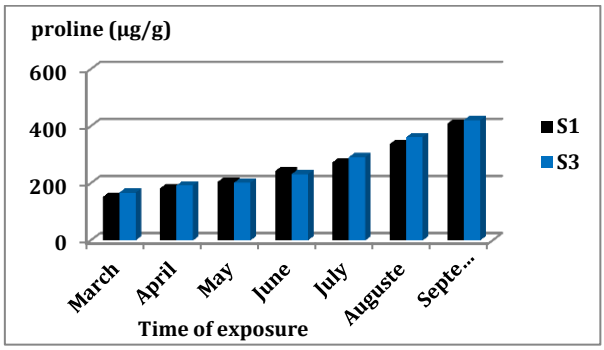



**Fig. 16:** Variations spatiotemporal of proline at *Fraxinus angustifolia*

Analysis of the variance in two criteria of model classification crossed fix relative in the spatiotemporal variation of the proline at *Fraxinus angustifolia* show that it is very highly significant in the time **(p = 0,000***)** but it is not her in the space **(p = 0,093)**.

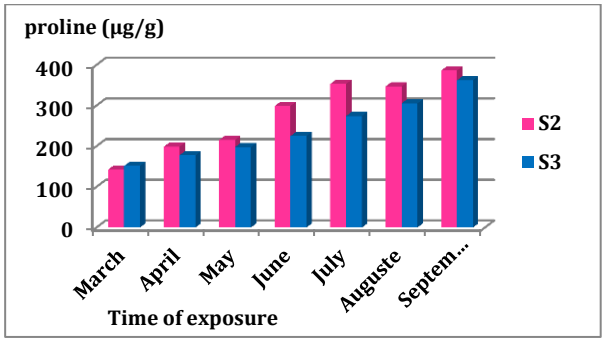



**Fig. 17:** Variations spatiotemporal of proline at *Eucalyptus camaldulensis.*

The **(Fig. 17)** reveals that the rate of the proline to *Eucalyptus camaldulensis* follows the same trend as that of the Fraxinus, because we notice an increase of this content according to the time of exhibition.

The comparison of the content average of her of the proline to *Eucalyptus camaldulensis* shows that it is very highly significant in the time **(p = 0,000***)** but it is only significant in the space **(p = 0,025*)**.

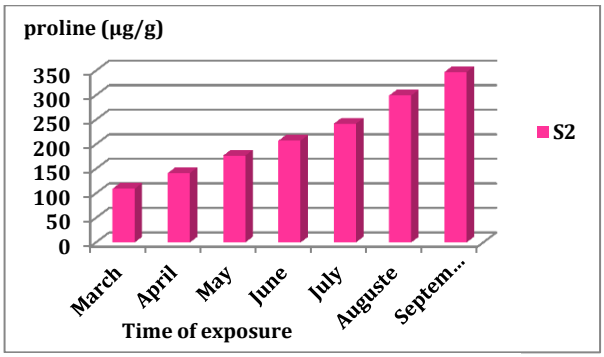



**Fig. 18:** Variations spatiotemporal of proline at *Eriobotrya japonica.*

According to the results appropriate to **Fig. 18**, we notice that the rate of the proline at *Eriobotrya japonica* increases from the first taking and persists until the last taking to affect **347.32 µg / g** a value which always remains lower than the content proline noticed to the other superior vegetables.

E. **Variation of the Report Fresh Material/Dry Material**

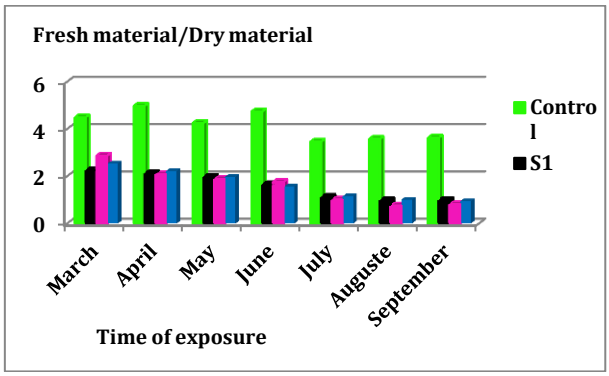



**Fig. 19:** Variations spatiotemporal of report Fresh material/Dry material at *Ramalina farinacea*

Dry material decreases also considerably with fluctuations in a site in the other one especially for the site 2 or the maximal value is affected the first taking with **2.87** and the minimal value is affected the sixth taking with **0.76**.

The comparison of the variation of the report FM / DM by means of the analysis of the variance to two criteria of classification, show that this spatial variation is not significant **(p = 0,917)** and that the temporal variation is very highly significant **(p = 0,000***)**.

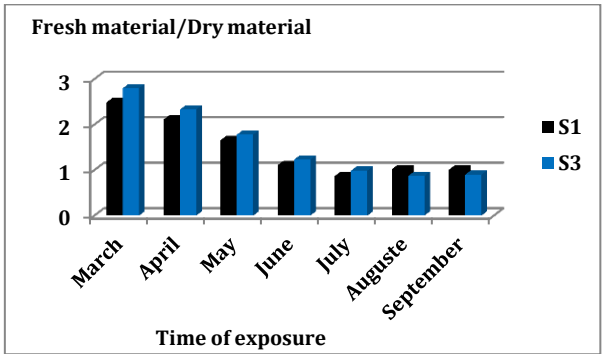



**Fig. 20:** Variations spatiotemporal of report Fresh material/Dry material at *Fraxinus angustifolia*

Also at *Fraxinus angustifolia*, we note fluctuations in the report fresh material/dry material from March till June followed by stability the rest of the months.

The comparison of the spatiotemporal variation of the report FM / DM at *Fraxinus angustifolia* shows that the latter is very highly significant in the time **(p = 0,000***)** but it is not it in the space **(p = 0,202***)**.

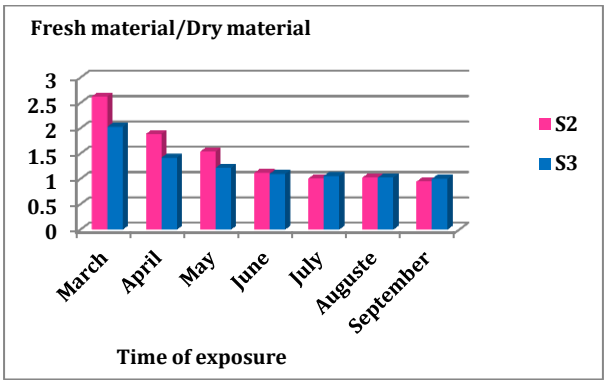



**Fig. 21:** Variations spatiotemporal of report Fresh material/Dry material at *Eucalyptus camaldulensis*

*sis* presents fluctuations during the first three months followed by stability at the level of both main highways the rest of the time.

The comparison of the spatiotemporal variation of the report FM / DM at Eucalyptus camaldulensis shows that this variation is very highly significant in the time **(p = 0,003***)** but it is not it in the space **(p = 0,120).**

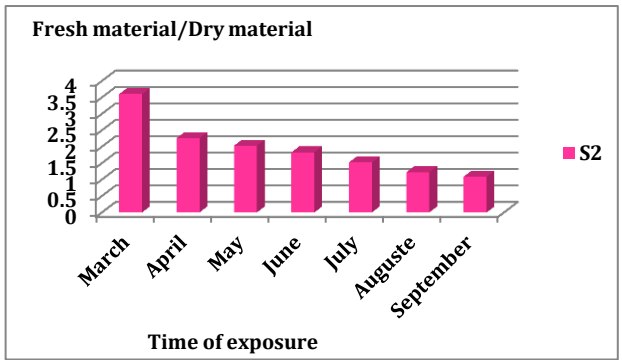



**Fig. 22:** Variations spatiotemporal of report Fresh material/Dry material at *Eriobotrya japonica*

Further to the data analysis appropriate to the **fig. 22**, we notice that the report FM / DM tends to decrease considerably after the second taking, this decrease stays function of several parameters to know the degree of pollution, the climatic parameters as well as other factors which interfering.

In comparison with the volume of road traffic at the level of three axes for the same schedules of counting, we notice that there is no difference between the axis 1 and 3 during the schedules of counting, on the other hand the latter is smelt at the level of the axis 2 where the road traffic seems more intense during rush hours. This gives some explanation by the importance of the urban areas and the infrastructures served by this strongly frequented axis. Indeed, the latter serves the city El Bouni, the urban areas of El Hadjar, Sidi Ammar, Chaïba, the University and the steel-making Complex as well as the other destinations towards Guelma and Souk Ahras.

Concerning the accumulation of the lead, the results which we obtained demonstrate well the presence of a strong pollution of lead by automobile origin, not only revealed by the use of transplants of lichen ( the most sensitive bio indicators) which accumulate approximately 268,33 µg / g, but also by certain phanerogamic species in situ which, in our sense present degrees different from sensibility face to face of this shape of pollution with an ascendancy at *Fraxinus angustifolia* and in this particular case at the level of the axis 1.

While at the level of the site 2 where the road traffic is the most intense, *Eucalyptus camaldulensis* situated along the main highway register **138.37 µg/g**, while *japonica Eriobotrya* accumulated only **58.8 µg/g** in 5 m. These results denote a specific difference as for the reaction towards the pollution of lead and consequently a strong accumulation is indicated at the species to the persistent foliage. This is confirmed by **Madany and al. (1990)** who demonstrates that the emitted polluting particles are better got by the rough surfaces with embossed; but the presence of a pilosity also favors their retention by the smooth skins where covered with cuticles and it for the same site and the same exposure in the automobile pollution **[28]**. While **little (1978),** notice that the rough leaves can collect ten times more lead than the smooth leaves **[29]**.

Besides, we register a net temporal lead accumulation between May and September during the period of drought. Generally, we consider that the precipitation during March and April tend to wash particulars pollutants at the level of the foliage, what influences the lead concentration accumulated. Thus dusts containing heavy metals accumulate on the air parties, particularly the leaves. This deposit of surface of leaves can be qualified as latent pollution, because the cuticle is considered as aim pervious barrier which opposes the penetration of pollutants in leaves. **Arvik and Zimdahl (1974)** showed that very fine lead particles could

penetrate into stomata, but it is improbable that big lead quantities penetrate in this way thus this process can be responsible only for a low part of the contamination of leaves by the lead **[30]**.

However, when leaves age, the efficiency of this barrier is altered; then it appears microphone cracks and pollutants which remain normally on-surface can penetrate easily **[31]**. But also, lead particles put deposited on the surface of leaves do not practically penetrate inside and can be easily washed. The most important of the ways of the harmful share of pollutants consist in their penetration in the organs of breath of vegetables represented by the stomata of leaves **[32].**
These vascular plants testify well of the air quality to be able to them accumulator. However the latter rest always function of the nature of the species (its morphology, its vegetative cycle), of the exposure time, the intensity of the pollution, and to the environmental factors such as the direction of winds, the precipitation, the humidity ....etc

The spatiotemporal follow-up of the moderate physiological parameters (content in chlorophyll,) testifies well of the air quality of every site.
The follow-up of the counting of vehicles on three road main trunk roads serving the urban area of Annaba demonstrated well the intensity marked with the road traffic at the level of the R.N.16 Annaba-El-Hadjar with regard to two other axes or it remains nevertheless not insignificant.

Besides, the variation of the physiological parameters of the used vegetables for which the accumulated lead content, is largely responsible in a parallel to other pollutants which can interfere seen the presence of several polluting infrastructures. However, we deduct that all the species of a perimeter, affected by a pollution do not react in the same way to pollutants. However there are intrinsic factors in plants, morphological where physiological, which determine the resistance, the tolerance where the sensibility of plants. Other factors biotic aged-related, at the physiological stage can intervene also in the sensibility of vegetables in this pollution of lead **[31].**

## IV. CONCLUSION

Considering the lead important contents accumulated by the used bio indicators we can extract two main conclusions:

- ✓ The species used in our study have proved of very good bio accumulative of lead, nevertheless the species of lichen: *Ramalina farinacea* present a power much higher accumulator that of the vascular plants.
- ✓ There is a strong urban pollution especially of lead in the region of Annaba particularly at the level of three main highways serving the urban area with ascendancy at the level of the axis 2.

## V. ACKNOWLEDGEMENTS

The thanks of the authors are sent to the national office of meteorology salt works to Annaba to have put in them measure the climatic data and to the experts of the review for their critical readings of the text.

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# The Relative Gene Expression of GAPDH in Mice Fed with a Short-Term High Fat Diet

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## ABSTRACT

The important role of nutrition in health and disease prevention is well recognized. The glyceraldehyde-3-dehydrogenase phosphate gene (GAPDH) is considered as a housekeeping gene: it encodes a key enzyme of the glycolysis, and seems to affect the metabolism of fatty acids. The purpose of this work was to study the relative expression of the GAPDH gene in mice subjected to a short-term high fat diet. The expression of the GAPDH gene has been analyzed in the livers of C57BL / 6N mice: 5 control mice and 5 mice subjected to a short term high-fat diet. The quantification of the mRNA has been performed by a real-time PCR using specific primers designated and validated *in silico*. The data have been analyzed by the REST software. The results showed a significant down expression ($p < 0.005$) of the GAPDH gene in mice subjected to high-fat diet. This suggests that a lipid diet may have an effect on the expression of the GAPDH gene. Thus the use of GAPDH as a reference gene should be reconsidered notably in gene expression studies.

**Keywords:** GAPDH, high-fat Diet, Housekeeping Gene, Lipid Metabolism, Mice

## I. INTRODUCTION

Currently, the prevalence of health problems related to nutrition in humans like obesity and diabetes are increasing. This phenomenon is strongly associated with a sedentary lifestyle. Also, these problems may be the consequence of a gene expression variation, especially at constitutive genes commonly considered as housekeeping gene.

The housekeeping genes mainly code for proteins essential to basic cell functions, e.g. beta actin, tubulin alpha (cytoskeleton) and Beta microglobulin (Major Histocompatibility Complex type I). These genes are stably expressed in all cells and play an important role in the metabolism and homeostasis [1]. Among them, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) also represents a gene traditionally used as a reference in expression studies [8]. In the liver, the glycerol 3-phosphate derived from the lipolysis is essentially converted to dihydroxyacetone phosphate (DHAP) by GAPDH before joining the glycolysis; which makes GAPDH a key enzyme in glycolysis and also in fatty acid metabolism [2]. Thomas. D *et al* (2000) demonstrates that expression of beta actin and GAPDH was affected in quantitative serum-stimulation studies [8].

Hence the aim of this preliminary work was to investigate the effect of a high-fat diet on the GAPDH gene expression in mice.

## II. METHODS AND MATERIAL

We used mice C57BL/6N 3 weeks old submitted or not to a high-fat diet as described previously [3]. The relative expression of the GAPDH gene has been analyzed in mice liver samples: 5 controls were

subjected to a normal diet, and 5 mice subjected to a high-fat diet (HF). The livers tissues were sampled and preserved at -80 °C until RNA extraction for gene extraction analysis by real time PCR (Applied Biosystems 7500) using Syber Green. The beta-glucuronidase (GUSB) and hypoxanthine phosphoribosyltransferase (HPRT) were used as reference genes [4].

The primers sequences (Table 1) were designed across consecutive exons using Primer3 software (http://frodo.wi.mit.edu/).

Table 1: Primers sequences

| Gene name | Primer sequence | PCR Product Size | Tm |
|---|---|---|---|
| GAPDH | Fw: GGAGAAACCTGCCAAGTATG<br>Rev: AGGAGACAACCTGGTCCTCA | 100 pb | 60°C |
| GUSB | FW: CGAACCAGTCACCGCTGAGA<br>Rev: CTTCCGAAACACTGGGTTCT | 100 pb | 60°C |
| HPRT | FW: ATTATGGACAGGACTGAAGC<br>Rev: AGGAGACAACCTGGTCCTCA | 120 pb | 60°C |

Legend: Tm: Annealing temperature

Table 2: Relative GAPDH gene expression after normalization

| Gene | Type | Reaction Eff | F.d | Std Erro | C.I 95% | P(H1) | Result |
|---|---|---|---|---|---|---|---|
| GAPDH | TAG | 1 | 0,270 | 0,059-1,401 | 0,015-1,401 | 0,005 | Down |

Legend:
TRG: Gene Target
F.D: Fold Change
C.I: Confidence Interval
P (H1): Probability of alternate hypothesis that difference between Sample and control groups is due only to chance.

Interpretation
GAPDH is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0,270 (S.E. range is 0,059 - 1,401).
GAPDH sample group is different to control group. P(H1)=0,005

Relative gene expression was calculated using the Data-AssistTM v2.7 beta software (Applied Biosystems) and REST 2009 (V2.0.7; Corbett Research and Pfaffl; (Pfaffl et al. 2002 )). For target gene, expression data was expressed as fold change between HF group and CTR group (Table 2). Differences were considered as significant at $p < 0.05$.

## III. RESULT AND DISCUSSION

Our results showed a significant down expression ($p < 0.005$) in mice fed with a short-term high-fat diet (Table 2). Barroso *et al* (1999) showed also a variation of the GAPDH expression after insulin stimulation in adipose tissue [5]. However, Robert. D *et al* (2005) showed a constant GAPDH expression in 72 human tissues in various situations [6]. Thus, as recommended by Caradec J *et al* (2010) and Thomas D. *et al* (2000) the expression of any gene should be accurately and systematically evaluated before their use as a reference gene.

## IV. CONCLUSION

Our preliminary results suggested that a high fat diet might have an effect on GAPDH gene expression. If the further analysis on a larger sample confirms these results, thus the use of GAPDH as a housekeeping gene should be reconsidered

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# Characterization of the Habitat of *Rosa Canina* L

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## ABSTRACT

The rosebush, genus Rosa, is the first ornamental species economically. It is part of the Rosaceae family which includes fruit and forest species. This preliminary work aims the study of *Rosa canina* L. existing in the mountains of Tessala (western Algeria). We wanted, through this study, to contribute to this species by characterizing its climate and substrate. The topics, on which the study was conducted, belong to three stations at different altitudes and exhibitions. The results we have Successful show that on all three of these stations, the species studied thrives on loamy soils rich in organic material, and low wet limestone and basic pH.

**Keywords:** *Rosa canina* L, climate, substrate, the mountains of Tessala

## I. INTRODUCTION

This preliminary work aims the study of *Rosa canina* L. existing in the mountains of Tessala (western Algeria genus Rosa includes 150-200 species of wild roses. These roses have a wide morphological diversity and use wide enough. These are species exposed directly to all the biological factors leading to erosion (climate, pollution, farming techniques ...) [1]. Among the species of the genus Rosa, *Rosa canina* L. there is a shrub prickly, hermaphrodite, which is reproduced individually or in groups. Its large flowers are designed to pollination by insects and the crossing is probably common [2].

A complementary basis of this work, we are interested in the context of this present work, a preliminary study on *Rosa canina* L. part of the vegetation of Mount Tessala (wilaya of Sidi Bel Abbes, Algeria Western ) . It is essentially characterize the environment within which this species

## II. METHODS AND MATERIAL

This work involves three main steps. Initially, we did a survey in Mount Tessala (wilaya of Sidi Bel Abbes ) to seek feet of *Rosa canina*.

### Selecting Stations

The mathematical editor on which along with text you can also write

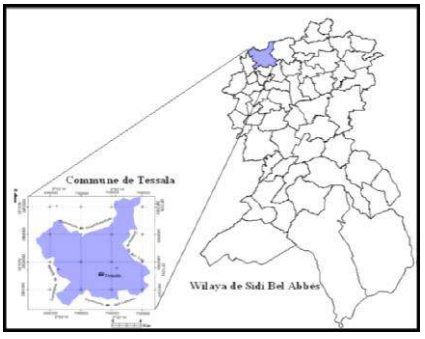


**Figure 1:** Location of the common Tessala(Algeria)

Tessala is an area where espouse very steep mountainous landscapes, steep to steep slopes and scenery of hills and plains. A Panoramic extraordinary view from the top of Jebel contemplated Tessala which

has a high biological diversity sector. Our study is based in the mountains of Tessala(Fig.1).

## Bioclimatic Study

The climate is a very important factor because of its major influence on vegetation. Presenting the climatology of the study area in order to place it relative to the general climatic conditions is a necessity. Among the limiting factors for the presence and distribution of forest species, the temperature is the most important determinant in characterizing the vegetation. Rain and temperature are climate hinge, they directly affect vegetation. Each species has a minimum or maximum threshold allowing it to stay alive. Beyond these limits the species' survival may be compromised [3].We used period (1980-2010) (ONM, 2011) to study the climate of the study area

## Soil tests

Sample sites were for three selected stations. The samples were taken at a depth of 20 cm. These samples were all accompanied by a fact sheet informing on all soil characterization parameters from field observations (structure, color limits, humidity, etc.). They are then placed in the open air for 15 days. Once dried, the earth is sifted through a 2 mm sieve to separate the coarse elements of fine soil lower than 2 mm. After the screening, we will access to the physico- chemical analysis.

Soil samples of each station have been physico-chemical analyzes.

These analyzes were performed in soil science laboratory at INRA (National Institute for Agricultural Research) of Sidi Bel Abbes (Algeria).

# III. RESULT AND DISCUSSION

### A. Bioclimatic Variables

Precipitation [4] defines rainfall as the primary factor that determines the type of climate. Indeed, it affects the maintenance and distribution of the vegetation on the one hand, and environmental degradation by the phenomenon of erosion on the other hand especially in early spring.

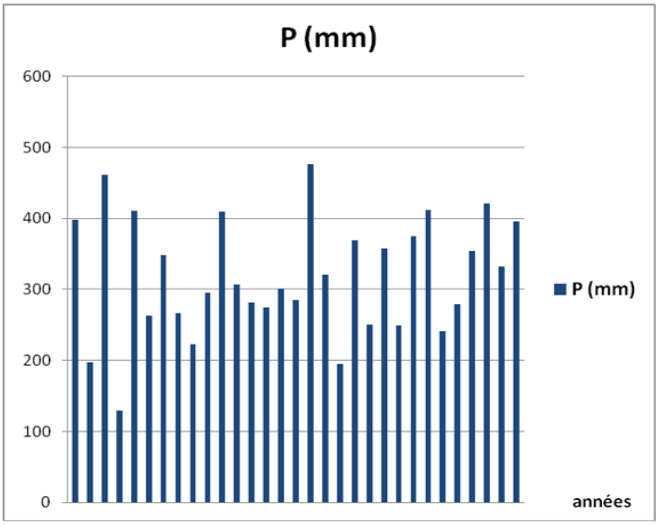


**Figure 2 :** Histogram comparison of annual rainfall (in mm) Town Tessala (1980-2010) (ONM, 2011)

Regarding annual precipitation in the region Tessala is low. The average annual rainfall recorded since 1980 to 2010 is indicative of the decrease in rainfall ; over a time duration of 30 years only the years ( 1982-1984-1990-1996-2004- 2008 and 2010 ) has registered more than 400mm ; the minimum is recorded in 1983with 129.5mm and the maximum is de530 mm in 2010(Fig.2)

### Temperature

Each plant grows in temperatures and temperature variations that suit them. Low or high critical values cause the shutdown or restart of the vegetation. Vegetation starting temperatures are generally between 0 and 15 ° C. Below growth is often stopped or negligible. The optimum growth is generally between 25 and 35 ° C and the growth is stopped between 40 and 45 ° C; Note also that the temperature requirements are not constant . Some plants need cold periods to initiate processes such as dormancy (wake the seeds) or flowering .effect exercised temperature on biochemical reactions is well known

From the study of the knowledge of the following variables: the maximum temperatures (M), the minimum temperatures (m) and average monthly temperatures (M + m / 2) can characterize the temperature of a given location.

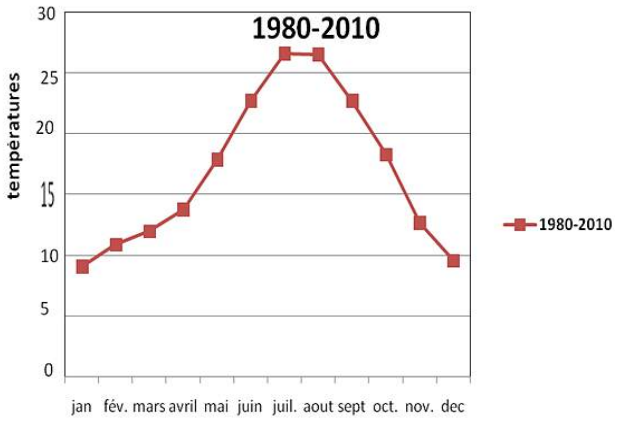


**Figure 3 :** Average monthly temperatures on the period 1980-2010 (ONM, 2011)

Monthly mean temperatures given by the formula (M + m / 2) show a maximum in July (26.6 ° C) and minimum (9.1° C) for January (Fig.3)

**Aridity index of De Martonne**

To evaluate the intensity of the drought index From Martonne , offers more ease and efficiency calculations

I = P / (T + 10)

P: average annual rainfall (mm)

T: Average annual temperature (° C)

WITH:

20 <I <30 the climate is temperate,

10 <I <20 the climate is semi-arid,

7.5 <I <10 the climate is steppe,

5 < I < 7.5 the climate is desert,

I < 5 the climate is hyper.

So I = 360.9 / (16.9 +10) = 13.42

The aridity index is around 13.42, registered Tessala the region in a semi-arid climate.

**Ombrothermic diagram of the period (1980-2010)**

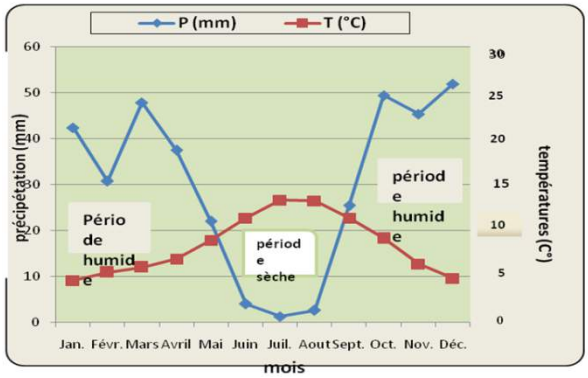


**Figure 4:** Diagram ombrothermic (p = 2t) of the Tessala station the period (1980-2010)

**B. Soil Tests**

**The physical parameters**

**Color and structure**

The color and structure of the soil of the three stations of study are shown in Table 1.

The st1 and st3 stations are similar in color and structure while st2 sol is dark brown with a lumpy structure (Tab.1).

| stations | color | structure |
|---|---|---|
| St1 | Dark brown | lumpy |
| St2 | Dark brown | polyhedral |
| St3 | Dark brown | lumpy |

Table 1.Color and structure of Soil samples

**Moisture**

The soil moisture varies from one station to another for the first station (st1) is 19.5 % for the second (st2) 10.76 % station and the third station (st3) 16%.

**Elemental composition and texture**

The results of the granulometric analysis of the various soil samples show that:

The proportions of sand for soil samples of the three stations is 30 % for st1 and st3 stations and for ST 2 it is 35%; silts on their rate is heterogeneous with 45 % for the first station; 38% for the second station and 55% for the third station; clays also vary with amounts of between 25% to st1; 27% to 15% st2 and st3. After projection of the particle size results on the triangle textures, we noticed that the texture of the three stations is silty (Tab.2)

**Table 2.** Textural characterization of soil samples from selected stations

| **Stations** | Granulometry (%) | | | Texture |
|---|---|---|---|---|
| | sand y | cla y | loam | |
| **St1** | **30** | **45** | **25** | silty |
| St2 | **35** | **38** | **27** | silty |
| St3 | **30** | **55** | **15** | silty |

**C. Chemical parameters**

The hydrogen potential (pH) of the samples was almost the same: 8.00 for st1 and st 3; 8.02 for st2.

The electrical conductivity (Ce) is between 0.09 and 0.14 (ms / cm). After projection of the results on the scale of salinity, we find that the different soils of the three stations belong to the category of non-saline soils (Ce <0.6 ms / cm).

Soil organic matter rate of the three stations, projected on the scale of determining the organic content, shows that the samples belong to the category of soils rich in organic matter (MO%> 4) with respectively 14.5% ; 14.33% and 12.5% for st3, st1 and st2. This links the work of Michel Caron (2005) showing that *Rosa canina* L. grows in any soil, but with still a preference for humus-rich land, exposed to the sun

The projection results on the total limestone scale interpretation carbonates highlights low load calcareous soils ST3 and average charging station limestone for st1 and st2 stations. For the active limestone, we obtained the following results st1 of approximately 2.61%, st2 is 3.34%, while st3 is too small to be calculated.

## IV. CONCLUSION

Our job is to study a medicinal- plant, part of the floristic Mount Tessala (western Algeria). This is *Rosa canina* L. the study of climate and substrate *Rosa canina* L opens search fields to better study this vegetable for better value.

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# Contribution to the Convective Drying of Green Oak Glands of Aures (Quercus ilex)

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## ABSTRACT

This work deals with the drying four shapes of green oak glands in ventilated oven (LabTechOven) by natural convection at 40, 50 and 60 C°. This is to study the kinetics of water content, the mass diffusivity and the activation energy. The results show that the diffusion coefficient varies between $7{,}53 \times 10^{-10}$ and $9{,}89 \times 10^{-8}$ ($m^2/s$) with the increasing temperature. The values of the activation energy are respectively 65,96, 34,17, 29,88 and 22,59 (kJ/mol) for the whole oak glands, peeled glands, peeled half glands and gland powder. These results are in agreement with the literature.

**Keywords:** Oak, Drying, Ventilated oven, Diffusivity

## I. INTRODUCTION

Vegetables and green fruits are important sources of minerals, vitamins and fiber [3]. The majority of fresh fruit contains high moisture content. After picking, they undergo aging process followed by decomposition. The water removal technique is used in order to reduce the availability of water and minimize the possible damage of plants [4]. In the food industry there are several techniques and many devices ensure the drying of food products. The drying technique is still very important because of their effect in the preservation of the quality of food. The improvement of various devices and instruments specific of drying does ceased to develop and improve (electric oven, ventilated oven, microwave ...). The mass diffusivity is the basis of property in calculating the mass transfer for drying agricultural products [5].

## II. METHODS AND MATERIAL

### A. PROJECT DESCRIPTION

The aim of our work is the decreasing a water content in oak glands by drying technique through dry air. In the food industry, there are many tools and several devices provide drying function (microwave, electric oven, oven, etc.....). We used a ventilated oven (brand LabTechOven) for drying ours samples (whole oak glands, peeled glands, peeled half glands and gland powder) by convection at three temperatures 40, 50 and 60 C°, the transfer of heat and mass is controlled.

### B. VENTILATED OVEN PROPERTY:

Ventilated Air drying, electronic temperature control and digital display, an inner glass door and another exterior metal door (thermal insulation), programmable drying time for more than 12 hours, alarm at end of cycle, programmable temperature to the start and stop automatically adjustable in 1 C°. The Ventilation ensures good homogeneity of the internal temperature [2].

Ventilated oven has the principle of convection drying. The heating element is not located within the specimen chamber of the oven, but in a separate external envelope. This prevents radiant heat from affecting the specimen, but the resulting temperature of the oven walls is enough to heat and dehydrate a specimen. Convective heat transfer is achieved by gravity or mechanical convection. Air intakes and exhausts can be adjusted to withhold or release humidity, Insulation reduces the rate of thermal

transfer and is responsible for the energy efficiency of the oven [2].

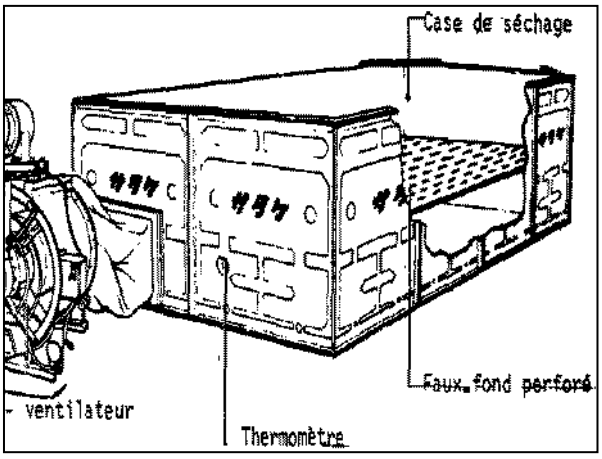


**Figure 1:** Composition of ventilated oven (LabtechOven) [2].

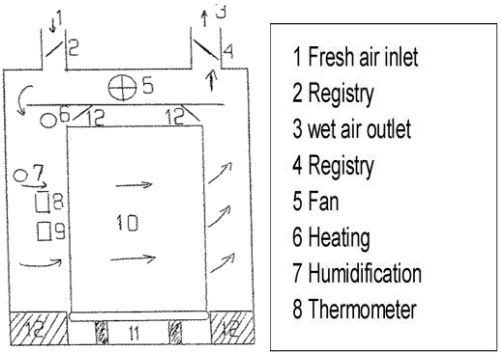


**Figure 2 :** The composition of the ventilated oven (Labtech) [2].

The transfer of moisture during drying is controlled by internal diffusion. The Fick's second law of diffusion was widely used to describe the drying process for most organic products. The relationship between the temperature and the mass diffusivity follows Arrhenius expression:

$$D = D_0 \cdot e^{(Ea/RT)}$$

The activation energy is calculated from the slope of the following equation:

$$LnD = LnD_0 - (Ea/RT)$$

The mass diffusivity of oak samples may be determined by the following relationship:

$$D = (K L^2) / \pi^2 \text{ [1].}$$

## III. RESULTS AND DISCUSSION

The values of the activation energy and the mass diffusivity are shown in Table 1.

The majority of agricultural products (92%) have a mass diffusivity in the range **$10^{-12}$ to $10^{-8}$ ($m^2/s$)**.

The values obtained ranged from **$7.53\times10^{-10}$** and **$9.89\times10^{-8}$ ($m^2/s$)**. The results show that the mass diffusivity increases with increasing of thickness samples and of drying temperature.

The values of the activation energy for most food material are in the range 12.7 to 110 kJ/mol. The values of the activation energy obtained are respectively **65,96**, **34,17**, **29,88** and **22,59** (kJ/mol) for the whole glands, peeled glands, peeled half glands and oak powder. The results show that the activation energy increases with increasing of diffusivity and of the sample thickness.

The values of the mass diffusivity and of the activation energy obtained are in agreement with the general line for drying food materials

TABLE I

ESTIMATED MASS DIFFUSIVITY AND ACTIVATION ENERGY OF OAK.

| **Sample Form** | **D ($m^2s^{-1}$)** | **Ea (kJ/mole)** |
|---|---|---|
| **Whole Glands** | $2{,}16\times10^{-8}$ to $9{,}89\times10^{-8}$ | 65,96 |
| **Peeled glands** | $2{,}08\times10^{-9}$ to $4{,}57\times10^{-9}$ | 34,17 |
| **Peeled half glands** | $2{,}08\times10^{-9}$ to $4{,}14\times10^{-9}$ | 29,88 |
| **Powder** | $7{,}35\times0^{-10}$ to $1{,}23\times10^{-9}$ | 22,59 |

Drying time decreases with increasing temperature. The temperature has an influence on the evolution of the moisture content during drying (fig 3). Strong oak drying time reduction was obtained when exposed to dry air at a temperature of 60 C°.

The various velocity curves for the three temperatures (40, 50, 60 C°) have shown a decreasing drying rate. The absence of the constant speed phase was noted. This result is in agreement with the results obtained for various plant products.

Reducing the thickness of the dried form causes an increase in the heat transfer surface and thus a lengthening of the drying time. The shortest drying time corresponds to the oak powder, then peeled half glands, next peeled glands, and finally the whole glands.

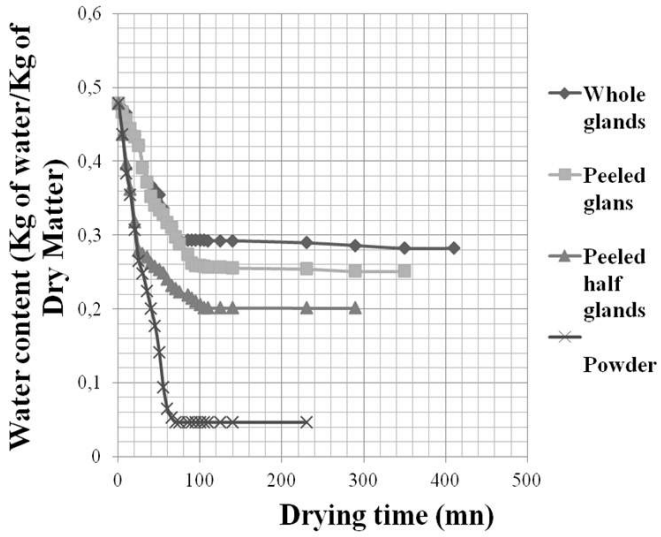


**Figure 3:** Evolution of the water content of the different samples of oak dried at 50 C°.

## IV. CONCLUSION

This work focuses on the direct drying of oak. The dried product has a long lifetime due to its low water content. The shape of the oak powder requires very short drying time and low activation energy. It is necessary to develop new drying devices to be well equipped, have lower cost and high energy efficiency to increase food production and to preserve the quality of the food.

## V. GLOSSARY

D: Mass diffusivity ($m^2$/s: square meter/second)
K: Drying constant ($s^{-1}$: $second^{-1}$)
D0: Arrhenius factor ($m^2$/s: square meter/second)
L: Product Thickness (m: meter)
Ea: Activation energy (kJ/mol DM: kilojoule/mol Dry Matter)
T: Air temperature (K: Kelvin)
R: Ideal gas constant (kJ/mol.
K: kilojoule/mol. Kelvin)
X: Water content (kg of Water/kg
DM: kilogram of water/kilogram Dry Mater)

## VI. ACKNOWLEDGMENT

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# Evaluating Foliar Responses of Sunflower Genotypes under Drought Stress

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## ABSTRACT

As a summer crop, sunflower (*Helianthus annuus* L.) is influences commonly by drought stress due to growing generally in rain fed regions without irrigation. Drought affects severely not only seed yield both also other important yield traits which plays important roles on yield formation in sunflower. Therefore, drought resistance became one of the most important goals in the sunflower breeding programs in the world. The study was conducted to determine drought tolerance levels of sunflower genotypes under controlled environmental conditions in Edirne which is a border city in Trakya region which is European part of Turkey and has about 50% of Turkish sunflower production areas. Sunflower restores lines which developed in National Project were evaluated foliar responses against drought stress. Based on study results, the most affected foliar trait was leaf area and it was reduced until 75%. Similarly, leaf number of plants also influenced severely especially from earlier droughts and plants responded about 60% leaf number decreases. On the other hand, on the chlorophyll content of plant leaves, sunflower genotypes responded differently both in earlier (R.3) and later (R.5.1) plant growth stages. While chlorophyll content of sunflower lines increased about 40-50%, some of them decreased about 30%. Similarly, sunflower genotypes responded differently drought stress in their foliar traits depending on when stress applied early or late. As results, sunflower genotypes had different tolerance to drought and higher tolerant ones will be considered to develop tolerant hybrids and will be used as initial material for further breeding purposes.

**Keywords:** Environmental Conditions, SPAD, Total Stress Response Index, Drought Response Index ,Seed , crops, Drought Stress

## I. INTRODUCTION

Sunflower is one the most important oil crops in the world. It grows generally in drylands so it is affected much from environmental conditions. Water stress is common factor limiting and reducing seed and oil yield especially in these rainfed and marginal regions [1]. To compete with other profitable crops in the rotation increasing hot temperature due global warming in recent years, new sunflower cultivars should be drought tolerant.

Sunflower plants protect themselves against drought stress altering some morphological, physiological or phonological responses based on genotypic capabilities. Since drought tolerance is so complex and quantitative trait, it is so difficult to improve through conventional plant breeding. Therefore, drought tolerance studies conduct generally under controlled conditions because it is not easy to understand of mechanism of drought and to define drought tolerant cultivars current environmental conditions where the occurrence because timing and severity of water stress may fluctuate from year to year [2], [3], [4].

Water deficiency lessens also net assimilation rate, dry weight of leaves, stem, and root and causes total dry weight and slow growth rate during the vegetation period in hot summer seasons. Plant responses to drought stresses involving processes at anatomical, cellular and molecular levels with resulting decreases in

plant photosynthesis too [5], [6], [7], [8], [9], [10], [11], [12].

Leaf area index is the most important growth indicator in sunflower because sunflower plants perform maximum photosynthesis with reaching the highest leaf area if there is not any stress [6]. In any water stress during these growing stages, crop growth rate could reduce because of decreasing in leaf area with falling leaves and with rapid aging of leaf especially after flowering stage [13], [14]. Especially in earlier growing period of sunflower (4 to 8 leaves), drought stress rapidly leads reduction of number and size of leaves, less leaf area index and less absorption at maturity stage, also shorter plants and lower plant dry matter. These earlier water stresses reduce leaf growth rate and leaf number among vegetative phase then it results initiating of decreases leaf area index after that [15], [16], [17]. Fereres et al. (1983) found that leaf area was decreased rapidly by drought stress and affected grain yield negatively and Goksoy et al. (2004) observed that restricted irrigations reduced leaf area due to yellowing and falling leaves too.

The chlorophyll content is one of the main indicators of plant foliage development and quantity changes could be considered for measuring of drought tolerance [18], [19]. However, when some of them indicated that the total chlorophyll content was reduced under drought conditions, especially after two weeks under water stress conditions, some of them observed increases on chlorophyll content after drought stress [20], [21].

The study was conducted to determine drought tolerance levels on foliar traits of sunflower male inbred lines developed in National Sunflower Project conducted by Trakya Agricultural Research Institute (TARI) under controlled environmental conditions in Edirne, Turkey.

## II. METHODS AND MATERIAL

The study was carried out in TARI research fields with fifty male inbred lines originated different genetic sources in 2014i Tunca commercial hybrid belonging Limagrain Co were used as control selected as one of the most stabile sunflower hybrids in different environments in Turkey. Trials were conducted with RCBD with one row and three replications. In each row, there were five plants and the distance between rows was 70 cm and 30 cm in rows. Trials were planted by hand in 29 May and plants were harvested and threshed by hand in 24 September.

The rainfall and humidity in 2014 is over longer year averages while average temperatures were the same and daily rainfalls in 2014 (Table 1) and applied irrigations to put water availability for field capacity during vegetation period were given Table 2. Drip irrigation was applied and as covering rain shelters, drought stress conditions were set up like below in the experiments.

Table 1. Daily rainfalls during the study (mm)

| Months and Rainfalls ( R ) (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5 | R | 6 | R | 7 | R | 8 | R |
| 31st | 28,0 | 4th | 38,7 | 4th | 0,9 | 7th | 11,2 |
| | | 5th | 6,6 | 5th | 0,3 | 18th | 5,6 |
| | | 6th | 2,2 | 16th | 39,5 | | |
| | | 26th | 42,2 | 17th | 40,1 | | |
| | | | | 20th | 3,0 | | |

Table 2. Irrigation amounts applied in the experiment plots (mm)

| Irrigation time | Irrigation (mm) | Irrigation time | Irrigation (mm) |
|---|---|---|---|
| 10.06.2014 | 50 | 10.08.2014 | 75 |
| 25.06.2014 | 70 | 18.08.2014 | 60 |
| 10.07.2014 | 65 | 28.08.2014 | 60 |
| 25.07.2014 | 40 | | |

Sunflower yield consist three main characters as plant per area, seeds per plant and seed weight. Consequently, if any biotic and abiotic stress in these critical stages, sunflower plants influence more or less depends on severity of stress [1]. Therefore drought stress groups were determined based on these vital periods in the study.

Stress group 1, 2 and 3 were set up in 23.06.2014, in 22.07.2014 and 04.08.2014, respectively. **Control:** All plant water requirement were supplied by drip irrigation (when field capacity reduced until 50%); **Stress group 1 ($S_1$):** When plants were 50 cm, **Stress group 2 ($S_2$):** at bud development, **Stress group 3 ($S_3$):** at the milky stage.

Leaf number (number/plant), leaf area ($cm^2$/plant), amount of chlorophyll as SPAD units at R3 and R5-1 growth stages were counted and measured with Hansatech Instruments (Chlorophyll Content Meter (CL-01).

Total Stress Response Index (TSRI) was calculated according to Singh et al. (2008) with minor modification. TSRI was calculated as the sum of drought response index (DRI) of individual plant attribute responses to the three levels of water deficit (Stress 1, 2 and 3) compared to the control. Positive or less negative value stated that more drought tolerance. DRI was calculated as:

DRI = [(RVt – RVc) / RVc] * 100;

RV = individual response variable for each measured parameter.

TSRI= DRILn + DRILa + DRIChlR3 + DRIChlR5

(Where Ln= leaf number, La= leaf area, ChlR3 and ChlR5= amounts of chlorophyll at R3 and R5-1 growth stages respectively)

## III. RESULT AND DISCUSSION

It was observed that drought stress seriously affected foliar development of male sunflower lines in the study. Based on observations, drought stress effects leaf development directly while water stress prolonged. In longer drought stress (S1), leaf numbers reduced about 20-60% comparing control application in the study (Table 3). However, in lower water stresses S2 and S3 groups), these influences observed about 0-40% levels.
Leaf number was the most influenced foliar trait after leaf area. The numbered of 38, 4, 8, 39, 7, 9, 11 and 25 males lines at S1 stress application, 8, 4, 7, 9, 20, 13, 11 and 38 numbered lines at S2 and the numbered 7, 4, 8, 5, 34, 11, 38 and 14 lines at S3 stress conditions affect less from drought stress (Table 3).

Leaf areas of sunflower male inbred lines were reduced 40-70% at S1 drought stage, 10-30% S2 and S3 stress groups comparing each of lines control applications in the study. Less influenced inbred lines were number of 8, 11, 10, 39, 49, 30, 7, 50 and 9 lines at S1 group, number of 4, 12, 8, 11, 7, 49, 34, 38 and 10 lines at S2 and number of 4, 8, 7, 11, 34, 10, 12 and 38 male lines at S2 stress application (Table 4).

The chlorophyll contents of sunflower male inbred lines responded differently drought stress applications both in earlier and late plant developments in the study (Table 5 and 6).

The changes among total chlorophyll contents of lines at R5-1 growth stage were insignificant while all other observed traits were significant statistically. At R5-1 stage, while total chlorophyll contents of 14 lines at S1 group, 17 lines of them at S2 and 20 lines of contents at S3 stress group were decreased about 10-30%, other lines of content were increased about 0-50% (Table 5). On the other hand, while the decreases on in total chlorophyll contents were observed in only five lines (10-30%) at S1 group and in 6 lines (10-20%) at S2 stress application, the increases were measured about 0-50% level in other lines of total chlorophyll contents in the study (Table 5).

Based on measured data of total chlorophyll contents in R5-1 stage, the numbered of 51, 49, 27, 41, 10, 46, 40 and 32 lines at stress 1 application; 5, 4, 7, 35, 20, 51, 43, 49 and 29 numbered lines at stress 2 and the numbered 30, 21, 16, 29, 50, 23 and 49 lines at stress 3 period were less affected from these stress conditions (Table 5).

Based on measured data of total chlorophyll contents in R3 stage, the numbered of 4, 1, 7, 43, 39, 26 and 50 lines at stress; 25, 50, 27, 26, 14 and 45 lines at S2 conditions were affected less from drought stress (Table 6).

The male inbred lines were categorized based on measured parameters against drought stress conditions with calculated total stress response index (TSRI) (Figure 1 and Table 7). While the having less negative valued lines were affected less from drought stress, higher negative valued sunflower male lines were influenced more from drought stress conditions. In other words, the inbred lines having values closer to 0 were labeled as more drought tolerant lines and distant valued lines could be more susceptible (Figure 1 and Table 7).
Based on the study results, while the male inbred lines were categorized five groups, especially three of them

(10004-2 R, 70352 R and 8129 R) were noticed as significant values (as less affected from drought stress) among other lines. 70352 R and 8129 R inbred lines were observed also the highest drought lines in previous study with measured other sunflower yield traits too [23].

Table 3: The effect of drought stress on leaf numbers (number/plant) in sunflower inbred lines

| # | Name of Line | Control | Stress 1 | Tolerance Index (%) | Stress 2 | Tolerance Index (%) | Stress 3 | Tolerance Index (%) |
|---|---|---|---|---|---|---|---|---|
| 38 | TT 199 R | 20,67 | 16,33 | **79,0** | 17,67 | **85,5** | 18,33 | **88,7** |
| 4 | 25712 R | 19,67 | 15,33 | **78,0** | 18,67 | **94,9** | 18,67 | **94,9** |
| 8 | 70352 R | 22,67 | 17,67 | **77,9** | 21,67 | **95,6** | 20,33 | **89,7** |
| 39 | TT 205 R | 25,67 | 19,33 | **75,3** | 21,33 | 83,1 | 21,00 | 81,8 |
| 7 | 6973 R | 22,00 | 16,33 | **74,2** | 20,33 | **92,4** | 21,33 | **97,0** |
| 9 | 7820 R | 21,67 | 16,00 | **73,8** | 19,67 | **90,8** | 18,67 | 86,2 |
| 11 | 8129 R | 21,33 | 15,67 | **73,4** | 18,33 | **85,9** | 19,00 | **89,1** |
| 25 | 9947 R | 23,33 | 17,00 | **72,9** | 18,67 | 80,0 | 19,67 | 84,3 |
| 1 | 0536 R | 23,33 | 16,33 | 70,0 | 18,33 | 78,6 | 18,33 | 78,6 |
| 3 | 010018 R | 25,33 | 17,33 | 68,4 | 20,67 | 81,6 | 21,33 | 84,2 |
| 24 | 9889 R | 25,00 | 17,00 | 68,0 | 19,33 | 77,3 | 19,67 | 78,7 |
| 43 | TT 216 R | 24,33 | 16,33 | 67,1 | 19,33 | 79,5 | 20,00 | 82,2 |
| 26 | 9979 R | 26,67 | 17,67 | 66,3 | 21,67 | 81,3 | 20,33 | 76,2 |
| 2 | 01001 R | 24,67 | 16,33 | 66,2 | 19,33 | 78,4 | 21,33 | 86,5 |
| 40 | TT 207 R | 24,67 | 16,33 | 66,2 | 18,67 | 75,7 | 21,33 | 86,5 |
| 51 | Tunca | 28,33 | 18,67 | 65,9 | 23,00 | 81,2 | 23,33 | 82,4 |
| 14 | TT 326 R | 23,33 | 15,33 | 65,7 | 19,67 | 84,3 | 20,67 | **88,6** |
| 30 | 9993 R | 22,33 | 14,67 | 65,7 | 17,00 | 76,1 | 18,33 | 82,1 |
| 41 | TT 212 R | 25,00 | 16,33 | 65,3 | 20,33 | 81,3 | 21,67 | 86,7 |
| 27 | 9987 R | 24,00 | 15,67 | 65,3 | 19,00 | 79,2 | 21,00 | 87,5 |
| 44 | TT 317 R | 26,67 | 17,33 | 65,0 | 22,33 | 83,7 | 22,67 | 85,0 |
| 13 | 8267 R | 25,67 | 16,67 | 64,9 | 22,33 | **87,0** | 21,67 | 84,4 |
| 5 | 3510 R | 25,33 | 16,33 | 64,5 | 21,00 | 82,9 | 22,67 | **89,5** |
| 34 | 10004-2 R | 25,33 | 16,33 | 64,5 | 20,67 | 81,6 | 22,67 | **89,5** |
| 21 | 9759 R | 27,00 | 17,33 | 64,2 | 21,67 | 80,2 | 22,67 | 84,0 |
| 17 | 9753-1 R | 21,33 | 13,67 | 64,1 | 15,67 | 73,4 | 15,67 | 73,4 |
| 42 | TT 214 R | 23,33 | 14,67 | 62,9 | 17,67 | 75,7 | 17,00 | 72,9 |
| 20 | 9758 R | 26,67 | 16,67 | 62,5 | 23,33 | **87,5** | 21,33 | 80,0 |
| 16 | 9702 R | 21,33 | 13,33 | 62,5 | 16,33 | 76,6 | 17,33 | 81,2 |
| 35 | TT 119 R | 24,67 | 15,33 | 62,2 | 20,67 | 83,8 | 20,33 | 82,4 |
| 22 | 9761 R | 27,33 | 17,00 | 62,2 | 21,33 | 78,0 | 21,00 | 76,8 |
| 31 | 9997-7 R | 23,67 | 14,67 | 62,0 | 16,67 | 70,4 | 18,00 | 76,1 |
| 33 | 10004-1 R | 25,33 | 15,67 | 61,8 | 19,00 | 75,0 | 18,33 | 72,4 |
| 10 | 7887-1 R | 22,67 | 14,00 | 61,8 | 16,67 | 73,5 | 18,00 | 79,4 |
| 50 | CL 217 R | 20,33 | 12,33 | 60,7 | 15,67 | 77,0 | 17,00 | 83,6 |
| 23 | 9786 R | 23,67 | 14,33 | 60,6 | 17,33 | 73,2 | 17,67 | 74,6 |
| 12 | 8165 R | 25,33 | 15,33 | 60,5 | 20,00 | 78,9 | 19,67 | 77,6 |
| 28 | 9990 R | 25,33 | 15,33 | 60,5 | 19,67 | 77,6 | 18,67 | 73,7 |
| 49 | 98920 R | 24,33 | 14,67 | 60,3 | 19,67 | 80,8 | 20,33 | 83,6 |
| 37 | TT 138 R | 26,00 | 15,67 | 60,3 | 18,67 | 71,8 | 19,67 | 75,6 |
| 47 | K9 R SN 1 | 24,33 | 14,33 | 58,9 | 19,33 | 79,5 | 18,67 | 76,7 |
| 6 | 62301 R | 28,33 | 16,67 | 58,8 | 19,67 | 69,4 | 24,33 | 85,9 |
| 29 | 9992 R | 23,33 | 13,67 | 58,6 | 16,67 | 71,4 | 18,33 | 78,6 |
| 32 | 9999 R | 24,67 | 14,33 | 58,1 | 17,67 | 71,6 | 17,67 | 71,6 |
| 36 | TT 135 R | 27,00 | 15,67 | 58,0 | 19,33 | 71,6 | 21,00 | 77,8 |
| 48 | 9868 R | 23,00 | 13,00 | 56,5 | 17,33 | 75,4 | 16,67 | 72,5 |
| 46 | TT 330 R | 26,33 | 14,67 | 55,7 | 19,33 | 73,4 | 20,33 | 77,2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 15 | 9487 R | 25,33 | 13,67 | 53,9 | 18,33 | 72,4 | 19,67 | 77,6 |
| 45 | TT 321 R | 20,67 | 10,67 | 51,6 | 14,67 | 71,0 | 15,33 | 74,2 |
| 18 | 9753-2 R | 22,33 | 10,67 | 47,8 | 14,33 | 64,2 | 15,33 | 68,7 |
| 19 | 9753-3 R | 22,67 | 9,33 | 41,2 | 14,67 | 64,7 | 16,33 | 72,1 |
| $\bar{x}$: LSD (0,01):0,61 | | 24,18 A | 15,39 C | | 19,02 B | | 19,61 B | |

Table 4: The effect of drought stress on leaf area ($cm^2$/plant) in sunflower inbred lines

| # | Name of Line | Control | Stress 1 | Tolerance Index (%) | Stress 2 | Tolerance Index (%) | Stress 3 | Tolerance Index (%) |
|---|---|---|---|---|---|---|---|---|
| 8 | 70352 R | 2085,7 | 1351,3 | **64,8** | 1890,3 | **90,6** | 1936,4 | **92,8** |
| 11 | 8129 R | 3900,3 | 2361,0 | **60,5** | 3366,9 | **86,3** | 3546,9 | **90,9** |
| 10 | 7887-1 R | 2914,7 | 1761,1 | **60,4** | 2326,3 | **79,8** | 2549,3 | **87,5** |
| 39 | TT 205 R | 2626,0 | 1585,3 | **60,4** | 1953,3 | 74,4 | 1990,9 | 75,8 |
| 49 | 98920 R | 3929,0 | 2345,3 | **59,7** | 3241,0 | **82,5** | 3336,5 | 84,9 |
| 30 | 9993 R | 2547,7 | 1465,7 | **57,5** | 1909,8 | 75,0 | 2163,7 | 84,9 |
| 7 | 6973 R | 3151,7 | 1748,0 | **55,5** | 2612,5 | **82,9** | 2866,8 | **91,0** |
| 50 | CL 217 R | 2389,3 | 1279,3 | **53,5** | 1885,0 | 78,9 | 1962,4 | 82,1 |
| 9 | 7820 R | 3589,0 | 1909,0 | **53,2** | 2581,7 | 71,9 | 2698,0 | 75,2 |
| 38 | TT 199 R | 2716,7 | 1425,7 | 52,5 | 2170,3 | **79,9** | 2341,7 | **86,2** |
| 25 | 9947 R | 2794,7 | 1466,3 | 52,5 | 2186,8 | 78,2 | 2271,9 | 81,3 |
| 41 | TT 212 R | 4816,3 | 2472,0 | 51,3 | 3452,5 | 71,7 | 3570,7 | 74,1 |
| 34 | 10004-2 R | 2329,3 | 1173,3 | 50,4 | 1878,7 | **80,7** | 2092,0 | **89,8** |
| 13 | 8267 R | 3517,0 | 1769,3 | 50,3 | 2634,3 | 74,9 | 2541,9 | 72,3 |
| 21 | 9759 R | 3324,0 | 1652,7 | 49,7 | 1997,3 | 60,1 | 2125,6 | 63,9 |
| 51 | Tunca | 8563,7 | 4122,3 | 48,1 | 6566,7 | 76,7 | 6787,7 | 79,3 |
| 14 | TT 326 R | 3030,7 | 1439,3 | 47,5 | 2208,7 | 72,9 | 2353,0 | 77,6 |
| 44 | TT 317 R | 4203,0 | 1990,7 | 47,4 | 3158,7 | 75,2 | 3203,3 | 76,2 |
| 26 | 9979 R | 3054,3 | 1445,3 | 47,3 | 1942,9 | 63,6 | 2089,3 | 68,4 |
| 1 | 0536 R | 2829,4 | 1309,0 | 46,3 | 2111,1 | 74,6 | 2187,3 | 77,3 |
| 12 | 8165 R | 4231,7 | 1949,3 | 46,1 | 3931,0 | **92,9** | 3671,7 | **86,8** |
| 40 | TT 207 R | 3186,3 | 1467,3 | 46,1 | 2083,3 | 65,4 | 2241,6 | 70,4 |
| 24 | 9889 R | 3199,3 | 1404,7 | 43,9 | 1989,7 | 62,2 | 2215,2 | 69,2 |
| 4 | 25712 R | 2347,3 | 975,3 | 41,6 | 2186,3 | **93,1** | 2309,7 | **98,4** |
| 45 | TT 321 R | 2356,0 | 955,7 | 40,6 | 1747,3 | 74,2 | 1830,3 | 77,7 |
| 16 | 9702 R | 2279,7 | 904,0 | 39,7 | 1609,3 | 70,6 | 1795,9 | 78,8 |
| 42 | TT 214 R | 3075,3 | 1204,3 | 39,2 | 1806,9 | 58,8 | 1830,8 | 59,5 |
| 22 | 9761 R | 4003,3 | 1552,3 | 38,8 | 2350,4 | 58,7 | 2475,1 | 61,8 |
| 36 | TT 135 R | 2848,3 | 1099,3 | 38,6 | 1573,1 | 55,2 | 1847,3 | 64,9 |
| 20 | 9758 R | 4136,3 | 1574,7 | 38,1 | 2872,6 | 69,4 | 2832,9 | 68,5 |
| 23 | 9786 R | 3764,3 | 1431,0 | 38,0 | 2305,9 | 61,3 | 2316,2 | 61,5 |
| 48 | 9868 R | 3174,3 | 1194,7 | 37,6 | 1929,9 | 60,8 | 2010,1 | 63,3 |
| 33 | 10004-1 R | 2812,7 | 1033,3 | 36,7 | 1758,1 | 62,5 | 1807,8 | 64,3 |
| 6 | 62301 R | 3019,3 | 1106,0 | 36,6 | 1549,4 | 51,3 | 2247,2 | 74,4 |
| 28 | 9990 R | 3823,3 | 1378,7 | 36,1 | 2381,0 | 62,3 | 2404,3 | 62,9 |
| 29 | 9992 R | 3819,3 | 1329,0 | 34,8 | 1968,1 | 51,5 | 2070,6 | 54,2 |
| 47 | K9 R SN 1 | 6510,0 | 2213,0 | 34,0 | 3668,9 | 56,4 | 3574,4 | 54,9 |
| 17 | 9753-1 R | 2067,7 | 700,3 | 33,9 | 1250,7 | 60,5 | 1258,3 | 60,9 |
| 32 | 9999 R | 2702,3 | 903,0 | 33,4 | 1682,5 | 62,3 | 1757,3 | 65,0 |
| 31 | 9997-7 R | 3677,7 | 1223,7 | 33,3 | 1745,2 | 47,5 | 1882,7 | 51,2 |
| 37 | TT 138 R | 2492,0 | 822,7 | 33,0 | 1430,3 | 57,4 | 1470,3 | 59,0 |
| 46 | TT 330 R | 2169,3 | 696,7 | 32,1 | 1249,3 | 57,6 | 1312,7 | 60,5 |
| 2 | 01001 R | 2598,7 | 792,9 | 30,5 | 1476,4 | 56,8 | 1832,3 | 70,5 |
| 5 | 3510 R | 3624,7 | 1087,0 | 30,0 | 2294,3 | 63,3 | 2816,0 | 77,7 |
| 43 | TT 216 R | 2869,3 | 853,3 | 29,7 | 1530,3 | 53,3 | 1647,1 | 57,4 |
| 35 | TT 119 R | 3579,0 | 1052,3 | 29,4 | 1786,3 | 49,9 | 1845,0 | 51,6 |
| 27 | 9987 R | 4819,7 | 1394,0 | 28,9 | 2382,1 | 49,4 | 2631,9 | 54,6 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 19 | 9753-3 R | 1830,3 | 522,7 | 28,6 | 1330,0 | 72,7 | 1417,0 | 77,4 |
| 18 | 9753-2 R | 1820,7 | 506,0 | 27,8 | 1067,7 | 58,6 | 1429,7 | 78,5 |
| 3 | 010018 R | 3807,0 | 982,3 | 25,8 | 2511,7 | 66,0 | 2664,7 | 70,0 |
| $\bar{x}$: LSD (0,01):152,4 | | 3303,4 A | 1403,1 C | | 2226,0 B | | 2357,3 B | |

Table 5: The effect of drought stress on chlorophyll amount at R5-1 growth stage in sunflower

| # | Name of Line | Control | Stress 1 | Tolerance Index (%) | Stress 2 | Tolerance Index (%) | Stress 3 | Tolerance Index (%) |
|---|---|---|---|---|---|---|---|---|
| 47 | K9 R SN 1 | 13,93 | 20,37 | 146,2 | 17,34 | 124,4 | 16,79 | 120,5 |
| 11 | 8129 R | 13,93 | 18,90 | 135,6 | 16,67 | 119,6 | 15,30 | 109,8 |
| 34 | 10004-2 R | 11,77 | 15,03 | 127,7 | 14,16 | 120,3 | 14,91 | 126,7 |
| 36 | TT 135 R | 19,30 | 23,63 | 122,5 | 21,13 | 109,5 | 21,00 | 108,8 |
| 2 | 01001 R | 16,20 | 19,67 | 121,4 | 18,28 | 112,8 | 17,84 | 110,1 |
| 16 | 9702 R | 12,97 | 15,20 | 117,2 | 14,59 | 112,5 | 13,03 | **100,5** |
| 13 | 8267 R | 10,91 | 12,67 | 116,1 | 12,02 | 110,1 | 12,01 | 110,1 |
| 15 | 9487 R | 14,73 | 16,73 | 113,6 | 16,38 | 111,2 | 15,31 | 103,9 |
| 9 | 7820 R | 12,04 | 13,63 | 113,2 | 13,21 | 109,7 | 13,60 | 112,9 |
| 14 | TT 326 R | 14,73 | 16,63 | 112,9 | 15,87 | 107,7 | 14,99 | 101,7 |
| 8 | 70352 R | 15,77 | 17,73 | 112,5 | 16,55 | 104,9 | 16,62 | 105,4 |
| 22 | 9761 R | 12,90 | 14,30 | 110,8 | 13,94 | 108,0 | 12,37 | 95,9 |
| 31 | 9997-7 R | 15,53 | 17,18 | 110,6 | 16,45 | 105,9 | 15,78 | 101,6 |
| 23 | 9786 R | 13,20 | 14,59 | 110,5 | 14,03 | 106,3 | 13,10 | **99,2** |
| 30 | 9993 R | 13,73 | 15,14 | 110,2 | 15,13 | 110,2 | 13,93 | **101,4** |
| 29 | 9992 R | 18,70 | 20,60 | 110,1 | 19,15 | **102,4** | 18,71 | **100,1** |
| 1 | 0536 R | 10,65 | 11,69 | 109,8 | 11,67 | 109,5 | 12,18 | 114,4 |
| 48 | 9868 R | 15,07 | 16,55 | 109,8 | 15,89 | 105,5 | 16,17 | 107,3 |
| 20 | 9758 R | 14,97 | 16,37 | 109,4 | 14,70 | **98,2** | 15,24 | 101,8 |
| 21 | 9759 R | 16,50 | 18,01 | 109,2 | 17,23 | 104,4 | 16,68 | **101,1** |
| 50 | CL 217 R | 20,53 | 22,40 | 109,1 | 21,30 | 103,8 | 20,49 | **99,8** |
| 3 | 010018 R | 12,27 | 13,23 | 107,9 | 13,75 | 112,1 | 13,13 | 107,0 |
| 35 | TT 119 R | 15,37 | 16,53 | 107,6 | 15,27 | **99,3** | 15,73 | 102,4 |
| 12 | 8165 R | 17,07 | 18,31 | 107,3 | 18,27 | 107,0 | 16,28 | 95,4 |
| 5 | 3510 R | 15,80 | 16,88 | 106,8 | 15,80 | **100,0** | 15,03 | 95,1 |
| 37 | TT 138 R | 18,17 | 19,31 | 106,3 | 18,75 | 103,2 | 17,83 | 98,2 |
| 28 | 9990 R | 16,03 | 16,94 | 105,6 | 15,44 | 96,3 | 14,68 | 91,6 |
| 7 | 6973 R | 16,03 | 16,83 | 105,0 | 16,29 | **101,6** | 15,31 | 95,5 |
| 51 | Tunca | 14,27 | 14,59 | **102,2** | 13,97 | **97,9** | 13,88 | 97,3 |
| 49 | 98920 R | 12,20 | 12,40 | **101,6** | 12,47 | **102,2** | 12,06 | **98,9** |
| 27 | 9987 R | 12,80 | 12,57 | **98,2** | 12,08 | 94,3 | 12,31 | 96,2 |
| 41 | TT 212 R | 11,30 | 11,05 | **97,8** | 10,84 | 96,0 | 9,57 | 84,7 |
| 10 | 7887-1 R | 17,43 | 16,94 | **97,2** | 15,18 | 87,1 | 16,30 | 93,5 |
| 46 | TT 330 R | 12,70 | 12,33 | **97,1** | 11,94 | 94,0 | 11,47 | 90,3 |
| 40 | TT 207 R | 9,03 | 8,70 | **96,3** | 8,34 | 92,4 | 8,47 | 93,7 |
| 32 | 9999 R | 9,60 | 9,21 | **96,0** | 8,29 | 86,4 | 8,58 | 89,4 |
| 4 | 25712 R | 13,93 | 13,24 | 95,0 | 13,94 | **100,1** | 15,00 | 107,7 |
| 33 | 10004-1 R | 15,13 | 14,13 | 93,4 | 13,41 | 88,6 | 13,67 | 90,3 |
| 43 | TT 216 R | 14,17 | 13,20 | 93,2 | 13,86 | **97,8** | 12,71 | 89,7 |
| 26 | 9979 R | 15,20 | 13,93 | 91,7 | 13,47 | 88,6 | 13,28 | 87,4 |
| 38 | TT 199 R | 16,83 | 15,43 | 91,7 | 14,51 | 86,2 | 14,58 | 86,6 |
| 42 | TT 214 R | 15,37 | 14,00 | 91,1 | 13,38 | 87,1 | 13,08 | 85,1 |
| 6 | 62301 R | 18,13 | 16,43 | 90,6 | 16,09 | 88,7 | 16,60 | 91,5 |
| 18 | 9753-2 R | 12,17 | 10,84 | 89,1 | 10,48 | 86,1 | 10,53 | 86,5 |
| 25 | 9947 R | 19,73 | 17,37 | 88,0 | 17,93 | 90,8 | 18,46 | 93,5 |
| 17 | 9753-1 R | 16,13 | 14,03 | 87,0 | 12,83 | 79,5 | 12,42 | 77,0 |
| 44 | TT 317 R | 12,10 | 10,51 | 86,8 | 11,56 | 95,5 | 10,06 | 83,1 |
| 45 | TT 321 R | 17,47 | 14,97 | 85,7 | 15,87 | 90,8 | 15,20 | 87,0 |
| 19 | 9753-3 R | 12,33 | 10,53 | 85,4 | 9,26 | 75,1 | 8,53 | 69,1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 24 | 9889 R | 22,73 | 17,93 | 78,9 | 17,93 | 78,9 | 17,23 | 75,8 |
| 39 | TT 205 R | 15,73 | 10,96 | 69,7 | 11,05 | 70,2 | 12,17 | 77,3 |
| $\bar{x}$: | | 14,81 | 15,30 | | 14,66 | | 14,31 | |

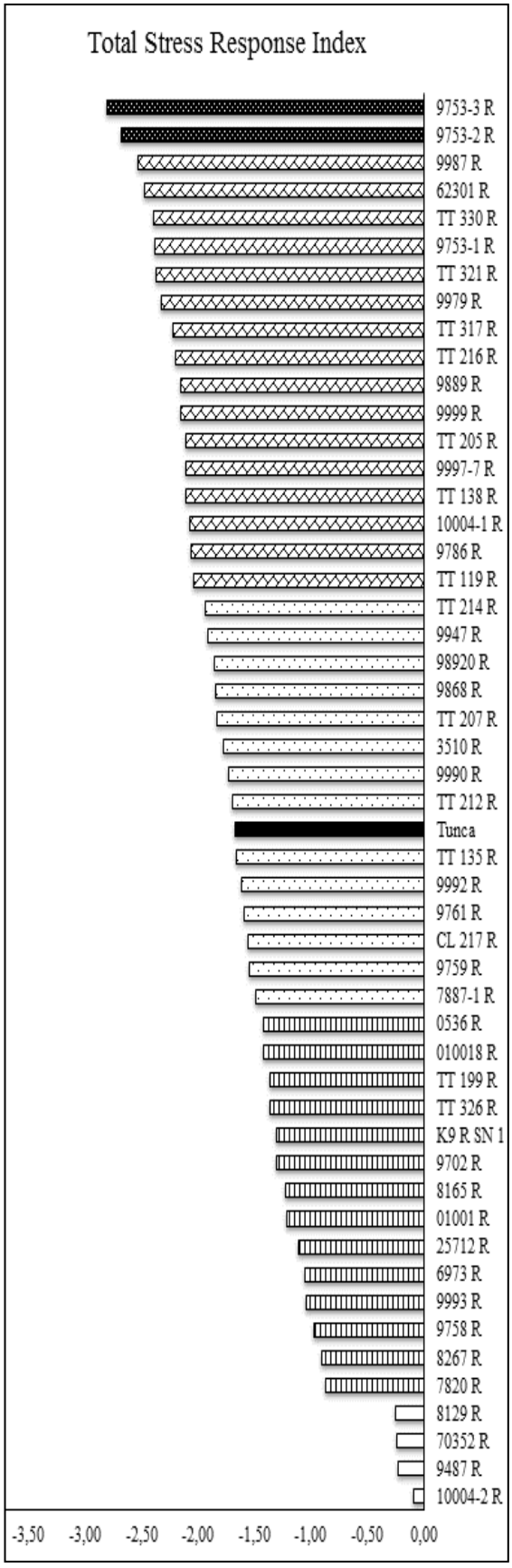


**Figure 1.** Total stress response index (TSRI) of leaf number, leaf area, amount of chlorophyll at R3 and R5-1 growth stages in sunflower lines subjected to drought stress.

Table 6: The effect of drought stress on chlorophyll amount at R3 growth stage in sunflower

| # | Name of Line | C | S1 | TI (%) | S2 | TI (%) |
|---|---|---|---|---|---|---|
| 20 | 9758 R | 6,58 | 9,83 | 149,5 | 9,11 | 138,6 |
| 29 | 9992 R | 9,25 | 13,28 | 143,6 | 12,33 | 133,3 |
| 8 | 70352 R | 7,94 | 11,07 | 139,4 | 8,18 | 103,1 |
| 28 | 9990 R | 9,08 | 12,15 | 133,7 | 11,51 | 126,8 |
| 32 | 9999 R | 5,14 | 6,75 | 131,2 | 6,18 | 120,1 |
| 42 | TT 214 R | 8,24 | 10,73 | 130,2 | 11,87 | 144,1 |
| 24 | 9889 R | 10,86 | 14,14 | 130,2 | 13,20 | 121,5 |
| 15 | 9487 R | 7,88 | 10,16 | 129,0 | 9,12 | 115,8 |
| 22 | 9761 R | 7,29 | 9,30 | 127,5 | 8,92 | 122,3 |
| 16 | 9702 R | 8,51 | 10,85 | 127,4 | 8,77 | 103,0 |
| 3 | 010018 R | 8,21 | 10,44 | 127,2 | 8,91 | 108,6 |
| 38 | TT 199 R | 9,01 | 11,19 | 124,3 | 9,31 | 103,4 |
| 13 | 8267 R | 6,99 | 8,64 | 123,6 | 8,09 | 115,8 |
| 30 | 9993 R | 7,27 | 8,96 | 123,1 | 7,97 | 109,6 |
| 17 | 9753-1 R | 8,82 | 10,83 | 122,8 | 11,40 | 129,3 |
| 21 | 9759 R | 10,93 | 13,36 | 122,2 | 11,63 | 106,5 |
| 33 | 10004-1 R | 5,25 | 6,29 | 119,8 | 6,71 | 127,8 |
| 48 | 9868 R | 8,69 | 10,40 | 119,7 | 9,31 | 107,1 |
| 9 | 7820 R | 6,51 | 7,77 | 119,4 | 6,98 | 107,2 |
| 37 | TT 138 R | 10,31 | 12,17 | 118,1 | 11,01 | 106,8 |
| 2 | 01001 R | 8,93 | 10,53 | 117,9 | 11,43 | 128,0 |
| 11 | 8129 R | 7,74 | 9,04 | 116,8 | 8,28 | 106,9 |
| 35 | TT 119 R | 8,71 | 10,09 | 115,8 | 9,73 | 111,8 |
| 36 | TT 135 R | 9,69 | 11,19 | 115,5 | 10,88 | 112,3 |
| 19 | 9753-3 R | 8,56 | 9,86 | 115,2 | 10,10 | 118,0 |
| 23 | 9786 R | 7,37 | 8,49 | 115,2 | 6,91 | 93,8 |
| 31 | 9997-7 R | 7,75 | 8,71 | 112,4 | 9,19 | 118,5 |
| 45 | TT 321 R | 10,74 | 11,99 | 111,6 | 10,56 | **98,3** |
| 46 | TT 330 R | 8,94 | 9,97 | 111,4 | 9,97 | 111,5 |
| 12 | 8165 R | 8,73 | 9,60 | 110,0 | 10,02 | 114,8 |
| 5 | 3510 R | 9,30 | 10,17 | 109,3 | 9,63 | 103,5 |
| 18 | 9753-2 R | 8,55 | 9,22 | 107,9 | 9,99 | 116,8 |
| 40 | TT 207 R | 6,82 | 7,34 | 107,7 | 7,96 | 116,7 |
| 51 | Tunca | 8,44 | 9,09 | 107,7 | 7,96 | 94,3 |
| 47 | K9 RSN1 | 9,60 | 10,30 | 107,3 | 10,60 | 110,5 |
| 10 | 7887-1 R | 9,00 | 9,63 | 107,0 | 11,18 | 124,2 |
| 41 | TT 212 R | 7,34 | 7,84 | 106,8 | 8,48 | 115,6 |
| 34 | 10004-2 R | 5,71 | 6,09 | 106,7 | 8,74 | 153,1 |
| 14 | TT 326 R | 9,39 | 9,98 | 106,2 | 9,25 | **98,5** |
| 4 | 25712 R | 8,83 | 9,32 | **105,6** | 7,11 | 80,5 |
| 1 | 0536 R | 9,58 | 10,01 | **104,5** | 9,10 | 95,0 |
| 7 | 6973 R | 8,56 | 8,94 | **104,4** | 8,22 | 96,0 |
| 43 | TT 216 R | 7,87 | 8,16 | **103,7** | 9,96 | 126,6 |
| 39 | TT 205 R | 8,90 | 9,13 | **102,6** | 10,53 | 118,4 |
| 26 | 9979 R | 8,98 | 8,79 | **98,0** | 8,88 | **98,9** |
| 50 | CL 217 R | 12,43 | 11,90 | **95,7** | 12,43 | **100,0** |
| 27 | 9987 R | 8,27 | 7,77 | 94,0 | 8,25 | **99,7** |
| 6 | 62301 R | 8,81 | 7,80 | 88,6 | 10,27 | 116,5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 25 | 9947 R | 14,00 | 12,00 | 85,7 | 14,30 | **102,1** |
| 44 | TT 317 R | 9,43 | 8,04 | 85,3 | 8,91 | 94,6 |
| 49 | 98920 R | 8,83 | 6,17 | 69,8 | 7,97 | 90,2 |
| x̄: LSD (0,05):0,56 | | 8,59 B | 9,71 A | | 9,55 A | |

Table 7: The effect of drought stress as TSRI on chlorophyll amounts at leaves in sunflower

| Lines / Treatments | Leaf number | | | | Leaf area *(cm²/plant)* | | | Chl R3 | | Chl R5-1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | S1 | S2 | S3 | S1 | S2 | S3 | S1 | S2 | S1 | S2 | S3 |
| 0536 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,5 | 0,7 | 0,8 | 1,0 | 0,9 | 1,1 | 1,1 | 1,1 |
| 01001 R | 1,0 | 0,7 | 0,8 | 0,9 | 0,3 | 0,6 | 0,7 | 1,2 | 1,3 | 1,2 | 1,1 | 1,1 |
| 010018 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,3 | 0,7 | 0,7 | 1,3 | 1,1 | 1,1 | 1,1 | 1,1 |
| 25712 R | 1,0 | 0,8 | 0,9 | 0,9 | 0,4 | 0,9 | 1,0 | 1,1 | 0,8 | 1,0 | 1,0 | 1,1 |
| 3510 R | 1,0 | 0,6 | 0,8 | 0,9 | 0,3 | 0,6 | 0,8 | 1,1 | 1,0 | 1,1 | 1,0 | 1,0 |
| 62301 R | 1,0 | 0,6 | 0,7 | 0,9 | 0,4 | 0,5 | 0,7 | 0,9 | 1,2 | 0,9 | 0,9 | 0,9 |
| 6973 R | 1,0 | 0,7 | 0,9 | 1,0 | 0,6 | 0,8 | 0,9 | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 |
| 70352 R | 1,0 | 0,8 | 1,0 | 0,9 | 0,6 | 0,9 | 0,9 | 1,4 | 1,0 | 1,1 | 1,0 | 1,1 |
| 7820 R | 1,0 | 0,7 | 0,9 | 0,9 | 0,5 | 0,7 | 0,8 | 1,2 | 1,1 | 1,1 | 1,1 | 1,1 |
| 7887-1 R | 1,0 | 0,6 | 0,7 | 0,8 | 0,6 | 0,8 | 0,9 | 1,1 | 1,2 | 1,0 | 0,9 | 0,9 |
| 8129 R | 1,0 | 0,7 | 0,9 | 0,9 | 0,6 | 0,9 | 0,9 | 1,2 | 1,1 | 1,4 | 1,2 | 1,1 |
| 8165 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,5 | 0,9 | 0,9 | 1,1 | 1,1 | 1,1 | 1,1 | 1,0 |
| 8267 R | 1,0 | 0,6 | 0,9 | 0,8 | 0,5 | 0,7 | 0,7 | 1,2 | 1,2 | 1,2 | 1,1 | 1,1 |
| TT 326 R | 1,0 | 0,7 | 0,8 | 0,9 | 0,5 | 0,7 | 0,8 | 1,1 | 1,0 | 1,1 | 1,1 | 1,0 |
| 9702 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,4 | 0,7 | 0,8 | 1,3 | 1,0 | 1,2 | 1,1 | 1,0 |
| 9753-1 R | 1,0 | 0,6 | 0,7 | 0,7 | 0,3 | 0,6 | 0,6 | 1,2 | 1,3 | 0,9 | 0,8 | 0,8 |
| 9753-2 R | 1,0 | 0,5 | 0,6 | 0,7 | 0,3 | 0,6 | 0,8 | 1,1 | 1,2 | 0,9 | 0,9 | 0,9 |
| 9753-3 R | 1,0 | 0,4 | 0,6 | 0,7 | 0,3 | 0,7 | 0,8 | 1,2 | 1,2 | 0,9 | 0,8 | 0,7 |
| 9758 R | 1,0 | 0,6 | 0,9 | 0,8 | 0,4 | 0,7 | 0,7 | 1,5 | 1,4 | 1,1 | 1,0 | 1,0 |
| 9759 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,5 | 0,6 | 0,6 | 1,2 | 1,1 | 1,1 | 1,0 | 1,0 |
| 9761 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,4 | 0,6 | 0,6 | 1,3 | 1,2 | 1,1 | 1,1 | 1,0 |
| 9786 R | 1,0 | 0,6 | 0,7 | 0,7 | 0,4 | 0,6 | 0,6 | 1,2 | 0,9 | 1,1 | 1,1 | 1,0 |
| 9889 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,4 | 0,6 | 0,7 | 1,3 | 1,2 | 0,8 | 0,8 | 0,8 |
| 9947 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,5 | 0,8 | 0,8 | 0,9 | 1,0 | 0,9 | 0,9 | 0,9 |
| 9987 R | 1,0 | 0,7 | 0,8 | 0,9 | 0,3 | 0,5 | 0,5 | 0,9 | 1,0 | 1,0 | 0,9 | 1,0 |
| 9990 R | 1,0 | 0,6 | 0,8 | 0,7 | 0,4 | 0,6 | 0,6 | 1,3 | 1,3 | 1,1 | 1,0 | 0,9 |
| 9992 R | 1,0 | 0,6 | 0,7 | 0,8 | 0,3 | 0,5 | 0,5 | 1,4 | 1,3 | 1,1 | 1,0 | 1,0 |
| 9993 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,6 | 0,7 | 0,8 | 1,2 | 1,1 | 1,1 | 1,1 | 1,0 |
| 9999 R | 1,0 | 0,6 | 0,7 | 0,7 | 0,3 | 0,6 | 0,7 | 1,3 | 1,2 | 1,0 | 0,9 | 0,9 |
| 10004-2 R | 1,0 | 0,6 | 0,8 | 0,9 | 0,5 | 0,8 | 0,9 | 1,1 | 1,5 | 1,3 | 1,2 | 1,3 |
| TT 119 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,3 | 0,5 | 0,5 | 1,2 | 1,1 | 1,1 | 1,0 | 1,0 |
| TT 135 R | 1,0 | 0,6 | 0,7 | 0,8 | 0,4 | 0,6 | 0,6 | 1,2 | 1,1 | 1,2 | 1,1 | 1,1 |
| TT 138 R | 1,0 | 0,6 | 0,7 | 0,8 | 0,3 | 0,6 | 0,6 | 1,2 | 1,1 | 1,1 | 1,0 | 1,0 |
| TT 199 R | 1,0 | 0,8 | 0,9 | 0,9 | 0,5 | 0,8 | 0,9 | 1,2 | 1,0 | 0,9 | 0,9 | 0,9 |
| TT 205 R | 1,0 | 0,8 | 0,8 | 0,8 | 0,6 | 0,7 | 0,8 | 1,0 | 1,2 | 0,7 | 0,7 | 0,8 |
| TT 207 R | 1,0 | 0,7 | 0,8 | 0,9 | 0,5 | 0,7 | 0,7 | 1,1 | 1,2 | 1,0 | 0,9 | 0,9 |
| TT 212 R | 1,0 | 0,7 | 0,8 | 0,9 | 0,5 | 0,7 | 0,7 | 1,1 | 1,2 | 1,0 | 1,0 | 0,8 |
| TT 214 R | 1,0 | 0,6 | 0,8 | 0,7 | 0,4 | 0,6 | 0,6 | 1,3 | 1,4 | 0,9 | 0,9 | 0,9 |
| TT 216 R | 1,0 | 0,7 | 0,8 | 0,8 | 0,3 | 0,5 | 0,6 | 1,0 | 1,3 | 0,9 | 1,0 | 0,9 |
| TT 317 R | 1,0 | 0,6 | 0,8 | 0,9 | 0,5 | 0,8 | 0,8 | 0,9 | 0,9 | 0,9 | 1,0 | 0,8 |
| TT 321 R | 1,0 | 0,5 | 0,7 | 0,7 | 0,4 | 0,7 | 0,8 | 1,1 | 1,0 | 0,9 | 0,9 | 0,9 |
| TT 330 R | 1,0 | 0,6 | 0,7 | 0,8 | 0,3 | 0,6 | 0,6 | 1,1 | 1,1 | 1,0 | 0,9 | 0,9 |
| K9 R SN 1 | 1,0 | 0,6 | 0,8 | 0,8 | 0,3 | 0,6 | 0,5 | 1,1 | 1,1 | 1,5 | 1,2 | 1,2 |
| 9868 R | 1,0 | 0,6 | 0,8 | 0,7 | 0,4 | 0,6 | 0,6 | 1,2 | 1,1 | 1,1 | 1,1 | 1,1 |
| 98920 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,6 | 0,8 | 0,8 | 0,7 | 0,9 | 1,0 | 1,0 | 1,0 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CL 217 R | 1,0 | 0,6 | 0,8 | 0,8 | 0,5 | 0,8 | 0,8 | 1,0 | 1,0 | 1,1 | 1,0 | 1,0 |
| Tunca | 1,0 | 0,7 | 0,8 | 0,8 | 0,5 | 0,8 | 0,8 | 1,1 | 0,9 | 1,0 | 1,0 | 1,0 |

## IV. CONCLUSION

As a conclusion, sunflower male inbred lines exhibited different tolerance to drought stress on foliar traits in the study. Higher tolerant ones such as 70352 R and 8129 R inbred lines will be considered to develop tolerant hybrids with crossing other tolerant female lines and they will be used as also initial material for further breeding purposes.

## V. ACKNOWLEDGMENT

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# Isolation of monosporal strains of *Fusicladium oleagineum*, the fungal causal agent of olive leaf spot from North-Western Algerian groves

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## ABSTRACT

Olive scab also called "peacock eye" desease caused by *Fusicladium oleagineum* (FO) is among the most important pathology of the olive tree (*Olea europaea*). Symptoms are initiated by the appearance of blackish circular spots on leafs, which turn to yellow and drop prematurely. Drastic foliar volume drop, shoots-death and general weakness of the tree leads to yield reduction. Under Algerian climate, the fruit is also infected. The scabbed olives eventually fall. This phytopathology known since 1923 was first considered of minor incidence. However, current intensive olive groves extension provides an ideal habitat for the FO development making it a major concern for farmers. Chemical or copper-based nature available treatments are source of pollution in ecosystems, although their limited effectiveness. Being biotrofic, FO has been poorly studied since *in vitro* cultures are hard to achieve. This work is related to isolation and conservation methods of Algerian FO native strains. This work would be a first step to a better understanding of the local FO agent.

**Keywords:** *Fusicladium oleagineum*, olive scab, isolation, identification.

## I. INTRODUCTION

The olive tree (*Olea europaea* subspecies *oleaceae*) is one of the main fruit species planted in Algeria with about 208 000 ha (33% of the trees area) [1]. The plantations are concentrated in the North, particularly in areas of the Tell. There are numerous olive varieties in Algeria (Var.), representing the basis of the livelihoods of several rural communities: Var. "Siguoise" found from Oued Rhiou to Tlemcen. Its expansion zone reaches the Mitidja. It is used mainly for the production of table green and black olives and for oil production. Var. “Sevillanas”, of Hispanic origin, with her very large fruits, is located in sub-coastal plains of Oran, used for olive green table production. Oil Var. “Rougette” is common in the Mitidja plain and the foothills of the Atlas, at low altitudes. Var. "Chemlal" is the most famous in Algeria, for its oil, her distribution goes from the Atlas of Blida to Bibans and Guergour regions. Thanks to her great vigour poor soils became profitable and provide oils of high quality. In Algeria there are also low extension varieties such as “Azeradj”, “Bouchouk”, “Aguenaou”, “Guergour”, “Limli” and “blanquette” of Guelma. Olive trees can be affected by many pests and diseases. They require a Mediterranean climate-type, characterized by cold (mean monthly t° ≥-3°C), wet but short winters and long, dry and hot (below 30°C) summer conditions. These diverse climatic conditions are favourable for olive leaf spot (OLS), which is actually the most significant leaf disease in olive-growing countries [2], [3], [4].

**Occurrence and importance:**

OLS is wide spread in the Mediterranean region (Fig. 1) such as Algeria, Morocco, Tunisia, Iran, Greece, France, Italy, and Spain, but also in the major olive-growing areas of the world; USA, South America, Australia, and New Zealand [6], [7], [8], [3].


**Figure 1:** Mediterranean olive groves expansion [5].

The damage that causes is the dieback of twigs, defoliation of branches and finally reduction of leaf coverage. Since OLS has been known for over a century in the Mediterranean countries, it is likely that these are the sources of this pathogen.

OLS is a foliar disease widespread in all olive growing regions of the world [9], [7], [6] and is also called peacock spot (because of the circular dark lesions with a chlorotic halo on the leaf) [10], [11] is caused by the fungus *Fusicladium oleagineum* (FO) (Castagne, Ritschel and U. Braun comb. nov., syn. *Spilocaea oleagina,* Castagne (Hughes)). This fungus (Emb. *Ascomycota,* S.Emb. *Pezizomycotina,* Cl. *Dothideomycetes,* S.Cl. *Pleosporomycetidae,* Ord. *Pleosporales,* Fam. *Venturiaceae,* Gen. *Cycloconium*) is mitosporic, since no sexual stage has been demonstrated [12]. But recent phylogenetic analysis has shown that FO is an anamorphic phase of a yet unidentified *Venturia* species [13]. FO strains *in vitro* cultures are hard to obtain, since the fungus is endogenous parasite and requires particular culture conditions. Consequently, this phytopathology is poorly studied, despite its economic importance damages.

**FO Symptoms**

FO causes a leaf-spot (2,5 to 12,5 mm in diameter) disease with symptoms on the upper surface of the leafs (Fig. 2).


**Figure 2 :** FOL symptoms on Var. "Siguoise" of Algeria.

The infective hypha enters the leaf by piercing and enzymatically degrading the thick cuticle, and then grows parallel to the leaf surface as hyaline, septate, branched subcuticular mycelium. Lesions are primary inconspicuous then scarcely detectable, later enlarge to form dark-brown, circular, mostly annular that become lightly velvety and are often surrounded by concentric, faint yellow, violent or pale brown halos [6]. The colonies remain localized in a cutinised layer of the epidermal cell wall until the leaf tissues decay. The effected leafs fall prematurely. Occasionally, under very wet conditions, small, sunken brown lesions can be found on the petioles, fruit peduncles and fruit. Once trees are infected, fungal spread is caused by rain-splashed [14], [15], or insects and wind carried conidia [16], [17]. The disease causes severe defoliation resulting in both reduced flower bud differentiation and fruit set in subsequent years. Serious yeald losses have been reported [7].

**Disease Cycle**

The olive is an evergreen perennial crop, leafs live generally for 2-3 years, dropping when the tree is putting on new growth, or when they are shaded. Under Algerian climates, the trees produce few or no new leafs in mid-summer, and resume growth again in the autumn, giving two peaks of leaf production [18]. It is this new leafs that are most susceptible to OLS disease [19].

An incubation period precedes the infection (Fig. 3). Its duration depends on the olive cultivar, environmental conditions, leaf age and trees seasonal growth. In our region, she is about 15 days under favourable temperature (below 20°C) and moisture conditions [20].

The inoculum of the primary infection comes from sporulating spots on the over wintered hanging leafs or aestivated on trees. When detached from their conidiophores, conidia lose their germinability in less than one week.

During infection early stages, the germ tubes of the conidia develop appressoria, to attach the pathogens to the leaf surface. Leaf infection is through the cuticle, which is pierced and enzymatically degraded by the hyphae. Further growth by radiating mycelia, composed of branched hyaline, septate hyphae, expand to form round, flat submerged colonies between the outermost portions of the epidermal cell wall and the cuticular layer. Conidiophores develop at this phase producing easily dispersible conidia [6], [20].

There are two periods of main infections during first-autumn and last-spring. The existence of a sexual stage of FO has not been reported, and thus the role of sexual reproduction in the infection process of this pathogen remains unknown [21], [22], [23].

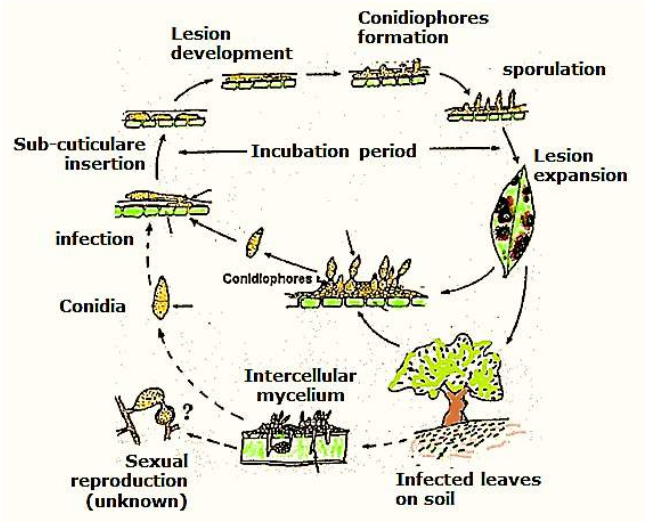


**Figure 3 :** Suggested life cycle of FO (Art by M., Mohamed Benkada).

**Control**

Pruning and chemical control schedules are the main measures. Chemical controls include the application of fungicides before and during the main infection seasons. In many areas with a dry Mediterranean climate, three spays are suggested.

## II. METHODS AND MATERIAL

FO strains were isolated from infected olive trees of "Sigoise" variety from the region of Sig (50 km Eastern of Oran, Algeria). Leafs with sporulating lesions were collected from trees chosen at random at least 10m apart, selecting only one lesion per tree to reduce the likelihood of clones. The leafs were dried for 3 days on the bench at room temperature and then stored at 4 °C until required for producing the single-spore isolates, which were grown on a mixture of three antibiotics-amended media; malt extract agar (MEA), potato dextrose agar (PDA) and our "special olive leaf extract " (SOLE) without and agar-amended at 15°C. Isolates were identified by their morphological and cultural characteristics when grown on agar SOLE medium. Colony morphology and pigmentation were compared with the published descriptions of Graniti [6].

## III. RESULT AND DISCUSSION

The fungal colonies are visible to the naked eye as dark, olive-brown spots after 2 to 3 weeks of growth at 15°C. After just one month of growth, the aerial mycelium of the colony appears as a hemispherical greyish green, felt-like stromatic body. The older colonies are differentiated into three layers: a basal submerged layer of loosely interwoven hyphae rich in chlamydospores, a medial layer above the surface of the agar formed of dark-brown closely interwoven hyphae, and an upper aerial layer of light brown felt-like mycelium (Fig. 4 a).

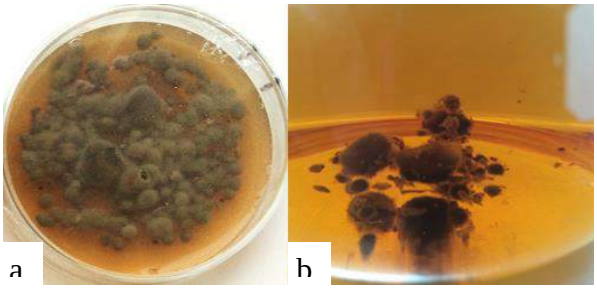


**Figure 4 :** Mycelial cultures of monospora FO on (a) agar and (b) liquid SOLE medium.

Mycelium growth was also obtained in static liquid cultures of SOLE (Fig. 4b). Monosporal fungal growth is practically absent on PDA and MEA media (Fig. 5a and b) compared to solidified SOLE (Fig. 5c).

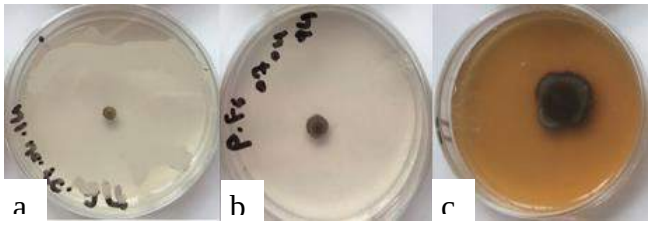

**Figure 5 :** Culture of FO on solidified (a) MEA, (b) PDA and (c) SOLE.

Culture conditions described in this work using a simple technic and an easy low-coast culture medium permit the reduction of incubation period from 6 months [24] to less than 2 months allowing a precious win of time.

## IV. CONCLUSION

These encouraging results open the way to comparative studies of different population genotypes of FO from Algeria and of neighbouring countries for a better knowledge of the biology of this pathogen. Due to increasing labour costs of harvesting of olives, the worldwide olive industry, including Algeria, is moving towards easily mechanized planting systems (i.e., ultra-high density plantings). These new systems, however favour disease occurrence with susceptible cultivars easily becoming infected by OLS [6]. The development of biological control research against FO would limit the environmental pollution due to fungicide use. In fact, environmental conditions favouring OLS development, copper-containing fungicides may neither be sufficiently effective nor sustainable for complete dependence on them alone because of the ecological impact of copper deposits in the soil. One of the main implications arising from the use of any fungicides is the behaviour of their residues, which give rise to important health considerations, especially in products destined for human consumption (Obanor, 2006). This would be an important component of the project that we already started to prepare with colleagues from Tunisia, Morocco and France.

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# Anatomical features of *Atriplex halimus* L. to Saline Environments

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## ABSTRACT

In arid and semi-arid regions, the genus *Atriplex* has agronomic and ecological interest. Indeed, in addition to their good forage quality, they ensure fixation and soil enrichment. Halophytic plants are able to tolerate saline environments. They often show a diversity of structural and physiological adaptations that include salt bladders, salt glands or hairs and multiple rows of hypodermis (probable with accumulation and storage role). Related to higher salinity of soil, the halophytes react also by some adaptive, anatomical features. In order to elucidate the anatomical variability level, root, steam and Leaves of two species of *Atriplex* were used: *Atriplex halimus* L. and *Atriplex canescens* (Pursh) Nutt. The results show anatomical differences that can be linked to their degrees of adaptation to external conditions.

**Key words**: halophytes, anatomy, blader vegetative organs, *Atriplex*, salinity.

## I. INTRODUCTION

*Atriplex* species are members of the family Amaranthaceae and contain both C3 and C4 plants. More than 200 species of plants belong to the *Atriplex* genera and most of them are salt bushes and highly tolerance to salt and drought [1]. *Atriplex halimus* L. (Mediterranean saltbush) is a halophytic shrub that is widely distributed in arid and semi-arid regions around the Mediterranean basin. *Atriplex halimus* L. grows naturally throughout Macronesia, the Mediterranean basin and beyond into western Asia: including southern Portugal, France, southern and eastern Spain (and the Canary Islands), Italy, Greece, Malta, Turkey, Cyprus, Israel, Syria, Lebanon, Jordan, Tunisia, Morocco, Algeria, Libya, Egypt and Saudi Arabia [2];[3];[4];[5]. Throughout its distribution, *Atriplex halimus* L. is exposed to high light intensity and temperature and varying degrees of drought and salinity; it can also withstand sub-zero winter temperatures or soil contamination by trace elements. Some of its physiological and biochemical tolerance mechanism such as adjustment of plant water relations are common to all or several of these environmental stresses, but others are specific to particular stresses. The importance of *Atriplex halimus* L. in the functioning of ecosystems is reflected in its promotion of soil biota, while it also acts as a food plant for mammals and arthropods. Its deep root system decreases soil erosion in arid zones, due to stabilization of the soil. The protein-rich shoot material of *Atriplex halimus* L. makes it an important fodder species for livestock, particularly sheep and goats. However, its low energy value means that it should be supplemented with carbohydrate-rich material, such as cereal straw. Potential new uses of this versatile plant species include the phytoremediation of soils contaminated by trace elements and the exploitation of its biomass as a source of renewable energy. Such applications, together with its continued use in low-intensity farming systems, should ensure that *Atriplex halimus* L. remains a vital plant species in low-rainfall regions. Due to its varied uses, particularly as livestock forage, it has been introduced elsewhere: for example, Oman, Iran, Iraq, Pakistan, South Africa, Chile, Argentina, New Zealand and the U.S.A. In countries closer to the Equator where it has been introduced, like

Kenya and Ethiopia, the small photoperiod variation during the year means that it does not flower. [4] classified *Atriplex halimus* L. as a "euhalophyte", able to withstand soil salinity levels equivalent to saturated paste EC values of 25-30 dS $m^{-1}$. [6] demonstrated that the seed germination of a coastal population of *Atriplex halimus* L. was more salt-tolerant than that of a population from a non-saline site; complete inhibition occurred at 700 and 350 mM NaCl, respectively, which represents very-high tolerance in both cases. Thus, we hypothesized that these species has developed specific morpho-anatomical and physiological features which enable it to grow under saline- conditions. Numerous studies have suggested that Atriplex spp. can be comparable to alfalfa in nutritional quality, with the advantage that many Atriplex spp. are xerohalophytes, capable of growing under saline irrigation or in dryland conditions [7]; [4]; [8]; [9]; [10]. Despite initial pessimism about the production potential of halophytes, these examples (*Salicornia*, *Atriplex* and *Distichlis*) show that euhalophytes can maintain high productivity of useful agricultural products up to a root-zone salinity of 70 g L−1 TDS, double the salinity of seawater. [11]

The objective of the present investigation was to uncover the anatomical features that aid in adaptation of salt tolerant of *Atriplex halimus* L. saline condition.

## II. METHODS AND MATERIAL

### A. Plant Material

The sample material subjected to our analysis is represented by leaves, stems and roots of *Atriplex halimus* L. Seeds of the specie were surface sterilized with sodium hypochlorite solution (1% available chlorine) for 10 min. After washing several times with distilled water, seeds were placed on wet filter paper soaked in distilled water in plastic cups in loam at 25◦C for two weeks. Plants were germinated from seeds and grown in a soil mixture of loam and sand (1V2V) for 4 months in plastic cups in the University of Poitiers greenhouses [16-h light (25◦C, 100 _E m−2s−1)/8-h dark (20◦C) cycle and 60% relative humidity] with Peter's solution until the 5th month. The nutrient solution was applied twice a week. Salt treatment was initiated by adding NaCl in the nutrient solution at concentrations of 0, 100 or 600 mM in a progressive way since 1 month.

To examine the roots, stems and leaves structure several segments (with length of 2–5 mm) were immediately fixed.

### B. Methods

Sectioning of the samples was fixed into RWL (photonic microscope) and glutaraldehyde (electronic microscope MEB). Cross sections were performed using the ultra-microtome technique. Histological observations and micrographs were performed with a ZEISS (AXIOPLAN) Light and electronic microscope (840 A de JEOL**)**.

## III. RESULT AND DISCUSSION

### Root anatomy

The examination of transverse sections of the young roots of the studied species (Fig. 1) revealed this specie have a more or less spherical form; They have the same general structure which consists of two separate parts, a considerably larger cortex and a central cylinder.

Symmetry is axial. We meet from outside to the inside following tissues: epidermis, parenchyma Cortical or mesophyll, endoderm, pericycle (hardly visible), phloem xylem (protoxylem and metaxylem).

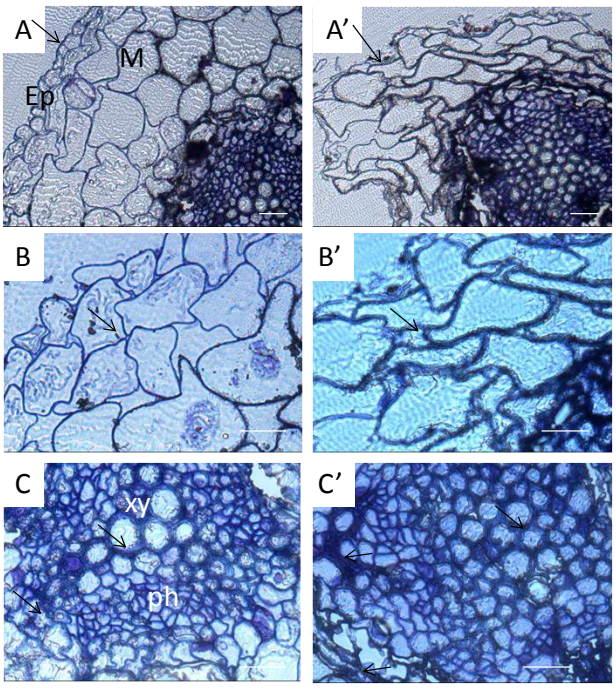

**Figure 1 :** transversal sections in roots of *Atriplex halimus* L. controls (A, B, C) and stressed (A ', B', C ') stained with toluidine blue. A, A ': Overview of a root (GX 20x10) B, B' detailed view in the cortex (GX 40 x10) C, C ': detailed view in the central cylinder (G X40x10).

**Stem anatomy**

This report will insist mostly on some histo-anatomical features which we consider to be more revealing on the adaptation of the plants to the soil salinity, as a major ambient factor which acts upon halophytes. Anyway, that does not mean that the other features are not relevant for one species or another, but just the fact that subsystems of the biologic system - the plant - have different values in clarifying the whole structure. Therefore, as a consequence of our investigation (Fig. 2), we could observe the typical secondary structure in *Atriplex halimus* L. stem.

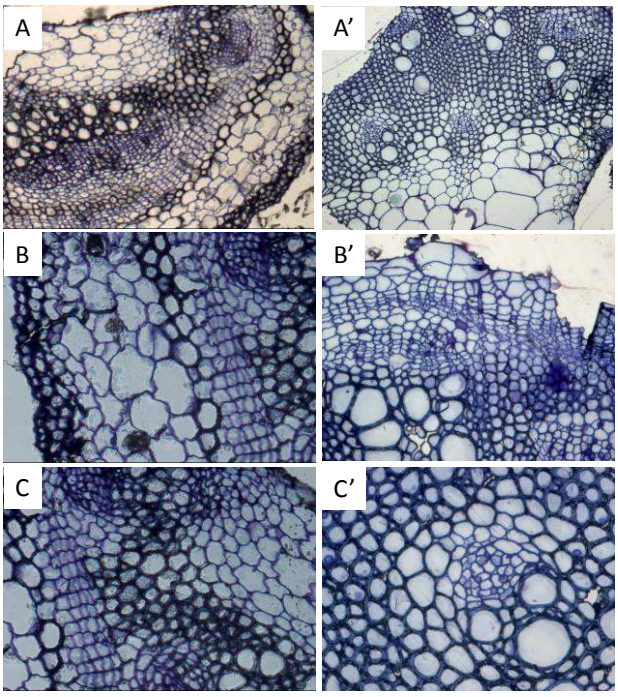


**Figure 2** : transversal sections in stems of *Atriplex halimus* L. controls (A, B, C) and stressed (A ', B', C ') stained with toluidine blue. A, A ': Overview of a stem (GX 20x10) B, B' detailed view inthe bark (GX 40 x10) C, C ': detailed view in the central cylinder (G X40x10).

**Leaf anatomy**

Concerning the leaves, there is a thickness (Fig. 3B') of the cortical parenchyma with development of spongy parenchyma with particular form as has been observed for roots**.**

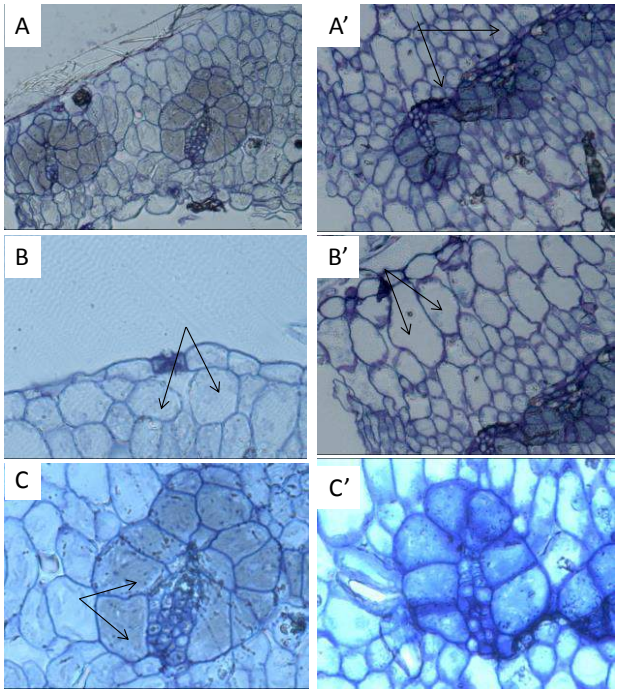


**Figure 3 :** transversal sections in leaves of *Atriplex halimus* L. controls (A, B, C) and stressed (A ', B', C ') stained with toluidine blue. A, A ': Overview of a leave (GX 20x10) B, B' detailed view in the cortex (GX 40 x10) C, C ': detailed view in the central cylinder (G X40x10).

The number of conductive beams forms a continuous sheath (Fig. 3 A'C').

*Atriplex halimus* L. possesses the C4 photosynthetic pathway [12]; [13], in which CO2 is incorporated into phosphoenolpyruvate (PEP) to form oxaloacetate through the action of PEP carboxylase. Accordingly, its leaves have the "Kranz" anatomy with a layer of bundle sheath cells surrounding each vascular bundle and radially-arranged palisade cells, although the bundle sheath is open [12]. Our observations are in agreement with the latter feature (Fig.3CC'). $C_4$ plants represent about 5% of Earth's plant biomass and 3% of its known plant species (Bond et al., 2005). Despite this scarcity, they account for about 30% of terrestrial carbon fixation

(*Osborne et Beerling, 2006)*. Increasing the proportion of $C_4$ plants on earth could assist bio sequestration of $CO_2$ and represent an important climate change avoidance strategy. Present-day $C_4$plants are concentrated in the tropics and subtropics (below latitudes of 45°) where the high air temperature contributes to higher possible levels of oxygenase activity by RuBisCO, which increases rates of photorespiration in $C_3$ plants.

C4 photosynthesis is normally associated with the compartmentation of photosynthesis between mesophyll (M) and bundle sheath (BS) cells. The mechanisms regulating the differential accumulation of photosynthesis proteins in these specialized cells are fundamental to our understanding of howC4 photosynthesis operates. Cell specific accumulation of proteins in M or BS can be mediated by post transcriptional processes and translational efficiency as well as by differences in transcription. Individual genes are likely regulated at multiple levels. Although *cis*-elements have been associated with cell-specific expression in C4 leaves, there has been little progress in identifying *trans*-factors. When C4 photosynthesis genes from C4 species are placed in closely related C3 species, they are often expressed in a manner faithful to the C4 cycle. Next-generation sequencing and comprehensive analysis of the extent to which genes from C4 species are expressed in M or BS cells of C3 plants should provide insight into how the C4 pathway is regulated and evolved [14]. Today, plants using C4 photosynthesis are widespread and important components of major tropical and subtropical biomes, but the events that led to their evolution and success started billions of years ago (bya). A CO2-fixing enzyme evolved in the early Earth atmosphere with a tendency to confuse CO2 and O2 molecules. The descendants of early photosynthetic organisms coped with this property in the geological eras that followed through successive fixes, the latest of which is the addition of complex CO2-concentrating mechanisms such as C4 photosynthesis. This trait was assembled from bricks available in C3 ancestors, which were altered to fulfill their new role in C4 photosynthesis. The existence of C4-suitable bricks probably determined the lineages of plants that could make the transition to C4 photosynthesis, highlighting the power of contingency in evolution [15].

Morphology of bladder hairs Fig. 4 showed that young leaf laminae were covered with bladder hairs on the adaxial and abaxial surfaces. This observation suggests that bladder hairs are differentiated at the young leaf stage. Bladder hairs of *Atriplex* species are shown to be differentiated from epidermis of leaf primordium at the very early stages of leaf differentiation [16]. At later developmental stages bladder hairs eventually rupture, releasing the salt on to the surface of the leaf laminae [16].

The Fig. 4 show that lower epidermis of the leaves show more stressed vesicles with a larger volume (Fig.4 A', B', C'). The same changes are observed at the upper epidermis (Fig.5 A'). The two skins showed stomata closed to varying degrees (Fig.4 C, C'; Fig.5 B, B'). Remarkable connections are visible between the vesicles that are connected to the epidermal cells by a single stalk (Fig.4 B, B', C). According [17]; [18] NaCl treatment increased the size of the trichome cells .Vesicular trichomes were observed on both the adaxial and abaxial surfaces of *Atriplex nummularia* leaves. The vesicular trichomes It is common to find vesicular trichomes covering the entire epidermis in species of Atriplex [19]; [20]. However, the details of this morphology can vary among species. A. nummularia is similar to Atriplex triangularis [21]. Microscopic observation of young leaves of *Atriplex gmelini* showed many bladder hairs on their surfaces, but their total number decreased along with leaf maturity. Sodium Green fluorescent approach revealed Na+ accumulation in bladder cells of young leaves when *Atriplex gmelini* was grown at high salinity (250 mM NaCl). Due to fewer bladder hairs in mature leaves, Na+ accumulation was mostly found in mesophyll cells of mature leaves under high salinity [22]. Differential accumulation of glycine betaine and choline monooxygenase in bladder hairs of *Atriplex gmelini* under high salinity [22]

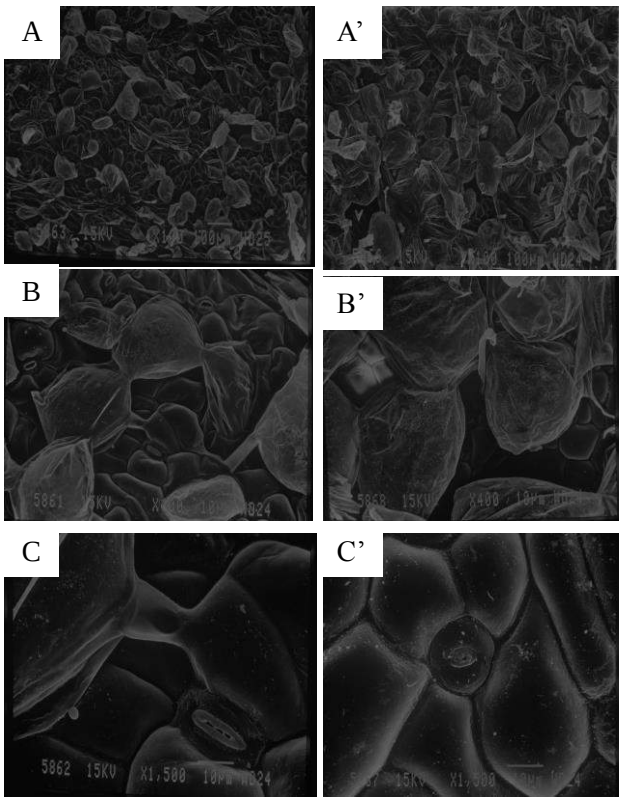


**Figure 4 :** Scanning electron microscopy photographs showing leaves abaxial surface: control (A,Band C) and stressed (A', B' and C') of *Atriplex halimus* L. (*different forms of vesicular or trichomes and stomata*)

However, only limited experiments have been conducted with this plant, and therefore its Important anatomical feature of *Atriplex halimus* L. (and other *Atriplex* spp.), particularly in relation to stress tolerance, are the vesiculated hairs[19] or vesicular trichomes [23] present on the leaf surface. These living cells consist of balloon-like hairs or bladder cells (Smaoui et al., 2011)[23], 80-200 mm in diameter and with a surface coating of a waxy material, attached to a stalk that is embedded in an epidermal cell.

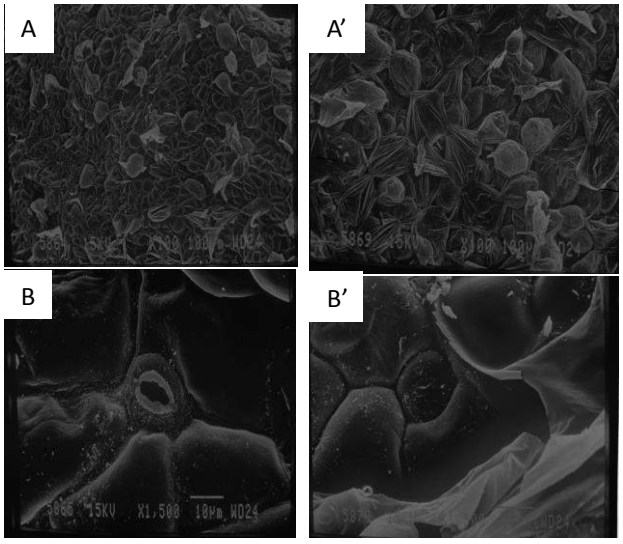


**Figure 5 :** Scanning electron microscopy photographs showing leaves adaxial surface: control (A,Band C) and stressed (A', B' and C') of *Atriplex halimus* L.

The number of salt glands, the size of the bladders and the percentage of collapsed bladders depends on the amount of salt present during plant growth [25],[26]. Salts crystallize on the leaf when the bladders burst, a process which appears to be related to leaf age [27],[28]**.** Like other halophytes, *Atriplex halimus* L. accumulates the main component ions of salinity Na+ [29]; [30] and Cl- and other anions [31]; [32]) in its tissues, storing them in the vacuole. The vesiculated hairs on the leaf surface also act as a sink for salt [19]; according to [1], more than 50% of the Naþ and Cl_ accumulated is translocated to the hairs. At very-high external salt concentrations (300 mM NaCl), damage appears, regarding stomatal conductance [33] the root plasma membrane permeability, root hydraulic conductivity and chlorophyll content 34], photosynthesis [35];[36]and intracellular organelles [37]; [38]. The latter species exhibits vesicular trichomes formed by a vesicular cell that binds to the epidermis through a single-celled stalk. In contrast, *Atriplex halimus* L. has a multicellular stalk. In adult trichomes of this species, the stalk can consist of up to three cells. *Atriplex* species accumulate the highest concentrations of salts in the vesicular trichomes. However, the stalk cell, unlike the vesicular cell, did not respond to differences in the level of water stress [39]. Because the stalk represents the connection between the epidermal and vesicular cells [40], changes in the structure of the stalk cell could affect the plant's ability

to transport salt from the epidermal cells to the vesicular cells and store it in the vesicular cells.

Total number of bladder hairs was observed to decrease with increased maturity of leaves and the distribution of bladder hairs becomes sparse as leaves develop and expand. This view is in line with a previous study on other Atriplex species [41]. However, the effect of NaCl treatment on distribution or concentration of the bladder hairs was not observed. Numerous halophytes possess leaf trichomes and salt glands that are able to remove large amounts of salt from the photosynthetically active tissues. In several cases, ion excretion is not specific for $Na^+$ or $Cl^-$ ions and high amounts of divalent cations, especially $Ca^{++}$, were also found in those structures [42].

## IV. CONCLUSION

At roots level, there is thickening of some cells of the central cylinder and the formation in the cortex of spongy parenchyma with particular form is very marked. At stems level, no differences have been revealed between the control and the stressed plants but the presence of calcium oxalate in plant control, indicate that this Cristal is not an indicator of salt tolerance. Concerning the leaves, there is a thickness of the cortical parenchyma and the number of conductive beams forms a continuous sheath. Stressed leaves of *Atriplex halimus* L. show dominance of the vesicles to the upper face, an important size in the underside's vesicles and presence of small stomata.

All these differences between the control and the stressed plant in the anatomy level show that there is some adaptations that can be related to Na Cl stress and specie on one hand and to their degrees of adaptation to external conditions on the other hand.

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# Biofilm production and antibiotic susceptibility profiles of Staphylococcus strains isolates from urinary catheter at the university hospital center of Tlemcen (Algeria)

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## ABSTRACT

Staphylococci are a major cause of infections associated with urinary catheterization and other medical devices. The ability of Biofilm production is an important step in the pathogenesis of these *Staphylococci* and depends on the expression of the *ica*ADBC operon leading to the synthesis of a polysaccharide intercellular adhesion.
In this study , 44 staphylococci isolates obtained from urinary catheter at the university hospital center (UHC) of Tlemcen (North-West Algeria) were analyzed for detecting the presence or absence of the intercellular adhesion *ica*A and *ica*D genes by polymerase chain reaction, phenotypic biofilm production was examined by tissue culture plate (TCP) and Congo red agar (CRA) methods. 17of 44 isolates were shown to carry ica -specific DNA, 18 produced slime on CRA plates but only 8 produced biofilm spontaneously on the polystyrene surfaces. Staphylococci strains isolated from urinary catheter showed high levels of resistance to penicillin (98%) and gentamicine (75%). The data reported indicate an important role of *ica* genes, phenotypic variability of biofilm production and antibiotic multiresistance as virulence factors in staphylococcal from urinary catheters.
**Keywords:** Staphylococcus; Urinary catheter; Biofilm; ica operon; slime; TCP.

## I. INTRODUCTION

Staphylococcus, a commensal microorganism routinely found on the human skin and in the hospital environment, has become the most important cause of nosocomial infections in recent years **(Chokr et al.,2007)** . They are generally associated with chronic infections related to implanted medical devices such as urinary catheterization **(Espinasse et al.,2010).**

However, bacterial colonization of implanted foreign material can cause chronic infections which are difficult to treat, lead to longer hospitalization time, and can result in much higher treatment costs **(Gad et al.,2009)** . The major virulence factor associated with this organism's ability to cause infections is dependent on adherence to medical devices and formation of a biofilm **(Cerca et al.,2005)**.

The biofilm consists of a multiple layers of sessile cells that adhere to the implant surface as well as to each other. Once a biofilm is formed, it can be very difficult to treat clinically the associated infection. This is due to the fact that the bacteria within the biofilm are well protected from the host immune response as well as antibiotic agents **(Hoyle and Costerton, 1990; Cramtonet al., 1999).** In fact, biofilm formation proceeds in two phases: primary attachment of staphylococcal cells on a biomaterial **(Macka et al., 2004)** followed by cell-cell adhesion, forming the multiple layers of the biofilm. This latter process is associated with the polysaccharide intercellular adhesin (PIA)( **Cramtonet al., 1999)** .

The synthesis of PIA is mediated by the products of the chromosomal ica gene (intercellular adhesion), which are organized in an operon structure. This operon contains the icaADBC genes, in addition to the icaRgene which exerts a regulatory function and is transcribed in

the opposite direction. Once this operon is activated, four proteins are transcribed, IcaA, IcaD, IcaB and IcaC, which are necessary for the synthesis of PIA **(Cafiso et al.,2004)**. PIA is synthesized from UDP-N-acetylglucosamine by N-acetylglucosaminyltransferase, which is encoded by the ica locus, particularly icaA. The expression of this gene alone induces low enzymatic activity and the production of low amounts of polysaccharide. However, the simultaneous expression of icaA and icaD promotes a significant increase in N-acetylglucosaminyltransferase, with a consequent increase in the amount of polysaccharide, forming oligomers of 10-20 b-1,6-Nacetylglucosamine residues **(Dobinski et al.,2002; Gotz,2002; Oliveira et al.,2010)**. IcaB is the deacetylase responsible for the deacetylation of mature PIA. In addition, the transmembrane protein IcaC seems to be involved in externalization and elongation of the growing polysaccharide **(Diemond-Hernández et al .,2010)** .

The expression of the ica operon and as a result, the formation of biofilms seems to be highly variable among staphylococci. Thus, biofilm expression is influenced by environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotic **(Ziebuhr et al.,1997; Mempel et al.,1994; Cho et al.,2002)**.

The differentiation of staphylococci with respect to its biofilm phenotype might help to elucidate the impact of staphylococci in diagnosis of infections related to biomedical devices**.** These observations can be useful in the prevention of device related infections **(Mathur et al.,2006)** .

Several studies have been published on the detection of the gene Ica among the staphylococcal strains **(Gad et al.,2009; Cho et al.,2002; Touati et al.,2007; Chaieb et al.,2005; Wang et al.,2010; Duran et al.,2010)** . However despite the increasing interest in the subject in recent years, the collect of data from Algerian hospital an institution is relatively difficult, hence, the low number of related studies.

The objective of the present study is to characterize Staphylococci strains isolates from urinary catheter at university hospital of Tlemcen in terms of antibiotic susceptibility, biofilm formation and presence of the icaA and icaD genes.

## II. METHODS AND MATERIAL

### 1. Bacterial strains

The strains selected in this study were isolated from urethral catheterization obtained from the Intensive Care Unit, urology and neurology services at the University Hospital Center (CHU) of Tlemcen (North-West Algeria). Urinary catheters studied were in latex without antibiotics; they were transported at 4°c, and analyzed immediately at the laboratory.

### 2. Identification

Microbiological analysis after removal of the catheter was performed by the technique of "Brun-Buisson"**(Brun-Buisson, 1994).** This technique consists in rinsing the lumen of the catheter with saline water and to vortex its extremity intravascular before culturing on Chapman agar plates allowing selection of staphylococci. Moreover, all isolates were identified by classic microbiological methods including: colonial morphology, Gram staining, catalase test, coagulase test and the Api-Staph test (BioMérieux®).

### 3. Antibiotic sensitivity test

Antimicrobial susceptibility testing was performed in accordance with the guidelines established by the antibiogram committee of the French Microbiology Society (**CASFM, 2010) using** 17 antibiotic discs including :

Penicilin**(10ug)**, Oxacillin **(5ug),** Cefoxitin **(30ug)**, Gentamicin **(10ug)**, Tobramycin **(10ug)** Amikacine **(30ug)**, Vancomycine **(30ug),** Rifampim**(30ug)**, Fosfomycin**(50ug)**, Fusidic Acid **(10ug)**, Clindamycin **(2ug)**, Pristinamycin **(15ug)**, Erythromycin**(15ug),** Ofloxacin**(5ug),** Tetracycline**(30ug),** Chloramphenicol **(30ug),** Triméthoprime /sulfaméthoxazole **(25ug).**

### 4. Detection of Biofilm Formation

#### 4.1 Tissue culture plate method (TCP)

Quantitative determinations of biofilm formation in 96-well tissue-culture plates (Sigma, UK) were performed based on the method of Christensen *et al.* **(Christensen et al.,1985)** with a modification in duration of incubation which was extended to 48 hours. Therefore, we had evaluated biofilm production in three different media, BHIB, BHIB with 2% sucrose, and BHIB with 1% glucose.

Bacteria were grown overnight in respective media, cultures were then diluted 1:100 and incubated in a microtiter polystyrene plate at 37°C. Microtiter wells were washed 3 times with distilled water, dried in an inverted position, and stained with 0.5% (w: v) crystal violet solution **(Mathur et al.,2006)**. The adherent cells were resuspended in 95% ethanol solution and the absorbance was measured at 540 nm by using a micro ELISA autoreader (model 680, Biorad, UK). The isolates were classified into three categories: a) non adherent, optical density lower than 0.120; b) weakly adherent, optical density higher than 0.120 or equal to or lower than 0.240; c) strongly adherent, optical density higher than 0.240.

### 4.2 Congo Red Agar method (CRA)

Phenotypic characterization of biofilm production was performed by culture of the staphylocoques isolates on CRA plates. This technique proposed by **Freeman *et al.*** requires the use of a specially prepared solid medium-brain heart infusion broth (BHIB) supplemented with 5% sucrose and Congo red.

The medium was composed of BHIB (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

According to the authors, biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes (**Jain and Agarwal, 2009).**

### 4.3 Detection of icaA and icaD loci

Extraction of bacterial DNA was made by thermal shock. After overnight culture on Luria Bertani agar plates (Bio-Rad, Marnes-la-Coquette, France), 5 colonies were suspended in 500yl of DNase- and RNase-free water (Invitrogen, England), the suspension was boiled at 100°C for 10 min in thermal block (Polystat 5, French), then centrifuged at 15000 rpm for 5min. An aliquot of 2 µL of the supernatant was used as DNA template for PCR.

The presence of *ica*A and *ica*D DNA were detected by polymerase chain reaction (PCR) using forward and reverse primers for *ica*A and *ica*D. For *ica*A, the forward primer (corresponding to nucleotides 1337–1356) had the following sequence: 5'-TCT CTT GCA GGA GCA ATC AA-3'; and the reverse primer (corresponding to nucleotides 1505–1524) had the following sequence: 5'-TCA GGC ACT AAC ATC CAG CA-3'. The primer sequences for *ica*D were: forward (nucleotides 1963–1982), 5'-ATG GTC AAG CCC AGA CAG AG- 3'; and reverse (nucleotides 2138–2160), 5'-CGT GTT TTC AAC ATT TAA TGC AA-3'. PCR amplification was carried out according to the parameters described by **Arciola *et al.***, and visualization of the amplified products by a 2% gel electrophoresis.

## III. RESULT AND DISCUSSION

### A. Results

#### 1. Characterization of Staphylococci Isolates from Urinary Catheter

A total of 44 strains have been obtained from urinary catheter used more than 48 hours at the University Hospital of Tlemcen. After biochemical identifications, all 44 strains were staphylococci species and included: *21 S.epidermidis*, 11 *S.saprophyticus*, 11 *S.aureus, and 1 S.hominis*.

#### 2. Antibiotic Sensitivity Test

Antibiotic susceptibility testing showed that majority of Staphylococci strains was resistant to more than nine antibiotics and were found to be susceptible to four

major antibiotics: Rifampim **,** Fosfomycin **,** Clindamycin and Chloramphenicol**.**

Moreover, no strains resistant to vancomycin and pristinamycin were found. The total percentage of resistance against each antibiotic is represented in Figure 1.

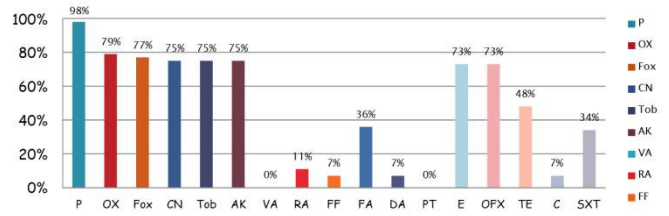


**Figure 1 :** Antibiotic resistance of Staphylococcus strains isolates from urinary catheter.

## 3. Detection of biofilm production

### 3.1. Detection of slime-producing Staphylococci Strains.

Phenotypic production of slime by all strains under study was assessed by culture on CRA plates. Slime producing strains appear as black colonies, and non-slime-producing strains appear as red colonies (Figure2).

Among the clinical isolates, 18 of 44 (41%) staphylococci strains were slime producers it included 6/11 S.saprophyticus, 5/11 S.aureus, 1/1 S.hominis and 6/21 S.epidermidis. The remaining 26 strains were non-slime producers.

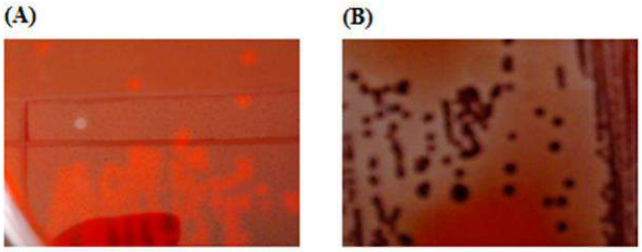


**Figure 2:** CRA plate test**. (A):** Non slime producing strains / **(B):** slime-producing strains

### 3.2. Study of biofilm production by the tissue culture plate method (TCP)

Quantitative determinations of biofilm formation were made by measuring adherence of broth cultures to 96-well tissue culture plates as outlined in Materials and Methods. Under standard growth conditions in BHI broth only 8 of 44 (18%) isolates were found to be biofilm forming.

To evaluate the impact of environmental growth conditions on biofilm formation by clinical isolates we performed biofilm assays using growth media supplemented with 1% glucose or 2% sucrose as described previously. This resulted in an increase in the numbers of isolates capable of biofilm formation with 15 of 44 (34%) of isolates producing biofilm in the presence of one of these media supplements. Thus, the overall rate of biofilm-forming strains raised from 18 to 34% after stimulation (Figure3 and4).

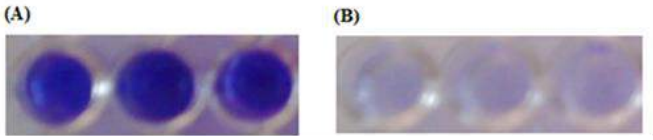


**Figure3:** Screening of biofilm producers by Tissue culture plate method (TCP).
**(A)** Biofilm producers / **(B)** non biofilm producers

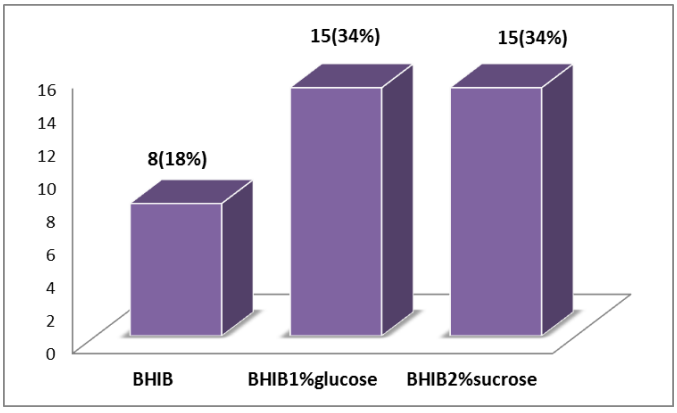


**Figure 4**: Biofilm formation of Staphylococcus strains on BHIB, BHIB 1% glucose and BHIB 2% sucrose.

### 3.3. PCR detection of ica A and ica D loci

The PCR technique was applied to the 44 staphylococcal strains. The *ica*A and *ica*D genes were detected concomitantly in 17 (38, 5 %) of the 44 staphylococci isolates giving a 188-bp band for the *ica*A gene and a 198-bp band for the *ica*D gene (Figure5). Furthermore, 2 Staphylococci strains presented only the loci *ica*D.

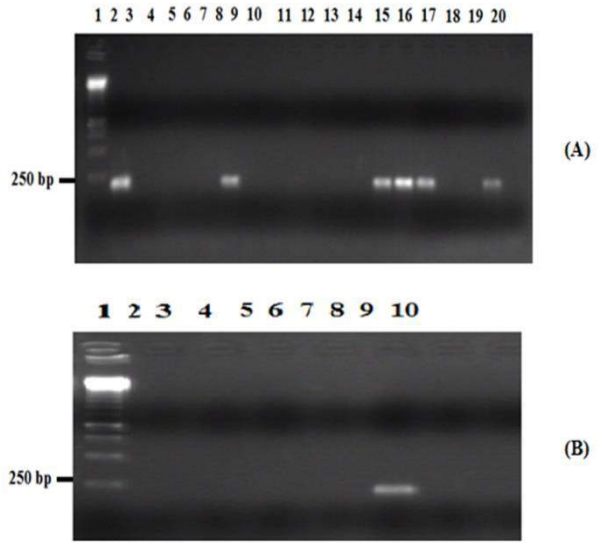


**Figure 5:** PCR detection of *icaA* and *icaD* genes. (A) PCR results with primers for *icaA.* Lane 1, 250-bp DNA molecular size marker; lanes 2–20, PCR amplicons obtained with DNA of Staphylococcus strains: 2 , S1 ; 3, S78 ; 4 , S51 ; 5 , S54; 6, S59; 7 , S60; 8 , S62; 9 , S64; 10, S69; 11, S73; 12, S77; 13, S79; 14 , S80; 15 , S84 ; 16 , S58;17 , S89; 18 , S91; 19 , S94 ; 20, negative control. (B) PCR results with primers for *icaD.* Lane 1, 250-bp DNA molecular size marker; lanes 2–10, PCR amplicons obtained with DNA of Staphylococcus strains: 2 , S96; 3 , S104; 4 , S105; 5 , S106; 6 , S107; 7 , S108; 8 , S109; 9, S110; 10, negative control.

## 4. Relationships between presence of the ica operon, slime production and TCP method

16 of the 17 *ica* A+ and *ica* D+ positive strains were slime producing and 8 produced a visible biofilm on the polystyrene surfaces under standard growth conditions. After stimulation by sugar supplementations 7 of 9 of the formerly *ica* A+/*ica* D+ positive and biofilm-negative formed a visible biofilm on polystyrene tissue culture plates. However, two ica-positive isolates remained biofilm negative even after exposure to these biofilm-inducing growth conditions. Two strains was *ica*A-/*ica*D- and biofilm negative was a producer of slime and the two strains *ica* A-/*ica*D+ was slime negative and biofilm negative.

All 23 *ica*A- /*ica*D-negative strains were unable to produce slime on CRA and biofilm on polystyrene tissue culture plates. The results obtained with all the strains are summarized in Table 1.

**Table 1:** relationships between the presence of the ica operon and biofilm production

| Strain | Species | Production of Slime | Biofilm | | | Presence of icaA /icaD |
|---|---|---|---|---|---|---|
| | | | **BHIB** | **BHIB** 1%glu | **BHIB** 2%sac | |
| S106 | ***S.saprophyticus*** | - | - | - | - | **icaA-/icaD-** |
| S107 | ***S.saprophyticus*** | + | + | + | + | **icaA+/icaD+** |
| S110 | ***S.saprophyticus*** | + | + | + | + | **icaA+/icaD+** |
| S104 | ***S.saprophyticus*** | + | - | - | - | **icaA-/icaD-** |
| S60 | ***S.saprophyticus*** | + | - | + | + | **icaA+/icaD+** |
| S84 | ***S.saprophyticus*** | + | + | + | + | **icaA+/icaD+** |
| S78 | ***S.saprophyticus*** | - | - | - | - | **icaA-/icaD-** |
| S86 | ***S.saprophyticus*** | - | - | - | - | **icaA-/icaD-** |
| S66 | ***S.saprophyticus*** | - | - | - | - | **icaA-/icaD-** |
| S52 | ***S.saprophyticus*** | - | - | - | - | **icaA-/icaD-** |
| S1 | ***S.saprophyticus*** | + | + | + | + | **icaA+/icaD+** |
| S105 | ***S.aureus*** | - | - | - | - | **icaA-/icaD+** |
| S89 | ***S.aureus*** | - | - | - | - | **icaA-/icaD+** |
| S62 | ***S.aureus*** | - | - | - | - | **icaA-/icaD-** |
| S80 | ***S.aureus*** | + | - | + | + | **icaA+/icaD+** |
| S65 | ***S.aureus*** | + | + | + | + | **icaA+/icaD+** |
| S54 | ***S.aureus*** | - | - | - | - | **icaA-/icaD-** |
| S79 | ***S.aureus*** | - | - | - | - | **icaA -/icaD-** |
| S77 | ***S.aureus*** | - | - | - | - | **icaA-/icaD-** |
| S57 | ***S.aureus*** | + | - | + | + | **icaA+/icaD+** |
| S100 | ***S.aureus*** | + | + | + | + | **icaA+/icaD+** |
| S83 | ***S.aureus*** | + | - | - | - | **icaA+/icaD+** |
| S108 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S109 | ***S.epidermidis*** | + | - | + | + | **icaA+/icaD+** |
| S103 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S91 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S92 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S95 | ***S.epidermidis*** | + | - | + | + | **icaA+/icaD+** |
| S94 | ***S.epidermidis*** | + | - | + | + | **icaA+/icaD+** |
| S7 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S96 | ***S.epidermidis*** | - | - | - | - | **icaA -/icaD-** |
| S74 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S51 | ***S.epidermidis*** | - | - | - | - | **icaA -/icaD-** |

| | | | | | | |
|---|---|---|---|---|---|---|
| S59 | ***S.epidermidis*** | + | - | - | - | **icaA -/icaD-** |
| S87 | ***S.epidermidis*** | - | - | - | - | **icaA -/icaD-** |
| S72 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S58 | ***S.epidermidis*** | + | + | + | + | **icaA+ /icaD+** |
| S69 | ***S.epidermidis*** | - | - | + | + | **icaA+ /icaD+** |
| S73 | ***S.epidermidis*** | + | + | + | + | **icaA +/icaD +** |
| S64 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S68 | ***S.epidermidis*** | - | - | - | - | **icaA -/icaD-** |
| S71 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S90 | ***S.epidermidis*** | - | - | - | - | **icaA-/icaD-** |
| S99 | ***S.hominis*** | + | - | - | - | **icaA+ /icaD+** |

## B. Discussion

In the last two decades, with the increased use of indwelling medical devices, nosocomial infections caused by Gram-positive bacteria, in particular staphylococci have become more prevalent as a cause of hospital-acquired infection **(Fitzpatrick et al.,2002).**

The major pathogenic factor is the ability to produce an extracellular slime and constitute a biofilm, making clinical treatment extremely difficult. The biofilm development process requires polysaccharidic intercellular adhesin, which is synthesized by the enzymes encoded by the intercellular adhesion cluster (*ica*) **(Martín-López et al., 2002)**.

Detection of biofilm production in staphylococcal strains isolated from indwelling medical devices is important. It helps to know virulence factors for bacterial pathogenicity. Therefore, in the present study, we have isolate 44 staphylococci strains from urinary catheter in order to test the occurrence of slime genes, biofilm production and slime production in staphylococci by PCR, TCP method and Congo red agar method respectively.

The results revealed *S. epidermidis* as the most frequently isolated species corresponding to 48% of all strains isolated. Other staphylococci species were also identified, including *S. saprophyticus*, *S.aureus* and *S.hominis*. These results are close to those obtained by **Diemond-Hernández *et al.***

It has been noticed in several studies that the *S. epidermidis* is the most frequently isolated species. That is that *S. epidermidis* makes up a significant part of the normal bacterial flora of the human skin and mucous membranes. It is probably easily introduced as a contaminant during the surgical implantation of the polymeric device (**Otto, 2008).**

In this study, *ica* A and *ica* D were detected concomitantly in 17 of the 44 staphylococci strains isolated from urinary catheter and the *ica* D gene alone in 2 staphylococci strains.
These results are close to those obtained by **Cafiso *et al.*** who also investigated the presence of genes involved in biofilm production. In that study, 35% of the isolates were positive for the *ica* A and *ica* D genes and some isolates only carried the *ica*D gene.

In the TCP assay with BHIB used as standard growth media, 8 of 17 *ica*A/D positive strains exhibited a biofilm. This was in agreement with observations of other investigators **(Cho et al.,2002; Mathur et al.,2006; Johannes et al.,2002)** in which only few or no biofilm producing isolates could be detected using this medium. Surprisingly, supplementation of BHIB media with different sugars (BHIB $_{2\%suc}$, BHIB $_{1\%glu}$) increased biofilm formation significantly, and 34% of the investigated isolates formed biofilm in at least one of the used media. Furthermore, two isolates of staphylococci *ica* D+ /*ica* A- did not form biofilm in both medium.

According to our results and those found in the literature, the co-expression of *ica*A with *ica*D increases the enzyme responsible for N-acetylglucosaminyl transferase activity considerably and is related to phenotypic expression of the capsular polysaccharide. **(Chaieb et al., 2005; Arciola et al., 2001; Gerke et al., 1998).**

Moreover, the expression of the *ica* operon and as a result, the formation of biofilms seems to be highly variable among staphylococci **(Ziebuhr et al.,1997; Mempel et al.,1994)**. Thus, biofilm expression is influenced by environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotics (**Cho et al., 2002)**. **Cramton *et al.,*** suggested that anaerobiosis strongly increases biofilm expression. The expression of biofilm

is also regulated by iron, with maximum expression occurring at low concentrations (**Chaieb et al.,2005** ). However, two staphylococci strains *ica* A+/*ica* D+ remained biofilm negative even under PIA-expression-stimulating growth conditions. The detection of no biofilm, despite the presence of *ica*, could be due to several reasons such as inactivation of the *ica* operon by insertion of an IS256 element in the *ica*C gene **(Ziebuhr et al.,1999)** , the action of the *Ica*R repressor **(Conlon et al.,2002)** , or post-transcriptional regulation **(Dobinsky .,2003)** .

The Comparison of the CRA test and the results obtained by PCR revealed that among the 17 *ica*A+ and *ica*D+ positive strains 16 were slime producing. In fact, these results correspond to those obtained by **Aricola *et al.,*** and **El- Mahallawy *et al*** ., In their study, a strong correlation was found between *ica* gene positivity and the ability to produce slime by CRA test (P <0.001) compared to the TCP method.

Two isolates of staphylococci were slime + /biofilm- and *ica* A-/*ica*D-. **Chokr *et al*** reported this phenomenon and suggested that in this strains variability in the *ica* locus sequence exists allowing production of a polysaccharide which reacts with the anti-PIA serum.

Furthermore, Staphylococci Strains isolates from urinary catheter showed high levels of resistance to different classes of antibiotics except for vancomycine and pristinamycin. They were significantly resistant to the penicillin (98%), oxacilline (79%), gentamicine (75%) and ofloxacine (73%). These results are close to those obtained by **Touati *et al*** who reported that Staphylococci isolates from catheter-related infection are significantly resistant to oxacilline (76,8%), gentamicine (46,4%) and ofloxacine (75%).

The high frequency of antibiotic resistance among strains isolates for urinary catheter is associated with high pressure antibiotic selection. The multicellular organization bacterium in biofilm gives them the advantage to acquire new genes. The biofilm is a perfect environment for exchange resistance plasmids (**Touati *et al.,* 2007)** since it includes both the greater probability of contact between cells and the negligible effect of shear forces **( Donlan,2001).**

**Gilbert *et al*** reported that biofilm producers were to be 10-1000 times less susceptible towards antibiotics than are the equivalent cells growing planktonically. Biofilm hampered penetration of antimicrobial and the concentrations required to eradicate biofilm producing bacteria are higher than those required to eradicate strains that did not produce biofilm **(Seif El-Din et al.,2011)**.

## IV. CONCLUSION

The presence of *ica* A and *ica* D genes in most of staphylococci strains allows the production of biofilm. The latter facilitates the development of infections by compromising the immune system of the patient and contributing to the failure of antibiotic therapy. This may result in recurrent infections and the emergence of multiresistant pathogens. Therefore, the analysis of the presence and expression of *ica* genes can clarify the different adhesion mechanisms in the pathogenesis of infections associated with medical devices and it could also be of value in the development of new preventive and therapeutic measures to eradicate biofilm in hospitals.

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# Comparative Study of the Pharmaceutical Activity of two plants of the Moroccan Spontaneous Flora: Mentha Pulegium (L) and Marrubium Vulgare (L.) (Lamiaceae)

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## ABSTRACT

The medicinal herbs are a natural source for a large variety of antioxydants. In our work, we conducted a comparative study on the aqueous extract of the sheets of two medicinal herbs which develop under the same natural conditions, The area of Casablanca located west of Morocco, and belong to the same botanical family these two plants are: Mentha pulegium (L) and Marrubium vulgare (L.) of the family of Lamiaceae. The two plants are largely used in traditional medicine by the local population. Sight their interest we evaluated the quantity of the phenolic compounds and flavonoïdes total, as well as the bacterial sensitivity which revealed important a disinfectant activity against several disease-causing agents, Moreover, the antioxydant activity of these two plants was led by the use of test VCEAC (Vitamin C Are equivalent Antioxydant Capacity). The results show that these two plants are rich in natural antioxydants, although the sheets of Mentha pulegium (L) richer than those of Marrubium vulgare (L.).

**Keywords :** Spontaneous Plants; Antibacterial Activity; Antioxydant Capacity are Equivalent; Moroccan Traditional Medicine.

## I. INTRODUCTION

The importance of the Moroccan pharmacopeia is based on two aspects raise, A great botanical diversity an ancestral tradition to know to make containing the medicinal herbs , However, the Moroccan medicinal flora remains ignored until our days, on the 4500 species and under plant species, the counted medicinal species do not exceed the 356 to 600 species.In spite of that, traditional medicine always occupied an important place in the traditions of medication in Morocco and the area of large Casablanca in is a concrete example.The analysis of the Moroccan medicinal bibliography shows that the regional ethnopharmacologic data very fragmentary and are dispersed, in the same way the knowledge to make is held currently only per few people.

The purpose of the present study, carried out in the circle of large Casablanca is to contribute to the knowledge of some medicinal herbs, to carry out a biological and phytochimic study of two spontaneous plants of the flora in the aforementioned area and to try to trace a safety margin for the therapeutic uses practised by the local population, the fact of being able to insulate, purify and analyze, the extracts of these plants, made it possible to know the composition and to validate the uses of it.

## II. METHODS AND MATERIAL

***Mentha pulegium (L.)***

also called cosmopolitan mint pouliot of distribution, in Morocco it is rather common, it pushes in the wet places on the rich ground one meets it until 1800 m of altitude .nC' is a plant from 20 to 55 cm height, long-lived by rhizomes, with hairy, strongly aromatic stems and with prickly odor. Its period of flowering extends from June until October.

***Characteristics***: The plant has properties antispasmodic, antalgic - calming and sedative antioxydant, disinfectant - antiviral and antibacterial, para-sympathicotonique powerful (hypotensive, vasodilatatrice, anaphrodisiaque), diuretic, expectorante, and vulnerary.

For the population, area of Casablanca, marjoram is indicated in difficult digestions, the influenzas, coughs, bronchitides. Its infusion in hot milk is very much used to make sleep the children. In local use, it is used in the care of the oral ignitions and the plaices.

***Marrubium vulgare (L)***

Very widespread plant throughout the year, one finds it on the edges of ways, the meadows dry and the waste grounds, but generally on grounds limestones, its thyme odor distinguishing it from the other plants. employee especially in a fresh state to prepare infusions and decoctions.

It is a hardy perennial of color greying resembling slightly mint, odorous, measuring from 30 to 80 cm according to the places, its sheets very fluffy from 2 to 5 cm have a crushed aspect, flowering extends from April to July the flowers are white (1 to 2 mm), sometimes a little dew,

***Characteristics:*** this very widespread plant throughout the year, is especially employed in a fresh state to prepare infusions and decoctions. Managed in the cases of typhus or paludism, it makes fall the fever. One employs it like expectorating at the time of bronchitides and other lung diseases. With strong amount, it is vermifuge. The convalescents or weakened absorb it like reconstituting, tonic and stimulant for the local population, the plant was traditionally employed in the manufacturing of the remedies against cough. It is also used like antidiabetic, only or associated with the fenugreek, the white lupin, thyme, with the white wormwood and the street, it in fact a true pharmacy is, except in the event of diabetes the decoction, which is very bitter, is edulcorated with honey or raisins

***Method used:*** for this work, the inhibition of the bacterial growth by the extracts of our plants, is studied by the method of diffusion in solid medium. With a punch of the wells are dug in the agar of Mueller-Hinton run in limp of Kneaded and sown by a germ-test. The diameters of inhibition are then measured around the wells after a 45 minutes preincubation to room temperature and an incubation with the drying oven with 37°C during 18 hours, and any zone with a diameter higher than 2 mm indicates an antibacterial activity (**Vanden Berghe and Vlietinck, 1991)**

***Preparation of the inoculum:*** the method of preparation of the inoculum is that recommended by the French company of microbiology (SFM) (official statement of 2005) which consists in preparing, starting from a culture of 18-24h of the bacterium studied on the agar medium, a suspension in saline solution (NaCl with 0.9%) equivalent to the standard McFARLAND 0.5 (| 108 UFC/ml). Thereafter, 1ml of the suspension of the inoculum, is spread out by flood on the surface of one limps of Petri containing of the agar of Mueller-Hinton, which will be let dry in the septic zone of the Bunsen burner **(SFM, 2005).**

***Stocks of reference***: during our study, several bacterial strains will be studied, for the majority are stocks of reference, i.e., beforehand determined stocks, thus for their culture one carries out only the colouring of Gram to see whether these of Gram () or (-). Other cultures on the other hand, result from wild stock of environmental origin (of CHU de Rabat). For these cultures, an identification of the stock is obligatory, thus the various tests of identification are carried out: api plate 20th, ODC, Mannitol mobility, Oxydase, Catalase (H2O2), Indol, LDC, ADH, TSI, Coagulase etc, and finally the colouring of Gram (**Cowan and Steel, 1974**).

***Methods of antioxydant test:*** Several methods are used to evaluate, in vitro and in vivo, From methodological point of view, the test with free radical DPPH is recommended for compounds containers HS, NH- and OH- groups. It is carried out with room temperature, this making it possible to eliminate any risk from thermal degradation of the molecules thermolabile (Salah and al., 1995; Sharma and Bhat, 2009)

***TestDPPH:*** chemical compound DPPH, was one of the first free radicals used to study the relation antioxydant structure-activity of the phenolic compounds. It has an electron not paired on an atom of the nitrogen bridge. Because of this delocalization, the molecules of the radical do not form dimers, the DPPH remains in its relatively stable monomeric form at ordinary temperature. The delocalization causes also the blue quite characteristic of the solution of DPPH. The measure of the efficiency of an antioxydant is done by measuring the reduction in colouring blue, due to a recombination of radicals DPPH, measurable by spectrophotometry with 515-518 Nm (Sharma and Bhat, 2009).

***B-carotene test:*** β-carotene, is a hydrocarbon having 11 double combined connections what explains its orange color, it neutralizes the free radicals effectively and prevents the peroxidation of the fatty-acids in chemical solution, it is also a powerful trapper of oxygen singulet (Pavia and Russell, 1999).

The antiradicalaire activity of the extracts by β-carotene is given by measuring the inhibition of oxydative degradation (discolouration) by the products of oxidation of the linoleic acid. Thus the kinetics of the discolouration of the emulsion in presence or not of antioxydant (negative control in which the sample is replaced by 25 methanol µl) is measured, at initial time (T = 0) and successively with T = 30 and 60 min, by spectrophotometry with 470 Nm (**Mansour and Khalil 2000**).

## III. DISCUSSION AND CONCLUSION

The present study related to the identification of the various chemical groups, the research of the activity antibacterial and antioxydant, the extracts of two spontaneous plants of the area of Casablanca. From the profitability point of view, the aqueous extracts gave the highest proportions in comparison with the extracts ethanolic, that peuts' to explain by the fact that the ethanol is a not very polar organic solvent thus more volatile than water, this property assure him the aptitude to degrease the powder instead of penetrating inside (**Perry and Green, 2007**).

The description, in vitro, of the various chemical entities, enabled us to note the presence, the wealth of flavonoïdes, tannins, triterpenes and coumarins at the two desLamiacées plants of the family, etparticulièrement the richesseen sterols and mucilage chezMarrubium vulgare.

The results revealed by in vitro characterization are confirmed by the chromatographic and spectrophometric analyses, and are quoted in the literature.

Indeed, the chromatographic analysis of the extract alcooliquedeMarrubium vulgare (L.) allowed identifierdes bitter principles, in particular marrubiine, acid phenols: cafeic acid, myristic, palmitic, stearic, oleic, linoleic and linoleic, of the mineral matter in the form of salt (of potassium, sodium and iron), of tannins of the saponosides, choline and essential oil in traces (**Kurbatova and al., 2003; Giordani and al., 2008).**

However, the chromatographic analysis of Mentha pulegium (L.) is related particularly to the chemical composition of its essential oil, this analysis shows the existence of pulégone like principal component, besides acetate of pulégone, menthone, isomenthone, neo-menthol, piperitone, γ-terpinene and β-caryophyllene (**Mahboubi and Haghi, 2008)**

The pharmaceutical interest of our treated plants, is proven by their biological activity, consequently the study of the bacterial sensitivity shows a broad spectrum of action against pathogenic germs tested.

Determination of the parameters of inhibition (CMI and CMB) allowed to us not only to confirm, quantify and compare the activities, but also to characterize the nature of the effect exerted by an extract on a given micro-organism (**Guerin-Faublée and Carret, 1999**).

The comparison of the results got by the method of diffusion in solid medium on the one hand and the method of dilution in liquid medium on the other hand, raises that: the values of CMI agree generally with those of the diameters of inhibition, the extracts having induced an important zone of inhibition present smallest CMI on the corresponding stocks. This comparison was carried out according to the method of Fauchere and

Avril (2002), for which a substance is bactericidal when report CMB/CMI is, and bacteriostatic when this report is. If report CMB/CMI is considered, the extract of Mr. vulgare, is especially bacteriostatic with regard to K. pneumoniae and S. enteritidis that it either clear dessouchesprélevées référencesou of the hospital medium.

To our knowledge, it is the first time that such results are thus clearly shown for these two plants. ((**Moussaid and al., 2011**)

Our work having been realized starting from the aqueous extracts (décoctés) and alcoholics (ethanol), this could thus justify the therapeutic uses of Marrubium vulgare, and Mentha pulegium in traditional medicine, and which are used as anti-infectious by the majority of the population near which we carried out the investigation. (**Moussaid and al., 2012**)

The antibacterial activity observed, is in addition explained by the results of the chemical analysis of the plants, this analysis reveals the presence of the compounds such as: alkaloids, tannins, saponins and the flavonoïdes, whose antimicrobic properties were already shown (**Cowan, 1999**).

Most these compounds were highlighted in the extracts by order of importance chezMentha pulegium and Marrubium vulgare, which makes it possible to as well explain their important activities on the Gram bacteria (-) as on Gram (+).

The antioxydant test that we have réaliséin vitroa given a notable result, the extracts of our plants are able to reduce radical DPPH, with a better inhibition by decreasing order Marrubium vulgare then Mentha pulegium. These results revealed that the extracts present anti-ridicalizing powers very important, nevertheless, this activity remains slightly lower than that of the ascorbic acid.

In the same way, the extracts showed a remarkable inhibiting activity of the coupled oxidation of the linoleic acid/β-carotene for the two plants, however this activity significantly remains lower than that of positive control (Propyl gallate).

We notice that the results differ appreciably according to the test used. That could be explained by the specific sensitivity to each test. The use of two different tests enabled us to have a better reading of the anti-ridicalizing activity of our extracts. Indeed we note that the activity of inhibition of the discolouration of β-carotene is lower compared to the results got by the DPPH.

According to the literature, the phenolic compounds present a strong potential antioxydant, made up which were highlighted in the extracts, with a strong concentration at Lamiacées. (**Marvin, 2010**).

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# Study of Contamination by Copper and Zinc of some Spontaneous Plant at Telamine Lake

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## ABSTRACT

One of the major current issues in environment is soil contamination by many toxic elements and compounds including heavy metals. These contaminated sites, often have a very diverse flora tolerating high levels of heavy metals. The objective of this work is to study the behavior of some wild plants towards copper and zinc contamination and to test the hypothesis that the zinc (Zn) and copper (Cu) concentration may be explained by the availability in the soil and biomass of the organ. The obtained results showed that the metal concentration in plants is weakly correlated with its presence in soil. Our survey, in agreement with many previous studies, shows that there is no direct link between the total content of Cu and Zn in soil and concentration in plants. In fact, for an element to be assimilated by a plant, it must be in the soil solution and bioavailable. As this study also confirmed, absorption capacity vary according to plant species and, in a plant, according to the considered organ.

**Keywords:** Heavy metals, contamination, plants, flora, zinc, copper.

## I. INTRODUCTION

Heavy metals are naturally present in rocks, they are released during it alteration to form the geochemical background [11]. Natural concentration of these heavy metals in soils varies depending on the nature of the rock, its location and its age. However, the major source of contamination is anthropogenic. In recent decades, the heavy metals intake in soil in the world extended [5].The atmosphere, water, and soil contamination by many toxic elements and compounds has become a major environmental problem.

These contaminated sites, often have a very diverse flora tolerating high levels of metals. The study of these resistant plants, for their detoxification, immobilisation or absorption capacity of heavy metals, could be an interesting tool, not only to estimate the risk of potential transfer of heavy metals in the ecosystem, but also as soil remediation tool. The challenge is not only the preservation of soils for agricultural production for a growing population, but also the recovery of degraded and contaminated soils.

It is in this context of sustainable development that this study was undertaken, the main objective being to select species of native flora able to accumulate metals in their plant tissues in the purpose of their subsequent use in phytoremediation.

## II. METHODS AND MATERIAL

### A. Presentation of the Study Area

Télamine lake (figure 1), is located in the east of Oran, commune of Benfreha, at an altitude of 95 m, while the village lies at an altitude of 107m. It occupies an elliptical depression oriented SSW –NNE whith coordinates of $0^{o}42$ 'W longitude and $35^{o}38$' N latitude. It is divided into three separate bowls extending over 6 km long and 1 km wide, with an area of 2400 ha.


**Figure 1:** Study Area

### B. Collection and plant packaging

The plants are collected in dry weather with roots, stems and leaves. They are cleaned of coarse earth elements stick, before being packaged separately in closed plastic bags. Soil samples are collected in the same time under the around rhizosphere plants area and are put in bags of the same type. They are then transported to the laboratory in a cooler to undergo mineralization.

The identification of plant taxa was made with the help of some taxonomic works such as «La nouvelle flore d'Algerie» of Quezel et Santa (1962-1963), «La Flore du Sahara» of Ozenda (1977), «La flore d'Afrique du nord» of Maire (1952), «La Flore descriptive et illustrée de la Corse» of Coste (1937).

The collected plants were cleaned, then separated into stems, leaves and roots.They were then dried in an oven for 72 hours at a temperature of 70 °C to be finally reduced to a fine powder using a mortar and pestle. 30 mg of powder was dissolved in 3.5 ml of mixture (HNO3 / HClO4) at the rate of (7V/1V), incubated in the dark for 24h and then diluted in 5 ml of HNO3 0.2% [9].

Soil samples were also dried in an oven for 24 hours at a temperature of 105 ° C, then sieved through a sieve with round holes of 2 mm diameter to separate the fine earth (fraction less than 2 mm), on which are performed the analyzes.

The assay is performed by Atomic absorption spectroscopy with flame equipped with a graphite oven of type AA 30/40 Zeeman, Statistical analysis was performed with the help of Microsoft STATISTICA software.
The results were expressed by their average and their error, the significant differences were established at p <0.05 in accordance with post-hoca LSD test.

## III. RESULT AND DISCUSSION

### A. Botanical identification

Five plant species were chosen from the spontaneous flora, the kept species are the most numerous on the assumption that these species are best suited to the conditions in situ.

The identified species are: E1: *Chenopodium album*, E2: *Spergularia salina*, E3: *Malva sylvestris*, E4: *Suaeda fructicosa* and E5: *Urtica pilulifera*.

### B. Changes of zinc and copper concentrations in plant parts

The assessment of contaminant levels is performed in leaves, stems and roots. The average of concentration is calculated for each plant. Figures 2 and 3 represent the mean concentrations of zinc and copper in each organ for the five sampled plants.

#### 1. Variation in zinc concentrations

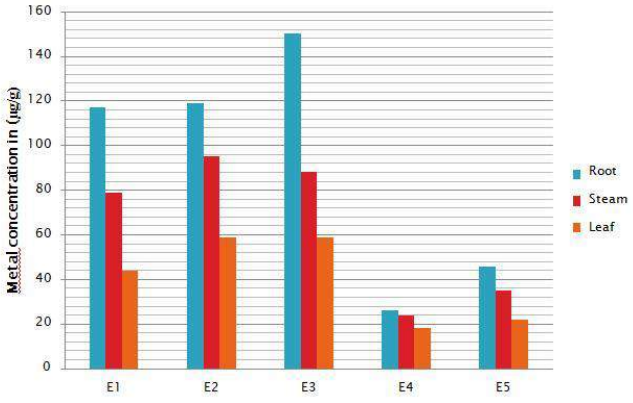


**Figure 2 :** Comparative studies of changes in zinc concentrations in plants (roots, stems and leaves)

The obtained results show high concentrations of zinc in underground parts of the five plants (Figure 2) with a greater accumulation in *Malva sylvestris* followed by *Spergularia salina* and *Chenopodium album*. These concentrations are higher than the rate found in soil (5.99 and 31.63 mg.kg-1.) and that for all the sampled plants. This can highlight a bioaccumulation power of zinc in these three plants.

According to Dauguet (2010), for an element to be assimilated by a plant, it must be in the soil solution and bioavailable. Zinc concentration in the soil solution depends on the amount of zinc in this soil, of the solubility of the particular compound of zinc and the adsorption extent. The solubility varies significantly among the various zinc compounds; zinc sulfat is very soluble in the soil solution, while zinc oxide is relatively insoluble. Zinc can be absorbed in the clay minerals, but also can form stable compounds with the soil organic matter, including the hydroxides, oxides and carbonates.

That is why a study detailing sufficiently the behavior of metals in the soil and in the plant must be undertaken in order to understand the behavior of these metals in the SOIL-PLANT matrix.

## 2. Variation in copper concentrations

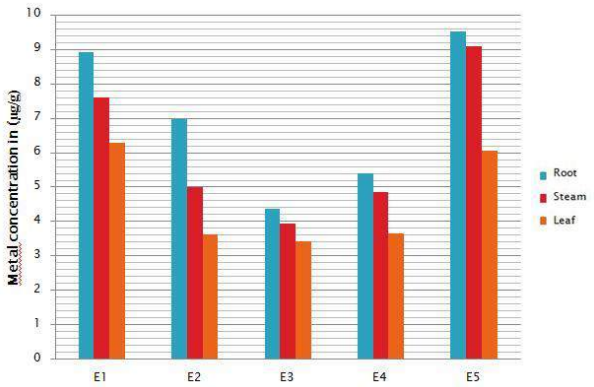


**Figure 3 :** Comparative studies of changes in copper concentrations in plants (roots, stems and leaves)

Copper concentrations in soil vary considerably depending on the soil type, Soil amendments, proximity to anthropogenic sources, Proximity to natural ore veins and composition of bedrock and parent material [2].

As for Zn and according to Figure 3 a bioaccumulation of copper is also observed in the roots compared to aerial parts, only this bioaccumulation is low compared to the levels of copper in the soil (25,3 et 59,73 mg·kg-1), this phenomenon can be explained by the fact that copper is strongly adsorbed to soil particles; therefore, it is much less mobile than other trace elements [1]. The result that the deposited copper tends to accumulate in the ground [16]. Different types of soil have limited retention capacity for copper ions, and leaching can occur when deposited copper levels exceed this capacity [4].

Factors that influence the availability of copper in the soil are, pH, cation exchange capacity (CEC), the organic content, the presence of iron oxides, manganese and aluminum and the redox potential [4]; [16].

Adriano (1986) demonstrated that the soil's ability to adsorb the copper increases with increasing pH, according to Bahi (2012), the soils of Telamine Lake have a pH ranging from 7.9 to 8.4, This would explain its high adsorption capacity of copper.

## C. Interspecific Variations in trace metal concentrations

In order to identify the species that accumulates more trace metals in Télamine lake, we collected and analyzed samples of five plants that dominate the plant community of our site. It should be noted that no study reports the trace metal contamination in Télamine Lake.

The figures (4 and 5) represent the variation of copper and zinc concentrations in the tested species. Statistical analysis reveals significant variations between species E4: *Suaeda fructicosa* whith E1: *Chenopodium album* and E2: *Spergularia salina* with E3: *Malva sylvestris* (E3>E2>E1>E4) ($p<0,05$) and between E5 : *Urtica pilulifera* with E2 : *Spergularia salina* and E3: *Malva sylvestris* (E3>E2>E5) ($p<0,05$).

### 1. Interspecific Variations in zinc

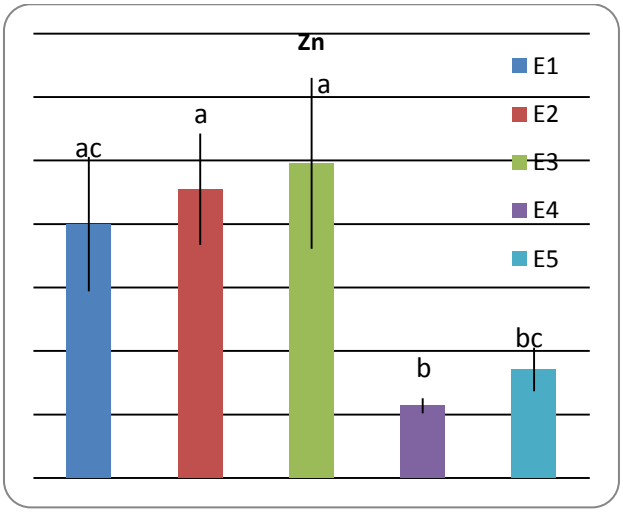


**Figure 4** : Comparative Study of the interspecific variation in Zinc concentrations

According to the results the levels of zinc in plants follow the following order: E3 > E2 >E1>E5>E4. The higher rate was obtained at both plants E3 and E2 identified as *Malva sylvestris* and *Spergularia salina* with rate of 99.13 and 90.96 ppm by dry material respectively. Both species may show an interesting bioaccumulating potential and a laboratory experimental study can be considered.

### 2. Interspecific Variations in copper

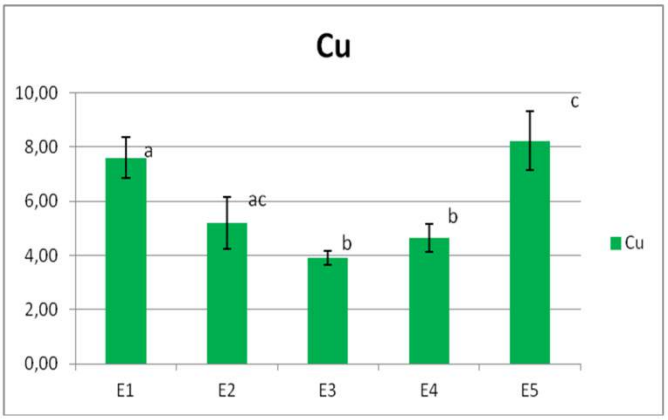


**Figure 5 :** Comparative Study of the interspecific variation in copper concentrations

Based on our, results copper contents in studied plants follow the following order: E5> E1> E2> E4> E3. The higher rate was obtained at both E1 and E5 plants identified as *Urtica pilulifera* and *Chenopodium album* with rates of 8.22 and 7.60 ppm respectively of dry matter.

Copper rates obtained are considerably lower than those of zinc this can be explained by the fact that it has a high affinity for organic matter and it binds more strongly than other trace elements [7]; [4]; [16]; [1]. According to Fuller (1977) and Gibson and Farmer (1984), it occurs in soil solutions often tied to dissolved organic matter and will be released in ionic form only under severe oxidation conditions or by microbial decomposition of organic matter.

## IV. CONCLUSION

This study demonstrated that the MTE concentration in plants is weakly correlated with its presence in the soil, because for an element to be assimilated it must be bioavailable. The transition in solution is under the influence of various factors, the most important is the PH and the organic matter content [15].

Removal ability of an MTE varies according to the targeted metal, the plant species and, in a plant, from an organ to another.

In our situation *Malva sylvestris* present the greatest potential to investigate for Zn contamination whereas for Cu *Urtica pilulifera* would be the best choice.
In conclusion, for a sustainable technological development, it is essential to continue research that not only must involve soil science, agronomy, physiology, ecophysiology and genetics but also disciplines able to answer questions by running a particular biomass and possible recycling of metals in metal production. In this context, process engineering certainly has a key role to play in building this new industry.

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# Effects of the Glyphosate Application on the Physio-Biochemical Parameters of *Xanthoria Parietina*

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## ABSTRACT

The extended use of Glyphosate as herbicide in weed's control can lead to the damage of the ecosystem. Ecological indicators can be used as early warning signals to assess the environmental problems. In order to evaluate the performance of the lichen as an air pollution bio monitoring, we investigated if treatment of the lichen *Xanthoria parietina* with Glyphosate causes bio physiological alterations. In this respect, the effect of various herbicide doses on the lichen's bio physiology was tested in the laboratory during 7 days under controlled conditions. Samples of lichen, naturally grown, were collected from different forest's sites and were treated at the recommended doses as well as with double doses. The findings have shown a clear reduction in the photosynthetic pigments. Of particular importance in this respect, are the pigments chlorophyll (a), (b), (a+b) and carotenoid. However, an increase in the total protein, sugar and proline products levels was observed. Finally this study shows that *Xanthoria parietina* is an appropriate organism for the bio monitoring of undesirable effects of the Glyphosate .

**Keywords:** Biomonitoring, Lichen, Glyphosate, Herbicide, Air Pollution.

## I. INTRODUCTION

Glyphosate is non-selective and systematic organ phosphorus herbicide which is used widely in order to control weeds [1]. Glyphosate targets a key enzyme (EPSPS) which is essential for the growth of most plants, inhibiting nucleic acid metabolism and protein synthesis [2]. Lichens are symbiotic bi- or tripartite organisms, it involves heterotrophic component mycobiont (ascomycetes) and autotrophic photobiont (green alga or a cyanobacterium) [3]. Lichens can be used as sentinels of air pollution because of the fact that they do not have real root and are strictly dependent on the atmosphere for their metabolism. Glyphosate is widely used by aerial spraying, so it may for sure reach a non-target species such as lichens, thus, the understanding of its possible impact is very important. It can be noticed that there is not enough studies on the effect of herbicide on lichens because they are considered as non-target organisms. However there are some few studies which indicate the possible negative effect of glyphosate treatment on the lichens. The aim of our study is to test the toxicity of glyphosate on the lichen *Xanthoria parietina* as well a physiological and chemical alterations consequences.

## II. METHODS AND MATERIAL

Samples of the *X. parietina* were collected from their natural environment located at 48˚ 86' 67" latitude and 2˚ 33' 33" longitude in the East of Oran (USTO University). The choice of this species was based on their previous uses in biomonitoring studies as well as in laboratory experiments. After collection, lichens were left in the cytology laboratory where the marginal lobes were removed, cleaned from extraneous material and used for the experiment. Glyphosate was provided using Rophosate 480 (Rotam agrochemical), a common commercial glyphosate-based herbicide. Three treatment solutions of 0 (deionized water: control), treatment corresponding to the lowest usage dose recommended by the producer and a treatment with two times higher

than the highest suggested usage dose. Samples were sprayed with the treatment solutions within seven days. The experiment was replicated three times.

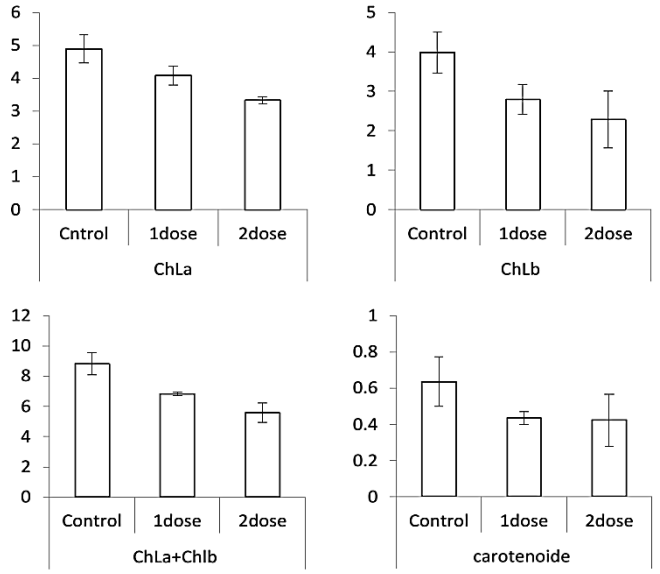


**Figure 1 :** Concentration of photosynthetic pigments (mg/g FM) in samples of *X. parietina*. Vertical bars indicate standard deviation.

The physiological effects were determined by quantifying chlorophyll a, b and a+b content as well as proline; total protein and soluble sugars concentration.

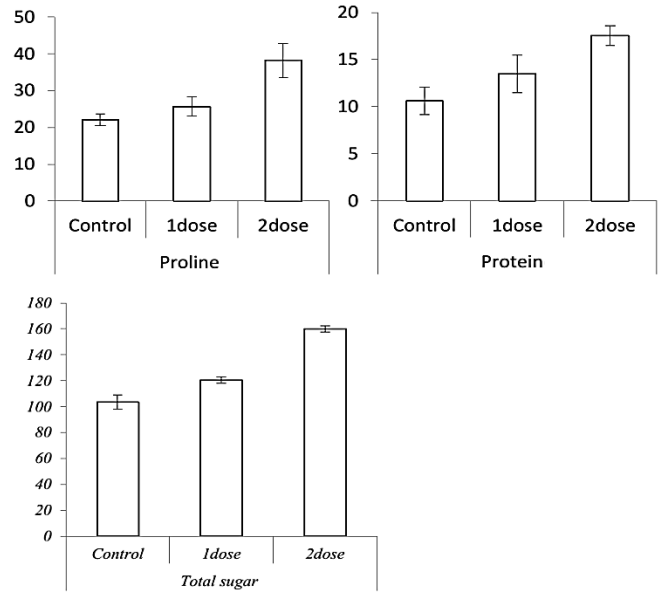


**Figure 2 :** Concentration of protein, proline and sugar (µg/100mg FM) in samples of *X. parietina*. Vertical bars indicate standard deviation.

Statistical analysis of resulted data is performed by the Student test (t test) by comparing the means of two populations using data from two independent samples.

## III. RESULT AND DISCUSSION

The physio-biochemical parameters investigated resulted negatively for some parameters and positively for the others, irrespective of the treatment doses, for the photosynthetic pigment content were decreased at the lowest and the highest treatment dose after seven days period. For the other parameters: proline protein and sugar we observed an increase in the content of this parameters. Interaction between doses the results was significant for all parameters. Fig.1 and Fig.2 represent the Changes of chlorophyll a, b, carotenoids, protein, proline and sugars rates in function of the concentrations of Glyphosate used.

For the photosynthetic pigments their variations are minimal. The value of the rate of chlorophyll (a + b) in control sample is 8.83 mg / g FM, in the one of the sample treated with a single dose is 6.84 mg / g of FM and finally in the treated with a double dose is 5.58 mg / g of FM. In contrary, carotenoids have not substantially changed. The Measure of the average levels of chlorophylls (ab) and carotenoid obtained indicates the existence of a state of stress due to the presence of a pollutant. Chlorophyll is sensitive to oxidative processes initiated by stress, such as photochemical oxidation [4]. In a study carried out by Mascher et al. (2002), it is found that the decrease in chlorophyll's concentration and carotenoid is a signal of poisoning [5]. Silberstein et al. (1988) reported that the degradation of chlorophyll is one of the clearest indications of the damage caused by air pollutants [6]. The decrease in the concentration of chlorophyll "a" and chlorophyll'b' can be explained by the decrease in the photosynthetic activity of the treated samples. The significant increase of protein is a sign of a possible infringement of other basic metabolism and it can be explained by the fact that the pesticide inside tissue stimulates protein synthesis of many enzymes especially those involved in detoxification process.

For the protein, proline and sugars content, a small increase was observed, depending on the concentration. The average values of these rates are summarized in the Fig. 2. The dosage of the proline has allowed us to

detect a phenomenon of stress under the effect of treatment with the pesticide. A metabolic disorder caused by the treatment was observed within the lichens. The increase in the rate of proline is due to the oxidation inhibition caused by the mitochondrial dysfunction. The results obtained allowed us to note that treatment with the pesticide, affect disorderly the proteins rates in both lichens and mousse. This disturbance is a sign of possible impairment of basic metabolism and reflects the toxicity of pesticides used. The dosage of the soluble proteins shows that treatment of the samples with a pesticide is behind an increase soluble proteins' concentration [7] which can be explained by the stimulation of protein synthesis of many enzymes known as an enzyme detoxifying. According to [8] the process of accumulation of soluble sugars and / or proline in leaf tissue of stressed plants is known as an adaptive characteristic. This confirms our results where we found increased levels of soluble sugars after treatment in both species. The ability of plants to respond to changes in the levels of soluble sugars can act as control mechanism incorporating the external environmental conditions such as biotic and abiotic stress [9].

## IV. CONCLUSION

This work allowed us to verify that the lichen species *xanthoria parietina* is sensitive to the effects of the glyphosate because of physiological parameters damages found. The study proved that the negative effects of pesticide depend on its concentration, we have shown that *xanthoria parietina* can be used as bioindicator in agricultural ecosystems, in addition to its known qualities as urban bioindicator.

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