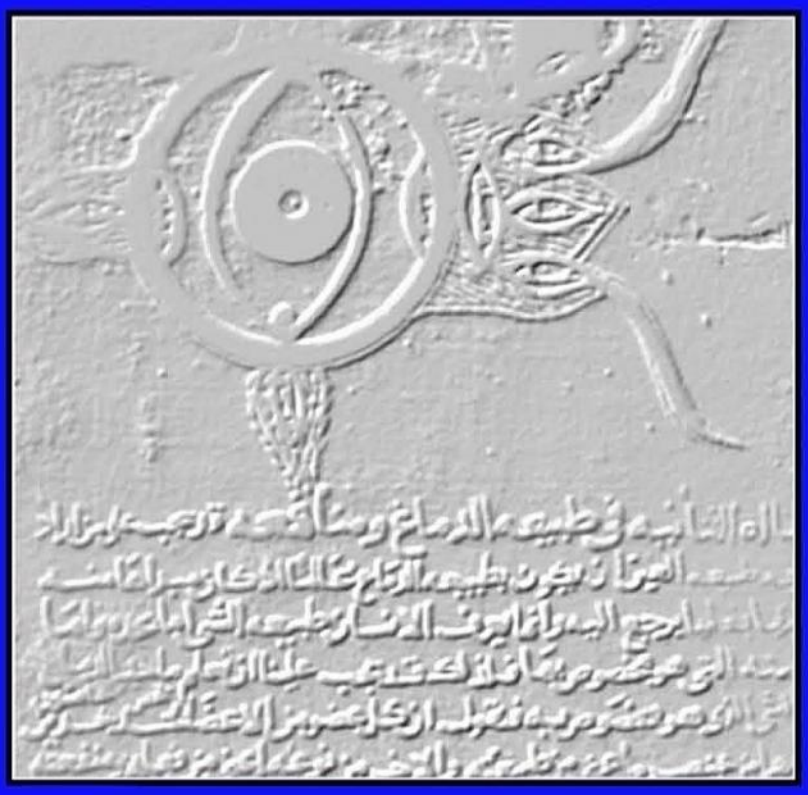
# BIOLOGIA

## TUNISIE



Décembre 2012, Vol, 2 ; N° 7 ; pp 1-122
Revue de l'Association Tunisienne des Sciences Biologiques
*Revue éditée par le Professeur Mohamed Amri*

*Biologia Tunisie Décembre 2012 ; N°7 ; pp 1 –122*

**BIOLOGIA TUNISIE**

# SOMMAIRE

**1. BEN KHALIFA Mekki1*, ALDAGHI Magid 2, ACHECHE Hajer1, KUMMERT Jean2, MARRAKCHI Mohamed1 and FAKHFAKH Hatem1**

PRESENCE OF 'CANDIDATUS PHYTOPLASMA PRUNORUM' IN APRICOT ORCHARDS IN TUNISIA
( PAGE 1- 5 )

**2. BEN TAMARZIZT HEND, BARAKET GHADA, BEN MUSTAPHA SANA, TRIFI MOKHTAR & SALHI-HANNACHI AMEL1***

MORPHOLOGICAL AND POMOLOGICAL VARIABILITY ANALYSIS OF TUNISIAN PLUM CULTIVARS
(PAGE 6- 13)

**3. MASMOUDI-KOUKI OLFAA, KADDOUR HADHÉMIA, HAMDI YOSRAA, VAUDRY DAVIDB,C,D, TONON MARIE- CHRISTINE B,C,D AND AMRI MOHAMEDA**

THE PROTECTIVE EFFECT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON ASTROGLIAL CELLS IS MEDIATED THROUGH ACTIVATION OF OCTADECANEUROPEPTIDE RELEASE
(PAGE 14- 19)

**4. HAMDI YOSRA A, KADDOUR HADHEMI A, JENHANI FAOUZIB, LEPRINCE JÉRÔME B,C,D, TONON MARIE-CHRISTINE B,C,D, AMRI MOHAMED*A AND MASMOUDI-KOUKI OLFAA.**

OCTADECANEUROPEPTIDE ODN PROTECTS CULTURED ASTROCYTE AGAINST HYDROGEN PEROXIDE-INDUCED CELL DEATH
(PAGE 20 – 25)

**5. REBEI OLFA[A,B*], BELKHIR MANEL[A,B], AMRI MOHAMED[A] AND FATTOUCH SAMI[B]**

ANTIOXIDANT ACTIVITY AND PHENOLIC EXTRACTS OF BLACK *(MORUS NIGRA L.)* AND WHITE *(MORUS ALBA L.)* MULBERRY FRUITS
(PAGE 26- 29)

**6. LEILA KALAI1, MONIA MNARI-HATTAB[2], NAJLA SADFI-ZAOUI[3], MOHAMED RABEH HAJLAOUI[2]**

CARACTERISATION DE L'AGENT CAUSAL DU MAL SECCO DE L'ORANGER ET EVALUATION DE L'ANTAGONISME BACTERIEN VIS-A-VIS DU PHOMA *TRACHEIPHILA*
(PAGE 30- 36 )

**7. BARAKET GHADA.[1], CHATTI KHALED[1]., SADDOUD OLFA[1]., BEN ABDELKRIM AHMED.[1], MARS MESSAOUD.[2], TRIFI MOKHTAR[1] AND SALHI-HANNACHI AMEL[1*].**

INTRA-SPECIFIC VARIABILITY IN RIBOSOMAL DNA SEQUENCE IN THE ITS2 REGION OF TUNISIAN FIG CULTIVARS
(PAGE 37-42)

**8. LOTFI RABAOUI *, SABIHA TLIG-ZOUARI & OUM KALTHOUM BEN HASSINE**

MORPHOMETRY AND WEIGHT CHARACTERIZATION OF *PINNA NOBILIS* (MOLLUSCA: BIVALVIA) ALONG THE NORTHERN AND EASTERN TUNISIAN COASTLINE
(PAGE 43-47)

**9. BEN ABDELKRIM AHMED, BARAKET GHADA, CHATTI KHALED, SADDOUD DABBEBI OLFA, TRIFI MOKHTAR & SALHI-HANNACHI AMEL[1*]**

MORPHOLOGICAL VARIABILITY OF SUB-SPONTANEOUS TUNISIAN FIG (FICUS CARICA L.) POPULATIONS
(PAGE 48- 54)

**10. HAMLAOUI-GASMI S., MOKNI M., AOUANI E., AMRI M., MARZOUKI L.**

EFFECT OF GARLIC'S ADMINISTRATION ON ERYTHROCYTES AND PLASMA PARAMETERS IN RAT
(PAGE 55-58)

**11. ELEUCH L, REZGUI S AND DAALOUL A**

INDUCED PHYSIOLOGICAL TRAITS BY NACL IN TWO BARLEY CULTIVARS
(PAGE 59 – 66)

**12. SINDA FEDHILA[1] & DIDIER LERECLUS[2]**

ANALYSE GENETIQUE DE LA VIRULENCE DES BACTERIES PATHOGENES DU GROUPE *BACILLUS CEREUS*
(PAGE 67- 79)

**13. HAMMAMI IMEN[1,2], CLAIRE MAUDUIT[2], AMRI MOHAMED[3], BENAHMED MOHAMED[2], MICHELE VERONIQUE EL MAY[1].**

*ALLIUM SATIVUM* FEEDING INDUCES APOPTOSIS OF GERM CELLS THROUGH CASPASE-3 PATHWAY
(PAGE 80- 84)

**14. IMEN HADJI SFAXI [1], FETEN BELHADJ [1] FERID LIMAM [2] AND M. NEJIB MARZOUKI [1]**

SCREENING OF ENZYMATIC ANTIOXIDANT ACTIVITIES IN ALLIUM SATIVUM L.
(PAGE 85- 88)

**15. RANIA JBIR[1,2], SALWA ZEHDI[1], NÉJIB HASNAOUI[1,2], MOHAMED MARRAKCHI[1], MESSAOUD MARS[2] & MOKHTAR TRIFI[1*],**

STUDY OF MOLECULAR POLYMORPHISM OF TUNISIAN POMEGRANATE (*Punica granatum* L.) CULTIVARS USING SSRs AND AFLPs MARKERS
(PAGE 89 – 95)

**16. KADRI. K [(1,2)], CHEIKH MED. H[(2)] ABDELLAWI. R [(2)], & BEN NACEUR. M [(2)]**

MORPHOLOGICAL AND MOLECULAR EVALUATION OF THE GENETIC DIVERSITY OF TUNISIAN LOCAL BARLEY ACCESSIONS (HORDEUM VULGARE L.)
(PAGE 96–103)

**17. REHAIEM Amel, GHRAIRI Taoufik, MANAI Mohamed***

CARACTERISATION ET PURIFICATION D'UNE BACTERIOCINE PRODUITE PAR UNE SOUCHE LACTIQUE ISOLÉE A PARTIR D'UN DERIVE LAITIER ARTISANAL TUNISIEN
(PAGE 104–112)

**18. KADDOUR HADHEMI, HAMDI YOSRA, VAUDRY DAVID, TONON MARIE-CHRISTINE, AMRIMOHAMED* AND MASMOUDI-KOUKI OLFA**

OCTADECANEUROPEPTIDE, ODN, PROMOTES CELL SURVIVAL IN RAT CEREBELLAR GRANULE NEURONS
(PAGE 113- 118)

**19. Manel BELKHIR[1-2*,] Olfa Rebai[1-2], Mohamed AMRI 1 & Sami FATTOUCH 2**

ANTIOXIDANT ACTIVIY OF PULP AND PEEL POLYPHENOLIC EXTRACTS FROM TUNISIAN AZEROLE (CRATAEGUS AZAROLUS) FRUIT
(PAGE 119–122)

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 1 – 5*

**BIOLOGIA TUNISIE**

# Presence of '*Candidatus* Phytoplasma prunorum' in apricot orchards in Tunisia

**BEN KHALIFA Mekki[1*], ALDAGHI Magid [2], ACHECHE Hajer[1], KUMMERT Jean[2], MARRAKCHI Mohamed[1] and FAKHFAKH Hatem[1]**

[1] Laboratoire de Génétique Moléculaire Immunologie et Biotechnologie; Faculté des Sciences de Tunis; Campus Universitaire, Tunis El-Manar, 2092 Tunis, Tunisia.
[2] Unité de phytopathologie, Département de Biochimie et de Biologie Appliquées-FUSAGX Passage des déportés, 2; B-5030 Gembloux, Belgium.

**Keywords:** Apricot, '*Candidatus* Phytoplasma prunorum', restriction fragment length polymorphism.

## ABSRACT

'*Candidatus* Phytoplasma prunorum' was detected, for the first time, in apricot trees in Tunisia. Infected trees showed early leaf reddening in autumn and off-season growth in winter followed with dieback. Infected trees developed small and tasteless fruits. Phytoplasma was detected by nested polymerase chain reaction (PCR) using universal phytoplasma primer pairs R16mF2/R1 and R16F2n/R2. A band with expected size was observed in samples collected from symptomatic, but not healthy apricot trees. PCR products (1.2 kbp) were used for restriction fragment length polymorphism analysis (RFLP) after digestion with endonuclease *Mse*I, *Rsa*I and *Taq*I. RFLP patterns obtained were similar to that previously reported for the European stone fruit yellows (ESFY, 16SrX-B). Identification has been further confirmed by PCR using ESFY specific primer pairs (ECA1/ECA2). This is the first report on the occurrence of '*Ca.* Phytoplasma prunorum' in Tunisia (GenBank accession no. AY688362).

## INTRODUCTION

Phytoplasma cause a range of persistent diseases in many fruit trees in the world (Ogawa et al., 1995). In Europe, many economically important decline diseases of stone fruit species have been associated with European stone fruit yellows (ESFY) phytoplasma (Jarausch et al., 1998) that belong to the subgroup B of the apple proliferation group (16SrX-B) (Lee et al., 1998). Seemüller & Schneider (2004) proposed that ESFY phytoplasma be designated as '*Candidatus* Phytoplasma prunorum'. The most common hosts of ESFY phytoplasma include apricots (Morovan, 1977), plum (Giunchedi et al, 1982), peach (Pollini et al, 2001) and almond (Seemüller et al., 1998). The characteristic disease symptoms of ESFY on apricots include early leaf reddening and new sprouting in winter (Morovan, 1977).

Apricots (*Prunus armeniaca* L.) are among the most common stone fruit trees planted, in Tunisia. Apricots represent 18.5% of stone fruit production. Most of it is for local consumption and only 1% is exported to neighboring countries and European Union (FAO, 2007).

During 2007, disease symptoms resembling those caused by ESFY phytoplasma were observed on apricots in Northern Tunisia where 17% of apricot plantations are located (FAO, 2007).

The aim of this study was to detect and identify the phytoplasma associated with apricot syndrome in Tunisia. To achieve this, PCR

analysis using universal and ESFY-specific primer pairs and restriction fragment length polymorphism analysis (RFLP) have been applied.

## MATERIAL AND METHODS

### Plant materials and phytoplasma reference strains

Leaf and phloem tissues were collected during autumn and winter 2003 from apricot trees showing ESFY-like disease symptoms from 3 apricot orchards in a district of Northern Tunisia (Ras-Jebel). Phytoplasma reference strains were maintained on the experimental host *Catharanthus roseus* (Periwinkle) and used as positive control in this study. These strains included apple proliferation (AP, 16SrX-A); European stone fruit yellows (ESFY, 16SrX-B) and pear decline (PD, 16SrX-C) phytoplasma (Lee *et al.,* 1998).

### DNA extraction

Total DNA, used as template in PCR reactions, was extracted from approximately 1g of freshly prepared bark tissue. DNA from phytoplasma strains used as controls was obtained from petioles and midribs of infected *Catharanthus roseus* plants. Nucleic acids were isolated according to the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992).

### PCR analysis

PCR amplification of phytoplasma 16S rDNA was carried out with universal primers R16mF2/R1 (Gundersen and Lee, 1996) and R16F2n/R2 (Lee *et al.,* 1995). Non ribosomal primers ECA1/ECA2 (Jarausch et *al.,* 1998) were also used for ESFY-specific detection. The PCR reaction was performed in 25 µl reaction volumes containing 20 ng DNA, 0.4 mM of each primer , 0.25 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 units of HotGoldStar DNA polymerase (Eurogentec, Belgium) with the manufacturer's supplied buffer. Thermocycling was performed using a Peltier thermocycler (Hybaid, Teddington, UK) for 35 cycles with 1 min each for denaturation, annealing and extension. In the final cycle the extension step was extended to 10 min. In phytoplasma-universal nested PCR, DNA amplified in PCR primed by R16mF2/R1 was diluted 1:25 with sterile distilled water and used as template in PCR primed by R16F2n/R2. PCR products were separated on 1.5 % agarose gels containing 0.5 µg $mL^{-1}$ ethidium bromide and visualised using a UV transilluminator. The molecular weight of the PCR products was determined by comparison with 100 pb DNA Ladder (Fermentas, Lithuania).

### RFLP analysis

Nested PCR products (10–12 µl) were digested with *Mse*I, *Rsa*I or *Taq*I endonucleases (Fermentas, Lithuania) according to the manufacturer's instructions. Digested fragments were separated on 2 % agarose gels containing 0.5 µg $mL^{-1}$ ethidium bromide and visualised under the UV light. The molecular weight of the fragments was determined by comparison with 100 pb DNA Ladder (Fermentas, Lithuania). RFLP patterns were compared with those obtained from the phytoplasma reference strains and the RFLP patterns previously published (Davis et al., 1997; Lee et al., 1993; Lee et al., 1998; Marcone et al., 1996; Vibio et al., 1996).

## RESULTS

### Disease symptoms

Apricot trees from different locations of Tunisia showed disease symptoms similar to those caused by European stone fruit (ESFY) phytoplasma. Disease symptoms consisted of early reddening leaf in autumn and off-season growth in winter in addition to sudden death of infected branches or the entire crown of trees. These symptoms are similar to those previously reported by Pastore et al. (1997). Infected trees developed small and tasteless fruits, which resulted in great yield losses.

### Detection of phytoplasma by PCR

Nested-PCR using the primer pairs R16mF2/R1 and R16F2n/R2 from symptomatic trees yielded a characteristic band of approximately 1.2 kb (Fig. 1). No DNA bands were observed when DNA from healthy trees was used.

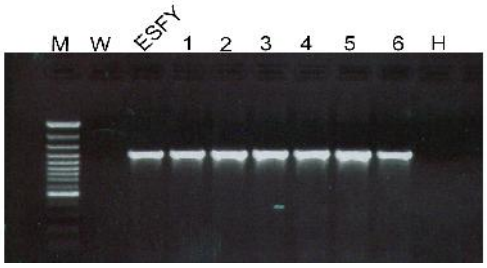



**Figure 1:** Detection of ESFY phytoplasma by nested PCR using universal primer pairs (R16mF2/R1; R16F2n/R2). The templates consisted of DNA extracted from asymptomatic apricots (H), symptomatic apricots (1-6) or from periwinkle plants infected with ESFY. W, water control; M, 100 bp DNA ladder.

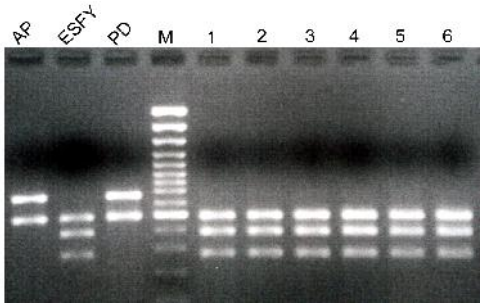



**Figure 2:** *Rsa* I restriction profiles of ribosomal DNA amplified using the universal primer pairs R16mF2/R1 and R16F2n/R2. The template DNA was from symptomatic apricots (1-6) or from periwinkle plants infected with the following phytoplasma strains: AP, apple proliferation; ESFY, European stone fruit yellows and PD, Pear decline. M, 100 bp DNA ladder.

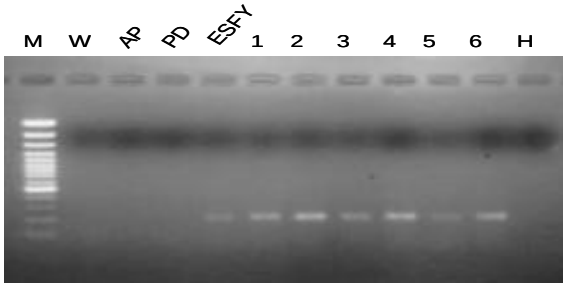



**Figure 3:** Detection of ESFY phytoplasma using ESFY specific primer pair ECA1/ECA2. Template DNA was obtained from asymptomatic apricots (H), symptomatic apricots (1-6) or from periwinkle plants infected with the following phytoplasma strains: AP, Apple Proliferation; ESFY, European Stone fruit Yellows and PD, Pear decline. W, negative control; M, 100 bp DNA ladder.

**Phytoplasma identification**

RFLP analysis of universal nested PCR products amplified from diseased apricot trees, showed restriction patterns (Fig. 2) identical to those previously reported for ESFY phytoplasma (Marcone et al., 1996).

PCR analysis using ESFY-specific primer pair ECA1/ECA2 revealed an amplification of 273 bp from all apricot samples tested and from ESFY phytoplasma used as positive control, no amplification was observed with AP and PD phytoplasma positive controls (Figure 3). These results are in agreement with infection of apricot trees tested with European stone fruit yellows phytoplasmas.

## CONCLUSION

Stone fruit diseases caused by phytoplasma have been reported in several countries in the world (Lee et al., 1995; Ogawa et al., 1995; Kison and Seemüller, 2001; Paltriniere et al., 2001; Pollini et al., 2001; Abou-Jawdah et al., 2002; Blomquist and Kirkpatrick, 2002; Anfoka and Fattash, 2004). However, the occurrence of these diseases on fruit trees has never been previously reported in Tunisia. Molecular techniques were applied in this study to confirm the aetiology of the disease observed on apricot trees in Tunisia. Results of nested PCR assays and RFLP analysis showed that all apricot samples were infected with '*Candidatus* Phytoplasma prunorum'. Therefore, data presented in this study demonstrate, for the first time, the infection of apricot trees in Tunisia with '*Candidatus* Phytoplasma prunorum' (GenBank accession no. AY688362).

'*Ca.* Phytoplasma prunorum' infections are among the most economically important diseases of apricot orchards in Europe. Despite pulling the infected apricot trees, there were no effective measures to control this disease. Since this is the first report on the occurrence of '*Ca.* Phytoplasma prunorum' in apricot trees in Tunisia, further investigations of the epidemiology of this disease must be undertaken. Since, this pathogen was reported to infect different stone fruit species (Giunchedi et al, 1982; Morovan, 1977; Nemeth, 1986; Pollini et al, 2001; Seemüller et al., 1998),

investigations concerning incidence and distribution of '*Ca.* Phytoplasma prunorum' in peach and almond, which represent with apricot the most common stone fruit trees planted in Tunisia, are justified.

## AKNOWLEGMENTS

This work was partially supported by grants from the Tunisian "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie " Projet "Lab B02".

## REFERENCES

Abou-Jawdah, Y.; Karakashian, A.; Sobh, H.; Martini, M. and Lee, I. M. (2002) An epidemic of almond witches_-broom in Lebanon: classification and phylogenetic relationships of the associated phytoplasma. Plant Dis. 86: 477–484.

Ahrens U, Seemuller E. (1992). Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies the sequence of the 16S rRNA gene. Phytopathol. 82: 828-32.

Anfoka, G. H. and Fattash, I. (2004). Detection and identification of Aster yellows (16SrI) phytoplasma in peach trees in Jordan by RFLP analysis of PCR-amplified products (16S rDNAs). J. Phytopath. 152 : 210–214.

Blomquist, C. L. and Kirkpatrick, B. C. (2002). Identification of phytoplasma taxa and insect vectors of peach yellow leaf roll disease in California. Plant Dis. 86 : 759-763.

Davis, R. E.; Dally, E. L.; Gundersen, D. E.; Lee, I. M. and Habili, N. (1997). `Candidatus Phytoplasma australiense', a new phytoplasma taxon associated with Australian grapevine yellows. Int. J. Syst. Bacteriol. 47 : 262-269.

FAO, Food and Agriculture Organisation of the unitated Nations, (2007). Données FAOSTAT, année 2007, http://www.fao.org/ag/agp/ressource/production.html.

Giunchedi, L.; Poggi-Pollini, C. and Credi, R. (1982). Susceptibility of stone fruit trees to the Japenese plum-tree decline causal agent. Acta. Hort. 130 : 285-290.

Jarausch, W.; Lansac, M.; Saillard, C.; Broquaire, J. M. and Dosba, F. (1998). PCR assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France. Eur. J. Plant Pathol. 104 : 17-27.

Kison, H. and Seemüller, E. (2001). Differences in strain virulence of the European stone fruit yellows phytoplasma and susceptibility of stone fruits trees on various rootstocks to this pathogen. J. Phytopathol. 149 : 533–541.

Lee, I. M.; Hammond, R. W.; Davis, R. E. and Gundersen, D. E. (1993) *Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms.* Phytopathol. 83 : 834-842.

Lee, I. M.; Bertaccini, A.; Vibio, M. and Gundersen, D. E. (1995). Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathol. 85 : 728–735.

Lee, I. M.; Gundersen, D. E.; Davis, R. E. and Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasma based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48 : 1153-1169.

Marcone, C.; Raggozzino, A. and Seemuller, E. (1996). European stone fruit yellows phytoplasma as the cause of peach vein enlargement and other yellows and decline diseases of stone fruits in Southern Italy. J. Phytopathol. 144 : 559-564.

Morvan, G. (1977). Apricot chlorotic leaf roll. E. P. P. O. Bull. 7 : 37–55.

Nemeth, M. (1986). Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees. Dordrecht, The Netherlands: Martinus-Nijhoff.

Ogawa, J. M.; Zehr E. I.; Bird, G. W.; Ritchie, D. F.; Uriu, K.; Uyemoto, J. K. (1995). Compendium of Stone Fruit Diseases. APS Press, St. Paul, Minnesota.

Paltrinieri, S.; Martini, M.; Stefani, E.; Pondrelli, M.; Bertaccini, A. and Fideghelli, C. (2001). Phytoplasma infection in peach and cherry in Italy. Acta. Hort. 550 : 365–370.

Pastore, M.; Vibio, M.; Murari, E. and Bertaccini, A. (1997). Incidenza, diagnosi e rospettive di difesa relatve alla fitoplasmosi dell'accartocciamento clorotico foliare dell'albicocco. Frutticol. 78 : 67-71.

Pollini, C. P.; Bissani, R. and Giunchedi L. (2001). Occurrence of European stone fruit yellows phytoplasma (ESFYP) infection in peach orchards in Northern-Central Italy. J. Phytopathol. 149 : 725–730.

Seemüller, E. & Schneider, B. (2004). 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and 'Candidatus Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *Int.* J. Syst. Evol. Micr. 54 : 1217-1226.

Seemüller, E.; Marcone, C.; Lauer, U.; Raggozzino, A. and Göschl, M. (1998). Current status of molecular classification of the phytoplasmas. *J. Plant. Pathol.* 80 : 3-26.

Vibio, M.; Bertaccini, A.; Lee, I. M.; Davis, R. E. and Clark, M. F. (1996). Differentiation and classification of aster yellows and related European phytoplasmas. *Phytopathol. Mediterr.* 35 : 33-42.

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 6 – 13*

**BIOLOGIA TUNISIE**

# MORPHOLOGICAL AND POMOLOGICAL VARIABILITY ANALYSIS OF TUNISIAN PLUM CULTIVARS

**BEN TAMARZIZT Hend, BARAKET Ghada, BEN MUSTAPHA Sana, TRIFI Mokhtar & SALHI-HANNACHI Amel[1*]**

Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences of Tunis, Campus University, 2092 El Manar Tunis, Tunisia
*Author for correspondence: e-mail: Amel.SalhiHannachi@fsb.rnu.tn
Phone: +216 71 87 26 00; Fax: +216 70 86 04 32

**Keywords:** Phenotypic variability, morphological, pomological traits, Plum, Tunisie**.**

## ABSRACT

To set up a rational collecting strategy for plum germplasm, a study was conducted using twenty-five morphological and pomological traits. The morphological study was conducted to assess intra et inter genetic variability of 13 local plum cultivars and to determine variables in biological characteristics, tree growth leaf and fruit descriptors. The analysis of variance showed a significant difference between cultivars and demonstrates that parameters relying on leaves, trees and fruits showed an important degree of variability and permit to discriminate plum cultivars. Principal component analysis pointed out a variation among individuals, mainly based on fruit, leaf, seed and tree growth. Ward's dendrogram and Principal Component Analysis (PCA) allowed clustering of cultivars according to their pomological traits and undependably of their geographic origin and. However, these analyses made it possible to resolve problem of mislabelling of cultivars (homonymy). The aim of this work was the morphological characterization of local plum varieties in order to establish a national ex situ collection and to start a improvement program of plum germplasm.

## INTRODUCTION

The plant family Rosaceae consists of over 100 genera and 3,000 species that include many important fruit, nut, ornamental, and wood crops. Modern breeding programs have contributed to the selection and release of numerous cultivars having significant economic impact. Plums hold the central position within the genus *Prunus* with numerous species with a basic number x=8 (Badenes and Parfitt 1995) and having originated in Europe, Asia, China, and North America (Zohary, 1992; Smartt and Simmonds, 1995). European plums belong to *Prunus domestica* L., *P. cerasifera* Ehrh., *P. spinosa* L. and *P. insititia* species. Although Japanese plums include *P. salicina* Lindl, and American ones contain *P. Americana* Marsh., *P. munsoniana* Wight and *P. angustifolia* Marsh. In the case of *Prunus domestica*, *P. spinosa* and *P. cerasifera* were proposed as the possible progenitor species (Crane and Lawrence, 1930; Salesses, 1975; Watkins, 1976; Watkins, 1981). Therefore, several studies were conducted to describe the genetic diversity and determine their taxonomic status of *Prunus* species involving physiological, pomological parameters and molecular markers (Ertekin et al. 2006). Some other works related to phenolic were applied in to many species and

were used to establish morphological diversity and to obtain many descriptors utilized into varietals identification (Groh et al. 1994). However, plum species have been classified primarily on the basis of morphological characteristics. In addition, biochemical and molecular characterization of plum have been reported. (Mnejja et al. 2004; Byrne and Littleton, 1988; Bellini et al. 1998, Gregor et al. 1994, Ortiz et al. 1997, Shimada et al. 1999; Heinkel et al. 2000; Goulao et al. 2001; Salesses et al. 1994; Cipriani et al. 1999; Groh et al. 1994; Mohanty et al. 2002; Mohanty et al. 2003). In Tunisia, the major cultivated cultivars result from Japanese (2n=16) and European species (2n=6n=48) (*P. salicina*, *P. domestica*). The local germplasm is characterized by numerous ecotypes mainly distinguishable by the fruit characteristics, skin colour, and fruit shape. However, the exact number of cultivars is still unknown since problems of synonymy and homonymy are frequently observed. It is noteworthy that a number of these cultivars have been destroyed as a consequence of the intensive urbanization in recent decades. In addition, severe genetic erosion is also threatening the remaining germplasm like diseases, use of introduced cultivars and monovarietal cultivation. In fact, commercial production is limited to some locations, where usually one or two cultivars account for most of the production and represented buy introduced ones recognised for their fruit quality, performance and productivity like Santa Rosa and Golden Japon cultivars (Anonyme, 2002) belonging to *Prunus salicina* (2n=16).

. However, local varieties well adapted to bioclimatic conditions are cultivated in traditional orchards. Thus, available information about the ancient landraces is restricted. To our knowledge, no detailed study has been devoted to genetic diversity of local germplasm. For these raisons, it is imperative to start genetic diversity study to characterize local plum germplasm.

The goals of the present study, based on morphological and pomological traits were as follows: (1) to estimate the amount of genetic diversity within and among plums cultivars (2) to determine the degree of genetic differentiation; and (3) to examine the phylogenetic relationship between cultivars to start conservation and improvement programs. In fact, to satisfy the future needs in these genetic resources, it is necessary to collect and conserve representative stocks of plum genetic diversity. Indeed, the chance for fulfilling future demand of genetic resources is better when a high level of genetic diversity is conserved and made available for breeders. This challenge should not be missed, particularly for the crops such as neglected and underutilised by national research programs.

## MATERIAL AND METHODS

### Plant materials

A total of 13 cultivars collected from three regions in northern Tunisian were studied. These consisted of local cultivars originating from Rafraf, Ras Jebel and Sounine. Table 1 summarizes the nomenclature and the ecotype labels.

**Table 1**: Origin and label of the Tunisian plum cultivars studied.

| Name | Code | Locality |
|---|---|---|
| Bedri 1 | BED1 | Ras Jebel |
| Bedri 2 | BED2 | Ras Jebel |
| Jabonia safra | JBS | Rafraf |
| Cidre | CID | Rafraf |
| Adham Hmém | ADH | Rafraf |
| Baydha Arbi | BYA | Rafraf |
| Meski Hamra 2 | MSH2 | Rafraf |
| Meski hamra 2 | MSH2 | Rafraf |
| Meski kahla 1 | MSK1 | Rafraf |
| Meski kahla 2 | MSK2 | Rafraf |
| Meski safra 1 | MSS1 | Rafraf |
| Meski safra 2 | MSS2 | Rafraf |
| Ain Tasstouria | ANT | Sounine |

### Morphological Descriptors

Twenty five qualitative and quantitative traits chosen from plum descriptors published by the International Plant Genetic Resources Institute (IPGRI-CEC, 1984) were studied (Table 2).

**Table 2:** Result of one-Way analyses of variance (ANOVA) applied on pomological and morphological parameters referring to growth, leaf, and fruits descriptors.

| Parameter | Label | Mean square | F observed | P |
|---|---|---|---|---|
| **Leaf length** | LL | 1.5176 | 5.58NS | 0.0651 |
| **Leaf width** | LWI | 1.4889 | 14.58** | 0.0128 |
| **Petiole length** | PL | 0.2153 | 6.98* | 0.0456 |
| **Fruit length** | FL | 1.7547 | 126.69** | 0.0002 |
| **Fruit width** | FWI | 0.9478 | 41.03** | 0.0018 |
| **Stalk length** | SL | 0.1777 | 4.12NS | 0.1026 |
| **Fruit weight** | FWE | 261.0099 | 80.28** | 0.0005 |
| **Fruit form** | FF | 4.0000 | 99999.99** | 0.0001 |
| **Skin colour** | SC | 4.5000 | 99999.99** | 0.0001 |
| **Pulpit colour** | PC | 4.5000 | 99999.99** | 0.0001 |
| **Firmness** | F | 4.0000 | 99999.99** | 0.0001 |
| **Juicy** | J | 5.3333 | 99999.99** | 0.0001 |
| **Acidity** | AC | 5.8333 | 99999.99** | 0.0001 |
| **Aroma** | A | 9.8333 | 99999.99** | 0.0001 |
| **Sweeten** | S | 13.8333 | 99999.99** | 0.0001 |
| **Seed length** | SL | 0.6275 | 478.13** | 0.0001 |
| **Seed width** | SWI | 0.1322 | 83.95** | 0.0005 |
| **Seed weight** | SWE | 8.9680 | 1.49NS | 0.3456 |
| **Seed Length / Width** | SL /WI | 0.2096 | 60.12** | 0.0009 |
| **Seed form** | SF | 2.1666 | 8.67* | 0.0318 |
| **Branch length 2006** | BL06 | 296.9605 | 2.42NS | 0.2060 |
| **Branch length 2007** | BL07 | 796.7915 | 35.47** | 0.0024 |
| **Enter-node 2006** | EN06 | 0.0465 | 0.24NS | 0.8636 |
| **Enter-node 2007** | EN07 | 0.4533 | 9.80* | 0.0258 |
| **Final bud diameter** | FBD | 0.0111 | 3.79NS | 0.1152 |

NS: Not significant; **: High significant; *: Significant. k1=p-1=4-1=3 ; k2=p-(n-1)=4(2-1)=4
Fth à 5%= 6,59 ; Fth à 1%= 16,70

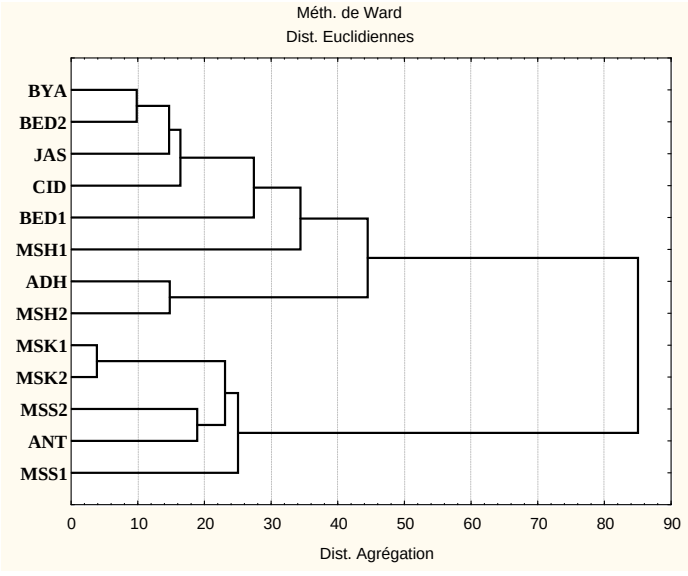



**Figure 1:** Ward's dendrogram of 13 Tunisian plum cultivars based on 25 morphological and pomological traits based on Euclidean distances.

**Data analysis**

Phenotypic data were used to approximate contribution of the measured traits in the genetic diversity among the considered cultivars. In order to elucidate genetic diversity structure and to establish relationships between cultivars, several statistical procedures were conducted. Data were computed using the SAS software (Statistical Analysis System, version V.6.07; SAS, 1990) to perform one-way analysis of variance (ANOVA) and multivariate analyses: principal components analysis (PCA) The multivariate analysis was applied to assess differences between cultivars and elucidate partitioning and structurations of phenotypic variability at inter- and intra varietal levels.

The Euclidean distance matrix was estimated and hierarchical cluster was constructed according to Ward's method (Ward, 1963) by module in the statistical packages STATISTICA 6.0 (StatSoft Inc, 2001) to establish relationships among cultivars.

## RESULTS

Morphological parameters corresponding to biological characteristics, growth of the tree leaves, and fruit were measured in order to describe the genetic diversity and its partitioning.

**One-way variance analysis: ANOVA**

The one-way ANOVA analysis showed a signifying diversity between cultivars, except for six parameters (Leaf length, Stalk length, Seed weight, Branch length 2006, Enter-node 2006, Final bud diameter). The ANOVA shows significant differences among cultivars for 19 traits out of 25. Table 2 illustrates that out of the 25 parameters 16 were highly significant (i.e., Leaf width, Fruit length, Seed Length / Width and Branch length 2007) and 3 were significant: Petiole length, Seed form and Enter-node 2007. We note that the most discriminator parameters are those related to fruit especially the skin and fruit pulpit colour, also those related to fruit qualitative parameters.

**Hierarchical clustering: Ward's method**

The dendrogram obtained in the basis of Ward's analysis was constructed. Figure 1 shows two major clusters (I) and (II). The first one (I) is composed by 'Meski Kahla1' [MSK1], 'Meski Kahla2' [MSK2], 'Meski Safra2' [MSS2], 'Ain Tasstouria' [ANT] and 'Meski Safra1' [MSS1]

cultivars. The second group (II) contains the remaining cultivars. Morphological method also made it possible to sort out the mislabelling and homonymy problems of different ecotypes based on local denomination. This is well exemplified in the case of 'Bedri1' [BED1] and 'Bedri2' [BED2]; 'Meski Kahla1' [MSK1] and 'Meski Kahla2' [MSK2]; 'Meski Safra1' [MSS1] and 'Meski Safra2' [MSS2]; 'Meski Hamra1' [MSH1] and 'Meski Hamra2' [MSH2] cultivars.

**Multivariate analysis: Principal component analysis (PCA)**

**Inter-varietal genetic variability**

We have performed a PCA analysis taking into account the vegetative traits and pomological parameters. Correlation coefficients computed for all pairs of traits (data not shown) revealed significantly positive values observed especially between seed length/width (SL/WI) and seed form (SF) parameters (0.98), fruit width (FWI) and fruit weight (FWE) (0.94) parameters. However, negative values were calculated for fruit acidity (AC) and seed form (SF) (-0.84) parameters.

Results of PCA analysis based on growth and leaf descriptors show that 65.06% of the global variability is accounted by the three first principal components (PCs) with 27.04%, 20.74% and 17.28% the absorbance of the three axes respectively (Table 3).

**Table 3:** Eigenvectors, Eigen values and percent variance explained by the first three principal components (PCs) for 25 traits analysed in 13 plum cultivars.

| **Eigenvectors** | **PC1** | | **PC22** | | **PC3** | |
|---|---|---|---|---|---|---|
| Cumulative % of variance explained | 27,04 | | 47,78 | | 65,06 | |
| | **cha** | **e.i** | **cha** | **e.i** | **cha** | **e.i** |
| | PL | +0.331 | SWI | +0.345 | FL | +0.375 |
| | SL/WI | +0.297 | SC | +0.314 | FF | +0.355 |
| | SF | +0.295 | FWI | +0.268 | BL07 | +0.287 |
| | INL07 | +0.274 | F | +0.240 | SL | +0.283 |
| | LL | -0.219 | STL | -0.245 | PC | -0.266 |
| | J | -0.271 | S | -0.214 | AC | -0.266 |

Cha: characters; e.i: Eigenvalue

The first principal component (PC1) is correlated positively with petiole length of (PL) and 2007 inter-node (INL07), length and fruit width ratio (SL/WI) and by the seed form (SF) and negatively with leaf length of (LL) and fruit juicy (J) (Table 3). However, the second principal component (PC2) is defined positively by variables: width of seed and fruit (SWI, FWI), skin colour (CS) and fruit firmness (F). This component is also negatively correlated to length stalk (LST), and fruit sweeten (S). the third principal component (PC3), integrated character related to fruit length, form, acidity (FL, FF, AC) and pulpit colour (PC) also by the seed length and 2007 branch length (SL, BL07) (Table 3).

Dispersion of cultivars on the first plan (1-2) of the principal component analysis that absorb 47.78% of the total variability shows morphological cultivar divergences and demonstrates an important phenotypical variability of plum germplasm. According to the PC1, cultivars: 'Meski kahla' [MSK], 'Meski hamra' [MSH] and 'Adham hmém' [ADH] characterized by greater plant growth, small leaves with long petioles, lower juicy fruit and large seed, were differentiated and opposed to the others cultivars. In fact, 'Ain Tasstouria' [ANT], 'Cidre' [CID], 'Jabonia safra' [JBS], 'Baydha arbi' [BYA], 'Bedri'[BED] and 'Meski safra' [MSS] cultivars are characterized by flattened seed sweet and clear fruit. In the other hand, it was possible to differentiate, according to PC2, 'Adham hmém' [ADH], 'Baydha arbi' [BYA] and 'Meski safra' [MSS] cultivars characterized by sweeten and juicy fruit. However, 'Ain Tasstouria' [ANT], 'Cidre' [CID], 'Jabonia safra' [JBS], 'Meski hamra' [MSH] and 'Meski kahla' [MSK] cultivars are considered by large, acid, firm and dark fruit with width seed (Figure 2).

**Intra-varietal genetic variability**

A distinctly intra varietal distribution was observed with PCA (Figure 3). Grouping of plums genotypes using PCA was based mainly on the first three PCs that account for 88.86 % of the variability observed with 52.07%, 23.30% and 13.48% respectively the absorbance of the three axes correspondingly (Table 4). The most important variables integrated by PC1 were skin colour (SC), seed length (SL) fruit length (FL)

and petiole length (PL). PC1 was negatively correlated by juicy (J), aroma (A) and sweeten (S) parameters. The second axis (PC2) is correlated positively with leaf length and width (LL), (LWI), acidity (AC) and fruit weight (FWE) and negatively with seed length and width ration (SL/WI) and seed form (SF). The PC3 integrated characters related with the stalk length (STL), fruit form and width (FWI, FF) (Table 4). Figure 3 shows that PC1 separated cultivars having big fruit and seed with intense colour as 'Meski kahla' [MSK] and 'Meski hamra' [MSH] from those with small and less coloured ones as 'Meski safra' [MSS] and 'Bedri' [BED]. The distribution of cultivars as referred to the first axes showed an important similarity between the two varieties 'Bedri' [BED] and 'Meski safra' [MSS].

It was possible to differentiate, according to PC2, cultivars 'Bedri' [BED] and 'Meski hamra' [MSH] with large and acid fruits and big leaves from 'Meski safra' [MSS] and 'Meski kahla' [MSK] haracterized by smaller and sweet fruit enclosed smaller seed (Figure 3).

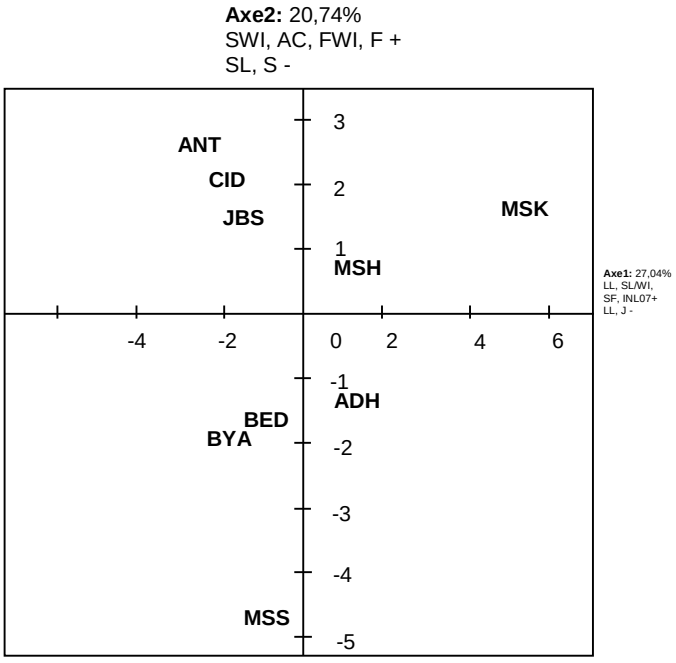



**Figure 2:** Dispersion of Tunisian plum cultivars in the plot (1-2) of the principal component analysis (47.78% of the global variability) based in phenotypic characters.

**Table 4:** Eigenvectors, Eigen values and percent variance explained by the first three principal components (PCs) for 25 traits analysed in 6 plum cultivars.

| **Eigenvectors** | **PC1** | | **PC2** | | **PC3** | |
|---|---|---|---|---|---|---|
| Cumulative % of variance explained | 52,07 | | 75,37 | | 88,86 | |
| | **cha** | **e.i** | **cha** | **e.i** | **cha** | **e.i** |
| | SC | +0,27 | LL | +0,35 | STL | +0,36 |
| | SL | +0,27 | LWI | +0,31 | FWI | +0,17 |
| | FL | +0,26 | AC | +0,31 | FF | -0,43 |
| | PL | +0,25 | FWE | +0,23 | LWI | -0,11 |
| | J | -0,25 | SL/WI | -0,30 | F | -0,38 |
| | A | -0,23 | SF | -0,30 | | |
| | S | -0,26 | | | | |

Cha: characters; e.i: Eigenvalue

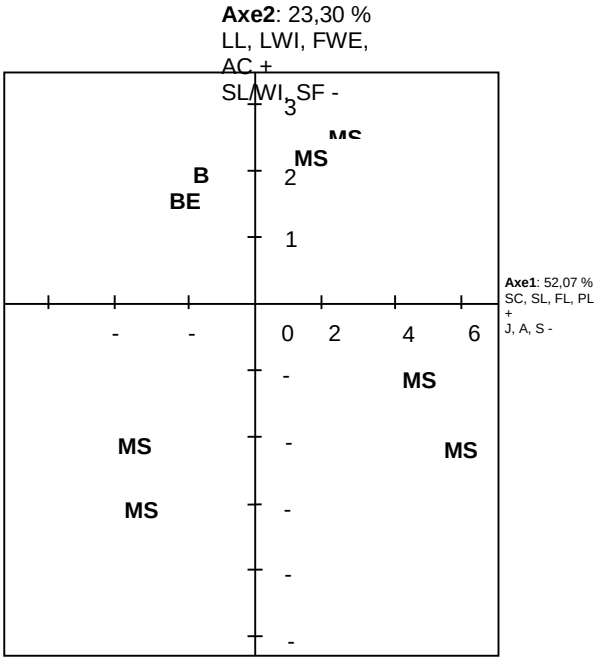



**Figure 3:** Intra-varietal phenotypic relationships among eight plum cultivars as given by the first two principal components (75.37% of the total variability) based on pomological on morphological parameters.

## DISCUSSION AND CONCLUSION

To maintain, evaluate and utilise germplasm efficiently, it is important to investigate the extent of genetic diversity available. Morphological characterisation is an important step in the description and classification of crop germplasm because a breeding programme mainly depends upon the magnitude of morpho-phenological variability. The morphological

traits analysed on Tunisian plum cultivars highlighted an important variability and allowed a clear distinction of the studied cultivars. These results will be very useful to characterize each cultivar and to create a catalogue of local cultivars. Results obtained by PCA analyses for inter- variability showed a distribution of genetic variability independent from their geographical origins.

To our knowledge, no studies reporting morphological diversity of plums are published. Consequently, we could not compare data obtained in the present study to others for a best appreciation of the morphological diversity of plums cultivar from Tunisia. The traits that contributed significantly to cultivars distinction are those related to sizes and weight of fruits, seeds, leaves and growth trees. The pattern of morphological variability observed in this study is similar to those from several studies demonstrating that in domesticated crops species such as fig (*Ficus carica* L.), morphological differences are often based on agronomic traits (Salhi Hannachi et al. 2003, Chatti et al. 2004; Saddoud et al. 2008). We could thus conclude that seed, fruit size and leaves parameters and tree's growth proved to be a useful tool for discriminating plum cultivars. More interestingly, the individuals analysed were grouped into two groups defined based on these parameters. The intra varietal diversity confirms results obtained by the dendrogram and it can be explained by an important somatic mutations or du to natural hybridization which could give rise to new subspecies as suggested by Shimada et al. (1999). Cluster analysis show that the considered cultivars are gathered independently from either geographical origin or appellation. Moreover, cultivars having the same denomination not cluster together. It is the case of cultivars 'Meski hamra' [MSH], 'Meski safra' [MSS] and 'Meski kahla' [MSK] originating from Rafraf and 'Bedri' [BED] collected from Ras Jebel in the north Tunisia. These may be considered as homonomies and the hypothesis of polyclonal varieties 'Meski' and 'Bedri' could be forwarded to explain this result.

As the high loss of global biodiversity continues, devoting efforts to the conservation of genetic resources for local crops are widely recommended. To achieve this objective, a better knowledge of the genetic diversity of the target crop is a prerequisite. From the investigation herein, it is worth mentioning that Tunisian plums displayed a wide range of diversity for most of the morphological traits studied. The traits related to fruit, seed, leaf and tree growth show the highest discriminating value and were, consequently, the most cultivar distinctive. Consequently, morphological and pomological traits could be used for cultivar identification during collecting missions. However, the low number of analysed individuals suggests that analysis of additional cultivars is required before a definitive conclusion can be revealed. It will be very useful to continue this study for all Tunisian plum cultivars. Such analyses allow the identification of most preferment genotypes. In addition, phenological characters must be associated to nuclear and cytoplasmic molecular markers such as SSR or AFLP in order to study this variability for one of the most widely consumed local fruit in Tunisia.

## AKNOWLEGMENTS

This work was partially supported by grants from the Tunisian "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie " Projet Lab B02.

## REFERENCES

Anonyme, (2002). Arboriculture fruitière. Variétés fruitières recommandées en Tunisie. Documents techniques INRAT N° 114.

Badenes, M.L., Parfitt, D.E. (1995). Phylogenetic relationships of cultivated *Prunus* species from an analysis of chloroplast DNA variation. Theoretical Applied Genetics 90, 1035-1041.

Bellini, E. Giordani, E. Nencetti, V. and Paffetti, D. (1998). Genetic relationships in Japenese plum cultivars by molecular markers. Acta Horticulturae. 478: 53-61.

Byrne, D. H. and Littleton, T.G. (1988). Electrophoretic characterization of diploid plums of the southeastern United States. Journal of the American Society for Horticultural Science. 113: 918-924.

Chatti, K., Salhi-Hannachi, A., Mars, M., Marrakchi, M., and Trifi, M. (2004). Analyse de la diversité génétique de cultivars tunisiens de figuier (*Ficus carica* L.) par les caractères morphologiques. Fruits 59 :49–61.

Cipriani, G. Lot, G. Huang, W.G. Marrazzo, M.T. Peterlunger, E. and Testolin, R. (1999). AC/GT and AAG/CT microsatellite repeats in peach (*Prunus persica* L. Batsch) isolation, characterization and cross-species amplification in *Prunus*. Theoretical and Applied Genetics. 99(1-2): 65-72.

Crane, M.B. and Lawrence, W.J.C. (1930). Studies in sterility. International Horticultural Congress. 9: 100-116.

Ertekin, C. Gozlekci, S. Kabas, O. Sonmez, S. and Akinci, I. (2006). Some physical, pomological and nutritional properties of two plum (*Prunus domestica* L.) cultivars. Journal of Food Engineering**.** 75(4): 508-514.

Goulao, L. Monte-Corvo, L. and Oliveira, C.M. (2001). Phenetic characterization of plum cultivars by high multiplex ratio markers: Amplified Fragment Length Polymorphisms and Inter-simple Sequence Repeats. Journal of the American Society for Horticultural Science. 126(1): 72–77.

Gregor, D. Hartmann, W. and Stosser, R. (1994). Cultivar identification in *Prunus domestica* using random amplified polymorphic DNA markers. Acta Horticulturae. 359: 33-40.

Groh, B. Bauer, H. and Treutter, D. (1994). Chemotaxonomical investigations of *Prunus domestica* by isoenzyme markers and phenolic compounds. Scientia Horticulturae. 58: 41-55.

Heinkel, R. Hatmann, W. and Stösser, R. (2000). On the origin of the plum cultivars `Cacaks Beauty', `Cacaks Best', `Cacaks Early' and `Cacaks Fruitful' as investigated by the inheritance of random amplified polymorphic DNA (RAPD) fragments. Scientia Horticulturae. 83: 149-155.

IPGRI (International Board for Plant Genetic Resources) and CEC (Commission of European Communities: Committee on disease resistance breeding and use of genebanks (1984) Descriptor list for plum and allied species. (Eds Cobianchi D, Watkins R) pp. 31

Mnejja, M. Garcia-Mas, J. Howad, W. Badenes, M.L. and Arùs, P. (2004). Simpel-sequence repeats (SSR) markers of Japanese plum (*Prunus salicina* Lindl.) are highly polymorphic and transferable to peach and almond. Molecular Ecology Notes. 4: 163-165.

Mohanty, A. Martin, J.P. and Aguinagalde, I. (2002). Population genetic analysis of European *Prunus spinosa* (Rosaceae) using chloroplast DNA markers. American Journal of Botany. 89: 1223–1228.

Mohanty, A. Martin, J.P. Gonzalez, L.M. and Aguinagalde, I. (2003). Assaciation between chloroplast DNA and mitochondrial DNA haplotypes in *Prunus spinosa* L. (Rosaceae) populations across Europe. Annals of Botany. 92: 749-755.

Ortiz, A. Renaud, R. Calzada, I. and Ritter, E. (1997). Analysis of plum cultivars with RAPD markers. Journal of horticultural sciences. 72: 1-9.

Saddoud, O, Baraket G., Chatti K., Trifi M., Marrakchi, M., Salhi-Hannachi, A., and Mars, M.. (2008). Morphological Variability of Fig (*Ficus carica* L.) Cultivars. International Journal of Fruit Science 8: 35-51

Salhi-Hannachi, A., Mars, M., Chatti, K., Marrakchi, M., Trifi, M. (2003). Specific genetic markers for Tunisian fig germplasm: Evidence of morphological traits, random amplified polymorphic DNA and inter simple sequence repeats markers. J. Genet. Breeding 57: 125–136.

Salesses, G. (1975). Some data on the cytogenetics of plums and the origin of plums. Acta Horticulturae. 48: 59–65.

Salesses, G. Grassely, C. Bernhard, R. (1994). Utilisation des espèces indigènes et exotiques pour l'amélioration des *Prunus* cultivés, variétés et porte-greffe. C. R. Académie d'Agriculture Française. 80: 77–88.

SAS (1990) SAS user's guide: SAS STAT, SAS BASIC. Version 6 fourth edition. Sas incl, BOX 8000. cary, NC 27512-8000, cary; NC: SAS institute Inc.

Smartt J, and Simmonds NW (1995) Evolution of crop plants, Ed 2nd edn. Longman Scientific &Technical, Harlow, UK.

Shimada, T. Hayama, H. Haji, T. Yamaguchi, M. and Yoshida, M. (1999). Genetic diversity of plums characterized by random amplified polymorphic DNA (RAPD) analysis. Euphytica 109: 143-147.

StatSoft, Inc. (2001) STATISTICA (data analysis software systems), version 6. StatSoft, Inc. (www.statsoft.com), Tulsa, OK. USA.

Ward, J. H. (1963). Hierarchical grouping to optimize an objective function. Journal of American Statistical Association. 58: 236-244

Watkins, R. (1976). Cherry, plum, peach, apricot and almond. In: Simmonds N.W. ed. Evolution of crop plants. Longman, London, UK. 242-247.

Watkins, R. (1981). Plums, apricots, almonds, peaches, cherries genus *Prunus*. In: Hora B. ed. The Oxford University encyclopaedia of trees of the world. Oxford University Press, UK. 196-201.

Zohary, D. (1992). Is the European plum, *Prunus domestica* L., a P. *cerasifera* EHRH x *P. spinosa* L. allopolyploid. Euphytica. 60: 75–77.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 14 – 19*

**BIOLOGIA TUNISIE**

# THE PROTECTIVE EFFECT OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE ON ASTROGLIAL CELLS IS MEDIATED THROUGH ACTIVATION OF OCTADECANEUROPEPTIDE RELEASE

**Masmoudi-Kouki Olfa[a], Kaddour Hadhémi[a], Hamdi Yosra[a], Vaudry David[b,c,d], Tonon Marie-Christine [b,c,d] and Amri Mohamed[a]**

**aLaboratory of Functional Neurophysiology and Pathology, Research Unit 00/UR/08-01, Department of Biological Sciences, Faculty of Science of Tunis, University Tunis El Manar, 2092 Tunis, Tunisia ; bInserm U413 /U982, Laboratory of Neuronal and Neuroendocrine Communication and Differentiation ; cInternational Associated Laboratory Samuel de Champlain ; dEuropean Institute for Peptide Research (IFRMP 23), Regional Platform for Cell Imaging of Haute-Normandie (PRIMACEN) , University of Rouen, 76821 Mont-Saint-Aignan,**

*Corresponding authors: ; Pr Amri Mohamed. Fax: (216) 216 71 87-16-66. ; E-mail address: mohamed.amri@fst.rnu.tn

**Keywords:** Neuropeptides, Astrocytes, Cell culture, Oxidative stress, Neuroprotection

## ABSTRACT

Oxidative stress in living cells results from accumulation of reactive oxygen species and free radicals such as hydrogen peroxide ($H_2O_2$) and initiates the cascade of events leading to apoptosis. Astroglial cells synthesize and release octadecaneurpeptide, a biological active peptide that has been implicated in cellular protection. The release of ODN is regulated by neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), known neuroprotective agent. The aim of the present study was to determine whether PACAP could prevent $H_2O_2$ toxicity by activating ODN receptor. Incubation of cultured astrocytes with graded concentrations of $H_2O_2$ for 1 h provoked a dose-dependent reduction of the number of living cells. Pretreatment of astrocytes with very low concentration of PACAP (0.1 nM) or ODN (0.1 nM) prevented cell death induced by $H_2O_2$. The effect of ODN was mimicked by the ODN metabotropic receptor agonist and the blockage of this receptor by a specific antagonist, $cyclo_{1-8}[Dleu^5]OP$, abolished totally the protective action of PACAP upon $H_2O_2$-induced astrocytes cell death. Taken together, these data indicate that PACAP, acting through ODN release, exerts a potent protective effect against astrocytes degeneration induced by oxidative stress.

## INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was initially isolated from the ovine hypothalamus for its ability to stimulate cAMP formation in rat anterior pituitary cells (Miyata et al., 1989). Soon after the discovery of PACAP, the presence of specific PACAP binding sites was demonstrated on astroglial cells (Tatsuno et al., 1991). Subsequent studies have shown that all three PACAP receptors are expressed in rat astrocytes (Ashur-Fabian et al., 1997; Grimaldi and Cavallaro, 1999), indicating that astrocytes are main target cells for PACAP in the central nervous system. It is well known that PACAP reduces brain damage after ischemia and ameliorates neurological deficits (Vaudry et al., 2000; Nakamachi et al., 2008; Ohtaki et al., 2008). In addition, some data suggest that neuroprotective activity of PACAP seems to involve an indirect mechanism requiring astrocytes (Brenneman et al., 2000; Brenneman et al., 2003; Shioda et al., 2004). PACAP-stimulated astrocytes secrete neuroprotective proteins, including activity-dependent neuroprotective protein and granulocyte colony-stimulating factor and a number of cytokines (Shioda et al., 2004; Masmoudi-Kouki et al., 2007). We have previously shown that PACAP, for very low doses and through activation of PAC1 receptor stimulates the release of endozepines (Masmoudi et al., 2003).

The endozepine, octadecaneuropeptide (ODN) is generated by proteolytic cleavage of an 86-amino acid precursor called diazepam-binding

inhibitor (DBI) (Guidotti et al., 1983) whose gene is mainly expressed in glial cells in the central nervous system (CNS) (Tonon et al., 2006). There is now evidence that ODN may act as an autocrine and paracrine factor modulating astroglial and neuron cell activity, respectively. At the cellular level, effects of ODN are mediated through central-type benzodiazepine receptors (CBR), i.e. glial cell proliferation (Gandolfo et al., 1999) and neurosteroid biosynthesis (Do-Rego et al., 2001), or by activation of the metabotropic receptor of ODN, i.e. neuropeptide expression in neuronal cells (Compère et al., 2004; Compère et al., 2005) and intracellular calcium increase in cultured rat astrocytes (Gandolfo et al., 1997; Leprince et al., 1998).

The observation that ODN, as well as PACAP, stimulates the release of neuroprotective agents from cultured astrocytes (Tokay et al., 2005) suggests that endozepines may contribute to PACAP-evoked cells protection. In order to test the hypothesis, we investigate the possible action of ODN on the protective effect of PACAP on oxidative stress-induced astroglial cells death.

## MATERIAL AND METHODS

### Cell culture

Secondary cultures of rat cortical astrocytes were prepared as previously described (Brown and Mohn, 1999) with minor modifications. Briefly, cerebral hemispheres from newborn Wistar rats were collected in Dulbecco's modified Eagle's medium/F12 (2 : 1; v/v) culture medium supplemented with 2 mM glutamine, 1% insulin, 5 mM HEPES, 0.4% glucose and 1% of the antibiotic–antimycotic solution. The tissues were dissociated mechanically and cells suspension was seeded at a density of 0.6 x106 cells/ml on 75- cm2 flasks.

When cultures were confluent, astrocytes were isolated from mixed glial cultures by shaking the flasks with an orbital agitator. Adhesive cells were detached by trypsination and pre-plated for 10 min to discard contaminating oligodendrocytes and microglial cells. Then, the non-adhering astrocytes were harvested and seeded in 24-well plates at a density of 0.2x106 cells/ml. The cells were incubated at 37°C in a humid atmosphere (5% CO2). After 4 days of culture, 100% of the cells were labelled with antibodies against glial fibrillary acidic protein. All experiments were performed on 5- to 7-day-old secondary cultures.

### Measurement of cell survival

Cells seeded into 24-well plates were subjected to various treatments and then incubated at 37°C for 10 min with 15µg/ml fluorescein diacetate (producing green fluorescence in living cells). Cells were washed three times with phosphate-buffered saline (PBS) and lysed with a Tris/HCl solution. Fluorescence intensity was measured (λ excitation = 485 nm and λ emission = 530 nm) with a FLx800 fluorescence microplate reader (Bio-Tek FL800TBI Instruments, USA).

### Statistical analysis

Data are presented as the mean SEM from three independent experiments performed in quadruplicate or quintuplicate. Statistical analysis of the data was performed by using Student's t test and ANOVA, followed by Bonferroni's test.

## RESULTS

Effect of PACAP on H2O2-induced astrocyte cell death

Incubation of cultured astrocytes with graded concentrations of H2O2 for 1 h provoked a dose-dependent decrease of the proportion of surviving cells (Fig. 1A). After 1 h of treatment, half the cells died when incubated with 0.3 mM H2O2, a concentration that was employed in all subsequent experiments. Co-incubation of astroglial cells with H2O2 and subnanomalar concentration of PACAP for 1 h induced a significant increase in the number of surviving cells (Fig. 1B).

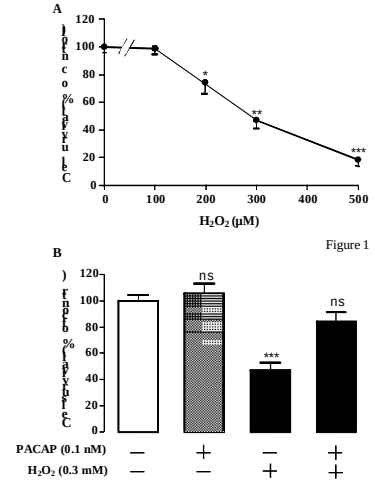



Figure 1

**Figure 1 :** Protective effect of PACAP on astroglial cell death induced by H2O2. (A) Cells were incubated in the absence or presence of graded concentration (100 to 500 µM) of H2O2. (B) Cells were preincubated for 10 min in the absence or presence of 0.1 nM PACAP and then incubated for 1 h with medium alone or with 0.3 mM H2O2 without or with PACAP. The results are expressed as percentages of the control value. Each value is the mean (± SEM) of at least three independent experiments performed in quadriplicate. ANOVA followed by the Bonferroni's test: *P< 0.05; **P < 0.01; ***P < 0.001; ns, not statistically different from the control.

Effect of ODN on protective action of PACAP against H2O2-provoked astrocyte cell death. Pretreatment of astrocytes with ODN for 10 min totally suppressed the toxic effect of 0.3 mM H2O2 on cells survival. Incubation of astrocytes with specific ODN metabotropic receptor agonist, cycloOP mimicked the protective action of ODN on H2O2-evoked cell death (Fig. 2A).

To determine if ODN is involved in the mechanism action mediated glioprotective effect of PACAP against H2O2 toxicity, we examined the effect of ODN receptors antagonists. Pre incubation of astrocytes for 30 min with the selective ODN metabotropic recepor antagonist, cyclo1-8[DLeu5]OP (1 µM) had no effect by itself on cell survival but totally abolished the glioprotective action of PACAP. In contrast, the CBR antagonist, flumazenil (1µM) had no effect (Fig. 2B).

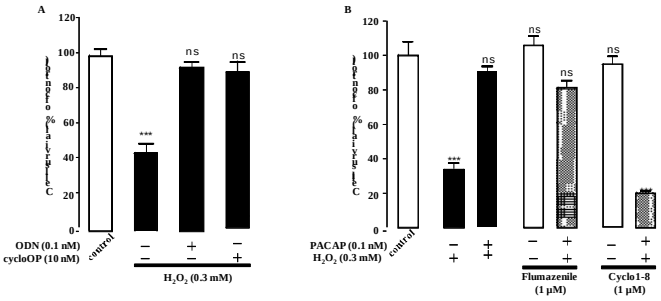



**Figure 2 :** (A) Protective effect of ODN and ODN metabotropic receptor agonist on astroglial cell death induced by H2O2. (B) Effect of central benzodiazepine receptor antagonist flumazenil and ODN metabotropic receptor antagonist cyclo1–8 [Dleu5] OP on PACAP-evoked glioprotection against H2O2-induced toxicity. Cells were preincubated for 30min in the absence or presence of flumazenil (1 µM) or cyclo1–8 [Dleu5] OP (1 µM) and then incubated for 1 h with medium alone or with H2O2 (0.3 mM) without or with PACAP (0.1 nM). The results are expressed as percentages of the control value. Each value is the mean (± SEM) of at least three independent experiments performed in quadruplicate. ANOVA followed by the Bonferroni's test: ***P < 0.001; NS, not statistically different from the control.

## DISCUSSION

It is clearly established that oxidative stress induced by H2O2 causes apoptosis in different cell types, including astroglial cells (Giffard and Swanson, 2005; Feeney et al., 2008). We have recently demonstrated that, PACAP, a neuropeptide that is known to protect neuron cells against neurotoxicity (Dejda et al., 2005), stimulates endozepines production by cultured rat astrocytes (Masmoudi et al., 2003; Masmoudi-Kouki et al., 2007). The present study demonstrates for the first time that PACAP exerts a protective effect on H2O2-treated astrocytes and this glioprotective effect of PACAP might be due in large part to the activation of ODN release.

Previous studies have shown that H2O2 induces neurotoxicity in cultured astroglial cells (Choi et al., 2007; Lu et al., 2008a; Lu et al., 2008b). In agreement with these reports, we observed that exposure of astrocytes to H2O2 provoked cell death in a concentration-dependent manner. The toxicity of H2O2 has also been described in other types of cells such as cortical neurons (Higgins et al., 2009) and hippocampal neurons (Feeney et al., 2008). In all these models, cell death exhibits the characteristic morphological and biochemical features of apoptosis and the neurotoxic effect of H2O2 on cultured cells can be prevented by neuroprotective agents (Brenneman et al., 2000; Shioda et al., 2004). The present study has shown that co-incubation of H2O2-treated astrocytes with the neuropeptide PACAP resulted in an increase in the number of surviving astrocytes, indicating that PACAP exerts a glioprotective action against oxidative stress-induced cells apoptosis.

There is now strong evidence that PACAP exerts direct neuroprotective actions on cultured neurons (Vaudry et al., 2000; Vaudry et al., 2002; Vaudry et al., 2003). In addition, PACAP can act indirectly by stimulating astroglial cells to generate various neurotrophic factors such as activity-dependent neurotrophic factor (ADNF), activity-dependent neuroprotective protein (ADNP), interleukin (IL) 1 and 6, neurotrophin-3, protease nexin-1 and RANTES (Brenneman et al., 2003; Dejda et al., 2005; Masmoudi-Kouki et

al., 2007). We have previously shown that cultured rat astrocytes contain and release substantial amounts of endozepines (Patte et al., 1999; Masmoudi et al., 2005; Tokay et al., 2008). Several lines of evidence indicate that the protective effect of PACAP against H2O2-induced astrocyte cells death could be mediated through activation of ODN release. We earlier found that subnanomolar concentrations of PACAP stimulates ODN-like peptides release from cultured astrocytes (Masmoudi et al., 2003). Here, we demonstrate that the gliopeptide ODN protects astrocytes upon H2O2 injury. Cyclo1-8OP mimicked the protective action of ODN, indicating that ODN prevents cells death through activation of its metabotropic rececptor. Addition of ODN metabotropic receptor antagonist totally suppressed the protective action of PACAP against H2O2 toxicity in cultured astrocyte. Taken together, these data indicate that the protective effect of PACAP against H2O2-induced cell death in astrocytes can be ascribed to activation of ODN receptors.

In conclusion, the present study has demonstrated that PACAP exerts a potent glioprotective effect against oxidative stress and indicate that, besides its direct protective effects, PACAP may also acts indirectly by stimulating the release of ODN that prevent astroglial cells death.

## Acknowledgments

This study was supported by the Research Unit 00-UR-08-01. Y.H. and H.K. were recipients of fellowships from the University of Tunis El Manar and a France-Tunisia exchange program CMCU-Utique program (to MA and MCT).

## REFERENCES

Ashur-Fabian, O., Giladi, E., Brenneman, D.E., Gozes, I., 1997. Identification of VIP/PACAP receptors on rat astrocytes using antisense oligodeoxynucleotides. J Mol Neurosci. 9, 211-222.

Brenneman, D.E., Spong, C.Y., Gozes, I., 2000. Protective peptides derived from novel glial proteins. Biochem Soc Trans. 28, 452-5.

Brenneman, D.E., Phillips, T.M., Hauser, J., Hill, J.M., Spong, C.Y., Gozes, I., 2003. Complex array of cytokines released by vasoactive intestinal peptide. Neuropeptides. 37, 111-9.

Brown, D.R., Mohn, C.M., 1999. Astrocytic glutamate uptake and prion protein expression. Glia. 25, 282-92.

Choi, J.H., Kim, D.H., Yun, I.J., Chang, J.H., Chun, B.G., Choi, S.H., 2007. Zaprinast inhibits hydrogen peroxide-induced lysosomal destabilization and cell death in astrocytes. Eur. J. Pharmacol. 571, 106-15.

Compère, V., Li, S., Leprince, J., Tonon, M.C., Vaudry, H., Pelletier, G., 2004. In vivo action of a new octadecaneuropeptide (ODN) antagonist on gonadotropin-releasing hormone gene expression in the male rat brain. Neuroscience. 125, 411–5.

Compère, V., Li, S., Leprince, J., Tonon, M.C., Vaudry, H., Pelletier, G., 2005. In vivo action of a new octadecaneuropeptide antagonist on neuropeptide Y and corticotropin-releasing hormone mRNA levels in rat. Mol. Brain Res. 141, 156–60.

Dejda, A., Sokolowska, P., Nowak, J.Z., 2005. Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. Pharmacol Rep. 57, 307-20.

Do-Rego, J.L., Mensah-Nyagan, A.G., Beaujean, D., Leprince, J., Tonon, M.C., Luu-The, V., Pelletier, G., Vaudry, H., 2001. The octadecaneuropeptide ODN stimulates neurosteroid biosynthesis through activation of central-type benzodiazepine receptors. J. Neurochem. 76, 128–38.

Feeney, C.J., Frantseva, M.V., Carlen, P.L., Pennefather, P.S., Shulyakova, N., Shniffer, C., Mills, L.R., 2008. Vulnerability of glial cells to hydrogen peroxide in cultured hippocampal slices. Brain Res. 1198, 1–15.

Gandolfo, P., Patte, C., Leprince, J., Thoumas, J.L., Vaudry, H., Tonon, M.C., 1997. The stimulatory effect of the octadecaneuropeptide (ODN) on cytosolic Ca2+ in rat astrocytes is not mediated through classical benzodiazepine receptors. Eur. J. Pharmacol. 322, 275–81.

Gandolfo, P., Patte, C., Thoumas, J.L., Leprince, J., Vaudry, H., Tonon, M.C., 1999. The endozepine ODN stimulates [3H]thymidine incorporation in cultured rat astrocytes. Neuropharmacology. 38, 725–32.

Giffard, R.G., Swanson, R.A., 2005. Ischemia-induced programmed cell death in astrocytes. Glia. 50, 299–306.

Grimaldi, M., Cavallaro, S., 1999. Functional and molecular diversity of PACAP/VIP receptors in cortical neurons and type I astrocytes. Eur J Neurosci. 11, 2767-72.

Guidotti, A., Forchetti, C.M., Corda, M.G., Konkel, D., Bennett, C.D., Costa, E., 1983. Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Proc. Natl. Acad. Sci. USA. 80, 3531–5.

Higgins, G.C., Beart, P.M., Nagley, P., 2009. Oxidative stress triggers neuronal caspase-independent death: endonuclease G involvement in programmed cell death-type III. Cell Mol Life Sci. 66, 2773-87.

Leprince, J., Gandolfo, P., Thoumas, J.L., Patte, C., Fauchere, J.L., Vaudry, H., Tonon, M.C., 1998. Structure-activity relationships of a series of analogues of the octadecaneuropeptide ODN on calcium mobilization in rat astrocytes. J. Med. Chem. 41, 4433–8.

Lu, L., Li, J., Zhu, Y., Mak, Y.T., Yew, D.T., 2008a. H2O2-induced changes in astrocytic cultures from control and rapidly aging strains of mouse. Int. J. Neurosci. 118, 1239–50.

Lu, M., Hu, L.F., Hu, G., Bian, J.S., 2008b. Hydrogen sulfide protects astrocytes against H(2)O(2)-induced neural injury via enhancing glutamate uptake. Free Radic. Biol. Med. 45, 1705–13.

Masmoudi-Kouki, O., Gandolfo, P., Castel, H., Leprince, J., Fournier, A., Dejda, A., Vaudry, H., Tonon, M.C., 2007. Role of PACAP and VIP in astroglial functions. Peptides. 28, 1753–60.

Masmoudi, O., Gandolfo, P., Leprince, J., Vaudry, D., Fournier, A., Patte-Mensah, C., Vaudry, H., Tonon, M.C., 2003. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates endozepine release from cultured rat astrocytes via a PKA-dependent mechanism. Faseb J. 17, 17–27.

Masmoudi, O., Gandolfo, P., Tokay, T., Leprince, J., Ravni, A., Vaudry, H., Tonon, M.C., 2005. Somatostatin down-regulates the expression and release of endozepines from cultured rat astrocytes via distinct receptor subtypes. J. Neurochem. 94, 561–71.

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun. 164, 567-74.

Nakamachi, T., Ohtaki, H., Yofu, S., Dohi, K., Watanabe, J., Hayashi, D., Matsuno, R., Nonaka, N., Itabashi, K., Shioda, S., 2008. Pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1R) co-localizes with activity-dependent neuroprotective protein (ADNP) in the mouse brains. Regul Pept. 145, 88-95.

Ohtaki, H., Nakamachi, T., Dohi, K., Shioda, S., 2008. Role of PACAP in ischemic neural death. J Mol Neurosci. 36, 16-25.

Patte, C., Gandolfo, P., Leprince, J., Thoumas, J.L., Fontaine, M., Vaudry, H., Tonon, M.C., 1999. GABA inhibits endozepine release from cultured rat astrocytes. Glia. 25, 404-11.

Shioda, S., Ohtaki, H., Suzuki, R., Nakamachi, T., Takenoya, F., Dohi, K., Nakajo, S., 2004. [Prevention of delayed neuronal cell death by PACAP and its molecular mechanism]. Nippon Yakurigaku Zasshi. 123, 243-52.

Tatsuno, I., Gottschall, P.E., Arimura, A., 1991. Specific binding sites for pituitary adenylate cyclase activating polypeptide (PACAP) in rat cultured astrocytes: molecular identification and interaction with vasoactive intestinal peptide (VIP). Peptides. 12, 617-21.

Tokay, T., Masmoudi, O., Gandolfo, P., Leprince, J., Pelletier, G., Vaudry, H., Tonon, M.C., 2005. Beta-amyloid peptides stimulate endozepine biosynthesis in cultured rat astrocytes. J. Neurochem. 94, 607–16.

Tokay, T., Hachem, R., Masmoudi-Kouki, O., Gandolfo, P., Desrues, L., Leprince, J., Castel, H., Diallo, M., Amri, M., Vaudry, H., Tonon, M.C., 2008. Beta-amyloid peptide stimulates endozepine release in cultured rat astrocytes through activation of N-formyl peptide receptors. Glia. 56, 1380–9.

Tonon, M.C., Leprince, J., Gandolfo, P., Compère, V., Pelletier, G., Malagon, M.M., Vaudry, H., 2006. Endozepines
In Kastin AJ, editor. Handbook of Biologically Active Peptides New York: Elsevier
813–819.

Vaudry, D., Gonzalez, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H., 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev. 52, 269-324.

Vaudry, D., Rousselle, C., Basille, M., Falluel-Morel, A., Pamantung, T.F., Fontaine, M., Fournier, A., Vaudry, H., Gonzalez, B.J., 2002. Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. Proc. Natl. Acad. Sci. USA. 99, 6398–403.

Vaudry, D., Falluel-Morel, A., Basille, M., Pamantung, T.F., Fontaine, M., Fournier, A., Vaudry, H., Gonzalez, B.J., 2003. Pituitary adenylate cyclase-activating polypeptide prevents C2-ceramide-induced apoptosis of cerebellar granule cells. J. Neurosci. Res. 72, 303 - 316

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 20 – 25*

**BIOLOGIA TUNISIE**

# OCTADECANEUROPEPTIDE ODN PROTECTS CULTURED ASTROCYTE AGAINST HYDROGEN PEROXIDE-INDUCED CELL DEATH

**Hamdi Yosra[a], Kaddour Hadhemi[a], Jenhani Faouzi[b], Leprince Jérôme [b,c,d], Tonon Marie-Christine [b,c,d], Amri Mohamed*[a] and Masmoudi-Kouki OLFA[a].**

aLaboratory of Functional Neurophysiology and Pathology, Research Unit 00/UR/08-01, Department of Biological Sciences, Faculty of Science of Tunis, University Tunis El Manar, 2092 Tunis, Tunisia

bLaboratory of Cellular Immunology and Cytometry, National Centre of Blood Transfusion, El Manar II 2092, Tunis, Tunisia

bInserm U982, Laboratory of Neuronal and Neuroendocrine Communication and Differentiation, Mont-Saint-Aignan, University of Rouen, 76128 Mont-Saint-Aignan, France

cInternational Associated Laboratory Samuel de Champlain dEuropean Institute for Peptide Research (IFRMP 23), Regional Platform for Cell Imaging (PRIMACEN), Rouen, France

**Keywords:** Astroglial cells, Cell death, Octadecaneuropeptide, Hydrogene peroxide.

## ABSRACT

Oxidative stress, resulting from accumulation of reactive oxygen species, plays a critical role on astroglial cell death associated with neurodegenerative diseases and stroke. Astroglial cells synthesize and release endozepines, a family of regulatory peptides including the octadecaneuropeptide ODN. It has been reported that endozepines are implicated in cellular proliferation and/or protection. Thus, the purpose of the present study was to investigate the potential protective effect of one ODN on H2O2-induced cell death in rat astrocytes. Incubation of cultured astrocytes with H2O2 (300 µM) for 1 h provoked a significant reduction of the number of living cells as evaluated by FDA and lactate dehydrogenase assay. Pretreatment of astrocytes with very low concentration of ODN (0.1 nM), prevents cell death induced by H2O2. Taken together, these data demonstrate that the endozepine for the first time that the endozepine ODN is a potent protective agent that prevents oxidative stress-induced apoptotic death.

## INTRODUCTION

Oxidative stress in living cells results from accumulation of reactive oxygen species (ROS) and free radicals such as hydrogen peroxide (H2O2) and hydroxyl radicals is implicated in several pathological processes including cerebral ischemia and neurodegenrative diseases (Halliwell, 1992; Hald and Lotharius, 2005). Studies conducted in various cell lines have shown that ROS can cause cell death by multiple mechanisms including damage of mitochondria leading to a decrease of ATP production, activation of caspases and DNA fragmentation (Vaudry et al., 2002; Chen et al., 2008). It is well known that astrocytes contain high levels of ROS scavengers molecules and antioxidant enzymes, which are not only involved in the protection of astroglial cells against the deleterious effects of ROS (Sokolova et al., 2001; Olesen et al., 2008), but may be also critical for neuron survival (Takuma et al., 2004; Watts et al., 2005). Although astrocytes are generally less susceptible to oxidative injury than neurons, there is strong evidence that oxidative stress also alters astrocyte functions (Feeney et al., 2008). Therefore, protection of astrocytes from oxidative assault appears essential to prevents neuronal damages in various pathological conditions involving oxidative neurodegeneration.

The octadecaneuropeptide (ODN) has been originally isolated from the rat brain and characterized as a endogenous ligand of benzodiazepine receptors (Ferrero et al., 1986).

ODN is generated by proteolytic cleavage of an 86-amino acid precursor called diazepam-binding inhibitor (DBI) (Guidotti et al., 1983) whose gene is mainly expressed in astroglial cells in the central nervous system (CNS) of vertebrates (Alho et al., 1995; Burgi et al., 1999). DBI and its processing products are collectively designated by the term endozepines (Tonon et al., 2006). The primary structure of ODN has been remarkably conserved during evolution (Tonon et al., 2006), suggesting that this peptide plays important biological functions. It has been reported that, ODN increase [3H]thymidine incorporation in cultured rat astrocytes (Gandolfo et al., 1999a). More recently we have shown that, exposure of cultured astrocytes to beta-amyloid peptide, the main constituent of senile plaques in Alzheimer Diseased (AD) brain, stimulates the biosynthesis and release of ODN-like peptide, suggesting that over production of ODN may contribute to astrocyte proliferation associated with AD (Tokay et al., 2005a; Tokay et al., 2008a). Altogether, these data suggest that endozepines may act as neurotrophic factors regulating proliferation and/or survival of astroglial cells under injury conditions. These observations prompted us to investigate the ability of ODN to counteract the neurotoxic effects of H2O2 on cultured astroglial cells.

## MATERIAL AND METHODS

### Cell Culture

Secondary cultures of rat cortical astrocytes were prepared as previously described (Brown and Mohn, 1999) with minor modifications. Briefly, cerebral hemispheres from newborn Wistar rats were collected in DMEM/F12 (2:1; v/v) culture medium supplemented with 2 mM glutamine, 1% insulin, 5 mM HEPES, 0.4% glucose and 1% of the antibiotic-antimycotic solution. Dissociated cells were resuspended in culture medium supplemented with 10% FBS, plated in 175-cm2 flask (Greiner Bio-one GmbH, Frickenhausen, Germany) and incubated at 37°C in a 5% CO2 / 95% O2 atmosphere. When cultures were confluent, astrocytes were isolated by shaking overnight the flasks with an orbital and plated on 35-mm Petri dishes at a density of 0.3 X 106 cells/ml. The cells were incubated at 37°C in a humid atmosphere (5% CO2). All experiments were performed on 5- to 7-day-old secondary cultures.

### Measurement of cell cytotoxicity

Astrocyte cells were incubated at 37°C with fresh serum-free medium in the absence or presence of test substances. At the end of the incubation, the cytotoxicity of H2O2 on astrocytes was determined by measurement of LDH activity in culture medium. The amount of LDH released into medium was measured by LDH assay kit (Bio-Maghreb) according to the manufacturer's instructions. The results were expressed as percentage of total LDH release after cell lysis with 1% Triton X-100 in PBS.

### Measurement of cell survival

Cultured cells were incubated at 37°C for 1 h with fresh serum-free culture medium in the absence or presence of H2O2 and/or ODN. FDA-AM (15 µg/ml) was added to the medium, and the cells were maintained at 37°C in the dark for 8 min. After washing twice with phosphate-buffered saline (PBS) cells were lysed with a Tris/HCl solution containing 1% SDS. Fluorescence was measured with excitation at 485 nm and emission at 538 nm, using a microplate reader (Bio-Tek FLx 800).

### Statistical analysis

Data are presented as the mean SEM from three independent experiments performed in quadruplicate or quintuplicate. Statistical analysis of the data was performed by using Student's t test and ANOVA, followed by Bonferroni's test.

## RESULTS

### Effect of ODN on H2O2-induced astrocyte cells death

Incubation of cultured astrocytes with 300 µM H2O2 for 1 h induced a significant increase of LDH levels in the culture medium. Pretratement of astroglial cells with ODN (0.1 nM) totally suppressed the effect H2O2 on LDH release (Fig. 1A), indicating that ODN increase in the number of surviving cells.

To determine whether ODN reduced H2O2-induced cell death, astrocytes were stained with FDA-AM and PI, markers of living and dying cells, respectively. Using flow cytometric

analysis, we observed very few cells labeled with PI in control (Fig. 2B), while incubation of astrocytes with 300 µM H2O2 induced a significant increase of PI fluorescence associated with a reduction of FDA fluorescence (Fig. 2C). Pretreatment of cells with 0.1 nM ODN for 10 min significantly reduced H2O2-induced cell death (Fig. 2D)

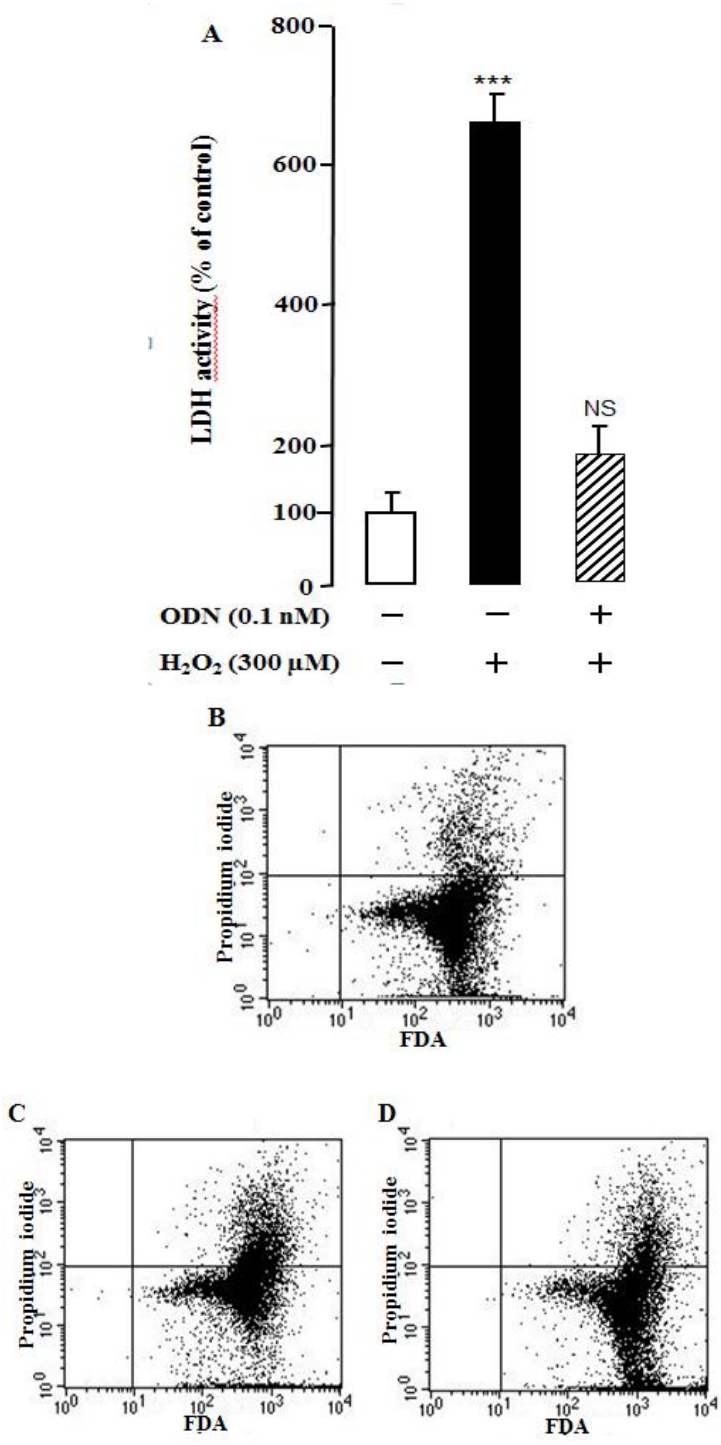



**Fig 1:** Effect of ODN on $H_2O_2$-induced cell death in cultured rat astrocytes. A. Cells were preincubated for 10 min in the absence or presence of 0.1 nM ODN and then incubated for 1 h with medium alone or $H_2O_2$ (300 µM) in the absence or presence of ODN. Each value is the mean (± SEM) calculated from at least 10 different wells from 3 independent cultures. ANOVA followed by the Bonferroni's test: ****P* < 0.001; NS, not statistically different from control. B. Cells seeded into Petri dishes were subjected to various treatments. At the end of the incubation, cells where loaded with FDA-AM and PI, and the fluorescence intensity was analyzed by flow cytometry.

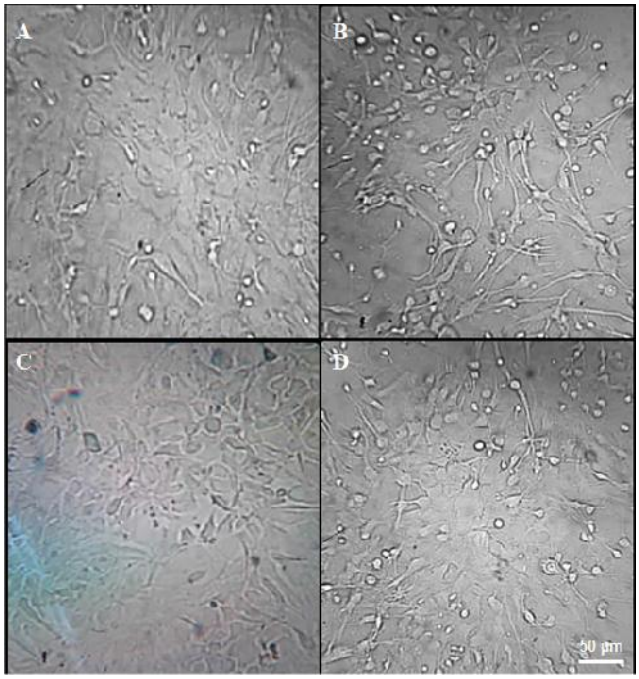



**Fig 2:** Phase-contrast images illustrating the effect of ODN on $H_2O_2$-induced changes in morphology of cultured rat astrocytes. Cells were preincubated for 10 min in the absence (A-B) or presence of 0.1 nM ODN (C-D), and then incubated for 1 h with 300 µM (B and D). Scale bar = 50 µm.

## DISCUSSION

It has been previously reported that oxidative stress induced by H2O2 causes apoptosis of different types of nerve cells, including glial cells (Giffard and Swanson, 2005; Lu et al., 2008b). We have recently demonstrated that ODN stimulates astrocytes proliferation (Tokay et al., 2005a), suggesting that ODN may be involved in the control of survival. In the present study we demonstrate that ODN can protect astroglial cells from apoptosis induced by exposure to H2O2.

In agreement with previous reports (Feeney et al., 2008; Lu et al., 2008a; Lu et al., 2008b), we observed that exposure of cultured astrocytes to a high concentration of H2O2 (300 µM) caused a massive cell death of astroglial cells. The sensitivity of astrocytes in this range of concentrations may be ascribed to their incapacity to clearing 300 µM H2O2 by catalase and/or peroxides activities. In accord with hypothesis, studies conducted on cultured astrocytes have shown that the rate of H2O2 clearance was strongly reduced in the presence of inhibitors of catalase and glutathione peroxidase activities (Dringen and Hamprecht, 1997b). Pretreatment of cultured astrocytes cells with the gliopeptide ODN totally suppressed H2O2-provoked LDH release. Visualization of living/dying cells by FDA-AM/PI staining by flow cytometry showed that subnanomolar concentrations of ODN significantly increase the number of surviving cells, indicating that the gliopeptide ODN can protect astrocytes against the deleterious effects of H2O2. Since Astroglial cells also possess an array of cellular defense systems, including glutathione peroxidase and catalase, to prevent damage caused by ROS, the protective effect of ODN against H2O2-induced cell death could be mediated through activation of the antioxidant enzyme system.

Examination of cultures by phase-contrast microscopy revealed that the cells death induced by H2O2 exhibited the characteristic features of apoptosis including cell shrinkage and DNA fragmentation (Joza et al., 2001). Pretreatment of cells with ODN (0.1 nM) restored the typical shape of differentiated astrocytes with flat polygonal cell bodies similar to that of untreated-astrocytes.

## CONCLUSION

In conclusion, the present study has demonstrated that ODN exerts a potent glioprotective effect against oxidative stress and might delay neuronal damages in various pathological conditions involving oxidative neurodegeneration.

## AKNOWLEGMENTS

Y.H., H.K. and R.H. were recipients of fellowships from the University of Tunis El Manar and a France-Tunisia exchange program CMCU-Utique. This study was supported by the Research Unit 00-UR-08-01 and the CMCU-Utique program (to MA and MCT).

## REFERENCES

Alho, H., Kolmer, M., Harjuntausta, T., Helen, P., 1995. Increased expression of diazepam binding inhibitor in human brain tumors. Cell Growth Differ. 6, 309–14.

Brown, D.R., Mohn, C.M., 1999. Astrocytic glutamate uptake and prion protein expression. Glia. 25, 282-92.

Burgi, B., Lichtensteiger, W., Lauber, M.E., Schlumpf, M., 1999. Ontogeny of diazepam binding inhibitor/acyl-CoA binding protein mRNA and peripheral benzodiazepine receptor mRNA expression in the rat. J. Neuroendocrinol. 11, 85–100.

Chen, S.H., Lin, J.K., Liu, S.H., Liang, Y.C., Lin-Shiau, S.Y., 2008. Apoptosis of cultured astrocytes induced by the copper and neocuproine complex through oxidative stress and JNK activation. Toxicol. Sci. 102, 138–49.

Choi, J.H., Kim, D.H., Yun, I.J., Chang, J.H., Chun, B.G., Choi, S.H., 2007. Zaprinast inhibits hydrogen peroxide-induced lysosomal destabilization and cell death in astrocytes. Eur J Pharmacol. 571, 106-15.

Dringen, R., Hamprecht, B., 1997a. Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. Brain Res. 759, 67-75.

Dringen, R., Hamprecht, B., 1997b. Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. Brain Res. 759, 67–75.

Dringen, R., Pfeiffer, B., Hamprecht, B., 1999. Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci. 19, 562-9.

Feeney, C.J., Frantseva, M.V., Carlen, P.L., Pennefather, P.S., Shulyakova, N., Shniffer, C., Mills, L.R., 2008. Vulnerability of glial cells to hydrogen peroxide in cultured hippocampal slices. Brain Res. 1198, 1–15.

Ferrero, P., Santi, M.R., Conti-Tronconi, B., Costa, E., Guidotti, A., 1986. Study of an octadecaneuropeptide derived from diazepam binding inhibitor (DBI): biological activity and presence in rat brain. Proc Natl Acad Sci U S A. 83, 827-31.

Gandolfo, P., Patte, C., Thoumas, J.L., Leprince, J., Vaudry, H., Tonon, M.C., 1999a. The endozepine ODN stimulates [3H]thymidine incorporation in cultured rat astrocytes. Neuropharmacology. 38, 725–32.

Gandolfo, P., Patte, C., Thoumas, J.L., Leprince, J., Vaudry, H., Tonon, M.C., 1999b. The endozepine ODN stimulates [3H]thymidine incorporation in cultured rat astrocytes. Neuropharmacology. 38, 725-32.

Gandolfo, P., Patte, C., Leprince, J., Do Régo, J.L., Mensah-Nygan, A.G., Vaudry, H., Tonon, M.C., 2000. The triakontatetraneuropeptide (TTN) stimulates thymidine incorporation in rat astrocytes through peripheral type benzodiazepine receptors. J Neurochem. 75, 701-707

Giffard, R.G., Swanson, R.A., 2005. Ischemia-induced programmed cell death in astrocytes. Glia. 50, 299–306.

Guidotti, A., Forchetti, C.M., Corda, M.G., Konkel, D., Bennett, C.D., Costa, E., 1983. Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Proc. Natl. Acad. Sci. USA. 80, 3531–5.

Hald, A., Lotharius, J., 2005. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? Exp. Neurol. 193, 279–90.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59, 1609–23.

Huang, J., Philbert, M.A., 1995. Distribution of glutathione and glutathione-related enzyme systems in mitochondria and cytosol of cultured cerebellar astrocytes and granule cells. Brain Res. 680, 16-22.

Jou, M.J., Jou, S.B., Chen, H.M., Lin, C.H., Peng, T.I., 2002. Critical role of mitochondrial reactive oxygen species formation in visible laser irradiation-induced apoptosis in rat brain astrocytes (RBA-1). J Biomed Sci. 9, 507-16.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L., Ferri, K.F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y.Y., Mak, T.W., Zuniga-Pflucker, J.C., Kroemer, G., Penninger, J.M., 2001. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. 410, 549-54.

Lu, L., Li, J., Zhu, Y., Mak, Y.T., Yew, D.T., 2008a. H2O2-induced changes in astrocytic cultures from control and rapidly aging strains of mouse. Int. J. Neurosci. 118, 1239–50.

Lu, M., Hu, L.F., Hu, G., Bian, J.S., 2008b. Hydrogen sulfide protects astrocytes against H(2)O(2)-induced neural injury via enhancing glutamate uptake. Free Radic. Biol. Med. 45, 1705–13.

Naval, M.V., Gomez-Serranillos, M.P., Carretero, M.E., Villar, A.M., 2007. Neuroprotective effect of a ginseng (Panax ginseng) root extract on astrocytes primary culture. J Ethnopharmacol. 112, 262-70.

Olesen, B.T., Clausen, J., Vang, O., 2008. Characterization of the transcriptional profile in primary astrocytes after oxidative stress induced by Paraquat. Neurotoxicology. 29, 13-21.

Raps, S.P., Lai, J.C., Hertz, L., Cooper, A.J., 1989. Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. Brain Res. 493, 398-401.

Sokolova, T., Gutterer, J.M., Hirrlinger, J., Hamprecht, B., Dringen, R., 2001. Catalase in astroglia-rich primary cultures from rat brain: immunocytochemical localization and inactivation during the disposal of hydrogen peroxide. Neurosci. Lett. 297, 129–32.

Takuma, K., Baba, A., Matsuda, T., 2004. Astrocyte apoptosis: implications for neuroprotection. Prog. Neurobiol. 72, 111–27.

Tokay, T., Masmoudi, O., Gandolfo, P., Leprince, J., Pelletier, G., Vaudry, H., Tonon, M.C., 2005a. Beta-amyloid peptides stimulate endozepine biosynthesis in cultured rat astrocytes. J. Neurochem. 94, 607–16.

Tokay, T., Masmoudi, O., Gandolfo, P., Leprince, J., Pelletier, G., Vaudry, H., Tonon, M.C., 2005b. Beta-amyloid peptides stimulate endozepine biosynthesis in cultured rat astrocytes. J Neurochem. 94, 607-16.

Tokay, T., Hachem, R., Masmoudi-Kouki, O., Gandolfo, P., Desrues, L., Leprince, J., Castel, H., Diallo, M., Amri, M., Vaudry, H., Tonon, M.C., 2008a. Beta-amyloid peptide stimulates endozepine release in cultured rat astrocytes through activation of N-formyl peptide receptors. Glia. 56, 1380–9.

Tokay, T., Hachem, R., Masmoudi-Kouki, O., Gandolfo, P., Desrues, L., Leprince, J., Castel, H., Diallo, M., Amri, M., Vaudry, H., Tonon, M.C., 2008b. Beta-amyloid peptide stimulates endozepine release in cultured rat astrocytes through activation of N-formyl peptide receptors. Glia.

Tonon, M.C., Leprince, J., Gandolfo, P., Compère, V., Pelletier, G., Malagon, M.M., Vaudry, H., 2006. Endozepines

In Kastin AJ, editor. Handbook of Biologically Active Peptides New York: Elsevier
813–819.

Vaudry, D., Pamantung, T.F., Basille, M., Rousselle, C., Fournier, A., Vaudry, H., Beauvillain, J.C., Gonzalez, B.J., 2002. PACAP protects cerebellar granule neurons against oxidative stress-induced apoptosis. Eur. J. Neurosci. 15, 1451–60.

Wang, X.F., Cynader, M.S., 2000. Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem. 74, 1434-42.

Watts, L.T., Rathinam, M.L., Schenker, S., Henderson, G.I., 2005. Astrocytes protect neurons from ethanol-induced oxidative stress and apoptotic death. J. Neurosci. Res. 80, 65–66.

*Biologia Tunisie Juillet 2012; N°7 ; 26 – 29*

BIOLOGIA TUNISIE

# ANTIOXIDANT ACTIVITY AND PHENOLIC EXTRACTS OF BLACK *(MORUS NIGRA L.)* AND WHITE *(MORUS ALBA L.)* MULBERRY FRUITS

Rebai Olfa[a,b*], Belkhir Manel[a,b], Amri Mohamed[a] and Fattouch Sami[b]

[a]Laboratory of Functional Neurophysiology and Pathology, 00/UR/08-01, Faculty of Science of Tunis, Tunisia.
[b]National Institute of Applied Sciences and Technology,Tunis, Tunisia. E-mail: olfa.rebai@yahoo.fr

***Keywords:*** Antioxidant; Flavonoïds; HPLC-DAD; Polyphenols; Mulberry.

**ABSTRACT**

Polyphenolic content (total phenolics, TP, and total flavonoids, TF) and antioxidant properties (TEAC) of black (*Morus nigra L.*) and white (*Morus alba L.*) mulberry fruits harvested from Tunisia were investigated. Black mulberry exhibited higher TP and TF content when compared to white mulberry. The TP contents of *M. nigra* and *M. alba* were 247 and 43 mg gallic acid equivalent in 100 g fresh weight (fw) basis, respectively. *M. nigra* had the richest amount of flavonoïds with 165.5 mg catechol equivalent/100g fw. Overall, both white and black mulberry aqueous-acetonic extracts had DPPH free radical scavenging effect with 44 and 36 mmol Trolox equivalent/100g fw, respectively. TEAC and TP were found significantly correlated ($r$ = 0, 97). The RP-HPLC-DAD technique allowed the identification of the phenolic compounds and revealed that catechin and chlorogenic acid were the major molecules.

## INTRODUCTION

Epidemiological studies have shown that diet rich in vegetables and fruits significantly reduce the incidence of chronic diseases such as cancer and cardiovascular disease (Riboli and Norat, 2003; Liu, 2003). The protective may be contributed by their antioxidant content (Lako *et al.*, 2007). Through additive and synergistic effects, the complex mixture of phytochemicals in vegetables and fruit may provide better protection than a single phytochemical (Liu, 2003; Heber, 2001). These highlight the importance of comprehensive antioxidants measurement in these foods.

Mulberry (*Morus sp*.) has been domesticated over thousands of years and has been adapted to worldwide areas of tropical, subtropical, and temperate zones. The most important grown polyphenols-rich mulberry species are *Morus alba*, and *Morus nigra*. Their fruits, roots and bark have been used in folk medicine (especially in Chinese medicine) to treat diabetes, hypertension, and anemia as well as arthritis. In Turkey, black mulberry fruits are used for treating mouth lesions.

Recently, white and black mulberries have gained an important position in the food industry due to their anthocyanins content (Ozgen *et al.*, 2007; Celik *et al.*, 2008; Zafra-Stone *et al.*, 2007). However, scientific studies on mulberry phytochemical properties are very limited and their biological and pharmacological effects remain poorly defined. In this study, white and black mulberry fruits harvested from Tunisia were investigated in a comparative manner.

## MATERIALS AND METHODS

### Plant material

Mulberry fruits of *M. nigra* and *M. alba* were sampled from across Tunisia. Fully mature fruits were harvested and transferred to the laboratory to store at -20C°.

### Extraction of total polyhenols

The modified method reported previously by Fattouch *et al.* (2007) was used. Briefly, sample was extracted with 4 volumes (w/v) of cold acetone/water (3:1). After centrifugation at 10000xg for 15 min at room temperature, the pooled collected

supernatants were rota-evaporated. The remaining aqueous solution was then vortexed for 5 min and filtered trough a 0.45 µm Teflon membrane (Millipore).To prevent polyphenols oxidation, extraction was achieved rapidly and extracts were immediately used. Samples were replicated three times.

## Determination of total phenolics (TP)

The Folin method was used as reported by Singleton and Rossi (1965). The extracts were combined with Folin-Ciocalteu phenol reagent, and incubated for 10 min followed by the addition of 7.5% sodium carbonate ($Na_2CO_3$). After incubation for 2 h, the absorbance was measured by a spectrophotometer at 725 nm. Gallic acid was used as standard and the results were expressed as mg gallic acid equivalent (GAE) per 100 g of fresh weight (fw).

## Determination of total flavonoids

Referring to the procedure described by Froehlicher *et al.* (2009), 1,5 mL of a 2% methanolic solution of $AlCl_3$ was added to 0,5 mL diluted sample and the mixture was kept in the dark for 10min. Absorbance was read at 430 nm using methanolic $AlCl_3$ as a blank. Catechol was used as standard and Flavonoïd amounts were expressed in mg catechol equivalent (CE)per 100 g fw.

## Phenolic compounds identification

Analysis of phenolic compounds was performed according to the method described by Fattouch *et al.* (2007) using a HPLC-DAD system. Optimum efficiency of separation was obtained using a 5% formic acid solution (solvent A), and pure methanol (solvent B) according to following sequence of linear gradients: start with 95 % (A) and 5% B, reach 75% (A) after 10 min, 65% (A) at 30 min, 55% (A) at 35 min, 55% (A) at 40 min, 50% (A) at 45 min, 45% (A) at 50 min, 30% (A) at 53 min, 25% (A) at 56 min, and 20% (A) at 60 min. Injection volume was 20 µL and detection was at 280 nm. Compounds were identified by comparing their retention times and spectral properties to those of standards, when available. Unknown chromatographic peaks were tentatively identified via their spectral features and referred to the literature.

## The total antioxidant activity (TEAC)

The Trolox equivalent antioxidant capacity was determined by the DPPH radical scavenging assay. A 25µL solution of extract appropriate dilution was added to 975 µL of a 40 µM methanolic solution of DPPH. After incubation for 30 min in the dark, the optical density was read at 517 nm using methanol as blank. Different dilutions of Trolox were used to set up the standard curve and TEAC was expressed in mmol Trolox equivalent/100 g fw.

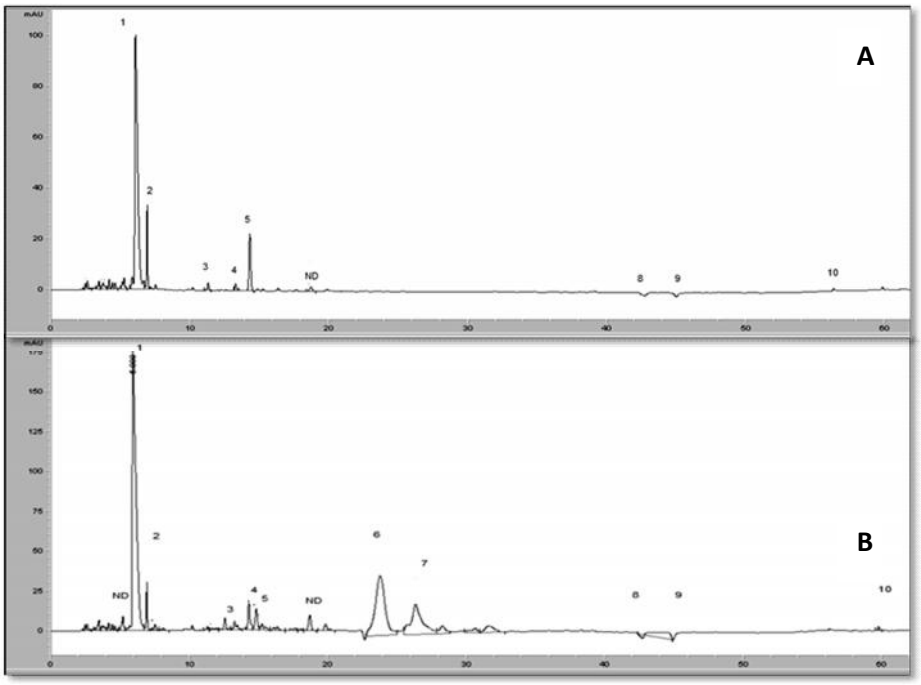



**Figure 1.** HPLC-DAD chromatograms (at 280 nm) of *Morus alba* (**A**) and *Morus nigra* (**B**) fruits aqueous-acetonic extracts. Peaks numbers corresponding to: **1**, (-)Cathechin; **2**, (+)Catechin; **3**, Chlorogenic acid; **4**, Cryptochlorogenic acid; **5**, Cathechin; **6**, Quercitine-3-di-glycoside; **7**, Quercitine dicoumaryl-glycoside; **8**, Kaempferol-3-rutinoside; **9**, Kaempferol; **10**, Isoquercitin.

## RESULTS AND DISCUSSION

The mulberry aqueous-acetonic extracts were subjected to a radical scavenging activity assay, employing the stable DPPH-radical, widely used to characterize the radical scavenging activity of a variety of natural polyphenols. In addition, we measured the gallic acid equivalent (GAE, also called 'total polyphenolic index') as a rough indication of the total amount of polyphenols, employing the Folin- Ciocalteu method. The total phenolics (TP), total flavonoïds (TF) and Trolox equivalent antioxidant capacity (TEAC) of the black and white Tunisian mulberry fruits were reported in Table 1. Here, it is worth noting that a fairly good correlation ($r$=0.79) was observed between the reciprocal of mmole equivalent Trolox and GAE values, suggesting that phenolic compounds are the major contributors to the observed TEAC.

**Table 1**: Total phenolics (TP), total flavonoïds (TF), and Trolox equivalent antioxidant capacity (DPPH scavenging activity) of black and white Tunisian mulberry fruits.

| | TPC (mg GAE/100g fw) | TF (mg CE/100g fw) | TEAC (mmol Trolox /100g fw) |
|---|---|---|---|
| *Morus nigra* | 247.05±72.2 | 165.6±10.2 | 44±7.01 |
| *Morus alba* | 43.33±4.76 | 13.22±5.22 | 36±6.01 |

Among the fruits investigated in this study, black mulberry had the greatest averages of TP, TF and TAC (Table 1). The white mulberry fruit extract had 83 % less TP content than *M. nigra*. Similar patterns were also observed for total flavonoïds, where *M. nigra* had a mean of 165.6 mg CE/100g fw, a finding in agreement with the report of Zadernowski *et al.* (2005). The range of TP between 43.33 and 247.05 mg GAE/100 g fw was comparable to what reported by Bae *et al.* (2007). These authors analyzed several cultivated mulberries and a wild variety and showed that this fruit contained relatively high levels of total phenolic substances (95.9-223.5 mg GAE/100 g fw). It is assumed that polyphenols contents of mulberry fruits can be somewhat variable, depending on the cultivar and the bioclimatic area of cultivation (Bae *et al.*, 2007). Besides, Ercisli and Orhan (2007) reported that total phenolics and total flavonoïds in fresh *M. alba* fruit were found about 181 mg GAE and 29 mg quercetin equivalent per 100 g on dry weigh basis, respectively. Polyphenolics and Flavonoïds are considered as functional components and effective antioxidants enhancing chances of using mulberry phenolic extracts for supplementation.

Individual phenolic compounds contents of Tunisian black and white mulberries polyphenolic extracts are presented in Figure1. Both aqueous-acetonic extracts contained phenolic acids and flavonols. (+)catechin and (-)catechin were the most abundant compounds, but slightly higher in white mulberry compared to the black fruit. Chlorogenic acid was detected to be the major phenolic acid in the two extracts; quercitine esters quercitine-3-di-glycoside and quercitine dicoumaryl-glycoside were only detected in black mulberry, suggesting a polymorphic scheme between the two tested species. Our results demonstrate that there are qualitative and quantitative differences between white and black mulberry fruits. In addition, *M. nigra* fruits had the highest TEAC, with an average of 44 mmol Trolox equivalent/100g fw using the DPPH scavenging method. Similarly, Ercisli and Orhan (2008) found that the antioxidant activity in selected black mulberries was important when using different antioxidant methods.

In our study, we noticed a high correlation coefficient between TP and TEAC. Such positive correlation is well established between antioxidant activity and polyphenols constituent as reported in earlier studies of small fruit phytonutrient contents (Konczak *et al.*, 2003).

## CONCLUSION

In this study, we compared and characterized the phenolic content and antioxidant capacity of two mulberries species, the black *M. nigra* and the white *M. alba*. Different characteristics were displayed by the two mulberry species. In addition to the high antioxidant capacity, black mulberry had high flavonoids and phenolics content, which may increase its popularity among the other mulberries and even among other fruits in general. These results, demonstrating superior horticultural and phytonutrient traits of mulberries, may also provide a basis for planning breeding strategies as well as selecting cultivars with high phytonutrient profiles and antioxidant capacities as functional foods for consumers. However, more detailed biological and pharmacological studies are still needed for additional clarification and better understanding of the health benefits of phenolic substances - rich mulberries.

## References

Celik, H., Ozgen, M., Serce, S. and Kaya, C. (2008). Phytochemical accumulation and antioxidant capacity at four maturity stages of cranberry fruit. *Science Horticulture*, 117: 345-348.

Ercisli, S. and Orhan, E. (2007). Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits. *Food Chemistry*. 103: 1380-1384.

Ercisli, S. and Orhan, E. (2008). Some physico-chemical characteristics of black mulberry (*Morus nigra* L.) genotypes from Northeast Anatolia region of Turkey. *Science Horticulture*, 116: 41-46.

Fattouch, S., Caboni, P., Coroneo, V., Tuberoso, C., Angioni, A., Dessi, S., Marzouki, N. and Cabras, P. (2007). Antimicrobial activity of Tunisian quince (*Cydonia oblonga* Miller) pulp and peel polyphenolic extracts. *Journal of Agricultural and Food Chemistry*, 55: 963-969.

Konczak, I.I, Okuno, S., Yoshimoto, M. and Yamakawa, O. (2003). Composition of phenolics and anthocyanins in a sweet potato cell suspension culture. *Biochemical Engineering Journal*, 14: 155-161.

Liu, R.H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal of Clinical Nutrition*, 78: 517S–520S.

Lako, J., Trenerry, V. C., Wahlqvist, M., Wattanapen-paiboon, N., Sotheeswaran, S. and Premier, R. (2007).Phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Food Chemistry*, 101: 1727-1741.

Ozgen, M., Serce, S., Gunduz, K., Yen, F., Kafkas, E. and Paydas, S. (2007). Determining total phenolics and antioxidant capacities of selected Fragaria genotype. *Asian Journal of Chemistry*, 19: 5573-5581.

Riboli, E. and Norat, T. (2003). Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *American Journal of Clinical Nutrition*, 78: 559-569S.

Zadernowski, R., Naczk, M. and Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *Journal of Agricultural and Food Chemistry*, 53: 2118-2124.

Shahidi, F. and Naczk, M. (2004). Antioxidant properties of food phenolics. In Phenolics in food and nutraceuticals. Boca Raton: CRC Press. p 403-437.
Bae, S-H. and Suh H-J. (2007). Antioxidant activities of five different mulberry cultivars in Korea. *LWT*, 40: 955-962.

Froehlicher, T., Hennebelle, T., Martin-Nizard, F., Cleenewerck, P., Hilbert, J-L., Trotin, F. and Grec, S. (2009). Phenolic profiles and antioxidative effects of hawthorn cell suspensions, fresh fruits, and medicinal dried parts. *Food Chemistry*, 115: 1747-1761.

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J.A. and Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition and Food Research*, 51: 675–683.

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 30– 36*

**BIOLOGIA TUNISIE**

# CARACTERISATION DE L'AGENT CAUSAL DU MAL SECCO DE L'ORANGER ET EVALUATION DE L'ANTAGONISME BACTERIEN VIS-A-VIS DU PHOMA TRACHEIPHILA

**Leila Kalai1, Monia Mnari-Hattab[2], Najla Sadfi-Zaoui[3], Mohamed Rabeh Hajlaoui[2]**

1 Laboratoire des Substances Bioactives, Centre de Biotechnologie de Borj Cedria, BP-901, 2050 Hammam-lif, Tunisie

2 Laboratoire de Protection des Plantes, INRA Tunisie, 2049 Hedi Karray, Tunis, Tunisie

3 Laboratoire Microorganismes et Biomolécules Bioactives, Faculté des Sciences de Tunis, Université Tunis El Manar, Tunisie

**Résumé**

Le mal secco est une trachéomycose acropétale dont l'agent causal s'attaque aux vaisseaux des citrus provoquant le dessèchement progressif de l'arbre. Cette maladie infecte généralement, les genres Citrus, Poncirus, Severinia, et Fortunella. Elle est plus virulente sur citronnier et cédratier et attaque très rarement les orangers et les clémentiniers. La caractérisation morphologique et moléculaire de l'agent causal isolé de l'oranger (cv. Thomson) a révélé une similitude du point de vue morphologie et virulence avec les isolats provenant du Citronnier.

Par ailleurs, l'alignement de la séquence de l'isolat tunisien HQ231345 avec 45 séquences d'isolats se trouvant dans les banques de gènes internationales a révélé une très forte homologie de séquence allant de 97 à 100%. Cette faible variabilité au niveau de la région séquencée de l'isolat tunisien confirme le développement clonale de cet agent pathogène.

D'autre part l'étude de l'antagonisme bactérien à l'égard de P. tracheiphila a montré que des isolats halophytes de Bacillus K2-5, M1-20 et J9 sont capables d'inhiber la croissance du pathogène de 2,87%, 26% et 52,97% respectivement. La cinétique d'inhibition a montré une meilleure efficacité de l'isolat J9 à inhiber la croissance du pathogène. Cette bactérie pourrait être un bon candidat pour la lutte biologique contre le mal secco des citrus.

## INTRODUCTION

Les Agrumes représentent la production fruitière la plus importante du monde et sont classés parmi les fruits les plus cultivés en Tunisie car ils occupent une place importante dans la vie socio-économique (Lakhoua, 1997). En dépit de la tradition tunisienne de produire les agrumes, le secteur agrumicole continue à affronter certains problèmes aussi bien à l'échelle de la production qu'à l'échelle de la commercialisation, en particulier au niveau du marché d'exportation.

Le dernier projet FAO (1995-1998) consacré au secteur Agrumes a permis une amélioration notable de l'assortiment variétale et l'étalement de la période de production d'Octobre à Juin. Toutefois, la production nationale en Agrumes et particulièrement en citrons est restée en deçà du niveau souhaité.

La fertigation et le contrôle phytosanitaire des Agrumes, deux aspects phytotechniques non encore maîtrisés par les producteurs, sont à l'origine de la faible productivité de ce secteur. En effet, l'intensification des vergers du citronnier a entraîné la recrudescence du mal secco (Phoma tracheiphila (Petri) L.A. Kantsch. & Gikaschvili) qui est devenue la principale menace de l'extension de la citriculture en Tunisie.

Cette maladie est une trachéomycose acropétale dont l'agent causal s'attaque au bois provoquant un dessèchement qui commence par les feuilles

des extrémités des rameaux pour finalement provoquer l'apoplexie de tout l'arbre. Le mal secco infecte en général, les genres Citrus, Poncirus, Severinia, et Fortunella. Mais il est plus virulent sur Citronnier et sur Cédratier (Whiteside et al., 1988). Généralement, toutes les espèces qui appartiennent aux genres cités ci-dessus, sont sensibles aux infections artificielles de P tracheiphila mais en vergers, leur degré d'infection est variable (Anonyme, 2003). Par contre, les oranges douces et les clémentiniers sont rarement attaqués par le mal secco (Whiteside et al., 1988, Hajlaoui et al., 2007).

Les moyens de détection couramment utilisées en Tunisie restent assez rudimentaires et se basent sur le diagnostic visuel, le test KOH et l'isolement du champignon (Chapot, 1963 ; Pionnat, 1982; Anonyme, 2003). En l'absence de moyens de lutte efficaces, cette affection parasitaire peut entraîner une mortalité totale des sujets peu de temps après leur contamination.

Devant l'ampleur des dégâts et en présence de chiffres aussi alarmants, les producteurs paraissent impuissants et les scientifiques restent perplexes. En effet, cette maladie demeure incurable et tous les moyens de lutte utilisés, restent vains. Ceci est dû à l'absence de produits actifs contre l'agent pathogène Phoma tracheiphila et à la sensibilité des variétés et des porte-greffes utilisés. Dans ce contexte, la lutte biologique pourrait être un nouveau moyen de lutte à tester.

Au cours du présent travail nous nous sommes proposés de caractériser l'agent du mal secco isolé sur oranger et de tester in vitro l'effet de bactéries halophytes à inhiber la croissance de l'agent pathogène.

## MATERIEL & METHODES

### Diagnostic du mal secco

Test KOH

Des rameaux provenant d'oranger (cv. Thomson) de la région du Cap Bon fraîchement prélevés à partir des brindilles symptomatiques, ont été découpés selon la transversale et trempés pendant 30 secondes dans une solution de KOH à 1% pour révéler la coloration rouge saumon caractéristique de la présence de Phoma tracheiphila (Chapot, 1963; Pionnat, 1982).

Isolement

L'isolement du champignon à partir du bois infecté a été réalisé selon le protocole établi par l'EPPO (2005). Les rameaux infectés ont été découpés en petits morceaux et désinfectés par l'eau de javel 1% suivi d'un rinçage à l'eau distillée stérile et séchés à l'aide de papier filtre stérile. Les morceaux de bois ont ensuite été repiqués sur milieu PDA (Potato Dextrose Agar) et mises en incubation à 25°C pour accélérer le passage du champignon du bois vers le milieu synthétique. Après purification des colonies, la conservation à long terme du champignon a été réalisée dans 30% de glycérol et 70% de PDB (Potato Dextrose Broth) à - 80°C et son maintien à court terme a été effectuée sur PDA à 4°C. Pour une utilisation de routine, les isolats sont cultivés sur PDA et incubés à 25°C.

### Pathogénie

Pour vérifier l'implication de l'isolat provenant d'oranger (cv. Thomson) dans la maladie, des inoculations foliaires de jeunes plants de bigaradier ont été assurées par des micro blessures infligées aux feuilles près des nervures principales au niveau desquelles, une gouttelette de suspension sporale ajustée à 106 spores/ml a été déposée délicatement par une micro seringue de façon à ce qu'elle adhère à la feuille.

Les plantes ont par la suite été ensachées pour créer une humidité saturante et transférées sous serre à 20°C les premières 24 heures puis à 25°C pour la suite de l'expérience.

### Caractérisation moléculaire du P. tracheiphila

Extraction et amplification de l'ADN

L'extraction de l'ADN à partir du mycélium de P. tracheiphila a été réalisée selon le protocole décrit par Kirkpatrick et al. (1987). L'amplification de la région ITS-5,8S-ADNr a été réalisée à l'aide des amorces universelles ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') et ITS4 (5'-TCCTCCGCTTATTGATATGC-3') développées par White et al. (1990). L'amplification a été réalisée dans un volume de 50 µl contenant 1 x tampon PCR (Promega), 200 µM de chaque dNTP (Promega), 1,5 mM MgCl2, 1 µM de chaque amorce (Opéron), 1U de green go Taq DNA polymerase (Promega) et 10 ng d'ADN génomique.

La PCR a été réalisée dans un thermocycleur Biometra programmé comme suit: dénaturation initiale à 94°C pendant 5 min; suivi de 31 cycles (dénaturation à 94°C pendant 1 min, hybridation à 50°C pendant 1 min et 30 s et extension à 72°C pendant 1 min et 30 s); et terminée par une extension finale à 72ºC pendant 7 min.

La migration des amplifias a été réalisée par électrophorèse sur gel d'agarose à 1,5% contenant 0,03 µl ml-1 de Bromure d'éthidium (BET), soumis à un voltage de 120v-100mA et visualisée par le gel doc (BIO -RAD) sous UV.

Séquençage de l'ADN fongique

L'isolat (Pt2), provenant d'oranger (cv. Thomson) de la région du Cap Bon a été sélectionné pour le séquençage et la suite de l'étude en raison de sa croissance et sporulation rapide. Un fragment d'environ 600 pb obtenu suite à l'amplification de la région ITS-5,8S-ADNr a été purifié (Wizard PCR DNA purification system, Promega). L'amplicon a été introduit par ligation dans le plasmide pGem-T Easy vector (Promega) ayant une taille de 3015 bp. Les cellules compétentes d'Escherichia coli (souche JM 109, Promega), ont été transformées avec le plasmide recombinant résultant puis cultivées sur le milieu LB (Luria-Bertani) additionné de X-gal pour la sélection des colonies bleues transformées.

Les deux plasmides recombinants issus de deux colonies indépendantes ont été digérés afin de vérifier la présence de l'insert puis purifiés en utilisant le Qiaprep Spin Miniprep kit (Qiagen) pour le séquençage. Ainsi, l'insert des deux clones différents a été séquencé dans les deux sens en utilisant les amorces du vecteur T7 Prom TAATACGACTCACTATAGGG et Sp6 ATT-TAG-GTG-ACA-CTA-TAG à l'aide d'un séquenceur automatisé type ABI3730 (Applied Biosystems) chez Génome express.

Analyse des séquences

Les séquences nucléotidiques obtenues ont été alignée avec ClustalW (Thompson et al., 1994) et comparée avec la base de données NCBI. Une seule séquence a été déposée dans les banques de gènes.

Isolement et identification des bactéries utilisées pour la lutte biologique

Trois isolats bactérien connus sous les diminutifs (J9, K2-5 et M1-20) isolés du Sud Tunisien (Chott Djerid) et identifiés comme appartenant à l'espèce Bacillus subtilis par Sadfi et al., (2008) ont été utilisées pour évaluer leur pouvoir antagoniste vis-à-vis de P. tracheiphila. Ces bactéries ont été cultivées sur un milieu TSA (Tryptic Soy Agar) et incubées à 30°C. La conservation des isolats a été réalisée dans un milieu liquide à base de Bouillon nutritif à 75% et de glycérol à 25% à une température de -20°C.

**Test d'antagonisme in vitro**

L'isolat Pt2 de P. tracheiphila dont la virulence a été vérifiée par inoculation artificielle, a été sélectionné pour la confrontation bactérie-champignon à raison de 5 répétitions par bactérie. Pour cela, deux disques mycéliens de 1 cm de diamètre ont été découpés à partir d'une jeune culture de Pt2 puis déposés sur milieu PDA équidistants d'un strie bactérien, réalisé au centre de la boîte, le champignon ayant été incubé 72 h avant la bactérie antagoniste. Les rayons d'inhibition (R1 et R2) ont par la suite été enregistrés quotidiennement pour le calcul du pourcentage d'inhibition G selon la formule suivante élaborée par Whipps, (1987):

G= [(R1-R2)/R1]*100 (R1= diamètre de la colonie du champignon, du centre du disque vers l'extrémité de la boite ; R2= diamètre de la colonie du champignon, du centre du disque vers la bactérie). Finalement, les niveaux d'inhibition ont été classés selon l'échelle élaborée par Korsten et al., (1995). L'effet antagoniste des 3 bactéries sur la croissance du P. tracheiphila a été estimé selon l'erreur standard ($\sigma n = \sigma/\sqrt{n}$ ; n : nombre de répétitions).

## RESULTATS

**Diagnostic du mal secco**

Le test KOH appliqué sur des rameaux montrant le début de dessèchement s'est révélé positif en donnant une coloration saumon du bois (Fig 1d). A partir de ces rameaux symptomatiques, des isolements ont été réalisés à partir du bois. Les premières colonies ont été obtenues au bout de 7 à 9 jours (Fig. 1a). L'identification de l'agent pathogène a été basée sur les critères morphologiques des pycniospores tels que décrits par Cicarone (1971) et Petri (Punithalingam et Holliday, 1973) (Fig 1 b et c) ainsi que sur les techniques traditionnelles de diagnostic (test de pathogénie et test KOH) et

confirmée par les techniques moléculaires. La pathogénie des isolats a été vérifiée par inoculation foliaire sur jeunes plants de bigaradier. Dix jours après inoculation, un halo jaune entourant le point d'inoculation se forme et progresse pour atteindre les nervures entraînant plus tard un dessèchement acropète de toute la plante (Fig 1e).

P. tracheiphila isolé de l'oranger parait donc très similaire du point de vue morphologie et virulence par rapport aux isolats obtenus précédemment à partir du genre Citrus.

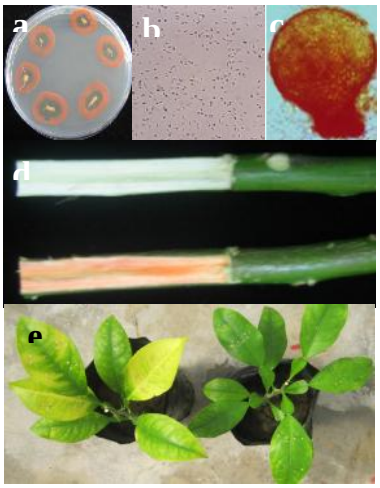



**Figure 1: Identification symptômatique du Phoma tracheiphila, agent du Mal secco**

a: Isolement du champignon à partir du bois infecté; b et c: Pycniospores et pycnide du Phoma tracheiphila; d: Test KOH positif sur un rameau infecté à gauche et négatif sur un rameau sain à droite. ; e : Test de pathogénie sur feuilles montrant le jaunissement des nervures chez le plant inoculé1 comparé au plant non inoculé 2.

## Caractérisation moléculaire du Phoma tracheiphila

La digestion du plasmide recombinant par Not1, a confirmé l'existence d'un insert de 630 pb (Fig 2 a et b). Le séquençage des deux clones transformés a permis l'obtention de 2 séquences identiques de 584 nucléotides. Une seule séquence a été déposée dans les banques de gènes sous le numéro d'accession HQ231345. Cette dernière présente 100% d'homologie avec les isolats de Phoma tracheiphila italiens du citronnier et du mandarinier respectivement AF272554- AF272552 (Fig 3).

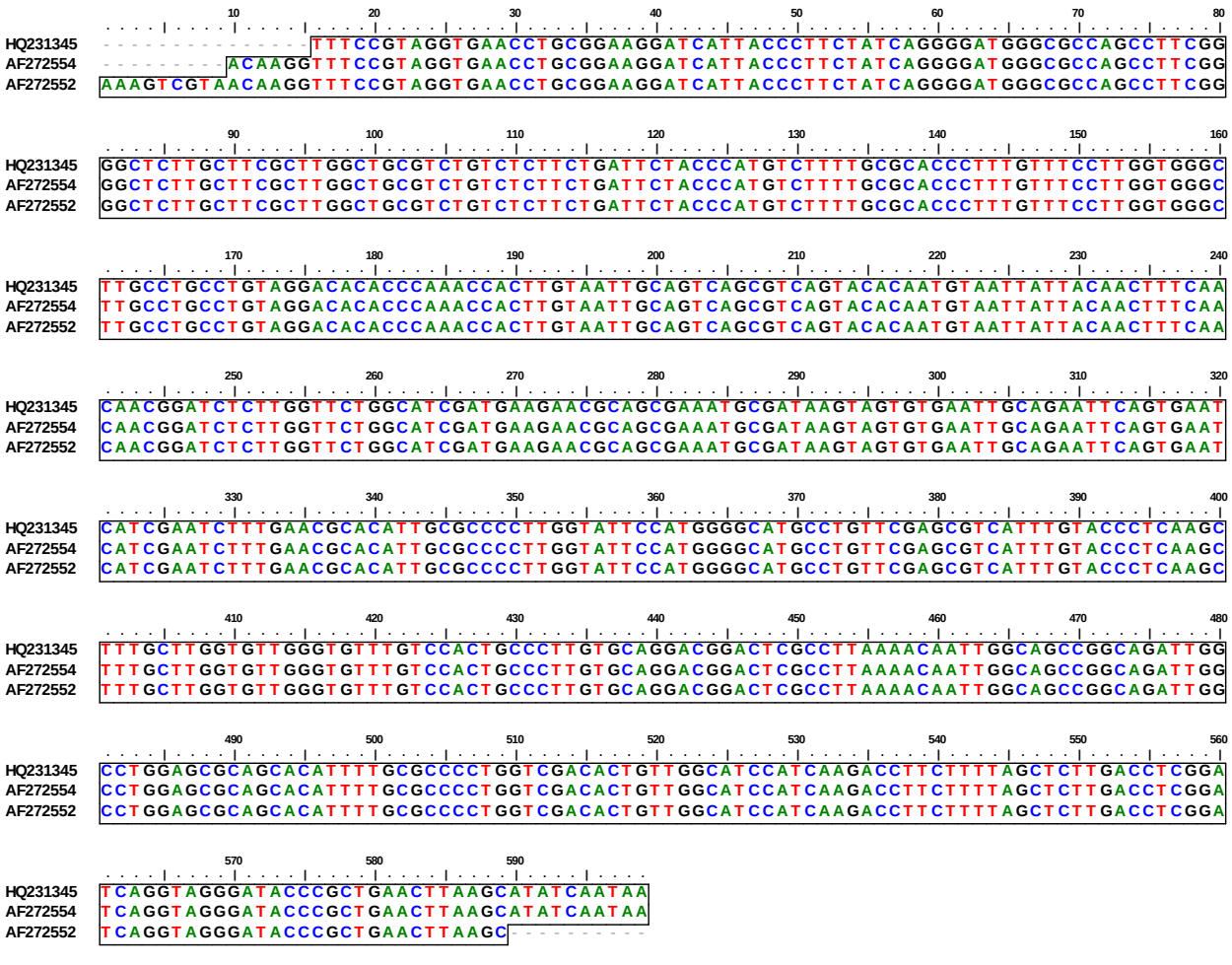


**Figure 3: Alignement multiples de la séquence** nucléotidique de l'isolat tunisien du *Phoma tracheiphila* Pt2 (HQ231345) par rapport à celle *du Phoma tracheiphila* italien isolé du citronnier AF272554 et du *Phoma tracheiphila* italien isolé du mandarinier AF272552.

Par ailleurs, l'alignement de la séquence de l'isolat tunisien HQ231345 avec au moins 45 séquences d'isolats se trouvant dans les banques de gènes internationales a révélé une très forte homologie de séquence allant de 97 à 100%. Cette faible variabilité au niveau de la région séquencé de l'isolat tunisien confirme les résultats de la caractérisation moléculaire publiés par Balmas et al., (2005) Ezra et al. (2007) et Kalai et al., (sous presse). Ainsi, soit le Phoma tracheiphila est naturellement très stable ou il faudra sélectionner d'autres régions afin d'étudier la variabilité des séquences en liaison avec le pouvoir pathogène de ce dernier. Cette homologie de séquence entre isolats de différentes régions géographiques et isolés sur différentes plantes hôtes montre le caractère clonale de ce Deutéromycètes.

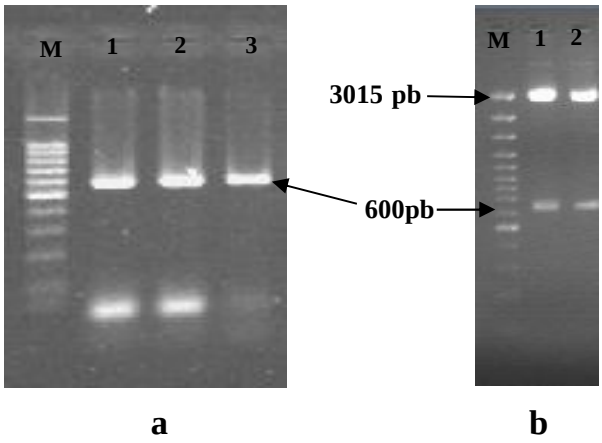



**Figure 2: Identification moléculaire du Phoma tracheiphila, agent du Mal secco** : a : Amplification de la région des ITS-5.8ADNr chez les deux clones1 et 2 de Pt2 (M1 : marqueur de taille 100pb (promega); b : Digestion du plasmide recombinant avec not1 (M2 : marqueur de taille (promega).

**Antagonisme bactéries-P. tracheiphila**

Devant l'inefficacité de la chémiothérapie contre l'agent du mal secco, la recherche d'autres moyens de lutte, en l'occurrence l'antagonisme microbien, reste la voie la plus plausible pour réduire l'ampleur de cette affection parasitaire des Citrus. La confrontation sur milieu gélosé du P. tracheiphila et des bactéries halophytes montre que les trois isolats bactériens se sont largement distingués à ce niveau. En effet, K2-5 n'a enregistré qu'un faible pourcentage d'inhibition (2,87%) correspondant au 1er niveau sur l'échelle de Korsten et al., (1995), alors que M1-20 avec un pourcentage atteignant les 26% est considérée comme appartenant au deuxième niveau d'efficacité. Finalement, la bactérie J9 a le pourcentage d'inhibition le plus important soit 52,97% (Fig 4).


**Figure 4: effet des différentes bactéries sur le Phoma tracheiphila vitro**
a: Phoma tracheiphila ; b : J9 et c: M1-20

L'évolution de la croissance radiale R1 et R2 du P. tracheiphila en co-culture avec les deux meilleures bactéries antagonistes respectivement M1-20 et J9 a été enregistrée en fonction du temps. Pour la souche M1-20, l'évolution de R1 et R2 ne présente pas de différences significatives les premiers jours. Une faible différence apparaît à partir du 13eme jour d'incubation, avec une nette différence au 16ème jour (Fig 5).

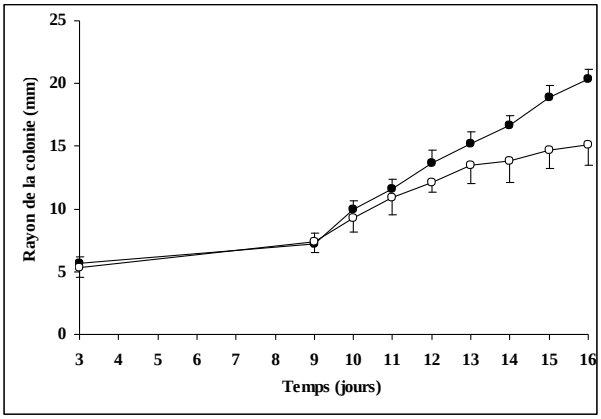
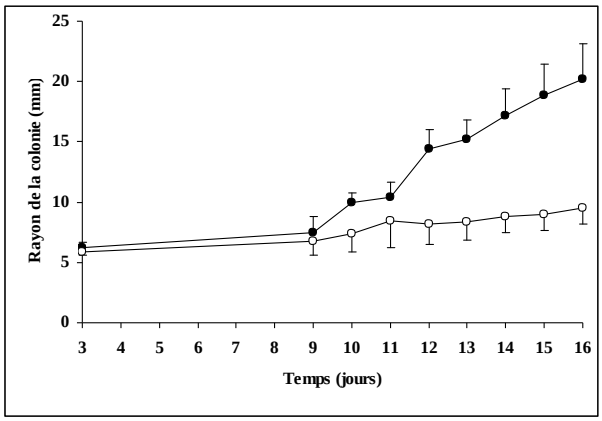



**Figure 5: Evolution de la croissance du *P. tracheiphila* en présence de la souche M1-20 de *Bacillus***
● Moyenne R1
○ Moyenne R2

Ceci pourrait être expliqué par le fait que la bactérie ne s'est mise à agir qu'après un certain délai, peut être en libérant des métabolites secondaires à action lente et localisée. En effet, il a été démontré dans des travaux antérieurs que la bactérie M1-20 est productrice de chitinase qui est une enzyme qui dégrade la chitine constituant majeure de la paroi des Deutéromycètes (Sadfi et al., 2008).

Concernant la confrontation J9 - P. tracheiphila, dès les premiers jours qui ont suivi le repiquage des deux protagonistes, les deux courbes correspondantes aux rayons R1 et R2 ont commencé à se distinguer avec une évolution plus rapide de R1. Cependant, c'est à partir du 10eme jour d'incubation qu'une nette différence entre ces deux paramètres peut être observée. En effet, comparativement, R2 évolue beaucoup plus lentement que R1 puisqu'en présence de J9, P. tracheiphila n'a pu gagner que 3,15 mm atteignant un maximum de 9,5 mm en 16 jours alors qu'à la même date le R1 a cumulé 13,95 mm c'est-à-dire 4,5 fois le R2 avec un maximum de 20,2 mm (Fig 6).




Cette bactérie agirait donc à distance sur l'agent pathogène via la production dans le milieu de culture de substances diffusibles. Etant donné que la J9 est aussi productrice de chitinase (Sadfi et al., 2008), la différence du mode d'action entre les deux bactéries testées serait donc le résultat d'une production beaucoup plus importante de chitinase chez ( J9) ou alors de la sécrétion d'autres substances bioactives qui sont connus pour leur effet fongitoxique ou encore les deux hypothèses à la fois. En effet, les Bacillus ont en moyenne 4-5% de leur génome dévoué à la synthèse d'antibiotique diverses. Parmi ces derniers, les lipopeptides sont les plus connus pour leur utilisation pharmaceutique et biotechnologique. Ils sont entre autre, responsables de la formation de pores au sein des parois cellulaires due à une perturbation osmotique de cette dernière comme c'est le cas pour les iturines ou alors de sa solubilisation causée par les surfactines (Aranda et al., 2005) ou encore de l'altération de sa structure causée par une interaction des fengycines avec la couche lipidique (Deleu et al., 2005).
Ces hypothèses méritent d'être plus étudiée pour parvenir à élucider le mode d'action de ces bactéries, afin d'extraire leur substances bioactives et d'optimiser leur production. L'isolat J9 de B. subtilis semble être le meilleur candidat pour des essais ultérieurs in vivo visant à combattre biologiquement le mal secco.

## CONCLUSION

Le Mal secco demeure la maladie des Citrus la plus préoccupante vu l'ampleur des dégâts qu'elle provoque sur toutes les variétés de citronnier, son incurabilité et son mode de transmission et de dissémination.
Devant l'absence de la résistance variétale et l'inefficacité de la lutte chimique contre cet agent pathogène, d'autres alternatives de lutte, en l'occurrence la lutte biologique pourrait être envisagée. Les résultats du test d'antagonisme in vitro, ont révélé que des bactéries halotolérantes du genre Bacillus ont inhibé la croissance de l'agent pathogène de plus de 53%. L'efficacité de ces agents de lutte biologique mérite d'être évaluée in planta sur des plants inoculés par l'agent pathogène.
Par ailleurs, il serait intéressant d'étudier le mode d'action de ces antagonistes, soit par purification des substances en question (lipopeptides ou chitinase) soit par détection moléculaire des gènes responsables de leur production.

**Remerciements**
Les auteurs remercient Monsieur Hattab Abdellatif, agrumiculteur sensibilisé au problème du Mal secco, pour sa précieuse collaboration lors des prélèvements d'échantillons à partir des arbres atteints

## Références Bibliographiques

Anonyme. 2003. Data sheets on quarantine pests. Deuterophoma tracheiphila. Prepared by CABI and EPPO for the UE: 1-3.

Aranda F.J., Teruel A., Ortiz A. 2005. further aspects on the hemolytic activity of the antibiotic lipopeptides iturin A. Biochim. Biophys. Acta 1713: 51-56.

Anonyme. 2005. Phoma tracheiphila. EPPO/OEPP Bull. 35: 307-311.

Balmas V., Scherm B., Ghignone S., Ould Mohamed Salem A., Cacciola S., Migheli Q. 2005. Characterisation of Phoma tracheiphila by RAPD, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for in planta PCR detection. Eur. J.Plant. Pathol. 111: 235-247.

Chapot H. 1963. Le Mal secco. Al Awamia, n° 9. Revue de la recherche agronomique marocaine INRA : 89-125.

Crossa-Raynaud P. 1960. Le mal secco. Extrait de vignobles, jardins et vergers de Tunisie. Imprimerie El Assia, Tunis : 1-7.

Ciccarone A. 1971. Il fungo del mal secco degli agrumi. Phytopath. Medit. 10: 68-75.

Deleu M., Paquot M., Nylander T. 2005 Fengycin interaction with lipids monolayers at the air-aqueous interface-implication of the effect of fengycin on biological membranes. J. colloid interface Sci. 283: 358-365.

Ezra D., Koroitor T. 2007. Molecular characterization of Phoma tracheiphila, causal agent of mal secco disease of citrus, in Israel. Eur. J. Plant. Pathol. 118: 183-191.

Hajlaoui M.R., Kalai L., Mnari Hattab M., Guermech A., Ben Abdelaal N. 2007. Occurrence of mal nero disease on mandarin and orange trees in Tunisia. New disease reports. 16: 2p.

Korsten, L., De Jager E.S., De Villurs E.E., Lourens A., Wehner F.C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. Plant. Dis. 79: 1149-1156.

Kirkpatrick B.C., Stenger D.C., Moris T.J., Purcell A.H. 1987. Cloning and detection of DNA from a non cultivable plant pathogenic mycoplasma-like organism. Science 238: 197-200.

Lakhoua H. 1997. Quelques réflexions sur le développement de l'agrumiculture en Tunisie. Revue de l'Institut National Agronomique de Tunisie.12: 179-193.

Pionnat J. C., Segur-fantino N. 1987. Influence de la nutrition potassique sur la réceptivité du citrus volkameriana au Phoma tracheiphila (Petri) Kanc. Ghik. Revue mensuelle de l'Institut de Recherches sur les fruits et Agrumes IRFA. Fruits. 42: 235-241.

Punithalingam, E., Holliday P. 1973. Deuterophoma tracheiphila. CMI descriptions of Pathogenic Fungi and Bacteria No. 399. CAB International, Wallingford, UK.

Sadfi N., Essghaier B., Hajlaoui M.R., Fardeau M.L., Cayaol J.L., Ollivier B., Boudabous A. 2008. Ability of Moderately Halophilic Bacteria to Control Grey Mould Disease on Tomato Fruits. Journal of Phytopathology, 156. pp: 42-52.

Solel Z., Oren Y. 1975. Outbreak of mal secco disease in Israel on normally tolerant Citrus cultivars. Plant. Dis. Reptr. 59: 946-947.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4973-4680.

Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. New. Phytol. 107: 127-142.

White, T.J., Bruns T., Lee S., Taylor J. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes tiré de PCR Protocols: A guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, eds. Academic Press. New York. USA: 322p.

Whiteside J.O., Garnsey S.M., Timmir L.W. 1988. Compendium of citrus diseases. APS PRESS, Minnesota, USA. 80p

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 37 – 42*

**BIOLOGIA TUNISIE**

# INTRA-SPECIFIC VARIABILITY IN RIBOSOMAL DNA SEQUENCE IN THE ITS2 REGION OF TUNISIAN FIG CULTIVARS

**BARAKET Ghada.[1], CHATTI Khaled[1]., SADDOUD Olfa[1]., BEN ABDELKRIM Ahmed.[1], MARS Messaoud.[2], TRIFI Mokhtar[1] and SALHI-HANNACHI Amel[1*].**

1: Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie. Faculté des Sciences de Tunis. Campus Universitaire, El Manar 2092. Tunisie
2 : U.R.Agrobiodiversité, Institut Supérieur Agronomique, 4042 Chott Mariem, Sousse, Tunisie.
*: Author for correspondence: e-mail: Amel.SalhiHannachi@fsb.rnu.tn, Phone: +216 71 87 26 00; Fax: +216 70 86 04 32

**Keywords:** ITS2, Tunisian, nrDNA, Genetic diversity.

## ABSRACT

Internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA (nrDNA) is a useful genomic region for establishing and understanding the evolutionary and genetic relationships among and within species. The current analysis portrays the utility of the ITS2 sequences in a set of Tunisian fig cultivars. Sequences analysis proved variation both in length and GC contents. Data shows that sequences length of the target spacer varied from 253 pb to 314 pb with an average of 261.4. Using the DAMBE and MEGA3 programs variation of GC and AT scored with an average of 62.3 and 37.6 respectively. Furthermore, genetic distances as well as cluster analysis have been successfully performed. The genetic distance estimated ranged from 0.038 to 0.576. In addition, Disparity Index Analysis was used to calculate the composition distance (Dc) and Disparity Index (ID). Results show that the Dc index varied from 0.02 to 2.68 and the ID ranged to 0.00 to 2.38. These parameters express high genetic diversity within the local fig germplasm at the ITS2 region of nuclear ribosomal DNA. This work allowed successfully estimating of genetic diversity on fig cultivars on the basis of ITS sequences. Results are discussed in relation to rational management of the fig genetic resources.

## INTRODUCTION

The common edible fig (*Ficus carica* L., Moraceae) is a gynodioecous species with two morphs: the female tree (common fig) and the hermaphrodite tree (caprifig) (Papadopoulou et al. 2002). *Ficus carica* is also one of the 700 species of the genus *Ficus*, originated mainly from the tropics (Berg, 2003). *Ficus Carica* grows wild in the Mediterranean basin since it is well adapted to the climate as in Tunisia where many varieties could be found. However, the local germplasm is currently threatened either by genetic erosion or by plagues such as the fig mosaic disease. For this reason, many researches have been raised to identify the relationship between cultivars and the estimation of genetic diversity, and many methods have been used in this process of either morphological traits or pomological characteristics to identify the Tunisian fig varieties (Chatti et al. 2004, Salhi Hannachi et al. 2003; Mars et al. 1994). Additionally, isozyme makers have been used to characterize these cultivars (Hedfi et al. 2003). This study portrays the achievements of molecular methods suitable in the surveying of

the molecular diversity and to resolve synonymy and homonymy problems. Consequently, DNA based techniques have been performed to provide efficient and useful markers to assess genetic diversity (Chatti et al. 2007, Saddoud et al. 2007, Salhi-Hannachi et al. 2003; 2004, 2005 and 2006). These analyses, report the development of random amplified polymorphic DNA (RAPD), inter simple
sequence repeats (ISSR) and simple sequence repeats (SSR). The feasibility of these methods is discussed with relation to characterize the local germplasm. The development of molecular data base is necessary to enhance selection and breeding programs and for the establishment of a national collection.
In this study we will evaluate the polymorphism analysis of the internal transcribed spacer (ITS) region. In fact, since the ITS2 is heterogeneous it became an ideal genomic region for the study of genetic diversity and to establish relationships among Tunisian fig cultivars.

## MATERIAL AND METHODS

### Plant material

The study was conducted on twelve fig cultivars, maintained in the collection established at the ISA of Chott Mariam (Table 1). The experimental materiel consisted of 11 common fig cultivars and 1 caprifig that are the main cultivated forms in the Sahel region of Tunisia.

### DNA extraction

Total cellular DNA was isolated from fresh leaves of single adult trees according to a protocol described by Dellaporta et al. (1983). DNA concentration was determined by both spectrophotometry at 260 nm and 0.8% agarose gel electrophoresis according to Sambrook et al. (1989).

**Table 1:** Tunisian fig genotypes used in this study.

| Label | Genotype | Origin |
|---|---|---|
| 1 | Besbessi 1 | Mesjed Aîssa |
| 2 | Besbessi 2 | Mesjed Aîssa |
| 3 | Besbessi 3 | Mesjed Aîssa |
| 4 | Zidi | Mesjed Aîssa |
| 5 | Soltani | Ouardanine |
| 6 | Bidhi 1 | Kalaa Kebira |
| 7 | Hemri | Ghadhabna |
| 8 | Bidh beghal | Mesjed Aîssa |
| 9 | Jrani* | Ghadhabna |
| 10 | Bidhi 2 | Khmara |
| 11 | Delgane | El Alia |
| 12 | Bither abiadh | Khmara |

* Caprifig: male tree

### DNA extraction

Total cellular DNA was isolated from fresh leaves of single adult trees according to a protocol described by Dellaporta et al. (1983). DNA concentration was determined by both spectrophotometry at 260 nm and 0.8% agarose gel electrophoresis according to Sambrook et al. (1989).

### Amplification of the ITS region and sequences analysis

Polymerase chain reaction (PCR) was used to amplify the ITS region using two external primers described by Weiblen et al. (2000). The amplification products were analysed by electrophoresis in 1.5% agarose gel and purified using the Kit <<Wizard SV Gel PCR Clean-Up System>> they were also automatically sequenced in both directions with the amplification primers by the automated fluorescent cycle sequencing method using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City CA, USA).
The ITS region boundaries were determined by comparison with various published sequences available in GenBank. By using the Kimura-2 method (Kimura, 1980), Nucleotide sequences had been aligned using the DAMBE and MEGA version 3.1 programs to calculate pairwise ITS sequence divergence between cultivars (Xia, 2000, Kumar, Tamura and Nei, 2004). The distance resultant was then computed in a matrix to generate dendrogram with reference to the neighbour- joining method (NJ) (Saitou and Nei, 1987) and unweighted pair group method with arithmetic averaging (UPGMA) algorithm (Sneath and Sokal, 1973). Internal support for groupings was assessed using 500 bootstrap replicates (Felsenstein, 1985).

## RESULTS AND DISCUSSIONS

The analysis of the whole ITS region showed that ITS2 was more heterogeneous and highlighted the diversity between Tunisian fig cultivars. In fact, the region has evolved single mutation events such as (indel zones (insertion/deletion), substitutions (transversion

and transition) and that had shaped the high degree of polymorphism. The analysis of the ITS2 was basically made on the level of the spacer size, the GC percentage and the genetic distance as showed below:

The analysis of the spacer's size showed that ITS2 spacer was extended between 253 bp in 'Besbessi2' to 314 bp in 'Zidi' (Table 2) data proved that ITS2 spacer is larger than ITS1 in *Ficus carica* except for 'Jrani' and 'Besbessi3' cultivars. The same results have been reported in other crops such as Butulaceae Cucurbitaceae, Fabaceae, Poaceae, Scrophulariaceae and Viscaceae (Baldwin et al. 1995).

In Tunisian fig the percentage of GC and AT showed that it varied from 55 - to 68 and from 32 to 45.5 with an average of 62.3 and 37.6 correspondingly for ITS2 (Table 2). The scored GC contents are almost similar to the reported ones in other plant species such as *Quercus* spp. (Baldwin et al. 1995, Bellarosa et al. 2005). In addition, the Disparity Index Analyses calculated show that: firstly, the Composition distance (Dc) estimated varied from 0.02 to 2.68. The lowest value scored between 'Bither abiadh' and 'Besbessi 1' cultivars and the highest value was calculated between 'Jrani' and 'Besbessi 2' cultivars (Table 3). Secondary, the Disparity Index (DI) calculated varied from 0.00 to 2.38. The maximum value of DI was scored between 'Jrani' and 'besbessi 2' and the minimum value was calculated between 'Bither abiadh'; 'Bidhi1'; 'Bidh beghal and 'Besbessi1'cultivars (Table 3).

The genetic distances based on the ITS2 spacer are ranged from 0.038 to 0.576. 'Bidhi2' and 'Zidi' cultivars are characterized by the maximum of divergence since they exhibited the greatest genetic distance value of 0.576. However, the smallest distance of 0.038 was observed between the caprifig 'Jrani' and 'Soltani' cultivar suggesting their great similarities (Table 4). The two type of dendrogram (UPGMA and N.J) illustrated identically the divergence between the studied cultivars (Figure (1a) and Figure (1b)). Two main clusters could be identified: the first one labeled (I), is composed of 'Bidhi 2'and 'Besbessi 2' that are significantly divergent from all the remaining varieties ranged in the second cluster labeled (II) that exhibited two sub-groups. The first sub-group (II-1) is consisted of 'Bither abiadh' cultivar while the second sub-group (II-2) is composed of the remaining cultivars. Thus, in both tree a great robustness of the branches was observed which was due to the high value of the bootstraps (85 and 100%).

**Table 2**: Nucleotide composition and length of ITS2 sequences of fig cultivars.

| Accessions | A | T | G | C | GC content (%) | AT content (%) | Length (bp) |
|---|---|---|---|---|---|---|---|
| Besbessi1 | 16.9 | 21.7 | 27.6 | 33.9 | 61.5 | 38.6 | 254 |
| Besbessi2 | 19.4 | 26.1 | 27.7 | 26.9 | 54.6 | **45.5** | **253** |
| Besbessi3 | 12.9 | 28.5 | 27 | 31.6 | 58.6 | 41.4 | 256 |
| Bither abiadh | 17.4 | 22.0 | 27.4 | 33.2 | 58.6 | 39.4 | 259 |
| Bidh beghal | 16.2 | 20.5 | 31.7 | 31.7 | 63.4 | 36.7 | 259 |
| Bidhi1 | 14 | 22.5 | 27.5 | 36 | 63.5 | 36.5 | 258 |
| Bidhi2 | 17.6 | 27.5 | 24.7 | 30.2 | **54.9** | 45.1 | 255 |
| Delgane | 15.2 | 19.8 | 29.2 | 35.8 | 65 | 35 | 257 |
| Solatani | 14.7 | 18.5 | 30.5 | 36.3 | 66.8 | 33.2 | 259 |
| Hemri | 16.7 | 18.3 | 30.4 | 34.6 | 65 | 35 | 257 |
| Jrani | 15.6 | 16.4 | 30.5 | 37.5 | **68** | **32** | 256 |
| Zidi | 16.6 | 17.8 | 30.3 | 35.4 | 65.7 | 34.4 | **314** |
| **Average** | **16.1** | **21.5** | **28.7** | **33.6** | **62.3** | **37.6** | **261.4** |

It was noticed, from this analysis that the caprifig 'Jrani', did not significantly diverge from the female trees which made us assume that our data is in agreement with the monoecious origin of the common fig (*Ficus carica* L.) that evolved later into a dioecious plant (caprifig and edible fig) (Machado et al. 2001). We also observed that grouping was made from their sex or denomination.

Similar results have been stated in common fig since an unstructured genetic variability have been registered using other molecular markers (Chatti et al. 2007, Salhi-Hannachi et al. 2005, 2006).

ITS2 polymorphism detected in *Ficus carica* L. accessions revealed a high level of variability in Tunisian fig crop suggesting that the ITS technology is a powerful and efficient approach in genetic relationships analysis at the intra-specific level.

**Table 3**: Disparity index analysis among twelve Tunisian fig cultivars based on ITS2 sequences: Composition distance (Dc) (lower diagonal) and Disparity Index (ID) (upper diagonal).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Besbessi 1 | | 0.648 | 0.320 | 0.000 | 0.000 | 0.000 | 0.299 | 0.127 | 0.361 | 0.078 | 0.467 | 0.385 |
| 2. Besbessi 2 | 0.939 | | 0.693 | 0.566 | 0.648 | 1.266 | 0.090 | 1.701 | 2.172 | 1.156 | 2.385 | 2.307 |
| 3. Besbessi 3 | 0.545 | 0.967 | | 0.414 | 0.631 | 0.275 | 0.148 | 0.889 | 1.311 | 1.037 | 1.701 | 1.230 |
| 4. Bither abiadh | 0.020 | 0.783 | 0.660 | | 0.000 | 0.061 | 0.344 | 0.098 | 0.352 | 0.000 | 0.434 | 0.340 |
| 5. Bidh beghal | 0.152 | 0.959 | 0.861 | 0.102 | | 0.135 | 0.689 | 0.152 | 0.275 | 0.000 | 0.373 | 0.340 |
| 6. Bidhi1 | 0.143 | 1.525 | 0.443 | 0.250 | 0.303 | | 0.730 | 0.016 | 0.209 | 0.230 | 0.402 | 0.168 |
| 7. Bidhi2 | 0.684 | 0.348 | 0.520 | 0.672 | 1.127 | 1.111 | | 1.299 | 1.934 | 1.119 | 2.160 | 1.980 |
| 8. Delgane | 0.225 | 1.992 | 1.057 | 0.299 | 0.279 | 0.139 | 1.668 | | 0.012 | 0.078 | 0.086 | 0.004 |
| 9. Soltani | 0.488 | 2.471 | 1.512 | 0.545 | 0.373 | 0.340 | 2.316 | 0.070 | | 0.123 | 0.004 | 0.000 |
| 10. Hemri | 0.209 | 1.439 | 1.234 | 0.172 | 0.070 | 0.348 | 1.516 | 0.152 | 0.184 | | 0.156 | 0.193 |
| 11. Jrani | 0.594 | 2.684 | 1.906 | 0.627 | 0.471 | 0.537 | 2.545 | 0.135 | 0.041 | 0.201 | | 0.029 |
| 12. Zidi | 0.533 | 2.611 | 1.447 | 0.619 | 0.447 | 0.307 | 2.381 | 0.078 | 0.012 | 0.266 | 0.086 | |

**Table 4**: Genetic distances matrix among a set of Tunisian fig cultivars based on ITS2 data and computed using the formula of Kimura-2 the bold characters correspond to the smallest and the greatest values respectively.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Besbessi 1 | | | | | | | | | | | | |
| 2. Besbessi 2 | 0.368 | | | | | | | | | | | |
| 3. Besbessi 3 | 0.268 | 0.342 | | | | | | | | | | |
| 4. Bither abiadh | 0.286 | 0.258 | 0.298 | | | | | | | | | |
| 5. Bidh beghal | 0.196 | 0.403 | 0.274 | 0.263 | | | | | | | | |
| 6. Bidhi1 | 0.185 | 0.317 | 0.190 | 0.217 | 0.190 | | | | | | | |
| 7. Bidhi2 | 0.542 | 0.316 | 0.517 | 0.431 | 0.660 | 0.532 | | | | | | |
| 8. Delgane | 0.106 | 0.368 | 0.190 | 0.234 | 0.139 | 0.134 | 0.508 | | | | | |
| 9. Soltani | 0.139 | 0.382 | 0.234 | 0.223 | 0.106 | 0.144 | 0.533 | 0.060 | | | | |
| 10. Hemri | 0.144 | 0.355 | 0.228 | 0.207 | 0.073 | 0.129 | 0.567 | 0.078 | 0.064 | | | |
| 11. Jrani | 0.139 | 0.382 | 0.239 | 0.223 | 0.105 | 0.149 | 0.542 | 0.051 | **0.038** | 0.047 | | |
| 12. Zidi | 0.165 | 0.389 | 0.257 | 0.246 | 0.115 | 0.154 | **0.576** | 0.078 | 0.051 | 0.078 | 0.060 | |

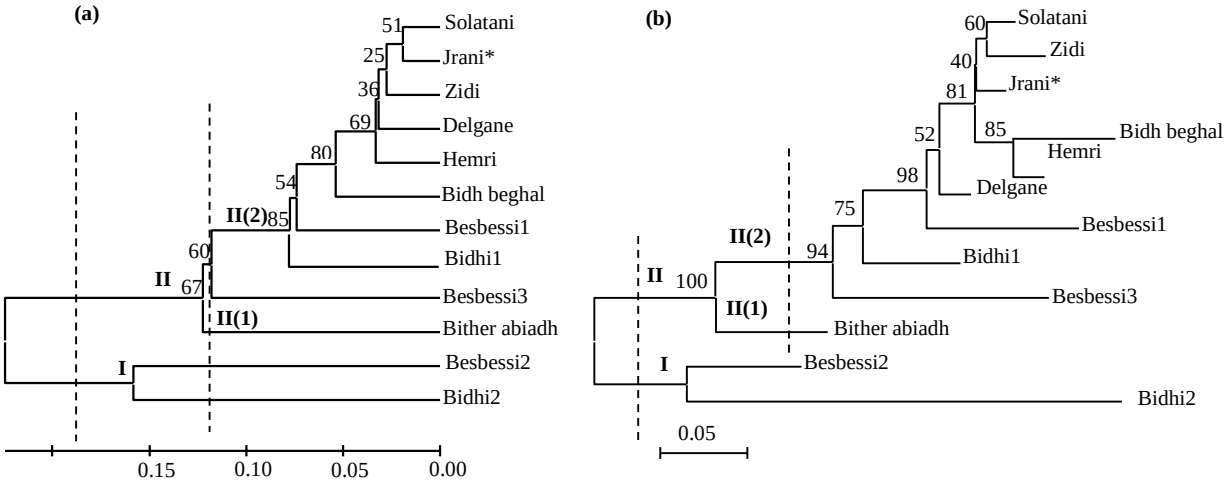



**Figure1**: Phenograms of 12 Tunisian fig genotypes based on ITS2 sequences: (a) unweighted pair group method with arithmetic averaging (UPGMA) dendrogram; (b) Neighbour- joining (NJ) tree.

**AKNOWLEGMENTS**

This work was partially supported by grants from the Tunisian "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie " Projet Lab B02.

**REFERENCES**

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue M.J. (1995). The ITS of nuclear ribosomal DNA: a valuable source of evidence on angiosperm pylogeny. Ann. Missouri. Bot. Gard. 82: 247-277.

Bellarosa, R., Simeone, M.C., Papini, A., Schirone, B. (2005). Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus spp*. Mol. Phyl. Evol. 34: 355-370.

Berg, C.C. (2003). Flora malesiana precursor for the treatment of Moracea 1: the main subdivision of *Ficus*: the subgenera. Blumea. 48:167-178.

Chatti, K., Saddoud, O., Salhi-Hannachi, A., Mars, M., Marrakchi, M., Trifi, M.(2007). Inferring of genetic diversity and relationships in a Tunisian fig (*Ficus carica* L.) germplasm collection by random amplified microsatellite polymorphisms. J. Int. Pl. Bio. 49 (3): 386-391.

Chatti, K., Salhi-Hannachi, A., Mars, M., Marrakchi, M., Trifi, M.. (2004). Analyse de la diversité génétique de cultivars tunisiens de figuier (*Ficus carica* L.) à l'aide de caractères morphologiques. Fruits. 59(1): 49-61.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19-21.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evol. 39: 783-79.

Hedfi, J., Trifi, M., Salhi-Hannachi, A., Ould Mohamed Salem, A., Marrakchi, M. (2003). Morphological and isoenzymatic polymorphism in Tunisian fig (*Ficus carica* L.) collection. Acta. Hort. 605: 319-325

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Kumar, S.,Tamura, K., and Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163.

Machado, C-A., Jousselin, E., Kjellberg, F., Compton, S-G., Herre, E-A. (2001). Phylogenetic relationships, historical biogeography and character evolution of fig pollinating wasps. Proc. Roy. Soc. Lond. Biol. 268: 685-694.

Mars, M., Carraut, A., Marrakchi, M., Gouiaa, M., Gaaliche, F. (1994). Ressources génétiques fruitières en Tunisie (poirier, oranger, figuier, grenadier). Plant. Genet. Res. New. 100 : 14-17.

Saddoud, O., Chatti, K., Salhi-Hannachi, A.,

Mars M., Rhouma, A., Marrakchi, M., and Trifi M. (2007). Genetic diversity of Tunisian figs (*Ficus carica* L.) as revealed by nuclear microsatellites. Hereditas 144: 149-157.

Saitou, N., Nei, M. (1987). The Neighbor-joining method. A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Salhi-Hannachi, A., Mars, M., Chatti, K., Marrakchi, M., Trifi, M. (2003). Specific genetic markers for Tunisian fig germplasm: Evidence of morphological traits, random amplified polymorphic DNA and inter simple sequence repeats markers. Journal of Genetics and Breeding 57, 125–136.

Salhi-Hannachi, A., Trifi, M., Zehdi, S., Mars, M., Rhouma, A., Marrakchi, M. ( 2004). Inter Simple Sequence Repeat fingerprintings to assess genetic diversity in Tunisian fig (*Ficus carica* L.). Genet. Res. Crop. Evol. 51 (3): 269-275.

Salhi-Hannachi, A., Chatti, K., Mars, M., Marrakchi, M., Trifi, M.(2005). Comparative analysis of genetic diversity in two collections figs cultivars based on random amplified polymorphic DNA and inter Simple Sequence repeats fingerprints. Genet. Res. Crop. Evol. 52 (5): 563-573

Salhi-Hannachi, A., Chatti, K., Saddoud, O., Mars, M., Rhouma, A., Marrakchi, M., Trifi, M.

(2006). Genetic diversity of different Tunisian fig (*Ficus carica* L.) collections revealed by RAPD fingerprints. Hereditas. 143:15-22.

Sambrook, J., Frithsch, E.F., Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold spring Harbor Laboratory, cold Spring Harbor, New York.

Sneath, P.M.A., Sokal, R.R., (1973). Numerical Taxonomy. W.H. Freeman and Company, San Francisco.

Papadopoulou, K., Ehaliotis, C., Tourna, M., Kastanis, P., Karydis, I., Zervakis, G. (2002). Genetic relatedness among dioecious *Ficus carica* L. cultivars by random amplified polymorphic DNA analysis, and evaluation of agronomic and morphologic characters. Genetica 114: 183-194.

Weiblen, G.D. (2000). Phylogenetic relationships of functionally diocious *Ficus* (Moraceae) based on ribosomal DNA sequences and morphology. Amer. J. Bot. 87(9), 1342-1357.

Xia, X. (2000). Data analysis in molecular biology and evolution. Kluwer Academic publishers, Boston. pp. 276.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 43 – 47*

**BIOLOGIA TUNISIE**

# MORPHOMETRY AND WEIGHT CHARACTERIZATION OF *PINNA NOBILIS* (MOLLUSCA: BIVALVIA) ALONG THE NORTHERN AND EASTERN TUNISIAN COASTLINE

**Lotfi Rabaoui ♠, Sabiha Tlig-Zouari & Oum Kalthoum Ben Hassine**

Unité de Recherche de Biologie, Ecologie et Parasitologie des Organismes Aquatiques – Département de Biologie, Faculté des Sciences de Tunis – 2092 El Manar – Tunis – TUNISIE. ♠: lrabaoui@gmail.com

**Keywords**: lagoon, marine, morphology, *Pinna nobilis*, shell, Tunisia.

**ABSTRACT**

The present study deals with the morphometric and weight characterization of *Pinna nobilis* populations along the northern and eastern Tunisian coastline. Metric and weight measurements were taken on each of one hundred fifty specimens collected from five different populations. Uni and multivariate analyses of metric indices showed segregation between lagoon and marine populations. As for weight indices, their statistical analysis did not show an inter-population discrimination either with respect to geographic localization or with respect to medium type. In addition, it seems that the morphometry and weight characterization of the fan shell primarily depends on environmental conditions.

## INTRODUCTION

The fan mussel *Pinna nobilis* Linnaeus, 1758 is a Mediterranean endemic species. It is the largest Mediterranean bivalve and one of the largest in the world, since it can reach more than one metre of total anteroposterior length (Zavodnik et al., 1991). It lives up to 20 years, while in Thermaikos Gulf (Greece) an age of 27 years has been reported (Galinou-Mitsoudi et al., 2006). During the last few decades, the populations of *P. nobilis* have strongly decreased because of abusive extraction by amateur divers, loss of their natural biotope (seagrass meadows) and negative effects of pollution on larvae (Moreteau & Vicente, 1982; Richardson et al., 2004). As a consequence, the mollusc has been included in the list of Mediterranean endangered species (Annex IV of the Habitat Directive and Annex II of the Barcelona Convention). *P. nobilis* occurs in coastal soft-bottom areas at depths between 0.5 and 60 m, mostly among meadows of the seagrasses *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera marina* or *Zostera noltii* (Zavodnik et al., 1991) but also in bare sandy bottoms (Katsanevakis, 2006). It lives partially buried by the anterior part of the shell (Zavodnik et al., 1991; Richardson et al., 1999) anchored with its developed byssus filaments that selectively attach to particles and solid structures existing in the substratum. In Tunisia, apart from the study carried out by Tlig-Zouari and Zaouali (1994) about this species in the archipelago of Kerkennah and its records in ancient malacofauna describing lists, there is no precise data about its distribution, current status or even its ecology. With the aim to fulfill these goals, a preliminary study was undertaken on this species along the Tunisian coastline, bearing on its biogeography in the northern and eastern sectors of Tunisia, on its growth as well on its interspecific relations (Rabaoui et al., 2007, 2008). The present study concerns the morphometric and weight characterization of *Pinna nobilis* in Tunisia, making a comparison between five populations located along the northern and eastern coasts of the country.

## MATERIAL & METHODS

During a study on the distribution of the fan shell in Tunisia (Rabaoui et al., 2008), 30 specimens of *Pinna nobilis* were taken from each of five dense sites distributed along the northern and eastern coasts of Tunisia: Echaâra (Bizerta lagoon), Njila (Bizerta lagoon), Sidi Rais (gulf of Tunis), Stah Jaber (bay of Monastir) and Teboulba (bay of Monastir) (fig. 1). Samples were randomly taken, with SCUBA diving, in a sampling surface of 200m². Each sampled individual was placed in a plastic bag bearing the station references and sampling date. At the laboratory, metric (19) and weight (5) measurements were taken on each individual. 19 metric measurements were taken into account: *Hs*: Height of the part above the sediment; *Hen*: Height of the buried part; *Ht*: Total shell height; *E*: Thickness; *Lc*: The largest width; *lc*: The smallest width; *D*: Outdistance between the higher corner of the hinge and the largest width; *Hned*: Height of the nacreous part (side with prints) of the right valve; *Hneg*: Height of the nacreous part (side with prints) of the left valve; *Hnpd*: Height of the pearly part (side without prints) of the right valve; *Hnpg*: Height of the nacreous part (side without prints) of the left valve; *Hnned*: Height of the non nacreous part (side with prints) of the right valve;

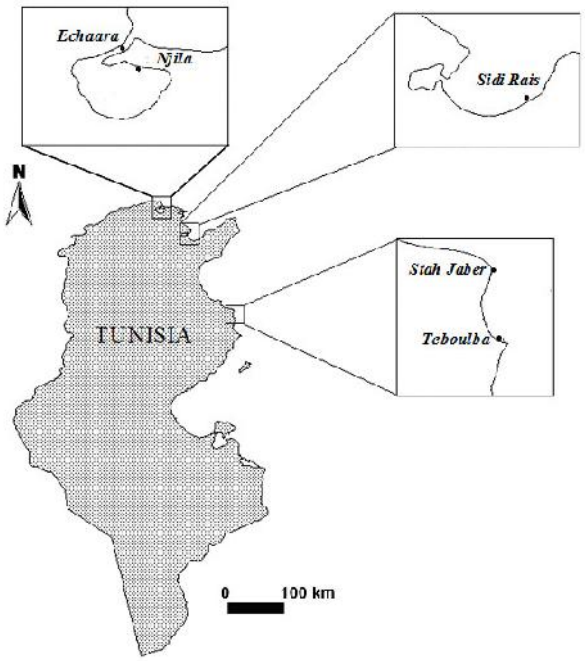



**Figure 1:** Location of the studied populations of *P. nobilis* along the northern and eastern coasts of Tunisia.

*Hnneg*: Height of the nacreous part (side with prints) of the left valve; *Hnnpd*: Height of the non nacreous part (side without prints) of the right valve; *Hnnpg*: Height of the non nacreous part (side without prints) of the left valve; *Lmned*: Maximum width of the nacreous part (side with prints) of the right valve; *Lmneg*: Maximum width of the nacreous part (side with prints) of the left valve; *Lmnpd*: Maximum width of the nacreous part (side without prints) of the right valve; *Lmnpg*: Maximum width of the nacreous part (side without prints) of the left valve. Five weight measurements were taken: *Pte*: Total weight of the individual with epibionts; *Pt*: Total weight of the individual (without epibionts); *Pcq*: Weight of the shell (without epibionts); *Pc_f*: Weight of fresh flesh; *Pc_s*: Weight of dry flesh. All metric indices (171) as well weight ones (10) were calculated on the basis of the measurements already taken on fan shells. Those which present values tending to a constant value or close/equal to 1 were excluded from the analysis. Thus, 29 metric indices (*Hs/Ht*, *Lc/Ht*, *lc/Ht*, *D/Ht*, *Hen/Ht*, *E/Ht*, *Hned/Ht*, *Hnpd/Ht*, *Hnned/Ht*, *Hnnpd/Ht*, *Lmned/Ht*, *Lmnpd/Ht*, *Hneg/Ht*, *Hnpg/Ht*, *Hnneg/Ht*, *Hnnpg/Ht*, *Lmneg/Ht*, *Lmnpg/Ht*, *D/Lc*, *E/D*, *E/Lc*, *Hnned/Hned*, *Hnnpd/Hnpd*, *Hnneg/Hneg*, *Hnnpg/Hnpg*, *Lmned/Hned*, *Lmneg/Hneg*, *Lmnpd/Hnpd* and *Lmnpg/Hnpg*) and 7 weight ones (*Pcq/Pt*, *Pc_f/Pt*, *Pc_s/Pt*, *Pc_f/Pcq*, *Pc_s/Pcq*, *Pc_s/Pc_f* and *Pt/Pte*) were taken into account during this study. The treatment of metric and weight indices was carried out by univariate (ANOVA and DUNCAN) and multivariate (Discriminating Canonical analysis "DCA") analyses.

## RESULTS

### *Analysis of metric characters*

The analysis of variance, carried out on metric indices showed a highly significant population effect for the majority of these indices. This is in favor of the existence of an important phenotypical variability between the five studied populations. According to the values of Snedecor-Fischer test "*F*", metric indices showing important heterogeneities are *D/Lc*, *Hnnpd/Hnpd*, *E/D*, *D/Ht*, *Hnnpg/Hnpg*, *lc/Ht* and *Hnnpd/Ht*. Duncan test showed, for the majority of metric indices, a clear separation between the studied populations. Indeed, for certain indices *Hned/Ht*, *Hnned/Ht*, *Hnnpd/Ht*, *Hneg/Ht*, *Hnneg/Ht*, *Hnned/Hned*, *Hnnpd/Hnpd*, *Hnneg/Hneg*, *Hnnpg/Hnpg*, *Lmnpd/Hnpd* and *Lmnpg/Hnpg*, Duncan test could discriminate between lagoon and marine populations. On the level of *Hned/Ht*, *Hnned/Ht*, *Hnnpd/Ht*, *Hneg/Ht*, *Hnneg/Ht*, *Hnned/Hned* and *Hnneg/Hneg*, average values recorded at Sidi Rais are significantly similar to those of Bizerta lagoon populations (Echaâra and Njila). For these indices, the test of Duncan separated these three populations. On the level of the indices *Hnnpd/Hnpd*, *Hnnpg/Hnpg*, *Lmnpd/Hnpd* and *Lmnpg/Hnpg*, the population of Teboulba is grouped with Echaâra and Njila. These three populations presented very close average values for these last metric indices (Tab. 1).

**Table I:** DUNCAN test applied on the averages of metric and weight indices. The populations presenting the same letters (*A*, *B*, *C*, *D*) form a group with significantly similar averages at the threshold of 5% of Duncan test. (EC: Echaâra; NJ: Njila; SJ: Stah Jaber; TB: Teboulba; SR: Sidi Rais).

| INDICES | CLASSIFICATION OF POPULATIONS | | | | |
|---|---|---|---|---|---|
| | ***METRIC CHARACTERS*** | | | | |
| ***Hs/Hat*** | SJ (***A***) | EC (***B***) | SR (***BC***) | NJ (***BC***) | TB (***C***) |
| ***Lc/Ht*** | SJ (***A***) | SR (***AB***) | NJ (***B***) | EC (***B***) | TB (***C***) |
| ***lc/Ht*** | SR (***A***) | TB (***AB***) | NJ (***AB***) | EC (***B***) | SJ (***C***) |
| ***D/Ht*** | TB (***A***) | SJ (***B***) | SR (***BC***) | NJ (***C***) | EC (***D***) |
| ***Hen/Ht*** | TB (***A***) | NJ (***A***) | SR (***A***) | EC (***A***) | SJ (***B***) |
| ***E/Hat*** | SR (***A***) | SJ (***AB***) | EC (***BC***) | TB (***CD***) | NJ (***D***) |
| ***Hned/Ht*** | NJ (***A***) | EC (***A***) | SR (***A***) | SJ (***B***) | TB (***B***) |
| ***Hnpd/Hat*** | TB (***A***) | NJ (***A***) | EC (***A***) | SR (***B***) | SJ (***C***) |
| ***Hnned/Ht*** | TB (***A***) | SJ (***A***) | NJ (***B***) | SR (***B***) | EC (***B***) |
| ***Hnnpd/Ht*** | SJ (***A***) | SR (***B***) | EC (***B***) | NJ (***B***) | TB (***C***) |
| ***Lmned/Ht*** | NJ (***A***) | SR (***AB***) | SJ (***AB***) | TB (***AB***) | EC (***B***) |
| ***Lmnpd/Ht*** | SJ (***A***) | TB (***A***) | SR (***AB***) | NJ (***AB***) | EC (***B***) |
| ***Hneg/Hat*** | NJ (***A***) | EC (***A***) | SR (***A***) | SJ (***B***) | TB (***B***) |
| ***Hnpg/Ht*** | TB (***A***) | NJ (***A***) | EC (***A***) | SR (***B***) | SJ (***B***) |
| ***Hnneg/Ht*** | TB (***A***) | SJ (***A***) | EC (***B***) | NJ (***B***) | SR (***B***) |
| ***Hnnpg/Ht*** | SJ (***A***) | SR (***B***) | EC (***C***) | NJ (***CD***) | TB (***D***) |
| ***Lmneg/Ht*** | NJ (***A***) | SJ (***A***) | SR (***AB***) | TB (***AB***) | EC (***B***) |
| ***Lmnpg/Ht*** | TB (***A***) | SR (***AB***) | SJ (***AB***) | NJ (***AB***) | EC (***B***) |
| ***D/Lc*** | TB (***A***) | SJ (***B***) | NJ (***B***) | SR (***B***) | EC (***C***) |
| ***E/D*** | EC (***A***) | SR (***B***) | SJ (***C***) | NJ (***C***) | TB (***D***) |
| ***E/Lc*** | TB (***A***) | SR (***AB***) | EC (***BC***) | SJ (***C***) | NJ (***D***) |
| ***Hnned/Hned*** | TB (***A***) | SJ (***A***) | SR (***B***) | EC (***B***) | NJ (***B***) |
| ***Hnnpd/Hnpd*** | SJ (***A***) | SR (***B***) | EC (***C***) | NJ (***C***) | TB (***C***) |
| ***Hnneg/Hneg*** | TB (***A***) | SJ (***A***) | EC (***B***) | SR (***B***) | NJ (***B***) |
| ***Hnnpg/Hnpg*** | SJ (***A***) | SR (***B***) | EC (***C***) | NJ (***C***) | TB (***C***) |

| | | | | | |
|---|---|---|---|---|---|
| ***Lmned/Hned*** | TB (***A***) | SJ (***AB***) | SR (***B***) | NJ (***B***) | EC (***C***) |
| ***Lmneg/Hneg*** | TB (***A***) | SJ (***A***) | NJ (***B***) | SR (***BC***) | EC (***C***) |
| ***Lmnpd/Hnpd*** | SJ (***A***) | SR (***B***) | TB (***C***) | NJ (***C***) | EC (***C***) |
| ***Lmnpg/Hnpg*** | SJ (***A***) | SR (***A***) | TB (***B***) | NJ (***B***) | EC (***B***) |
| ***WEIGHT CHARACTERS*** | | | | | |
| ***Pcq/Pt*** | SJ (***A***) | TB (***B***) | EC (***C***) | SR (***C***) | NJ (***C***) |
| ***Pc_f/Pt*** | NJ (***A***) | SR (***A***) | EC (***A***) | TB (***B***) | SJ (***C***) |
| ***Pc_s/Pt*** | EC (***A***) | SR (***B***) | NJ (***C***) | TB (***C***) | SJ (***D***) |
| ***Pc_f/Pcq*** | NJ (***A***) | SR (***A***) | EC (***AB***) | TB (***BC***) | SJ (***C***) |
| ***Pc_s/Pcq*** | EC (***A***) | SR (***B***) | NJ (***BC***) | TB (***CD***) | SJ (***D***) |
| ***Pc_s/Pc_f*** | EC (***A***) | SR (***B***) | TB (***C***) | SJ (***CD***) | NJ (***D***) |
| ***Pt/Pte*** | NJ (***A***) | TB (***A***) | SJ (***A***) | EC (***AB***) | SR (***B***) |

Discriminating Canonical Analysis showed that the first three axes explain 92.86% of total inertia. Axis 1 absorbs 51.83% of total variability. It is positively correlated with *lc/Ht*, *Hen/Ht*, *Hnpd/Ht*, *Hnpg/Ht*, *D/Lc*, *E/Lc* and negatively with the indices *Hs/Ht*, *Lc/Ht*, *Hnnpd/Ht*, *Hnnpg/Ht*, *Hnnpd/Hnpd* and *Hnnpg/Hnpg*. The second axis absorbs only 29.8% of total variability. The representation of individuals on the plan 2-1 (fig. 2) supported the separation observed previously by univariate analyses.

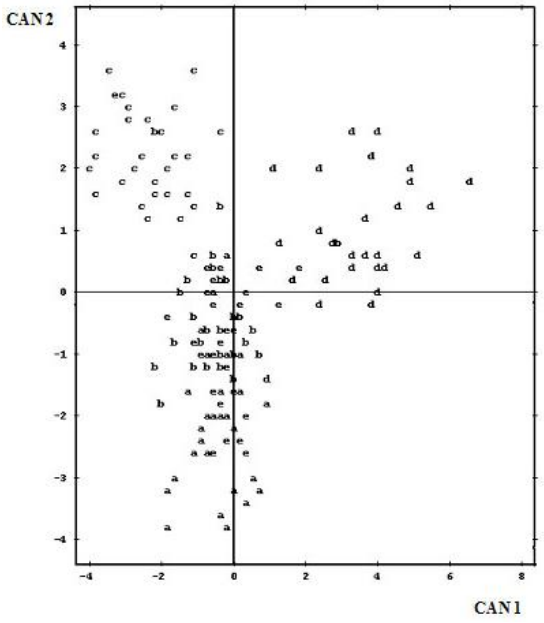



**Figure 2:** Discriminating Canonical Analysis of metric indices of *Pinna nobilis* populations of (a: Echaâra; b: Njila; c: Stah Jaber; d: Teboulba; e: Sidi Rais).

The two populations of Monastir bay were separated. The population of Stah Jaber (c) was located on the negative side of axis 1 while Teboulba (d) is located on the positive side of the same axis. The other populations were spread around the center of axis 1 and overlapped. As for Axis 2, it discriminated two groups: the first is formed by the two populations of Stah Jaber (c) and Teboulba (d) and the second consisted of the populations of Echaâra (a), Njila (b) and Sidi Rais (e). The latter populations appeared to be gathered on the negative side of axis 2. However, it is possible to detect discrimination between the two populations of Echaâra (a) and Njila (b). Sidi Rais population (e) overlap with these populations in the center of axis 1 (fig. 2). Mahalanobis distances, recorded between the five examined populations, supported the observed discrimination. The dendrogram of the whole populations of *Pinna nobilis* separated between marine and lagoon populations (fig. 3).

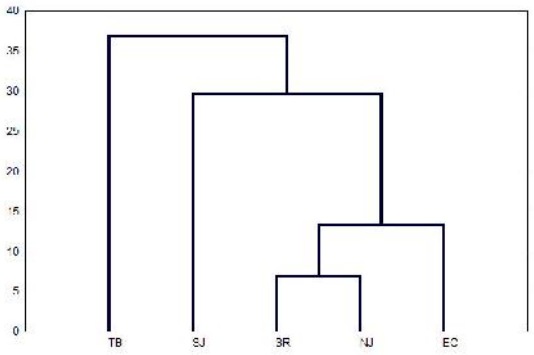



**Figure 3**: Dendrogram of Mahalanobis distances based on metric indices between the various populations of *Pinna nobilis*. (EC.: Echaâra; NJ: Njila; SJ: Stah Jaber; TB: Teboulba; SR: Sidi Rais).

***Analysis of weight characters***

The analysis of variance, carried out on weight indices showed a highly significant population effect. This is in favor of the existence of an important variability between the five studied populations. Weight indices which had an important heterogeneity were *Pc_s/Pc_f*, *Pc_s/Pt* and *Pc_s/Pcq*. Multiple comparison of averages using Duncan test could separate between populations and supported an inter-population segregation. For certain indices (*Pcq/Pt*, *Pc_f/Pt* and *Pc_f/Pcq*), Duncan test discriminated between the populations of Echaâra, Njila and Sidi Rais from those of Stah Jaber and Teboulba. The populations of Monastir bay were gathered together (Tab. 1). Concerning the DCA, the results showed that axis 1 absorbs 82.39% of total variability; it is defined positively by the indices *Pc_s/Pt*, *Pc_s/Pcq* and *Pc_s/Pc_f*. The second axis explained 12.38% of total variability. It is correlated with the indices *Pcq/Pt*, *Pc_f/Pt* and *Pc_f/Pcq*. Individuals' representation on axis 1-2 plan showed heterogeneity between the five populations *Pinna nobilis* (fig. 4). Indeed, two groupings were found according to axis 1: one consisted of Echaâra (a) and another formed by the populations of Njila (b), Stah Jaber (c) and Teboulba (d). Sidi Rais population (e) is dispersed in the two sides of axis 1(fig. 4). Mahalanobis distances calculated between populations for weight indices, showed a clustering of marine populations and a separation between lagoon populations of *Pinna nobilis* (Fig. 5).

## DISCUSSION

*P. nobilis* growth was investigated by several researchers (Moreteau & Vicente, 1982; Tlig-Zouari & Zaouali, 1994; Richardson et al., 1999, 2004; Galinou-Mitsoudi et al., 2006; García-March et al., 2002; Rabaoui et al., 2007). Concerning the morphology of its shell, Czihak and Dierl announced since 1961 important variations. More recently, Combelles et al., (1986) observed a phenomenon of ecomorphose on juveniles in relation to the presence or absence of *P. oceanic* herbarium. The present study is one of the investigations concerning this aspect on the northern and eastern Tunisian coastline. ANOVA of the 29 metric indices, taken separately, showed that according to the

considered index, there was a variable grouping of the five populations of *Pinna nobilis*.

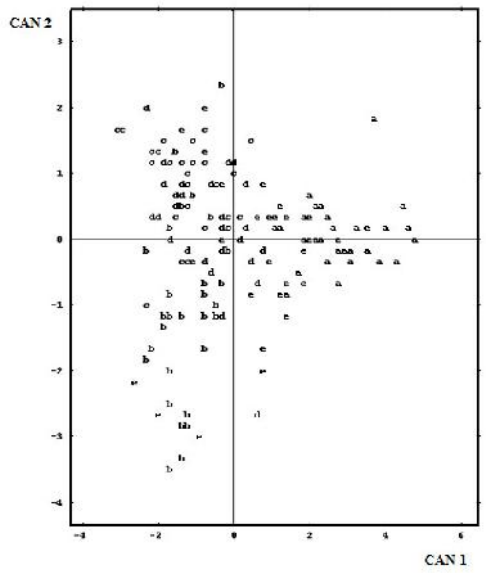



**Figure 4:** Discriminating Canonical Analysis of weight indices of *Pinna nobilis* populations (a: Echaâra; b: Njila; c: Stah Jaber; d: Teboulba; e: Sidi Rais).

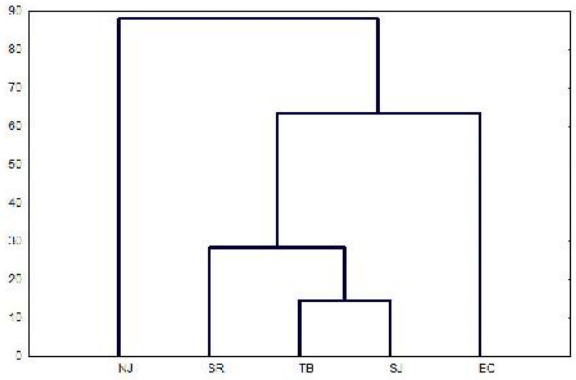



**Figure 5:** Dendrogram of Mahalanobis distances based on weight indices between the various populations of *Pinna nobilis* (EC.: Echaâra; NJ: Njila; SJ: Stah Jaber; TB: Teboulba; SR: Sidi Rais).

Certain indices showed that the five examined populations are completely heterogeneous, whereas others could separate the populations of Echaâra (a), Njila (b) and Sidi Rais (e) from the other populations. In spite of its marine location, Sidi Rais population (e) was grouped with lagoon ones (Echaâra (a) and Njila (b)). This seems to be due to the fact that the majority of sampled individuals were collected in the lagoonal zone which precedes the reef barrier formed by the herbarium of *Posidonia oceanica* (area of Sidi Rais). Indeed, the conditions which reign in this area attribute to this zone a lagoon character. The site is protected by the reef barrier; it seems that the influence of currents on this level is lower. So the indices discriminating these three populations can be regarded as discriminators of lagoon populations. In addition, the indices *Hned/Ht*, *Hnned/Ht*, *Lmned/Ht*, *Lmnpd/Ht*, *Hneg/Ht*, *Hnneg/Ht*, *Hnned/Hned*, *Hnneg/Hneg*, *Lmneg/Hneg* could arrange together the populations of Monastir bay. These indices discriminate thus the marine populations of *Pinna nobilis* (Tab. 1). The variability of population grouping according to the indices can be explained by the fact that the growth of certain metric variables depends on the environmental conditions and can thus differ from one site to another. They can also vary with the life cycle of the animal (Katsanevakis et al., 2007). For example, in Vouliagmeni Lake (Greece), *Pinna nobilis* showed an obvious change in the relative growth of shell width in relation to total length (Katsanevakis et al., 2007). The variability in relative growth of *P. nobilis* was also observed in other Mediterranean areas (Combelles et al., 1986). Comparable variations were also observed with the species *Mytilus galloprovincialis* (Gardner, 1992) and other marine species (Demir, 2003; Katsanevakis, 2006). The same segregations were also determined by multivariate analyses. Indeed, DCA could separate between marine and lagoon populations and could detect a separation even between the marine populations. This discrimination matched with the geographical locations of the populations (fig. 1). Similar observations were mentioned by García-March et al. (2007) who found differences even between the individuals of the same population which are subjected to different environmental conditions. Indeed, several environmental factors can affect the morphometry as well as the growth of several bivalve species. Among these factors, food availability, temperature, upwelling intensity, type of sediment and hydrodynamics are worth noting (Steffani & Branch, 2003; Philips, 2005). For *P. nobilis*, it was reported that severe disturbances of the sediments and high stress caused by hydrodynamics can cause a reduction of the growth and thus a change of the shell shape (García-March et al., 2007). For other bivalve species, the relative growth and morphology of the shell can be affected by the depth, hydrodynamics, predation, temperature and sediment type (Steffani & Branch, 2003). Regarding weight indices, univariate analysis allowed to gather the populations according to their biotope. Contrary to metric indices, weight indices did not show a marked variability between populations. DCA showed an overlapping between the five populations, in particular between Njila (b), Stah Jaber (c) and Teboulba (d). Such an inter-population discrimination seems to be in relation with "site" factor. Indeed, the environmental conditions can influence the biomass of the fan shell. The capture of suspended particles by this filter-feeding animal can change considerably from one site to another, because of the differences in swiftness and organic contents of water currents which generally affect the biomass of bivalves (Shimeta & Jumars, 1991). This observation was also reported by Arizpe (1995) for the species *Pinna rugosa*, cultivated in Bahia of Paz (Mexico). These authors announced that the variability in current velocity water and nutritive elements affects the condition index of the species. Similar variations were also reported for the species *Pinna bicolor* (Wu and Shin, 1998) and for the mussel *M. galloprovincialis* (Gardner, 1992). Summarizing, the study of morphometric and weight characters of *P. nobilis* enabled to characterize the fan mussel populations along the northern and eastern Tunisian coastline. Thus, the morphology and biomass of the fan mussel seem to be in close relation with the environmental conditions of, in particular hydrodynamics and nutritive elements. Further studies are necessary to see whether this

segregation is really due to environmental or genetic factors.

**REFERENCES**

Arizpe, O. (1995). Mortality, growth and somatic secondary production of the bivalve, *Pinna rugosa* (Sowerby), in suspended and bottom culture in Bahia de La Paz Mexico. Aquaculture Research. 26: 843-853.

Combelles, S., Moreteau, J. C. and Vicente, N. (1986). Contribution à la connaissance de l'écologie de *Pinna nobilis* L. (Mollusque eulamellibranche). Sci. Rep. Port-Cros Nat. Park. 12: 29-43.

Demir, M. (2003). Shells of Mollusca Collected from the Seas of Turkey. Turk. J. Zool. 27: 101-140.

Galinou-Mitsoudi, S., Vlahavas, G. and Papoutsi, O. (2006). Population study of the protected bivalve *Pinna nobilis* (Linnaeus, 1758) in Thermaikos Gulf (North Aegean Sea). Journal of Biological Research. 5: 47–53.

García-March, J.R., García-Carrascosa, A.M. and Peňa, Á.L. (2002). In Situ Measurement of *Pinna nobilis* shells for age and growth studies: a new device. Marine ecology. 23 (3): 207-217.

García-March, J.R., Garcia-Carrascosa, A.M. and Perez-Rojas, L. (2007). Influence of hydrodynamic forces on population structure of *Pinna nobilis* L., 1758 (Mollusca: Bivalvia): the critical combination of drag force, water depth shell size and orientation. Journal of Experimental Marine Biology and Ecology. 342: 202-212.

Gardner, J.P.A. (1992). *Mytilus galloprovincialis* (Lmk.) (Bivalvia: Mollusca): the taxonomic status of the Mediterranean mussel. Ophelia. 35: 219–243.

Katsanevakis, S. (2006). Population ecology of the endangered fan mussel *Pinna nobilis* in a marine lake. Endangered Species Research. 1: 51-59.

Katsanevakis, S., Thessalou-Legaki, M., Karlou-Riga, C., Lefkaditou, E., Dimitriou, E. and Verriopoulos, G. (2007). Information-theory approach to allometric growth of marine organisms. Mar. Biol. 151: 949–959.

Moreteau, J.C. and Vicente, N. (1982). Evolution d´une population de *Pinna nobilis* L. (Mollusca, Bivalvia). Malacologia. 22: 341-345.

Philips, N.E. (2005). Growth of filter-feeding benthic invertebrates from a region with variable upwelling intensity. Mar. Ecol. Prog. Ser. 295:79–89.

Rabaoui, L., Tlig-Zouari, S., Katsanevakis, S. and Ben Hassine, O.K. (2007). Comparison of absolute and relative growth patterns among five *Pinna nobilis* populations along the Tunisian coastline: an information theory approach. Marine Biology. 152: 537-548.

Rabaoui, L., Tlig-Zouari, S. and Ben Hassine, O.K. (2008). Distribution and habitat of the fan mussel *Pinna nobilis* Linnaeus, 1758 (Mollusca: Bivalvia) along the northern and eastern Tunisian coasts. Cahiers de Biologie Marine. 49: 67-78.

Richardson, C.A., Kennedy, H., Duarte, C.M., Kennedy, D.P. and Proud, S.V. (1999). Age and growth of the fan mussel *Pinna nobilis* from south-east Spanish Mediterranean seagrass (*Posidonia oceanica*) meadows. Mar. Biol. 133: 205-212.

Richardson, C.A., Peharda, M., Kennedy, H., Kennedy, P. and Onofri, V. (2004). Age, growth rate and season of recruitment of *Pinna nobilis* (L) in the Croatian Adriatic determined from Mg:Ca and Sr:Ca shell profiles. J. Exp. Mar. Biol. Ecol. 299(1): 1-16.

Shimeta, J. and Jumars, P.A. (1991). Physical-mechanisms and rates of particle capture by suspension-feeders. Oceanography and Marine Biology. 29: 191-257.

Steffani, C.N. and Branch, G.M. (2003). Growth rate, condition, and shell shape of *Mytilus galloprovincialis*: responses to wave exposure. Mar. Ecol. Prog. Ser. 246: 197–209.

Tlig-Zouari, S. and Zaouali, J. (1994). Croissance individuelle et saisonnière de *Pinna nobilis* dans les îles Kerkennah. Bull. Soc. Sci. Nat. Tunisie. 23: 69-74.

Wu, R.S.S. and Shin, P.K.S. (1998). Transplant experiments on growth and mortality of the fan mussel *Pinna bicolor*. Aquaculture. 163: 47-62.

Zavodnik, D., Hrs-Brenko, M. and Legac, M. (1991). Synopsis on the fan shell *Pinna nobilis* L. in the eastern Adriatic Sea. In: Boudouresque, C. F., Avon, M. and Gravez, V. eds: Les Espèces Marines à Protéger en Méditerranée. Gis Posidonie publ. Marseille. 11: 169-178.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 48 – 54*

**BIOLOGIA TUNISIE**

# MORPHOLOGICAL VARIABILITY OF SUB-SPONTANEOUS TUNISIAN FIG (*FICUS CARICA* L.) POPULATIONS

**Ben Abdelkrim Ahmed, Baraket Ghada, Chatti Khaled, Saddoud Dabbebi Olfa, Trifi Mokhtar & Salhi-Hannachi Amel[1*]**

Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences of Tunis, Campus University, 2092 El Manar Tunis, Tunisia
*Author for correspondence: e-mail: Amel.SalhiHannachi@fsb.rnu.tn
Phone: +216 71 87 26 00; Fax: +216 70 86 04 32

**Keywords**: *Ficus carica*; Variability; Morphological traits; Sub-spontaneous fig; Tunisia

## ABSRACT

Sixty-four of Tunisian sub-spontaneous fig were analysed based on morphological characters. One way ANOVA analysis show a significant difference between wild fig populations and demonstrates that parameters measured on leaves show an important degree of variability and permits to discriminate sub-spontaneous fig individuals. Principal component analysis pointed out a variation among individuals based on leaf. Ward's dendrogram and Principal Component Analysis (PCA) allowed clustering of trees studied according to their morphological traits and independently of their geographic origin. The aim of this work was the phenotypic characterization of Tunisian sub-spontaneous fig in order to start an improvement program of this species. In fact, estimation of gene flow and genetic diversity structuration of wild compartment permit to evaluate its proximity with the cultivate germplasm.

## INTRODUCTION

The genus *Ficus* (Moraceae) includes some 750 species of woody plants found in most tropical and subtropical forests throughout the world (Berg, 1989). These species of trees, shrubs, climbers, and hemi-epiphytic stranglers are recognized by a specialized inflorescence and pollination syndrome (Janzen, 1979; Berg, 1990; Weiblen, 2000). Fig trees (*Ficus carica* L., 2n=26) have been cultivated in the eastern Mediterranean zone since 4000 B.C. (Zohary, 1988). *Ficus carica* is indigenous to Persia, Asia Minor and Syria (Tous, 1996) and is probably the first intentionally cultivated plant during the Neolithic revolution. Its domestication preceded that of cereals by about a thousand years (Kislev, 2006).

In Tunisia, fig germplasm consists of numerous landraces mainly selected by farmers for their fruit qualities and maintained in orchards where fruit types are unequally represented (Saddoud, 2007). The fig is widely spread in all the climatic stages in Tunisia (Mars, 1998). Thus, a large phenotypic diversity characterizes the large number of ecotypes distinguishable by taste, colour and flavour of the fruit (Rhouma, 1996; Mars, 2007). In addition, where the fig is cultivated, we often find wild individuals because there are favourable conditions for their natural development. Indeed, spontaneous figs systematically develop in the rocks, usually along the rivers banks, in steep-sloped valleys

but not too deep, which means on well-lit slopes with a few trees. This topography is particularly common in areas of limestone's plates. Previous work found that the fig occupies this type of habitat in many Mediterranean regions (Khadari, 2005). The sub-spontaneous fig took place by a seed reproduction, and this happens in a natural way without man's intervention but using other factors such as the birds which consume the seeds. Due to the gastric juices enzymes these grains become fertile. So it is interesting to study their genetic diversity, because there will be a very important genetic mixture between either the individuals of the same region or even between those of the most remote ones during the birds' migration. Our aims in this work are to investigate genetic diversity and to establish relationships among Tunisian sub-spontaneous fig by using morphological traits analysis. The evaluation of morphological variations was based on the observation and the measurement of the morphological criteria on the leaves. Indeed, this information is important to have a first inventory of wild germplasm in order to identify the most discriminating and informative characteristics in the estimation of the amount and the organization of the variability of theses Tunisian sub-spontaneous fig populations. Results obtained from these markers are compared and discussed in order to start an improvement program of this species.

## MATERIAL AND METHODS

Plant material

A total of 64 wild fig trees were inventoried in northern and central Tunisia (11 individuals from Menzel Bouzelfa, 11 from El Haouaria, 7 from Kerkennah Islands, 6 from Ghar Elmeleh, 12 from Raf Raf, 5 from Ras Jebel and 18 from Siliana). The plant material consisted in leaves randomly taken from adult trees and used to undergo a morphological study.

Morphological characters

Morphological characterization of each individual was performed according to the guidelines provided by the international plant genetic resources institute (IPGRI, 2004) in the 'Descriptors of fig' (F. carica related Ficus ssp) (Table 1).

To study the morphological variability of Ficus carica sub-spontaneous 10 leaves were randomly taken at different part of the tree and eight variables were measured. All morphometric measurements were taken using a Vernier caliper with digital display.

Analysis of morphological data

In order to elucidate genetic variability and to establish relationships between sub-spontaneous trees, several statistical procedures were conducted. Phenotypic data were used to approximate contribution of the measured leaves traits in the genetic diversity among the considered individuals. Data were computed using the SAS software (Statistical Analysis System, version V.6.07; SAS, 1990) to achieve one-way analysis of variance (ANOVA) and principal components analysis (PCA) as a multivariate analysis. The PCA analysis was applied to assess differences between individuals and elucidate partitioning of phenotypic variability at inter-populations level. Parameters means were compared by DUNCAN'S multiple range test ($P< 0.05$) (Dangnelie, 1975).

The Euclidean distance were estimated between individuals and an hierarchical cluster was constructed according to Ward's method (Ward, 1963) by module in the statistical packages STATISTICA 6.0 (StatSoft Inc, 2001) to establish relationships among Tunisian sub-spontaneous fig trees

## RESULTS

*Determination of the dominant leaf type*

A variable number of leaves lobes are observed in the same tree. The dominant leaf type was determined for 10 or 14 leaves per tree. Result demonstrates the dominance of leaves with 3 and 5 lobes in the Cap Bon region, 5 and 7 lobes in the Kerkennah islands. Concerning Kesra and Sidi Mansour (Seliana) areas we recorded the dominance respectively of leaves showing 3 and 5 lobes. Results provide a distribution of leaves types according to the prospected geographical areas (Table 2).

**Petiole**

| S | H | F | E | D | C | G | A | B |
|---|---|---|---|---|---|---|---|---|
| 8,63 | 7,61 | 7,44 | 7,07 | 7,01 | 6,92 | 6,26 | 5,98 | 5,8 |

**Petiole**

| G | S | B | C | D | F | H | E | **A** |
|---|---|---|---|---|---|---|---|---|
| 0.64 | 0.52 | 0.40 | 0.38 | 0.37 | 0.36 | 0.35 | 0.34 | 0.34 |

**Leaf**

| S | F | D | G | A | B | C | E | H |
|---|---|---|---|---|---|---|---|---|
| 19.11 | 18.72 | 17.98 | 16.94 | 16.84 | 16.83 | 16.09 | 15.32 | 15.03 |

**Leaf**

| S | F | D | C | G | E | A | H | B |
|---|---|---|---|---|---|---|---|---|
| | 19.29 | 19.08 | 17.89 | 17.72 | 16.93 | 16.66 | 16.55 | 16.48 |

**Leaf length/ Leaf**

| B | A | F | G | D | H | E | C | S |
|---|---|---|---|---|---|---|---|---|
| 1.02 | 1.0 | 0.97 | 0.96 | 0.93 | 0.92 | 0.92 | 0.91 | 0.88 |

**Depth of the sine basal**

| S | F | E | C | G | D | H | B | A |
|---|---|---|---|---|---|---|---|---|
| 3.63 | 2.93 | 2.85 | 2.70 | 2.58 | 2.54 | 2.30 | 1.69 | 1.62 |

**Depth of left lateral sinus1**

| S | D | C | F | H | A | G | B |
|---|---|---|---|---|---|---|---|
| 7.97 | 7.90 | 6.83 | 6.57 | 6.17 | 5.59 | 5.24 | 5.00 | 4.96 |

**Depth of right lateral sinus2**

| S | D | C | F | H | A | G | E | B |
|---|---|---|---|---|---|---|---|---|
| 7.91 | 7.89 | 6.58 | 6.26 | 5.87 | 5.58 | 5.19 | 5.04 | 4.83 |

**Figure 1**: Result of Duncan test (0.05) conducted on morphological characters.

S: Raf Raf 2, H: Kesra, F: Ras Jebel, E: Raf Raf 1, D: Ghar Elmelh, C: Kerkennah island, G: Sidi Mansour, A: Menzel Bouzelfa, B: Haouaria

*One way Analysis of variance (ANOVA)*

The ANOVA analyses applied on the morphological parameters enabled the determination of the most discriminating variables between the studied populations. The characters used are very efficient to estimate phenotypic variability of these populations. Thus, the study shows that out of the 8 measured parameters only depth of left side sine 1 [SL1] and depth of right side sine 2 [SL2] give evidence of highly significant differences among the considered populations (Table 3). In addition, mean comparison by Duncan's test (at 5%) confirm this report and show that these parameters discriminate significantly the wild fig populations (Figure 1).

**Table 1**: Leaf characters retained for the study of sub-spontaneous *Ficus carica* L. populations diversity in Tunisia.

| Parameter | Abbreviation | Unit | Character |
|---|---|---|---|
| **Leaf descriptors** | PL | Cm | Petiole lenght |
| | PW | Cm | Petiole width |
| | LL | Cm | Leaf width |
| | LW | Cm | Leaf lenght |
| | LL /LW | Notification | Leaf Length/ Leaf width |
| | SB | Cm | Depth of the Sine Basal |
| | SL1 | Cm | Depth of left side Sine 1 |
| | SL2 | Cm | Depth of right side Sine 2 |

**Table2**: Percentage of leaf type observed in eight populations for sub-spontaneous phenotypic diversity of *Ficus carica* L. in Tunisia.

| | Leaves percentage(%) | | | | |
|---|---|---|---|---|---|
| **Populations** | **1 Lobe** | **3 Lobes** | **4 Lobes** | **5 Lobes** | **7 Lobes** |
| **Menzel Bouzelfa** | 2,81 | 38,73 | 1,42 | 45,07 | 11,97 |
| **El Haouaria** | 5,36 | 39,29 | 2,68 | 34,82 | 17,86 |
| **Kerkennah Islands** | 7,14 | 12,86 | 4,29 | 32,86 | 42,86 |
| **Ghar elmeleh** | 0 | 26,67 | 1,67 | 56,67 | 15 |
| **Raf Raf** | 0 | 10,94 | 10,94 | 46,88 | 31,25 |
| **Ras Jebel** | 2 | 34 | 4 | 60 | 0 |
| **Sidi Mansour** | 10 | 70 | 5 | 15 | 0 |
| **Kesra** | 0 | 17,5 | 1,67 | 80 | 0,83 |

The correlation matrix shows that depth left lateral sine1 [SL1] parameter is highly and positively correlated to depth of the right lateral sine2 [SL2] (0.97). Negative and significant

correlations were recorded between [LL/LW] and depth of the sine basal [SB] traits (-0.78) (Table 4).

**Table 3**: One way analyses of variance (ANOVA) applied on quantitative parameters referring to leaf descriptors.

* : Significant
NS : No significant

| Morphological traits | Mean square | F. observed | P |
|---|---|---|---|
| **Petiole length (PL)** | 5.18 | 1.89 NS | 0.07 |
| **Petiole width (PW)** | 0.07 | 1.17 NS | 0.33 |
| **Leaf length (LL)** | 11.98 | 1.79 NS | 0.09 |
| **Leaf width (LW)** | 14.60 | 1.39 NS | 0.22 |
| **Leaf Length/ Leaf width (LL / LW)** | 0.01 | 1.71 NS | 0.11 |
| **Depth of the sine basal (SB)** | 2.52 | 1.89 NS | 0.07 |
| **Depth of left lateral sinus1 (SL1)** | 8.01 | **2.47 *** | 0.02 |
| **Depth of right lateral sinus2 ( SL2)** | 7.71 | **2.47 *** | 0.02 |

The Euclidean matrix distance was established. The obtained matrix exhibits genetic distances ranging from 0.4 to 26.4 and shows a high phenotypic diversity among the studied individuals. The lowest distance is observed between the individuals 65 and 66 from Sidi Mansour population (Siliana). The distance that shows the maximum difference was noticed between the individuals 60 and 77 from Ras Jebel and Kesra populations respectively. As shown by Figure 2, the established Ward's dendrogram based on Euclidean genetic distances show two main groups. The first one is composed by trees from Ghar El Meleh population and the second one is essentially composed by trees belonging to Sidi Mansour, Kesra, El Haouaria and Kerkennah populations. Moreover, individuals from populations of Raf Raf, Menzel Bouzelfa and Ras Jebel are shared between the two groups. The Ward's dendrogram topology shows a considerable morphological variability no correlated to geographical origins of the prospected sites (Figure 2).

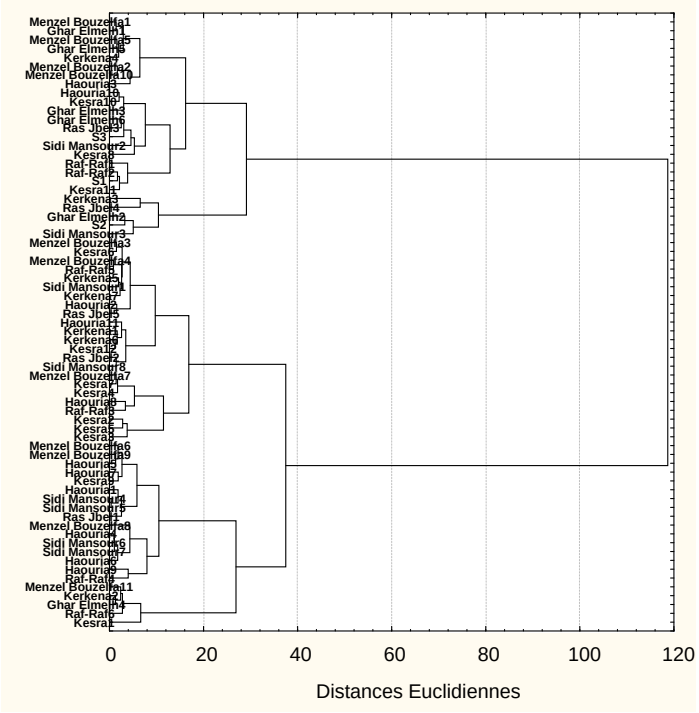



**Figure 2:** Ward's dendrogram of 64 Tunisian sub-spontaneous fig trees based on Euclidean distances applied on leaves parameters.

In order to confirm this assumption we have performed a PCA analysis. A correlations matrix between the measured characters was established. The analysis of this matrix highlights positive and negative correlations.

**Table 4**: Correlation matrix between all parameters measured on sub-spontaneous fig leaves.

| | PL | PW | LL | LW | LL/LW | SB | SL1 | SL2 |
|---|---|---|---|---|---|---|---|---|
| **PL** | 1.00 | | | | | | | |
| **PW** | 0.12 | 1.00 | | | | | | |
| **LL** | 0.58 | 0.28 | 1.00 | | | | | |
| **LW** | 0.70 | 0.39 | 0.79 | 1.00 | | | | |
| **LL/LW** | -0.29 | -0.24 | 0.13 | -0.48 | 1.00 | | | |
| **SB** | 0.47 | 0.37 | 0.28 | 0.73 | **-0.78** | 1.00 | | |
| **SL1** | 0.48 | 0.22 | 0.47 | 0.65 | -0.43 | 0.55 | 1.00 | |
| **SL2** | 0.46 | 0.18 | 0.47 | 0.64 | -0.43 | 0.52 | **0.97** | 1.00 |

The strongest positive correlation (0.97) was obtained between the [SL1] and [SL2] parameters, while the lowest positive correlation (0.12) was recorded between [PL] and [PW].

The negative correlations were ranged from (-0.78) observed between the parameters [LL / LW] and [SB] to (-0.24) between the parameters [LL / LW] and [PW] (Table 4).

ACP results show that the three first principal components accounted for 84.38% of the total variation and suggest a good structuration of the sub-spontaneous fig trees diversity. The first component absorbs (54.84%) of the total diversity and positively defined by [LW] and [SL1] parameters. This axis is negatively correlated to the parameter [LL / LW]. The second axis absorbs (17.12%) of the total variability and defined positively by [LL] and negatively by [SB] traits. The third axis absorbs (12.42%) of the total diversity and defined positively by [PW] and negatively by [SL2] characters (Table 5).

**Table5:** Variables relative contribution to the variation provided by the first three axis of the PCA**.**

| **Eigenvectors** | **PC1** | **PC2** | **PC3** |
|---|---|---|---|
| Absorption proportion (%) | 54,84 | 17.12 | 12.42 |
| Cumulative of variance explained | | 71,96 | 84,38 |

| Axes | **Var** | **e.i** | **Var** | **e.i** | **Var** | **e.i** |
|---|---|---|---|---|---|---|
| | LW | +0.44 | LL | +0.60 | DP | +0.79 |
| | SL1 | +0.40 | -SB | -0.37 | SL2 | -0.40 |
| | LP | +0.34 | | | | |
| | - LL/LW | -0.28 | | | | |

Var : variable ; e.i: Eigenvalue

The screening of all individuals in the plot defined by the two first components, which absorb (72 %) of the total variability, shows a very condensed dispersion (Figure 3).

This dispersion reveals the presence of a low variability between populations. In contrast, the intra-population variability is important in spite of the geographical origin of the studied individuals. Indeed, it was found that the population of Kesra is the most polymorphic and that Menzel Bouzelfa is the slightest diverse population.

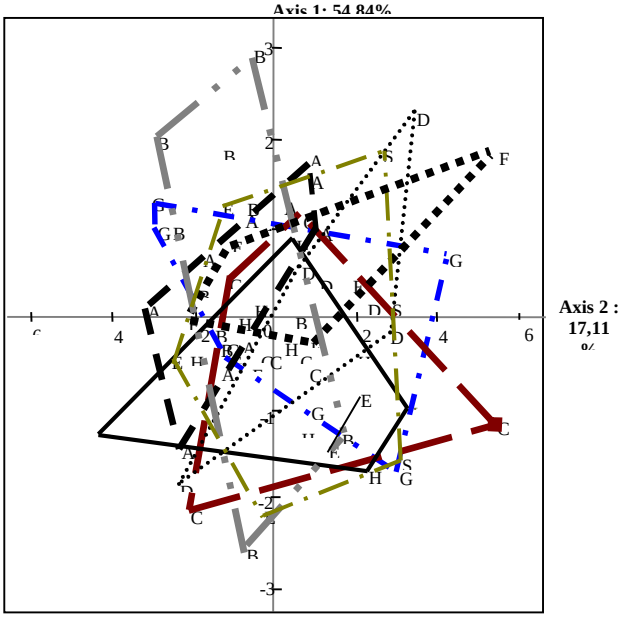



**Figure 3**: Dispersion of Tunisian wild fig trees in the plot (1-2) of the principal component analysis (72% of the global variability) based in phenotypic characters.

A : Menzel Bouzelfa B : El Haouaria C: Kerkennah D : Ghar El-Melh E : Raf-Raf F : Ras Jebel G : Sidi Mansour H : Kesra

## DISCUSSION

The purpose of the present study was to evaluate genetic diversity and characterize the sub-spontaneous fig tree using morphological parameters of leaves. Results exhibited considerable genetic variation among Tunisian sub-spontaneous fig populations. Our results suggest the most discriminating characters are depth of left lateral sinus1 [SL1] and depth of right lateral sinus2 [SL2]). In addition, various differences were detected on percentage of number of lobes. These traits will be very useful to characterize and discriminate sub-spontaneous fig populations. Indeed, prospections conducted permit to conclude that a great mixture of the lobe number was unregistered per prospected area. Results obtained by PCA and ward's dendrogram showed a distribution of trees genetic variability independent from their geographical origins. The pattern of morphological variability observed in this study is similar to those from several studies

demonstrating that for common fig cultivars morphological differences are often based on morphological and pomological as suggested by several works (Salhi Hannachi et al. 2003, Chatti et al. 2004; Saddoud et al. 2008, Papadopoulou et al. 2002).

It will be very useful to continue this study for enlarged the number of wild fig trees and attain other regions in Tunisia. In order to optimize and refine these results molecular markers such as ssrs must be revealed. Combined results will improve discrimination between sub-spontaneous *Ficus carica* individuals to elaborate domestication history of fig in Tunisia. Our results demonstrate that Tunisian spontaneous fig germplasm presents specific genetic resources. Clonal diversity, ancient cultivation, local gene pool and genetic structuring at a local scale imply a high potential to start local cultivars selection and breeding programs.

**AKNOWLEGMENTS**

This work was partially supported by grants from the Tunisian "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie " Projet Lab B02.

**REFERENCES**

Berg, C.C. (1989). "Classification and distribution of *Ficus*". Experientia. 45: 605-611.

Berg, C.C. (1990). "Reproduction and evolution of *Ficus* (Moraceae): traits connected with the adequate rearing of pollinators". Mem. NY Bot. Gard. 55:169–185.

Chatti, K. ; Salhi Hannachi, A. ; Mars, M. et al. (2004). Analyse de la diversité génétique de cultivars tunisiens de figuier (*Ficus carica* L.) à l'aide de caractères morphologiques. Fruits. 59: 49–61.

Dagneli, P. (1975). Théorie et methodes statistiques. Applications agronomiques. Volume II : Les méthodes de linférence statistique. Eds., Les presses agronomiques de Gembloux. 463 p.

IPGRI. (2004). Descriptors for fig (Ficus carica L.). IPGRI, CIHEAM. ISBN 92-9043-598-4.

Janzen, D.H. (1979). "How to be a fig" annual review of ecology and systematics 10: 13-51.

Khadari, B.; Grout, C. ; Santoni, S. ; Hochu, I. ; Roger J.P. ; Ater, M. ; Aksoy, U. and Kjellberg, F. (2005). " Etude préliminaire des origines de *Ficus carica* L. et de sa domestication » Les Actes du BRG. 5 : 53-65

Kislev, M. E.; Hartmann A. and Bar-Yosef O. (2006). "Early domesticated fig in the Jordan Valley." Science. 312: 1372–1374.

Mars, M.; Chatti, K.; Saddoud, O.; Salhi Hannachi A.; Trifi, M. and Marrakchi, M. (2007). "Fig cultivation and genetic resources in Tunisia, an overview", Acta Horticulturae. 798: 27-32.

Mars, M.; Chelbi, T. and Marrakchi, M. (1998). "Multivariate analysis of fig (*Ficus carica* L.) germplasm in southern Tunisia", Acta Hortic. 480: 75–81.

Papadopoulou, K.; Ehaliotis, C.; Tourna, M.; Kastanis, P.; Karydis, I. and Zervakis, G. (2002). Genetic relatedness among dioecious Ficus carica L. cultivars by random amplified polymorphic DNA analysis and evaluation of agronomic and morphological characters. Genetica. 1-12.

Rhouma, A. (1996). "Les ressources phytogénétiques oasiennes: le figuier (Ficus carica L.).– In: Proc. 3èmes Jour nées Nationales sur les Acquis de la Recherche Agronomique, Vétérinaire et Halieutique, Nabeul.

Saddoud, O.; Chatti, K.; Salhi Hannachi, A.; Mars, M.; Rhouma, A.; Marrakchi M. and Trifi, M. (2007). "Genetic diversity of Tunisian figs (*Ficus carica* L.) as revealed by nuclear microsatellites", Hereditas. 144: 149–157.

Saddoud, O.; Baraket, G.; Chatti, K.; Trifi, M.; Marrakchi, M.; Salhi-Hannachi A. and Mars M. (2008). "Morphological variability of fig (*Ficus carica* L.) cultivars" », Int. J. Fruit Sci. 8: 35–51.

Salhi Hannachi A.; Mars M.; Chatti K.; Marrakchi M. and Trifi M. (2003). "Specific genetic markers for Tunisian fig germplasm:

evidence of morphological traits, random amplified poly
morphic DNA and Inter simple sequence repeats markers." J. Genet. Breed. 5:125-136.

StatSoft, Inc. (2001). STATISTICA (data analysis software systems), version 6. StatSoft, Inc. (www.statsoft.com), Tulsa, OK. USA.

SAS (1990). SAS user's guide: SAS STAT, SAS BASIC. Version 6 fourth edition. Sas incl, BOX 8000. cary, NC 27512-8000, cary; NC: SAS institute Inc.

Tous, J. and Ferguson, L. (1996). Mediterranean fruits, in progress in new corps, edited by j.janick. ASHS Press, Arlington , V.A.

Ward J. H. (1963). Hierarchical Grouping to optimize an objective function. Journal of American Statistical Association. 58: 236-244.

Weiblen, G. D. (2000). Phylogenetic relationships of functionally dioecious *Ficus* (Moraceae) based on ribosomal DNA sequence variation and morphology. American Journal of Botany 87: 1342-1357.

Zohary, D. and Hopf, M. (1988). "Domestication of plants in the old world", Oxford, Clorendon Press.

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 55 – 58*

**BIOLOGIA TUNISIE**

# EFFECT OF GARLIC'S ADMINISTRATION ON ERYTHROCYTES AND PLASMA PARAMETERS IN RAT

**HAMLAOUI-GASMI S., MOKNI M., AOUANI E., AMRI M., MARZOUKI L.**

Laboratoire de Neurophysiologie Fonctionnelle et Pathologies, Faculté des Sciences de Tunis – Campus Universitaire Manar II 1060 Tunis, Tunisie

## INTRODUCTION

Garlic (*Allium sativum* L.) is a widely used medicinal plant which has been extensively studied as a nutritional supplement in acute as well as in chronic experiments (Lawson and Bauer 1998). Garlic exerted wide range effects such as antiviral and antibacterial (Taratinsev et al. 1992), antitumoral (Schaffer et al. 1996), antiatherosclerotic (Campbell et al. 2001), hypolipidemic (Yeh and Liu 2001) and antioxidant (Banerjee et al. 2002).
However several reported effects were divergent even contradictory according to experimental duration, garlic extraction and dosage and also mode of administration (Banerjee and Maulik 2002). Garlic is generally administered either orally (PO) or by intraperitoneal (IP) route. This latter way of administration which avoids the gastric barrier was previously shown to be more effective than gastric gavage. Recently special emphasis was made on the use of the IP route in chemotherapy treatment (Canistra, 2006).
The present study was undertaken to compare the two modes of garlic administration on erythrocytes and plasma parameters i-e PO and IP routes in chronic experiments of one month duration. Garlic was used at high dosage previously demonstrated to reduce plasma cholesterol when acutely administered by IP route (Mokni et al. 2006).

## MATERIALS AND METHODS

### Animals and treatment

Adult male wistar rats (180–200 g) procured from Pasteur Institute (Tunis) were kept in clean plastic cages and allowed to acclimatize in the laboratory environment from 7 days. Animals were randomly divided into four groups of 12 and treated for 30 days as follows:
Group I received standard diet and served as control. Group II received standard diet supplemented with aqueous extract of garlic at 5g/kg bw.
Group III received intraperitoneal injection (IP) of double distilled water (control).
Group IV received aqueous garlic by IP injection at 5g/kg bw.
Animals were regularly cheked for weight gain or loss. After one month treatment, blood was collected by cardiac punction and used for hematological and biochemical parameters determination.

### Preparation of garlic extracts

Garlic was purchased from local market, peeled and ground with an electric mixer. It was diluted in double distilled water at 4 g/ml on the basis of the weight of the starting material and centrifuged (Beckman J20, 15 min at 10 000 g and 4°C). Supernatant was aliquoted and stored at – 80°C until use.

### Hematological parameters

Erythrocytes counts, hemoglobin level, haematocrit, VGM, CCMH and TGMH were determined automatically using coulter counts apparatus.

### Plasma parameters

Glycemia and plasma content calcium, phosphate, sodium and potassium were given on plasma with the assistance of an automat (Coulter).

**Statistical analysis**

All data are expressed as mean ± SEM by factorial ANOVA, followed by Student test. Differences between groups were considered significant when $p < 0.05$.

## RESULTS

**Effect garlic on the hemoglobin rate**

Administered by PO, garlic induced a significant increase of 14% of the hemoglobin rate. This level, 14 g/dl at the control, passes to 15.96 g/dl in the treated animals.

However, injected in IP garlic induced a highly significant reduction in the hemoglobin rate rea[illegible]

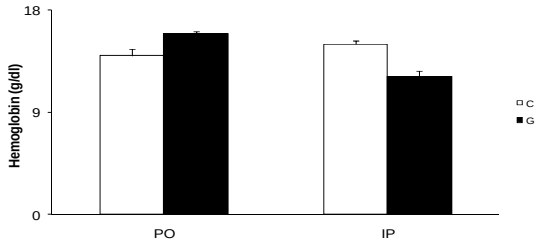



Figure 1: Effect of garlic on hemoglobin level

**Effect of garlic on the haematocrit rate**

Administered by PO, garlic supports a significant increase in the haematocrit (+18.16). Indeed, the haematocrit being of 40.20% at the control group reaches the value of 47.5% in the treated rats.

On the other hand in IP, garlic involves a highly significant reduction of the haematocrit (-12.54%). This erythrocyte parameter is 43.77% in the control group. It reaches a value of 38.28% in the treated animals.

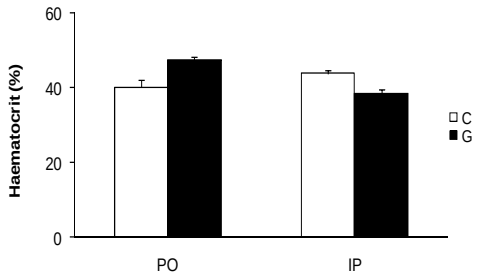



Figure 2: Effect of garlic on haematocrit

**Effect of garlic on the VGM, the CCMH and the TGMH**

The results carried by table 1 reveal that garlic administered by PO does not involve any significant variation on VGM, CCMH and TGMH. Nevertheless, in the animals treated by garlic by IP mode, we noted a clear increase in the VGM and a significant reduction in the CCMH. The TGMH remains invariable even in IP.

**Table 1:** Effect of garlic on VGM, CCMH and TGMH

| | Control by PO | Garlic by PO | Control by IP | Garlic by IP |
|---|---|---|---|---|
| VGM (µm3) | 52,85 ± 0,7 | 51,5 ± 0,5 | 52,32 ± 0,2 | 54,63 ± 0,7** |
| CCMH (g/l) | 33,6 ± 0,3 | 34 ± 0,1 | 34,78 ± 0,4 | 33,32 ± 0,4* |
| TGMH (pg) | 18,15 ± 0,3 | 17,52 ± 0,2 | 19,18 ± 0,1 | 18,20 ± 0,3 |

**Effect of garlic on glycemia and plasma electrolytes**

Table 2 shows that garlic does not induce any variation concerning the glycemia and the concentration of the plasma electrolytes whatever its mode of administration in the various treated animals.

**Table 2:** Effect of garlic on glycemia and plasma electrolytes

| | Control by PO | Garlic by PO | Control by IP | Garlic by IP |
|---|---|---|---|---|
| Glycemia (g/L) | 1,28 ± 0,03 | 1,35 ± 0,05 | 1,32 ± 0,02 | 1,38 ± 0,04 |
| Calcemia (mM/L) | 2,29 ± 0,08 | 2,44 ± 0,1 | 2,18 ± 0,01 | 2,20 ± 0,02 |
| Natremia (mM/L) | 132,54 ± 2 | 129,86 ± 2 | 130,9 ± 2,7 | 134,6 ± 0,6 |
| Kaliemia (mM/L) | 6,20 ± 1,10 | 5,90 ± 1,00 | 6,39 ± 0,30 | 6,31 ± 0,14 |

## DISCUSSION

The present study was undertaken in order to bring some clues to several discrepancies about the effectiveness of garlic beneficial health effects (Agarwal 1996). We used garlic in subchronic experiments of one month duration at high dosage previously shown to exert cholesterol and glucose lowering activities (Mokni M. 2006) and compared IP versus PO mode of administration.

Overall we found that garlic administration by PO route exhibited much more beneficial effects than the IP way. When orally administered garlic exerted positive growth effects evaluated by weight gain (not shown). Furthermore garlic increased the erythrocytes number (not shown) as well as hemoglobin content and haematocrit. All these data which fully corroborated previous work demonstrating further strengthened its putative use as an antifatigue agent (Murihara N. et al. 2007) by its ability to increase erythrocytes number. This last effect might have been provoked by garlic induced decreased

rigidity inherent to its hypolipidemic effect. In this respect it is recognized that osmotic fragility is a determinant of the deformability property of erythrocytes which is essential for their function and survival against destruction by the spleen (Kempaiah R.K. et al. 2005).
Conversely when IP administered garlic induced weight loss (no shown), slightly decreased erythrocytes number as well as their osmotic fragility (no shown). It also slightly decreased hemoglobin level and haematocrit. Noteworthy, that this result might have been the consequence of the lack of a clear garlic lipid lowering effect in these experimental conditions. All these data obtained in normoglycemic rats are comparable to a recent work using type 2 diabetic animals in which garlic was shown to have no influence on several parameters as final body weight and hemoglobin content (Shahidul and Haymie, 2008). Thus garlic mode of administration appeared essential. When orally administered garlic exhibited beneficial effects with no evident toxicity even at high dosage used in the present study. When IP administered, garlic exhibited overall toxic side effects as indicated by weight loss, high level in plasma transaminases or increased MDA content reflecting multiple organ failure especially the liver (data not shown).

In this respect, oral administration of low garlic concentration has been recently shown to exert antioxidant properties in plasma and erythrocytes of elderly subjects (Avc A. et al. 2008).
Although preliminary our data opened the way to the putative use of high garlic concentration either as antioxidant when orally administered or as prooxidant when IP administered. Furthermore this approach may found therapeutic applications in intraperitoneal chemotherapy protocols (Canistra S.A. 2006).
Garlic is commonly allowed like a hypoglycemiant agent and this which that is the mode of administration. We for our part showed that garlic does not exert any effect hypoglycemiant whatever the route of administration.
In this order Rosen et al (2001) mentioned the absence of hypoglycemiant effect of garlic after its administration by intragastric way. In the same way, it was shown (Liu et al., 2006) that a daily treatment of diabetics rats by garlic oil (100 mg/kg bw) or of the DADS by oral way during 16 weeks no effect the glycemia.

However, of the contradictory results showed (Chang et al., 1980) that the ingestion of garlic powder induced a reduction in blood glucose. This effect was also obtained in chronic conditions, after oral administration of 800mg of garlic powder/day what induces a reduction of 11.6% of the glycemia (Kiesewetter et al., 1991). Moreover, in diabetics rats treatment by garlic improves all the changes of these parameters on a level comparable with that reached by treatments with insulin or the glibenclamide (Sheela and Augusti, 1992).
We can conclude that the antianemic effects of garlic are original, interesting and could have promising therapeutic applications.

**REFRENCES**

H. Aebi. Catalase in vitro. Methds Enzymol.1984; 105:121-6.

K.C. Agarwal. Therapeutic actions of garlic constituents. Med. Res. Rev. (1996)16, 111-124.

A. Avc, T. Atl, I.B. Ergüder, et al. Effects of garlic consumption on plasma and erythrocytes antioxidant parameters in elderly subjects. Gerontology (2008) 54:173-6.

S.K. Banerjee and S.K. Maulik. Effect of garlic on cardiovascular disorders: a review. Nutrition J (2002) 1:4

S.K. Banerjee, M. Maulik, S.C. Mancahanda et al. Dose-dependent induction of endogenous antioxidant in rat heart by chronic administration of garlic. Life Sciences (2002) 70, 1509-18.

J.H. Campbell, J.L. Efendy, N.J. Smith and G.R. Campbell. Molecular basis by. which garlic suppresses atherosclerosis. J. Nutr (2001) 131: 1006S-1009S

S.A. Canistra, Intraperitoneal chemotherapy comes of age. N. Engl. J. Med. 354 (2006) 77-79.

M.L.W. Chang, M.A. Johnson. Effect off garlic one carbohydrate metabolism and lipid synthesis in rats, J Nutr. 110 (1980) 931-936.

A.K. Dinda, S.K. Gupta, S.K. Maulik, Dose-dependent induction of endogenous antioxidant in rat heart by chronic administration of garlic, Life Sciences 70 (2002) 1509-1518.

HA Hassan, SM El-Agmy, RL Gaur et al. In vivo evidence of hepato- and reno-protective effect of garlic oil against sodium nitrite-induced oxidative stress. Int J Biol Sci. 2009; 5: 249-55.

R.K. Kempaiah and K. Srinivasan. Influence of dietatary spices on the fluidity of erythrocytes in hypercholesterolaemic rats. Br J Nutr (2005) 93: 81-91.

H. Kiesewetter, F. Jung, G. Pindur, E.M. et al. Effect off garlic one thrombocyte aggregation, microcirculation and other risk factors, Int J Covering joint Pharmacol Ther Toxicol. 29 (1991) 151-155.

L.D. Lawson, R. Bauer, A review of its medicinal effects and indicated active compounds, In: phytomedicines of Europe. Chemistry and Biological activity 691 (1998) 176-209.

S.I. Liochev, I. Fridovich. The effects of superoxide dismutase on $H_2O_2$ formation. Free Radic Biol Med. 2007. 42:1465-9.

P.L. Liu, C.K. Wong, H. Lii, L.Y. Sheen. Antidiabetic effect off garlic oil drank not diallyl disulfide in rats with streptozotocin-induced diabetes, Food and chemical Toxicology 44 (2006) 1377-1384.

M. Mokni, F. Limam, M. Amri and E. Aouani. Acute effects of a partially purified fraction from garlic on plasma glucose and cholesterol levels in rats: putative involvement of nitric oxide. Indian J Biochem Biophys (2006); 43: 386-90.

N. Morihara, T. Nishihama, M. Ushijima et al. Garlic as an antifatigue agent. Mol Nutr Food Res 2007 51: 1329-34.

R.T. Rosen, R.D. Hiserodt, E.K. Fukuda et al. Determination off Al, S-Allylcystein and volatile metabolites off Garlic in breath, plasma gold simulated gastric fluids, J Nutr. 131 (2001) 968S-971S.

K. Satho, Serum lipid peroxidation in cerebrovascular disorders determined by a new colorimetric method, Clin. Chim. Acta 90 (1978) 37–43.

E.M. Schaffer, J.Z. Liu, J. Green, C.A. Dangler and J.A. Milner. Garlic and associated allyl sulphur components inhibit N-methyl-N-nitrosurea induced rat mammary carcinogenesis. Cancer Lett. (1996) 102, 199-204.

I. Shahidul and C. Haymie. Comparative effect of dietary ginger and garlic investigated in a type 2 diabetes model of rats. J. Med. Food (2008) 11: 152-159.

C.G. Sheela, K.T. Augusti. Antidiabetic effects off Dirties cysteine sulphoxide isolated from garlic Al sativum Linn, Indian J Exp Biol. 30 (1992) 532-526.

A.V. Taratinsev, P.V. Vrizheshch, A.A. Schegolev et al.. Ajoene antagonizes integrine-dependent processes in HIV-infected T lymphoblast. AIDS (1992) 6, 1215-1217.

Y.Y. Yeh and L. Liu. Cholesterol-lowering effect of garlic extract and organosulfur compounds: human and animals studies. J. Nutr. (2001) 131, 989S-993S.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7; 59 – 66*

**BIOLOGIA TUNISIE**

# INDUCED PHYSIOLOGICAL TRAITS BY NACL IN TWO BARLEY CULTIVARS

**Eleuch L, Rezgui S and Daaloul A**

Laboratoire de Génétique et Amélioration des Plantes, Département d'Agronomie et Biotechnologies Végétales. Institut National Agronomique de Tunisie, 43, Avenue Charles Nicolle, 1082 Tunis, Cité Mahrajène, TUNISIE

*Corresponding author Eleuch Lilia; E-mail: lilia.eleuch@voila.fr ; Phone number: +21621591696

**Keywords:** *Hordum vulgare*, salinity stress, physiological parameters.

## ABSRACT

Mechanisms of salinity tolerance were assessed using two barley (*Hordum vulgare* L.) cultivars (Acsad1230 and Arig8 originated from Lybia and Morocco, respectively). These cultivars were grown three salt treatments: 0, 70 mM and 140 mM of NaCl. Dry matter, relative water content, chlorophyll and glucose contents were measured from the leaves during juvenile and mature growth stages. Superior dry matter accumulation in Acsad 1230 at both growth stages was attributed to stable chlorophyll (a/b) ratio and a compartmentalization of $Na^+$ and $Cl^-$ ions. Increased relative water content, lower glucose rate and increased $Na^+$ and $Cl^-$ ions suggested that Acsad1230 is tolerant to salt stress. This cultivar appears to support salt stress with a minimum energy requirement. In Arig 8, a significant decline for the relative water content dry matter and chlorophyll (a/b) ratio was noted. Increased glucose synthesis was required in Arig 8 to ensure osmotic adjustment. This cultivar is characterized as salt susceptible.

## INTRODUCTION

Barley is the most important cereal crop in Tunisia covering over than one third of the total cereal areas. Most barley is cultivated on marginal growing areas of the semi-arid where drought events are prevailing and frequently encountered. Barley grown in the central and southern regions of Tunisia is often suited for both grazing and grain production. In these semi-arid areas, barley grain production is limited because of water deficit and droughts that are occurring frequently. Hence, irrigated farming systems are becoming a common cultural practice to alleviate drought effects. However these farming systems are particularly prone to Stalinization and alkalinisation (Munns et al., 2000) causing limited yielding ability of barley cultivars. Moreover; greater heat and longer photoperiod combined with limited rainfall are among the major causes of salt accumulation on the surface affecting a significant cereal growing areas in the

semi-arid (Ben Naceur et al., 2001).
Salinity is considered as an irreversible obstacle affecting the vegetative growth (Lachaal, 1998) of various plant species. Among the cultivated crops, barley was found the most tolerant cereal species because of its rapid vegetative growth and faster progression of phenological development. The ability to produce greater biomass of a cultivar under saline relative to that produced in control conditions (Munns et al., 2000) and differences on the rate of $Na^+$ and Cl- uptake (Greenway and Munns, 1980) were considered as a mechanism conferring salt tolerance in several barley cultivars (Arbaoui et al., 2000). Comprehensive assessment of the relationship between rates of $Na^+$ or $Cl^-$ and salt tolerance is however lacking. Therefore, establishing potential association between morphological selection criteria and physiological traits using salt tolerant and salt susceptible barley cultivars may contribute to characterize the mechanism of salt tolerance and potential implication of these traits in the expression of this tolerance.

## MATERIAL AND METHODS

Two six rows barley cultivars (*Hordeum vulgare* L.): Arig 8 and Acsad 1230 originated from Morocco and Lybia respectively were used in this investigation. These cultivars were grown in pots using three salt treatments: Control, 70 and 140 mM of NaCl. Irrigation of the pots was supplemented every 5 to 6 days using tap water added with salt treatment. One leaching irrigation is applied every other salt treatment. Each salt x cultivar treatment combination was replicated using three pots, thus a total of 18 experimental units were monitored during the different the whole experiment. At Z2.1 and Z4.5 growth stage, random samples of vegetal material were taken from aerial plant parts to determine fresh biomass, dry weight and mineral components. Fresh weight from leaves was determined from samples using weighing scale of 1/10 mg precision. Dry weight is determined after drying these samples at 80°C for a period of 48 hours. Relative water content was using the following equation RWC= (FW-DW)/(SW – DW) where FW= fresh weight, DW=dry weight and SW= turgescent tissue . Maximum turgescent plant tissue is placed during 24h at 4°C in distilled water.
Fine grinded plant tissue samples were ground with a cyclone mill. Powder samples were added with 5 ml acid nitro-perchlorique. The mattress is covered with funnels and mineralized up till complete discoloration. $Na^+$ cation is estimated using spectrophotometry (photometer Eppendorf) and $Cl^-$ ion content is determined using coulometer with Chloridometer (Buchler-Cotlove). Sugar extracts were determined using grinded 200mg fresh plant tissue from leaves at Z2.1 and Z4.5 growth stages for which 1ml of potassium ferro-cyanure 15% is added along with 3ml of distilled water and 1ml Zinc Acetate 30%. Final solution volume is then adjusted with distilled water to 10 ml. The content is filtrated and conserved at -20°C Glucose is determined (Dingeon, 1975) using enzymatic kits type Biomaghreb. Glucose content is then determined using 505 nm wavelength after an incubation of 10 min at 37°C.
Chlorophyll a and chlorophyll b were extracted using dimethyl-foramid and quantified by spectrophotometry at 663nm wavelength and 645nm representing maximum absorption for chlorophyll a and b respectively. Chlorophyll a and b rates expressed in mg/g of FW were estimated using the following formula (Arnon, 1949) using the following equations:
Chla (mg/ml) = (0,0127 * A663)-(0,00269*A645); Chl b (mg/ml) = (0,0229 *

A645)- (0,00468*A663) ; Chl totale (mg/ml) = (0,0202 * A645)- (0,00802*A663)
Data were analyzed as completely randomized design using proc anova of SAS (1995) with the option of $LSD_{0.05}$ to compare means of both main and interaction effects.

## RESULTS

The expression of physiological traits at both growth stages provided indication that barley cultivars used in this investigation are characterized by a different salt response at both growth stages. During juvenile growth stage (Z2.1), relative water (RWC); Sodium ($Na^+$), Chloride ($Cl^-$) and glucose contents are under the control of both genotype and NaCl concentrations of the substrates (significant cultivars x salt treatment interaction $p<0.01$) (Table 1 ). However, dry matter (DM) and chlorophyll ratio a/b (Chla/b) were not affected by either cultivar nor by salt treatment. This result may indicate that both barley cultivars are characterized by a comparable DM during early growth stage (Z2.1) and may have similar photosynthetic capacity. At later growth stage (Z 4.5), significant ($p<0.01$) cultivars x salt treatment interaction on RWC and glucose were noted (Table 2); whereas NaCl significantly ($p<0.01$) affected DM; $Na^+$ and Chl (a/b). These discrepancies between both growth stages could suggest important changes in the metabolic pathways within each cultivar to support incremental accumulation of salt within cell tissue. Hence, the magnitude of salt effects on the physiological traits may depict a differential mechanism conferring salt tolerance that is operating within each barley cultivar.

**Table 1.** Mean squares of physiological traits: dry matter (DM); relative water content (RWC); sodium content (Na+); chlorine (Cl-); chlorophyll (a/b) ratio Ch(a/b) and glucose content evaluated at Z2.1 growth stage for two barley cultivars assessed under salt treatments.

| Source of Variation | ddl | DM ($g10^{-3}$) | RWC (%) | Na+ (µeq/g DM) | Cl- (µeq/g DM) | Ch(a/b) | Glucose (mM/l) |
|---|---|---|---|---|---|---|---|
| Cultivars (C) | 2 | 0.6 $^{ns}$ | 77.44** | 1612057.17** | 622747.70** | 0.17 $^{ns}$ | 0.027** |
| Salt (S) | 1 | 0.3 $^{ns}$ | 5.29* | 2718. 20** | 2076.41 $^{ns}$ | 0.12 $^{ns}$ | 0.222** |
| S*C | 2 | 0.5 $^{ns}$ | 89.61** | 121344. 99** | 96341.06** | 0.27 $^{ns}$ | 0.063** |
| Error | 12 | 0.2 | 0.59 | 777. 53 | 2365.34 | 0.38 | 0.00002 |
| C.V | | 23.06 | 0.92 | 3.15 | 3.07 | 18.16 | 0.81 |
| $R^2$ | | 0.47 | 0.98 | 0.99 | 0.98 | 0.18 | 0.99 |

* ;** Significant at $p<0.05$ and $p<0.01$ probability levels respectively. ns no significant difference at p=0,05

**Table 2.** Mean squares of physiological traits: dry matter (DM); relative water content (RWC); sodium content (Na+); chlorine (Cl-); chlorophyll (a/b) ratio Ch(a/b) and glucose content evaluated at Z4.5 growth stage for two barley cultivars assessed under salt treatments

| Source of Variation | ddl | DM (g) | RWC (%) | Na+ (µeq/g DM) | Cl- (µeq/g DM) | Ch(a/b) | Glucose (mM/l) |
|---|---|---|---|---|---|---|---|
| Cultivars (C) | 2 | 0.48 $^{ns}$ | 26.79* | 22203.78 $^{ns}$ | 19.41 $^{ns}$ | 0.88 $^{ns}$ | 0.224 ** |
| Salt (S) | 1 | 4.59** | 6.90 $^{ns}$ | 884644.02** | 9233.70 $^{ns}$ | 2.02* | 0.024 ** |
| S*C | 2 | 0.60 $^{ns}$ | 51.98** | 44370.59 $^{ns}$ | 3843.96 $^{ns}$ | 0.12 $^{ns}$ | 0.016 ** |
| Error | 12 | 0.50 | 4.68 | 24022.52 | 14326.18 | 0.43 | 0.00003 |
| C.V | | 42.62 | 3.08 | 16.24 | 9.08 | 27.97 | 0.71 |
| $R^2$ | | 0.64 | 0.72 | 0.87 | 0.13 | 0.50 | 0.99 |

* ;** Significant at $p<0.05$ and $p<0.01$ probability levels respectively. ns no significant difference at p=0,05

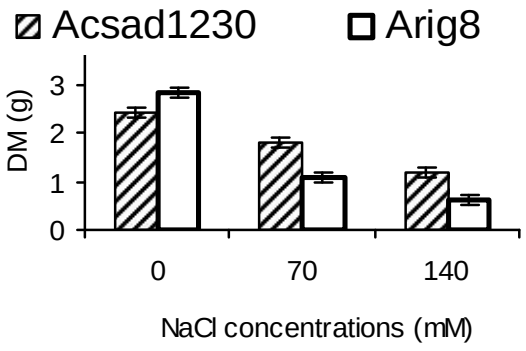



**Figure1.** Effect of salt treatment on dry weight of biomass in aerial plant parts evaluated for both barley cultivars ( Acsad 1230 and Arig 8) at Z4.5 growth stage.

**Dry Matter Accumulation**

At Z2.1 growth stage, both cultivars showed comparable biomass accumulation in unsalted growing condition ranging from 82 mg to 71 mg for Arig 8 and Acsad 1230 respectively. Although, this difference is not significantly different, it is apparent that increased level of salt content did not exert depressive effect on phytomass accumulation for Acsad 1230, whereas 46.3% dry matter reduction was noted for Arig8 when 140 mM of salt treatment was applied (Table 1). At Z4.5 growth stage a significant dry weight reduction ranging from 62.2% to 78.5% was noted for Arig8 with increased salt levels of 70 mM and 140 mM respectively. It is however noted that dry weight reduction was not significant and less pronounced in Acsad 1230 as compared to Arig8 (Figure 1) (Table 2).

**Leaves Moisture Content**

Relative moisture content in the leaves (Figure 2a) evaluated at Z2.1 growth stage is positively associated with increased levels of salt treatments relative to control growing conditions in Acsad 1230. Increases of water content of 7% and 4% were noted under low (70mM NaCl) and elevated (140mM NaCl) salt conditions respectively. Inversed trend is noted for Arig8 where relative moisture content decreased (14%) significantly with greater NaCl concentrations relative to control conditions (Table 1). At Z4.5 growth stage, relative water content was comparable for both cultivars grown under control conditions ranging 69.3% to 72.1% for Arig8 and Acsad1230 respectively. Significant reductions of the relative water content of 7.46 % were noted for Arig8; whereas increased relative water content (9%) was obtained for Acsad1230 (Figure 2b) when 140 mM of NaCl is applied (Table 2).

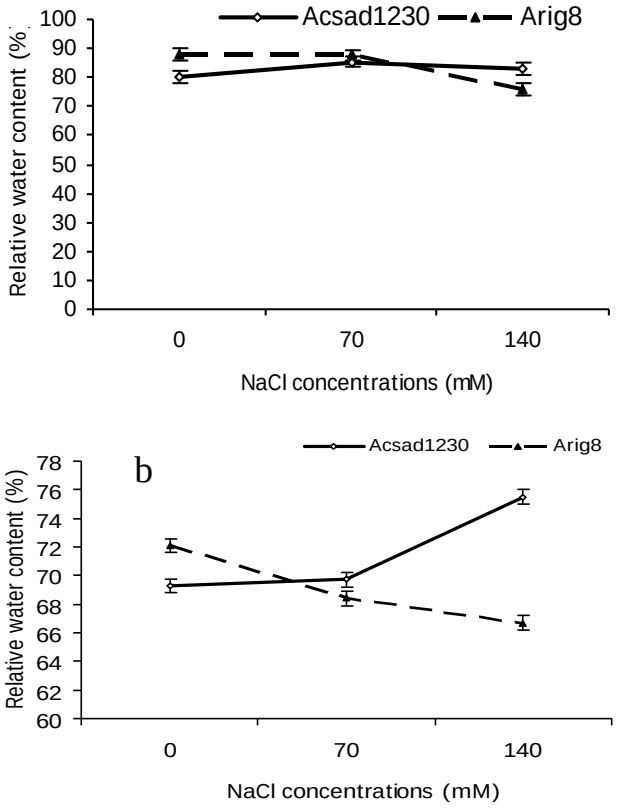



**Figure 2.** Effect of salt treatment on relative water content evaluated in two barley cultivars ( Acsad 1230 and Arig 8) at Z2.1 (a) and Z4.5 (b) growth stages**.**

**Sodium Content**

At early growth stage Z2.1 greater sodium content (366µeq/gDM) was found in Arig8 than in Acsad 1230 (240 µeq/g DM) when both cultivars were grown under control conditions. Sodium concentrations increased with greater salt treatment applications in both cultivars. This increase ranged from 331% and 522% for the cultivar Acsad 1230 treated with 70mM and 140 mM respectively. Whereas a slight increase of 196% is noted for Arig8 for both treatments (Figure 3a) (Table 1). At growth stage Z4.5, there were no genetic differences for $Na^+$ when both cultivars are grown non saline conditions; however, significant increases of sodium

contents of 160% and 189% were noted for Acsad1230 grown under 70mM NaCl and 140 mM NaCl respectively. These increases were only of 79% and 97% for Arig8 grown under similar saline conditions (Figure 3b) (Table 2).

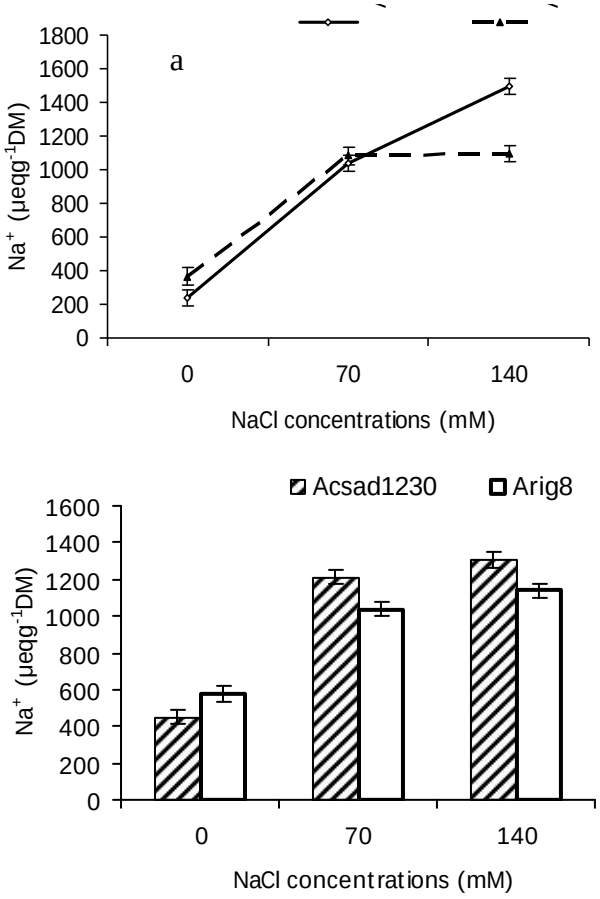



**Figure 3.** Effect of salt treatments on sodium content in leaves observed in two barley cultivars (Acsad 1230 and Arig 8) at Z2.1 (a) and Z4.5 (b) growth stages.

**Chloride Content**

Greater Chloride concentration was found during juvenile growth stage Z 2.1 in Arig8 (1332 µeq/g DM) as compared to Acsad1230 (1135 µeq/gDM) grown under control conditions; however; $Cl^-$ concentrations appeared to be positively associated with increasing salt stress. More pronounced effects were found for Acsad1230 where Chloride concentrations increased consistently from 39% to 76% at 70mM and 140 mM salt levels. In Arig8, Chloride increase of 30% is noted for both salt levels as compared to control growing conditions (Figure 4) (Table1). At growth stage Z 4.5 both cultivars grown under control showed comparable $Cl^-$ concentrations of 1248.08 µeq/g DM and 1300.76 µeq/g DM for Acsad 1230 and Arig 8 respectively. Increased levels of NaCl did not affect Chloride concentration in both cultivars at mature growth stage (Table 2).

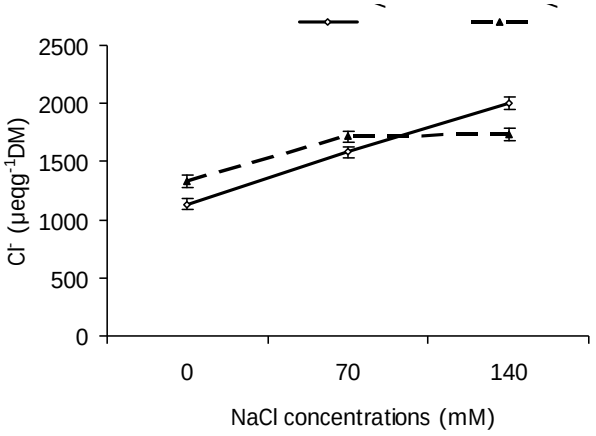



**Figure 4.** Effect of salt treatments on Chloride content in leaves observed in two barley cultivars (Acsad 1230 and Arig 8) at Z2.1 growth stage.

**Chlorophylls a/b Ratio**

A ratio of 3.1 mg/g of fresh weight was noted for both cultivars grown in non saline conditions at Z.2.1 growth stage. This ratio did not indicate a significant variation for both cultivars grown under the various slat treatment levels (Table 1). At Z4.5 growth stage, chlorophyll ratio was identical for both cultivars under control salt treatment. Increasing levels of NaCl did not affect this ratio in Acsad 1230 whereas induced reduction of 49.43% was noted for Arig8 grown under 140mM NaCl (Figure 5) (Table 2).

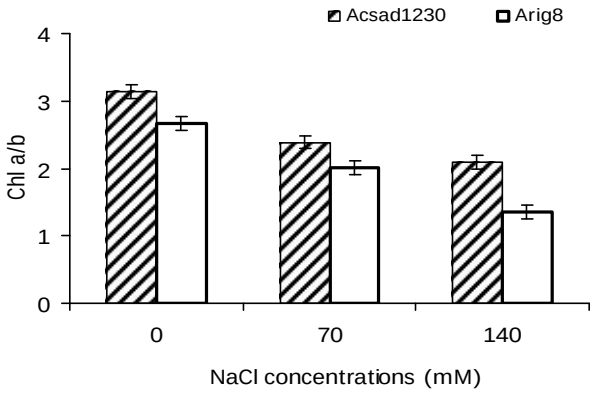



**Figure 5.** Effect of salt treatments on chlorophyll a/b ratio observed for in two barley cultivars (Acsad 1230 and Arig 8) at Z4.5 growth stage.

**Glucose Content**

Glucose content was significantly superior in Acsad 1230 (0.7mmol/l) than in Arig8 (0.3mml/l) grown under control condition at Z 2.1 growth stages. When increased levels of salt treatment are applied, a significant increase of glucose content is noted for Arig8 ranging from 10% and 86% for 70mM and 140mM NaCl applications respectively. In the opposite, reduced glucose content in Acsad 1230 ranging from 7% and 13% was noted for the same salt treatments (Figure 6a) (Table 1). At Z4.5 growth stage glucose content was 0.235 mM/l in Arig8 and 0.128mM/l in Acsad1230 grown under non saline condition. These glucose rates increased for about 81% in Arig8 in either 70mM or 140mM of salt treatment. A decrease of glucose of 9% is obtained when 70mM NaCl is applied and an increase of glucose content of 42% with 140mM salt treatment were noted for Acsad1230 (Figure 6 b) (Table 2).

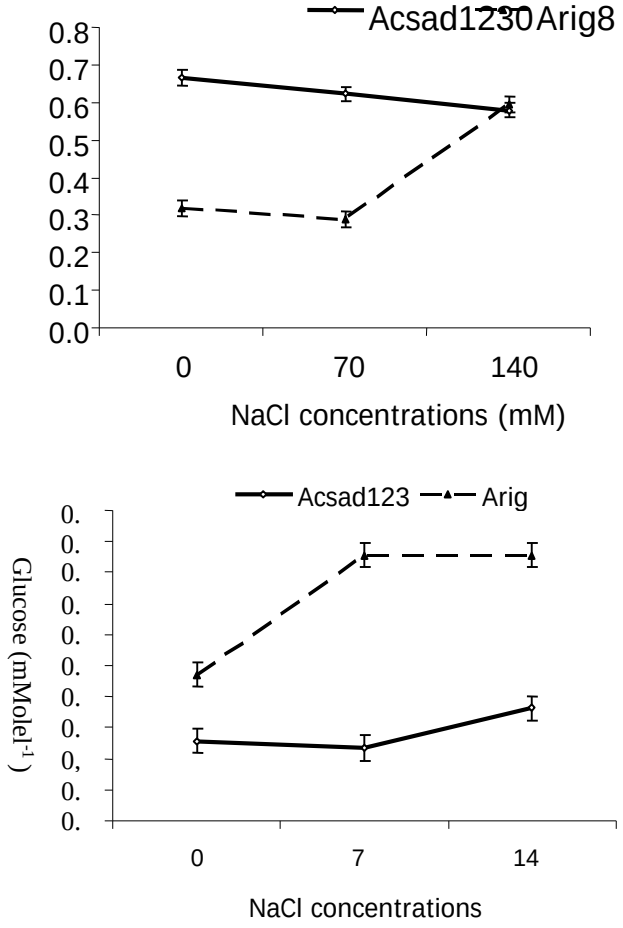



**Figure 6.** Effect of salt treatment on glucose content observed in two barley cultivars (Acsad 1230 and Arig 8) at Z2.1 (a) and Z4.5 (b) growth stages.

**DISCUSSION**

Improvement of salt tolerance may be possible if useable genetic variable exists. Results of this investigation indicated that both barley cultivars are characterized by a differential response to increased levels of salt as depicted by the magnitude of the effects on the expression of the various physiological traits and the changes of inorganic and organic solutes. However most of the mean values of these traits suggest that Acsad1230 is more tolerant than Arig8 to salt stress. Tolerance to saline conditions of Acsad 1230 is attributed mainly to the ability of this cultivar to maintain a constant to limited reduction of dry matter production suggesting adequate carbohydrate supply to the growing plant parts (Munns, 1993); and efficient salt sequestration within vacuoles. Several authors (Sooeveld et al., 1999) reported that reduced biomass caused by NaCl could be perceived as selection criteria for salt tolerance that is often associated with a limited effect on dry matter production and increased water status in the vegetative plant parts.

Two phases were identified during vegetative growth period Z2.1 and Z4.5 with different responses to salt stress. Several authors (Munns, 1993; Bounaqba, 1998; Fortmeier and Schubert, 1998) reported comparable results using selected wheat; barley and maize cultivars. Lower salt susceptibility and limited salt effects are noted at juvenile plant growth stage (Z2.1) and during advanced growth stage Z4.5 a greater salt effect is noted on both barley cultivars. The latter phase contributed to discriminate between both cultivars with regard to salt tolerance. The reduced growth rates noted during the first phase is attributed to salt osmotic effects operating on both barley cultivars. However the second phase could be explained by the toxic effects of the accumulated NaCl ions within plant tissues. These results are supported by a slow growth rates noted for Arig8 as compared to Acsad 1230.

Using three *Suaeda* species (Boucaud, 1972) demonstrated that during the juvenile growth cycle, greater accumulation of mineral nutrients; whereas, at mature growth stage there is an activation of protein synthesis after stabilizing intracellular with minerals. Therefore; salt susceptibility observed at mature growth stage of

plant growing in saline substrates could be attributed to the specific effects of $Na^+$ on the metabolism of cells. However the difference in response to salt tolerance noted for both barley cultivars during mature growth stage could be explained by the relative period required to the vacuole saturation of parenchyma cells and leaves senescence due to excess ions of $Na^+$ and $Cl^-$ that may cause a nutrient disorder and imbalance of other cation such as $K^+$. These phenomena are often observed in mature leaves and causing a major intoxication of these leaves and promote early senescence (Bounaqba**,** 1998). The repartition $Na^+$ and $Cl^-$ ions in younger and mature leaves was not uniform for both cultivars. Greater $Cl^-$ ion concentrations are found at juvenile growth stage. This declining gradient of $Cl^-$ in relation with plant age could be attributed to a reduced concentration of this ion in the xylem sap during its upward translocation.

Thus, higher Chloride rates were noted for Acsad 1230 than observed for Arig 8 during Z2.1 and Z4.5 growth stages. These phenomena could be perceived as a mean to maintain the acid-basic status of the cells. Hence, greater Chloride levels are associated with significant reduction of organic and mineral other than $Cl^-$ such as organic acids. Therefore, a more appropriate osmotic adjustment is taking place by an accumulation of both ions $Na^+$ and $Cl^-$ within plant leaves in Acsad1230.

Using both growth stages, the cultivar Acsad 1230 is considered as includer whereas Arig 8 is considered as partial exluder. This inclusive trait is considered as characteristics of salt tolerant plant species such as halophytes or glycophytes species that are characterized by a comparable response to halophytes (sugar beet, cotton). These species could absorb and transport large rates of $Na^+$ without affecting the metabolic process (Rush and Epstein, 1981). This is achieved by $Na^+$ ions a sequestration within vacuole. Nonhalophytes (glycophytes) are characterized by salt exclusion from the leaves to minimize toxicity but accelerates water deficit in plant parts (Alberico and Cramer, 1993). Hence; the relationship between sodium and the relative water content in the leaves observed for both growth stages (Z2.1 and Z4.5) prove that $Na^+$ excess is accumulated within the apoplasm causing cellular dehydratation (Bounaqba, 1998).Osmotic adjustment is realized by an accumulation of $Na^+$ for Acsad 1230 but not with organic solute explaining the relative tolerance of this cultivar to support increased NaCl concentrations in the substrates. In the opposite Arig 8 accumulates greater levels of organic solute (higher accumulation of glucose with increased salt levels in the substrates) to adjust osmotic pressure. This high energetic cost to synthesize important glucose quantity required for the osmotic adjustment in Arig8 could be prejudicial to the growth rates and vegetative development for these plants (Alarcon et al., 1994). Limited glucose synthesis was found in Acsad 1230 explaining better growth rates of this cultivar in substrates enriched with increased salt levels. Furthermore; the stable chlorophyll (a/b) ratio noted in Acsad1230 at both growth stages suggest that photosynthesis is not disrupted in this cultivar when exposed to elevated NaCl levels. However, lower chlorophyll (a/b) ratio noted at mature leaves for Arig8 suggests that photosynthesis may have been declining because of a significant drop in chlorophyll content per unit leaf area that was attributed to accumulation of salt levels in the leaves.

This investigation aiming to establish a comprehensive assessment of salt tolerance using two barley cultivars, indicated that the cultivar Acsad1230 is salt tolerant and characterized by a limited growth rates observed using control substrates. Moreover, Acsad1230, Greater water status in leaves and higher vacuolar compartmentalization appeared to confer salt tolerance in this barley cultivar. On the opposite Arig 8 is greatly affected by increased salt levels, although has greater growth rate in control substrates.

Enhanced ability for $Na^+$ compartmentalisation, the adjustment of the relative water content and substantial improvement of organic solutes appeared to be the most important selection criteria for salt tolerance. However; progress in elucidating the mechanisms that co-regulate the expression of these physiological parameters should be considered in order to efficiently select for salt tolerance.

## REFERENCES

Alarcon, J.J., M.C. Bolarin, M. Scanchez-Blanco and A.Torrecillas, 1994. Growth, yield and water relations of normal fruited and cherry tomato cultivars irrigated with saline water. J. Horti. Sci., 69: 283-288.

Alberico, G.J. and G.R. Cramer, 1993. Is the salt tolerance of maize related to sodium exclusion? I. Preliminary screening of seven cultivars. J. Plant. Nutri., 16: 2289-2303.

Arbaoui, M., M. Benkhelifa and M. Belkhoja, 2000. Réponses physiologiques de quelques variétés de blé dur à la salinité au stade juvénile. Options méditerranéennes. Série A. séminaires. Méditerranés, 40 : 267-273.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts Polyphenoloxidase in Beta vulgaris. Plant. Physiol., 24: 1-15.

BenNaceur, M., C. Rahmoune, H. Sdiri, M. Meddahi and M. Selmi, 2001. Effet du stress sur germination, la croissance et la production en grains de quelques variétés maghrébines de blé. Science et changements planétaires/Sécheresse, 12: 167-174.

Boucaud, J., 1972. Effects du NaCl sur l'activité de la nitrate réductase chez deux variétés halophiles: *Suaeda maritima var. macrocarpa* et *var. flexilis*. Physiol. Planta., 27: 37-42

Bounaqba, S., 1998. Analyses déterminants de la tolérance a NaCl chez le blé tender, le triticale, et l'orge. Utilisation de la fluorescence chlorophyllienne dans le diagnostic de l'état fonctionnel du photosystème II. Thèse de Doctorat d'Etat en Biologie, Université de Tunis II, Tunisie.

Dingeon, B., 1975. Annals of Biology and Chimistry. 33 (3). Biomaghreb.

Fortmeier, R. and S. Schubert, 1998. Salt tolerance of maize (Zea mays L.): the role of sodium exclusion. Plant .Cell. Environ., 18: 1040-1047.

Greenway, H. and R. Munns, 1980. Mechanism of salt tolerance in nonhalophytes. Annual. Revue. Plant. Physiol., 3: 149-190

Lachaal, M., 1998. Variabilité de la réponse à la salinité chez la lentille, et variation en fonction du stade de développement. Thèse de Doctorat d'Etat Es Sciences Naturelles, Université de Tunis II, Tunisie.

Munns, R., 1993. Physiological processes limiting plant growth in saline soil: some dogmas and hypothesis. Plant. Cell. Environ., 16: 15-24.

Munns, R., R.A. Hare and T.D. Colmer, 2000. Salt tolerance in wheat and barley In 8th International Barley Gentics Symposium. Volume 1. Ed. Dept. of Plant Science, Waite Campus, Adelaide University, pp 238-245.

Rush, D.W. and E. Epstein, 1981. Comparative studies on the sodium, potassium, and chloride relations of a wild halophytic and a domestic salt-sensitive tomato species. Plant. Physiol., 68: 1308-1313.

Sooeveld, C., R. Baas, H.M.C. Nijssen and J. Hoog, 1999. Salt tolerance of flower crops grown in soilless culture. J .Plant. Nutri., 12: 1033-1046.

SAS 1995. JMP (Version 3.2.1)**.** Statistical discovery software. SAS Institute, Inc., Cary, NC 27513.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7; 67 – 79*

**BIOLOGIA TUNISIE**

# ANALYSE GENETIQUE DE LA VIRULENCE DES BACTERIES PATHOGENES DU GROUPE *BACILLUS CEREUS*

**Sinda Fedhila[1] & Didier Lereclus[2]**

[1]Unité Protéomie Fonctionnelle et Biopréservation Alimentaire. Institut Supérieur des Sciences Biologiques Appliquées de Tunis. 9, avenue Zouhaïer Essafi. 1006. Tunis. Tunisie.
[2]Unité Génétique Microbienne et Environnement. Institut National de la Recherche Agronomique. La Minière. 78285 Guyancourt cedex. France.

**Mots clés:** pathogénie, facteurs de virulence, mutagenèse ciblée, mutagenèse transpositionnelle, IVET

**ABSTRACT**

Bacteria efficiently colonize various ecological niches including plants and animals. While invading these niches, they often cause more or less deleterious effects. These infection activities, designated "pathogenesis" or "virulence", result from the expression of virulence genetic determinants. Several genetic approaches have been developed in recent years in numerous pathogenic microbes in order to identify these determinants and to assess their expression and role in overall virulence. The resulting findings are a necessary prerequisite to better understand pathogenesis, molecular bases of host/parasite interactions and to develop new strategies preventing emergence of new pathogens. The purpose of this review is to highlight recent genetic technologies used as tools for studying molecular bases of *Bacillus cereus* group virulence and to discuss the many microbial virulence factors identified. Bacteria belonging to this group are of particular interest in that they possess both specific and opportunistic virulence features. Indeed, these environmental bacteria are able to secrete diverse metabolite enzymes and toxins which can be harmful to targeted or not organisms.

**RESUME**

Les bactéries sont capables de coloniser des niches écologiques aussi diverses que les végétaux et les animaux. Les bactéries pathogènes envahissent ces niches en causant différentes infections ayant des effets morbides plus ou moins graves. Ces phénomènes d'infection, désignés sous les termes "pathogénie" ou "virulence" sont le résultat de l'expression de plusieurs gènes dits "déterminants génétiques de virulence". Plusieurs approches génétiques ont été développées ces dernières années, chez de nombreux germes infectieux, en vue d'identifier des gènes de virulence potentiels et d'étudier leur expression et leur rôle dans la pathogénie. Ces connaissances sont un préalable nécessaire pour mieux comprendre un pouvoir pathogène, appréhender les interactions moléculaires hôte/microbe et, par conséquent, développer des stratégies visant à prévenir l'émergence de nouveaux pathogènes. Cette revue représente une analyse pratique et théorique des bases génétiques de la virulence des bactéries du groupe *Bacillus cereus sensu lato*. Ce groupe est particulièrement intéressant car il englobe des activités de virulences aussi bien spécifiques qu'opportunistes. Il s'agit de bactéries de l'environnement qui possèdent toutes les capacités de produire et de sécréter divers métabolites, enzymes et toxines qui peuvent avoir des effets nocifs sur des organismes visés ou non.

## INTRODUCTION

Le groupe *Bacillus cereus* sensu lato est composé d'une très grande variété de bactéries réparties en trois principales espèces : *Bacillus cereus* sensu stricto (*B. cereus*), *Bacillus*

*thuringiensis* (*B. thuringiensis*) et *Bacillus anthracis* (*B. anthracis*). Ces *Bacillus* partagent un fond génétique commun qui, associé à des toxines spécifiques, leur permet de coloniser des hôtes aussi divers que les plantes, les insectes et les mammifères. Ainsi, bien qu'étroitement apparentées, les trois espèces présentent des propriétés pathogènes extrêmement diversifiées.

*B. anthracis*, agent responsable de la maladie du charbon, ou "anthrax" en anglais, est une espèce composée de souches très monomorphes. Elle se distingue des autres espèces du groupe *B. cereus* par la présence de plasmides (pXO1 et pXO2) spécifiant la synthèse de toxines et d'une capsule d'acide -D-glutamique, principaux déterminants de la maladie du charbon. Ces facteurs confèrent à *B. anthracis* un pouvoir hautement pathogène pour tous les mammifères, y compris l'homme, par voie cutanée, par ingestion et principalement par inhalation. Cette espèce peut aussi être discriminée sur la base d'un certain nombre de caractéristiques telles que l'absence de mobilité des formes végétatives, l'absence de propriétés hémolytiques, et la sensibilité à l'ampicilline.

*B. thuringiensis* et *B. cereus* sont des espèces extrêmement proches qui, contrairement à *B. anthracis*, sont composées de souches très hétéromorphes, dont la seule marque réellement distinctive est la synthèse chez *B. thuringiensis*, d'un cristal protéique insecticide constitué de toxines appelées protéines Cry. Les thématiques portant sur le rôle de ces toxines spécifiques dans la pathogenèse et le profil d'expression de leurs gènes ont été largement étudiées et nous invitons les lecteurs a se référer aux revues correspondantes (Mock et Fouet, 2001; Schnepf et al., 1998). Les gènes spécifiant ces toxines Cry de *B. thuringiensis* sont également portés par des plasmides. Une souche de *B. thuringiensis* guérie pour le (ou les) plasmide (es) porteurs de gènes *cry* ne produit pas de cristal insecticide et perd ainsi son pouvoir pathogène spécifiquement dirigé contre les insectes. De ce fait, une souche de *B. thuringiensis* $Cry^-$ ne peut pas être distinguée d'une souche de *B. cereus*. Un fait remarquable est que, la perte de ces plasmides par *B. thuringiensis* ne se traduit pas par une perte totale de virulence. Les souches acrystallifères de *B. thuringiensis* (non productrices de cristaux insecticides), et donc de *B. cereus*, bénéficient de propriétés pathogènes opportunistes communes attribuées par des déterminants aspécifiques de virulence qui sont d'origine chromosomique. Ce domaine est relativement récent par rapport aux investigations concernant l'étude des protéines Cry. Il a commencé à être exploré à la fin des années 1990 avec le développement d'outils de génie génétique adaptés aux bactéries du groupe *B. cereus*.

Dans cette revue nous proposons de développer le thème de l'analyse génétique des propriétés pathogènes opportunistes de *B. cereus* et *B. thuringiensis*, à savoir, les stratégies moléculaires ayant permis d'identifier et de mettre en évidence le rôle des gènes de virulence aspécifiques potentiels de ces deux bactéries dans la pathogenèse. Nous proposons également de donner un aperçu sur les différents réseaux de régulation de l'expression de ces gènes. L'étude de ces mécanismes contribue remarquablement à la compréhension de l'intervention de ces facteurs dans la virulence.

## PROPRIETES PATHOGENES OPPORTUNISTES DE *B. THURINGIENSIS* ET *B. CEREUS*

Du fait de leur parenté phylogénétique, *B. cereus* et *B. thuringiensis* partagent un grand nombre de facteurs de virulence impliqués dans des propriétés opportunistes. La plupart des souches de *B. cereus* et *B. thuringiensis* sont hautement pathogènes pour les insectes par pénétration des cellules végétatives ou de spores dans l'hémocoele. Dans certains cas, l'injection de quelques cellules bactériennes suffit pour produire chez certaines espèces d'insectes une septicémie létale en moins de 24 heures (Zhang et al., 1993). Ces observations témoignent de la capacité de *B. thuringiensis*/*B. cereus* à se maintenir dans l'hémolymphe de son hôte et à s'y développer, contrairement à d'autres bacilles non entomopathogènes, tels que *Bacillus subtilis* ou *Bacillus megaterium*, pour lesquels, l'injection d'une dose dépassant $10^4$ bactéries est complètement inoffensive. D'autre part, il est fréquemment observé que, per os, la présence de spores ou cellules végétatives de *B. thuringiensis*/*B. cereus* synergise l'activité toxique de doses sublétales de protéines Cry.

*Galleria mellonella* (*G. mellonella*) représente l'exemple typique des insectes chez lesquels la mortalité n'est observée que lorsque des bactéries sont co-ingérées avec les toxines Cry (Salamitou et al., 2000).

Chez l'homme, certaines souches de *B. cereus* (et éventuellement de *B. thuringiensis*) sont des pathogènes opportunistes fréquemment identifiés comme cause de toxi-infection d'origine alimentaire. Deux formes de gastroentérite sont fréquemment rencontrées : un syndrome émétique et un syndrome diarrhéique (Kotiranta et al., 2000). Néanmoins, certaines infections systémiques opportunistes rares mais sérieuses telles que des endocardites, des pneumonies, des endophtalmites, ont été attribuées à *B. cereus* (Callegan et al., 1999; Hilliard et al., 2003; Miller et al., 1997; Strauss et al., 2001). Dans le cas des endophtalmites, *B. cereus* est capable de provoquer une cécité dans les 24 heures qui suivent l'infection oculaire (Kotiranta et al., 2000). Par ailleurs, des souches de *B. cereus* multi-résistantes aux antibiotiques ont été isolées à partir d'équipements hospitaliers (Meena et al., 2000). Récemment, une souche de *B. cereus* a été rapportée comme cause d'infections très sévères comparables à la maladie du charbon (Hoffmaster et al., 2004). Cette souche de *B. cereus* possède deux plasmides : l'un est similaire à pXO1 et le second porte des gènes responsables de la formation d'une capsule.

Dans le but d'identifier les déterminants génétiques potentiellement impliqués dans la pathogénie de ces bactéries, diverses approches génétiques ont été suivies:

1/ Mutagenèse ciblée de facteurs de virulence potentiels

2/ Caractérisation de mutations transpositionnelles conférant un phénotype avirulent

3/ Identification de gènes bactériens spécifiquement exprimés pendant le processus infectieux

Chez *B. thuringiensis* et *B. cereus*, les gènes identifiés sont testés par rapport à leur rôle dans la pathogénie par inactivation et analyse de la virulence des mutants, *in vivo*, dans divers modèles animaux d'infection (1) par injection intrahémocoelique chez *Bombyx mori* (*B. mori*); (2) par ingestion forcée des larves de *G. mellonella* et évaluation de l'effet synergique des bactéries sur la toxémie; (3) par instillation nasale chez le modèle murin; (4) par infection oculaire chez le lapin.

## GENETIQUE DE LA VIRULENCE DES BACTERIES DU GROUPE *B. CEREUS*

### Approche génétique directe par mutagenèse ciblée

Elle consiste à identifier chez *B. thuringiensis* et *B. cereus* des gènes de virulence potentiels (ou "candidats") dont les produits sont suspectés de jouer un rôle dans la virulence, en raison de leurs effets délétères *in vitro* ou de leur homologie avec des facteurs dont le rôle dans la pathogénie a été démontré chez d'autres bactéries pathogènes. Dans ce dernier cas il s'agit d'une approche comparative par analogie avec des bactéries pathogènes. Elle est très souvent utilisée dans l'analyse génétique de la virulence microbienne en raison de la disponibilité des séquences génomiques de nombreux microbes pathogènes. Les deux stratégies permettent non seulement de mettre en évidence le gène en question chez *B. thuringiensis* et *B. cereus*, mais également de l'inactiver par mutagenèse ciblée afin d'analyser son rôle dans la virulence. Une copie mutée du gène d'intérêt est construite, par remplacement d'une région interne par une cassette de résistance à un antibiotique, et est insérée dans un vecteur thermosensible intégratif (Arnaud et al., 2004; Lereclus et al., 1995). La connaissance du génome de *B. anthracis* (Read et al., 2003) et des génomes de différentes souches de *B. cereus* et *B. thuringiensis* (Ivanova et al., 2003; Rasko et al., 2004); www.intergratedgenomics.com) permettait d'amplifier les régions 5' et 3' du gène candidat pour réaliser une copie interrompue du gène. Le gène candidat est ensuite remplacé par sa version mutée par double recombinaison homologue entre le vecteur recombinant et le chromosome. Ainsi, divers gènes candidats ont été analysés pour leur rôle dans la virulence, tels que ceux spécifiant les flagellines, métalloprotéases, protéases de choc thermiques et énhancines.

### Mutagenèse ciblée de facteurs de virulence potentiels identifiés par leur activité biologique

De nombreux facteurs de virulence aspécifiques, ont été identifiés chez *B. cereus* et *B.*

*thuringiensis*. Il s'agit de toxines (entérotoxines hémolytiques Hbl et non hémolytiques Nhe, cytotoxine K, hémolysines) d'enzymes dégradatives (phospholipases C, PlcA et PlcB ; métalloprotéases), protéines pariétales (flagellines et couche S), etc. (Hansen et Salamitou, 2000). Ces facteurs ont été isolés et caractérisés biochimiquement à partir de surnageants de culture de *B. cereus* ou de *B. thuringiensis*. Ils sont sécrétés par ces bactéries pendant la phase stationnaire. L'étude de l'implication de ces facteurs dans la pathogenèse a, en partie, reposé sur l'analyse de leur effet, *in vitro*, sur des modèles cellulaires et tissulaires. Il en ressort que le complexe Hbl possède une activité entérotoxigénique, hémolytique et dermonécrotique ; il provoque en outre, une augmentation de la perméabilité vasculaire et une accumulation de fluides dans une anse iléale liguée de lapin (Beecher et al., 1995). Nhe est aussi toxique que Hbl et possède, en outre, un effet cytotoxique sur les cellules Vero (Lund et Granum, 1997). Quant à CytK, cette protéine s'est avérée fortement hémolytique contre les érythrocytes de bœuf et de lapin et très toxique pour les cellules de l'épithélium intestinal (Hardy et al., 2001; Lund et al., 2000). Néanmoins des résultats récents ont démontré que l'inactivation individuelle des gènes *cytK*, *plcB* et hblC n'affecte pas les propriétés cytotoxiques des surnageants de culture de *B. thuringiensis* et *B. cereus* vis-à-vis des cellules intestinales mammifères du type CaCo2 ou HeLa (Ramarao et Lereclus, 2006). Il a été suggéré que l'absence de l'un de ces facteurs pourrait être compensée par d'autres facteurs non encore identifiés. Par ailleurs, une régulation pléiotrope, responsable de la synthèse de la majorité de ces facteurs de virulence potentiels a été mise en évidence chez les deux bactéries. Cette régulation est assurée par un activateur transcriptionnel appelé PlcR. Ce régulateur a tout d'abord été identifié comme activateur du gène *plcA* codant pour la phospholipase C spécifique du phosphatidyl inositol (Lereclus et al., 1996). Il a été montré que PlcR active l'expression de ce gène à l'entrée des bactéries en phase stationnaire. L'expression du régulon PlcR est soumise à une double régulation : i) le régulateur PlcR est activé à l'entrée en phase stationnaire par l'interaction d'un petit peptide diffusible (PapR) agissant comme un effecteur du "quorum-sensing" (Gominet et al., 2001; Lereclus et al., 1996; Slamti et Lereclus, 2002) ; et ii) la forme phosphorylée de Spo0A, un facteur clé de déclanchement de la sporulation, réprime l'expression du gène *plcR* quand les conditions environnementales sont favorables à la sporulation (Lereclus et al., 2000). Il est à noter que chez *B. anthracis* le gène *plcR* est non fonctionnel, suite à la présence d'une mutation non-sens (Agaisse et al., 1999). PlcR active la transcription des gènes du régulon en se fixant directement sur un motif palindromique hautement conservé ($TATGNAN_4TNCATA$) appelé boîte PlcR (Agaisse et al., 1999; Slamti et Lereclus, 2002). Des analyses génétiques couplées à la recherche *in silico* de la boîte PlcR dans la séquence chromosomique de la souche ATCC14579 de *B. cereus* révèlent l'existence de plus de 40 gènes cibles (Gohar et Lereclus, résultats non publiés). La délétion du gène *plcR* provoque une forte diminution de la virulence de *B. thuringiensis*/*B. cereus* vis-à-vis du modèle *G. mellonella* en infection orale (Salamitou et al., 2000). Elle a, en revanche, un effet moins marqué sur le pouvoir pathogène des cellules bactériennes lorsqu'elles sont directement injectées dans l'hémocoele des larves de *B. mori*. Il est suggéré que les facteurs du régulon PlcR interviennent principalement au niveau de l'étape initiale de l'infection naturelle qui est le franchissent de la barrière protectrice de l'hôte, en l'occurrence l'épithélium du tube digestif dans le cas de l'infection par voie orale. En outre, l'interruption du gène *plcR* réduit le pouvoir pathogène de *B. thuringiensis* et *B. cereus* vis-à-vis du modèle souris par instillation de spores ce qui suggère l'implication de *plcR* dans les propriétés opportunistes de ces bactéries vis-à-vis des mammifères (Salamitou et al., 2000).

L'un des gènes régulés par PlcR, *inhA2*, s'est révélé fortement impliqué dans la synergie des spores de *B. thuringiensis*/cristaux chez *G. mellonella* (Fedhila et al., 2002). Cependant, des expériences de complémentation fonctionnelle du mutant *plcR* à l'aide du gène *inhA2* placé sous le contrôle d'un promoteur constitutif, ne restaurent pas le phénotype sauvage. Il a été suggéré que les membres du régulon agissent de concert dans le déroulement du processus infectieux (Fedhila et al., 2003a). InhA2 est une

métalloprotéase à zinc qui a été identifiée en raison de son homologie avec InhA1, une autre métalloprotéase à zinc, précédemment identifiée comme étant un inhibiteur de l'immunité des insectes (Dalhammar et Steiner, 1984; Edlund et al., 1976). InhA1 possède en outre trois propriétés qui soulignent fortement sont rôle dans la virulence : i) une activité toxique vis-à-vis des insectes *Trichopulsia ni* et *Drosophila melanogaster* par injection (Dalhammar et Steiner, 1984; Lövgren et al., 1990; Siden et al., 1979), ii) une activité collagénase (Chung et al., 2006; Dalhammar et Steiner, 1984) et iii) une cytotoxicité qui permet aux bactéries de sortir des macrophages (Ramarao et Lereclus, 2005). InhA1 constitue, en effet, un constituant majoritaire de l'exosporium des spores de *B. cereus* (Charlton et al., 1999).Cependant, contrairement à InhA2, InhA1 ne fait pas partie du régulon PlcR, et l'inactivation de son gène n'a pas encore permis de définir son rôle dans la pathogenèse de *B. thuringiensis* chez l'insecte. Par ailleurs, le gène *inhA1* est activé par Spo0A-P via le produit du gène *abrB* (Grandvalet et al., 2001). Les métalloprotéases InhA1 et InhA2 appartiennent, en effet, à une famille multigénique de deux à quatre copies, selon les souches *B. thuringiensis* et *B. cereus* (Fedhila, 2002).

**Mutagenèse ciblée de facteurs de virulence potentiels identifiés par analogie avec ceux de microorganismes pathogènes**

Le séquençage et l'annotation de la totalité des génomes des principales espèces des bactéries du groupe *B. cereus* ont ouvert le champ à diverses applications. De nombreux gènes de virulence potentiels ont ainsi pu être retrouvés.

**Les protéines de choc thermique Clp**

Chez *B. thuringiensis*, des gènes spécifiant des protéines de choc thermique de la famille Clp ont été identifiés par homologie aux protéines Clp de bactéries pathogènes et non pathogènes telles que *Listeria monocytogenes* et *B. subtilis*. Les protéines de la famille Clp présentent des propriétés de protéases ou de chaperons moléculaires en fonction de leur organisation (Gottesman et al., 1997a,b; Wickner et Maurizi, 1999). Ces deux fonctions, énergie-dépendantes, contribuent d'une part, à la renaturation ou à l'élimination de protéines présentes sous une forme non native suite à une mutation ou un stress ; et participent, d'autre part, à l'accomplissement de mécanismes physiologiques tels que l'activation ou la dégradation de régulateurs transcriptionnels. Ainsi, les protéines Clp sont connues pour intervenir dans la tolérance au stress, réponses adaptatives de la phase stationnaire, acquisition de l'état de compétence et également la virulence. Ce dernier volet a été rapporté par plusieurs études. Chez *B. thuringiensis*, deux copies du gène *clpP*, *clp1* et *clpP2* ont été trouvées et délétées afin d'étudier leur rôle dans la virulence. L'analyse de la virulence des mutants dans le modèle d'infection intrahémocoelique *B. mori*, a montré que seul le mutant Δ*clpP1* est avirulent. Cependant ce phénotype s'est avéré essentiellement lié au défaut de croissance du mutant dans des conditions expérimentales de basse température (25°C). La virulence est, en revanche, rapidement restaurée quand les insectes sont placés dans des conditions de température optimales (37°C). Les invertébrés étant des animaux à sang froid, il a été suggéré que ClpP1 pourrait être considéré comme un facteur de virulence facultatif ou "accessoire", en l'occurrence, un facteur d'adaptation de la bactérie à la température *in vivo* de son hôte (Fedhila et al., 2003b).

**L'énhancine des baculovirus EnhB**

Un deuxième gène candidat pour la contribution à la virulence a été identifié chez *B. thuringiensis* par analogie avec les baculovirus. Il s'agit du gène de l'énhancine. Ce gène a été identifié par recherche, *in silico* dans le génome de *B. cereus* (souche ATCC14579), de gènes régulés par PlcR qui interagiraient avec la barrière intestinale de l'hôte. Une ORF précédée par une boîte PlcR putative et dont la séquence déduite en acides aminés représente 23-25% d'identité avec les énhancines de divers baculovirus a été retrouvée (Li et al., 2003; Popham et al., 2001). Les énhancines virales sont considérées comme facteurs de virulence majeurs. L'énhancine du baculovirus granulaire de *Trichopulsia ni* (TnGV) a été la plus étudiée. Elle dégrade les glycoprotéines riches en chitine, composants majeurs de la matrice péritrophique du tube digestif (Wang et Granados, 1997). Par

ailleurs, l'effet synergique de l'énhancine de ce baculovirus sur la toxicité des protéines Cry de *B. thuringiensis* a été rapporté chez certains lépidoptères (Granados et al., 2001). L'analyse de la séquence génomique des bactéries pathogènes *Yersinia pestis* et *B. anthracis* a révélé l'existence de gènes codant pour des énhancines (Parkhill et al., 2001; Read et al., 2003). Cependant leur rôle dans la virulence n'a jamais été étudié. Tout comme la majorité de énhancines virales (Lepore et al., 1996), la séquence en acides aminés déduite du gène de l'énhancine de *B. thuringiensis* possède le motif de fixation de l'atome de zinc (HEXXH) caractéristique des métalloprotéase à atome de zinc (Hajaij-Ellouze et al., 2006). Des études transcriptionnelles ont montré que l'énhancine de *B. thuringiensis* appartient au régulon PlcR. Néanmoins son inactivation n'affecte pas la virulence de *B. thuringiensis* par voie orale (Hajaij-Ellouze et al., 2006).

**Le régulateur Fur des gènes impliqués dans l'acquisition du fer**

Une étude récente a identifié, chez la souche 569 de *B. cereus*, par recherche d'homologie de séquence protéique, l'homologue du régulateur Fur de *B. subtilis* (Harvie et al., 2005). Fur est un répresseur transcriptionnel de gènes impliqués dans l'acquisition du fer par les bactéries. Le régulon Fur a été identifié chez de nombreuses bactéries. Il regroupe plus de 40 gènes intervenant dans diverses voies métaboliques, notamment le métabolisme du fer, ainsi que dans des réponses aux stress oxydatifs. Fur réprime directement l'expression des gènes du régulon en se fixant sur une séquence consensus, appelée boîte Fur (5'-GATAATGATAATCATTATC-3'), localisée au niveau des promoteurs des gènes. Dans des conditions de concentration intracellulaire élevée de $Fe^{2+}$, Fur complexe les ions de fer. Cette interaction avec les ions métalliques lui confère une conformation spatiale qui lui permet de se fixer sur l'ADN, en l'occurrence sur sa boîte, ce qui provoque la répression du régulon. Chez plusieurs bactéries pathogènes, l'expression de nombreux gènes de virulence est régulée par Fur (Harvie et Ellar, 2005; Horsburgh et al., 2001; Litwin et Calderwood, 1993). Il a été suggéré que l'expression Fur-dépendante de ces gènes est déclenchée par la carence en fer dans le milieu *in vivo* de l'hôte. Le fer est un élément essentiel pour les bactéries. Dans l'environnement, le fer $Fe^{2+}$ est très peu disponible suite à son oxydation rapide en $Fe^{3+}$. Chez les animaux, hôte vertébrés ou invertébrés de nombreux pathogènes, le $Fe^{2+}$ est également limité, car complexé à des chélateurs de l'hôte tels que les transferrines, ferritines, haemoglobines, myoglobulines. Face à cette carence ferrique *in vivo*, la bactérie développe des stratégies d'acquisition de fer qui consistent à exprimer des récepteurs protéiques de surface qui fixent des composants contenant du fer et, à sécréter des sidérophores, chélateurs ayant une grande affinité de fixation du fer capables de concurrencer les chélateurs de l'hôte, et de réimporter le fer sous forme complexée dans la cellule bactérienne (Ratledge et Dover, 2000). Ces processus d'acquisition du fer sont soumis à une régulation négative par le régulateur Fur, afin d'éviter l'effet toxique d'une accumulation du fer intracellulaire (Touati, 2000). Fur semble ainsi être, pour la bactérie, un indicateur indirect de la nature de l'environnement dans lequel elle se trouve. En réponse à la carence en fer libre qui caractérise le milieu hôte, la répression de certains gènes de virulence par Fur est levée, ce qui permet à la bactérie d'initier la pathogenèse. Chez la souche 569 de *B. cereus*, le gène *fur* identifié a été inactivé. La virulence des mutants est significativement atténuée chez un modèle d'infection insecte. Ce phénotype résulterait de la perturbation de plusieurs fonctions cellulaires due à l'expression constitutive du régulon Fur et, par conséquent, à l'augmentation du taux de fer intracellulaire. En outre, le mutant est plus sensible aux réactions oxydatives caractéristiques des mécanismes de défense de l'hôte aux infections microbiennes (Ha et al., 2005; Harvie et al., 2005).

**Approche génétique aléatoire par mutagenèse transpositionnelle**

Cette approche consiste à rechercher, dans une banque de mutants insertionnels, des phénotypes ayant perdu leur propriété pathogène vis-à-vis du modèle d'infection utilisé. Ces banques sont généralement construites à l'aide de l'insertion aléatoire de transposons, ce qui implique un criblage individuel des mutants. Cette technique s'appelle RTM (Random Transposon

Mutagenesis). Holden et coll. (1995) ont conçu et développé une autre technique de mutagenèse, à l'aide de transposons génétiquement marqués. Cette technique, appelée STM (Signature-Tagged Mutagenesis), présente l'avantage de détecter, sur la base d'une sélection négative, les phénotypes avirulents au sein de pools de mutants insertionnels (Hensel et al., 1995). Holden et coll. ont utilisé cette technique afin d'identifier des gènes impliqués dans la virulence de *Salmonella typhimurium* dans un modèle murin de fièvre typhoïde. Cette technique a, depuis, été modifiée en fonction du pathogène et de l'hôte (Saenz et Dehio, 2005). Les deux techniques de RTM et STM ont été appliquées à *B. thuringiensis* et *B. cereus* en vue de rechercher des gènes impliqués dans la virulence et l'adaptation de ces bactéries à l'environnement intrahémocoelique de l'hôte insecte (Fedhila et al., 2004; Steggles et al., 2006).

Dans le cas de la technique STM, une banque de mutants transpositionnels a été divisée en pools. Le pool infectant (input) et le pool de mutants récupéré du modèle d'infection en post-mortem (output) sont hybridés au transposon. Ceci permet d'éliminer les clones n'ayant pu réussir le processus infectieux et donc, les clones contre-sélectionnés par le modèle d'infection. La comparaison ultérieure des profils d'hybridation de l'input et de l'output permet de ressortir les mutants ayant été éliminés lors de la sélection négative, en l'occurrence les clones avirulents. Grâce à la connaissance de la totalité de la séquence des transposons utilisés, il est possible de cartographier le site d'insertion et par conséquent d'identifier le gène potentiellement impliqué dans la virulence. La suite de la démarche consiste à reproduire la mutation insertionnelle par délétion remplacement du gène interrompu par double recombinaison homologue et à confirmer le phénotype atténué du mutant de délétion vis-à-vis du modèle d'infection. Des travaux récents ont ainsi permis d'identifier chez *B. thuringiensis*, grâce à la technique STM utilisant le transposon Tn917, différentes catégories de gènes de virulence potentiels vis-à-vis du modèle d'infection intrahémocoelique *Manduca sexta* : des régulateurs transcriptionnels de la famille ArsR, des protéines histone-like ainsi que diverses séquences codantes de fonction inconnue (Steggles et al., 2006).

Dans le cas de la technique RTM, une banque de mutants insertionnels a été construite, chez la souche 407 $Cry^-$ de *B. thuringiensis*, à l'aide d'un système de mutagenèse transpositionnelle permettant l'insertion du transposon mini-Tn10 (Gominet et al., 2001). Cette banque a été criblée par injection des transposants dans l'hémolymphe de *B. mori* en vue d'identifier des mutations conférant un phénotype avirulent. Des mutants insertionnels indépendants affectés au niveau de la virulence et dans lesquels le transposon s'est inséré dans le locus *yqgByqfZ* au niveau de la région 3' du gène *yqgB* ont été sélectionnés. Des délétions simples et doubles de ces gènes ont été construites et des expériences ont été réalisées pour déterminer les rôles respectifs de *yqgB* et *yqfZ* dans la virulence. Il en ressort que seul le double mutant présente une virulence retardée. Des études supplémentaires d'analyse de la réponse de ce mutant à divers stress ont permis de démontrer que ce dernier présente également une absence de motilité et un défaut de croissance à basse température (25°C). Par ailleurs, une restauration totale de la virulence du double mutant était observée lorsque les larves d'insecte infectées étaient placées dans des conditions de température optimales pour *B. thuringiensis* (37°C). Il a été suggéré que le locus *yqgByqfZ* était impliqué dans l'adaptation de *B. thuringiensis* à la température interne de son hôte poïkilotherme et représente, de ce fait, un facteur indirectement associé à la virulence (Fedhila et al., 2004).

Un autre mutant transpositionnel de *B. thuringiensis* contenant une insertion du miniTn10 dans le gène *flhA* a été isolé et caractérisé par Ghelardi et coll. (Ghelardi et al., 2002). Le gène *flhA* appartient à la famille des gènes flagellaires de classe II impliqués dans le système d'export de type III des composants flagellaires de *S. typhimurium* (Minamino et Macnab, 1999). La caractérisation phénotypique du mutant insertionnel indique que FlhA intervient dans la motilité, et dans l'expression de certains gènes du régulon PlcR (Bouillaut et al., 2005; Ghelardi et al., 2002). Cette étude rapporte donc, pour la première fois, un deuxième niveau de régulation des gènes de virulence appartenant au régulon PlcR, assuré

par le système flagellaire. Par ailleurs, le mutant Δ*flhA* est affecté dans sa cytotoxicité vis-à-vis des cellules HeLa et au niveau de sa virulence chez *G. mellonella* en infection orale et intrahémocoelique (Bouillaut et al., 2005). Des études ultérieures ont permis de montrer que le gène *flhA* joue un rôle essentiel dans l'adhésion de *B. cereus*/*B. thuringiensis* aux cellules CaCo2 et HeLa (Ramarao et Lereclus, 2006). Les auteurs ont suggéré que les propriétés adhésives de *B. cereus* contribueraient au syndrome diarrhéique causé par ce pathogène opportuniste. L'adhésion des bactéries aux cellules eucaryotes de l'hôte est souvent considérée comme un événement clé dans les étapes initiales de l'établissement d'une infection. Ces travaux sont en accord avec des études antérieures qui suggéraient que les flagelles des bactéries du groupe *B. cereus* interviennent dans les stades précoces de la pathogenèse (Lövgren et al., 1993; Zhang et al., 1993).

**Approche transcriptomique indirecte par IVET**

La méthode IVET (*In Vivo* Expression Technology) est l'une des approches génétiques les plus récentes, utilisées dans l'analyse de la pathogénie et d'autres phénomènes physiologiques bactériens. C'est une approche transcriptomique ciblée permettant de pratiquer une sélection positive de promoteurs spécifiquement activés dans des conditions de croissance particulières (Rediers et al., 2005). La toute première application de cette technologie a porté sur les bactéries pathogènes à Gram$^{-}$. Elle avait pour objectif d'identifier les gènes qui s'expriment spécifiquement durant la colonisation d'un hôte sensible (Camilli et al., 1994; Mahan et al., 1993; Osbourn et al., 1987). Une stratégie IVET, fondée sur la recombinaison site spécifique, a été appliquée chez *B. cereus* pour la première fois en 2006 (Fedhila et al., 2006). Ce système permet de détecter des promoteurs activés *in vivo*, de façon transitoire et conditionnelle. Le principe est de cloner une banque de fragments d'ADN de *B. cereus* en amont du gène *tnpI* spécifiant une recombinase spécifique de site. Quand ce fragment contient un promoteur activé dans les conditions étudiées, la recombinase est produite et conduit à la perte d'un gène de résistance à un antibiotique et à l'acquisition d'une nouvelle résistance. L'infection par inoculation orale du modèle invertébré *G. mellonella* a été choisie afin de mimer les infections gastrointestinales et d'identifier des déterminants génétiques *ivi* (*In Vivo* Induced) de *B. cereus* qui s'expriment spécifiquement durant tout le cycle infectieux, de la colonisation du tube digestif jusqu'à la septicémie finale. 20 gènes *ivi* ont été identifiés. Ils spécifient des fonctions aussi diverses que la régulation, métabolisme, réplication, division cellulaire, transport, virulence et adaptation. L'un de ces gènes, ivi29, fortement induit *in vivo*, a été étudié. Ce gène, baptisé *ilsA* (Iron-regulated Leucin-rich Surface protein), spécifie une protéine présentant des propriétés d'internaline et de sidérophore grâce à la présence de 4 domaines spécifiques : peptide signal N-terminal pour l'export, domaine NEAT (Near Fe3+ siderophore Transporter) de transport du fer, domaine LRR (Leucin-Rich Repeats) d'interaction avec les protéines et domaine SLH (Surface Layer Homology) d'ancrage aux peptidoglycanes membranaires. Les internalines et les sidérophores sont d'importants facteurs de virulence chez de nombreux pathogènes (Andrade et al., 2002; Braun et Cossart, 2000). Les internalines de *L. monocytogenes* jouent un rôle essentiel dans la pathogenèse. Elles assurent l'internalisation de ce pathogène intracellulaire dans les cellules de l'hôte. Ce rôle est cependant spécifique des internalines des bactéries invasives. Des études transcriptionnelles indiquent que *ilsA* appartient au régulon Fur. L'inactivation de *ilsA* par délétion/remplacement réduit la virulence de *B. cereus* vis-à-vis du modèle *G. mellonella*. Il a été suggéré que ce nouveau facteur de virulence, IlsA, est nécessaire pour l'adaptation de *B. cereus* à un contexte infectieux particulièrement carencé en fer (Fedhila et al., 2006). En effet, il a été rapporté que la concentration du taux des protéines hémolymphatiques complexant le fer (transferrines, apoferritines) chez les insectes augmente en réponse à infection microbienne. Ceci est le cas des larves de *Aedes aegypti* et de *D. melanogaster* infestées par des nématodes (Beerntsen et al., 1994) et par *Escherichia coli* (Yoshiga et al., 1999), respectivement. Le fait que *ilsA* soit un membre du régulon Fur rejoint les résultats des travaux cités plus haut qui

soutiennent que l'expression de nombreux gène de virulence est régulée par Fur.

## CONCLUSION & DISCUSSION

L'application de ces approches moléculaires à l'analyse génétique de la virulence du groupe *B. cereus* a permis d'identifier des facteurs de virulence de différents types. Wassenaar et Gaastra (2001) ont subdivisé les gènes de virulence microbiens en trois classes : **1)** les vrais gènes de virulence (gènes des toxines) ; **2)** les "life-style genes" permettant au pathogène d'accomplir la totalité de son cycle infectieux au sein de son hôte (gènes de colonisation : adhésines, invasines, fimbriae, flagellines et gènes de contournement du système immunitaire : couche S, protéases dégradant spécifiquement des immunoglobulines, lipopolysaccharides membranaires) ; **3)** les gènes associés à la virulence, dont les produits sont nécessaires à l'activité des facteurs de virulence appartenant au premier et au deuxième groupe (gènes de maturation, systèmes de sécrétion, gènes de ménage, régulation). Les toxines étant des facteurs de virulences propres aux vrais pathogènes, les facteurs identifiés chez *B. thuringiensis* et *B. cereus* appartiennent, pour la plupart d'entre eux, aux deux derniers groupes ce qui explique en partie leur intervention dans les propriétés opportunistes de ces bactéries. Bien que le rôle de la plupart de ces facteurs ait été bien élucidé sur le plan génétique, leur réelle contribution à la pathogenèse dans un contexte infectieux n'a pas été établie de façon approfondie. Ceci est du à l'occultation du système hôte dans l'analyse du rôle de ces facteurs dans la virulence. L'hôte constitue, en effet, un déterminant important des interactions hôte/pathogène et peut influencer de manière considérable les différents paramètres qui décrivent une infection. Actuellement, on assiste à une orientation de ces démarches génétiques vers des approches cellulaire (imagerie cellulaire, histopathologie, visualisation des facteurs de virulence au sein de l'hôte par fusion avec des rapporteurs…etc.) qui tentent de fournir des résultats complémentaires permettant de mieux élucider les bases moléculaires et cellulaires de la virulence microbienne.

## REFERENCES

Agaisse, H.; Gominet, M.; Økstad, O.A.; Kolstø, A.B. and Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. Mol. Microbiol. 32: 1043-1053.

Andrade, M.A.; Ciccarelli, F.D.; Perez-Iratxeta, C. and Bork, P. (2002). NEAT: a domain duplicated in genes near the components of a putative Fe3+ siderophore transporter from Gram-positive pathogenic bacteria. Genome Biol. 3: 0047.1-0047.5.

Arnaud, M.; Chastanet, A. and Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. 70: 6891.

Beecher, D.J.; Schoeni, J.L. and Wong, A.C. (1995). Enterotoxic activity of hemolysin BL from *Bacillus cereus*. Infect. Immun. 63: 4423-8.

Beerntsen, B.T.; Severson, D.W. and Christensen, B.M. (1994). *Aedes aegypti*: characterization of a hemolymph polypeptide expressed during melanotic encapsulation of filarial worms. Exp. Parasitol. 79: 312-21.

Bouillaut, L.; Ramarao, N.; Buisson, C.; Gilois, N.; Gohar, M.; Lereclus, D. and Nielsen-Leroux, C. (2005). FlhA influences *Bacillus thuringiensis* PlcR-regulated gene transcription, protein production and virulence. Appl. Environ. Microbiol. 71: 8903-8910.

Braun, L. and Cossart, P. (2000). Interactions between *Listeria monocytogenes* and host mammalian cells. Microbes Infect.2: 803-11.

Callegan, M.C.; Booth, M.C.; Jett, B.D. and Gilmore, M.S. (1999). Pathogenesis of gram-positive bacterial endophthalmitis. Infect. Immun. 67: 3348-3356.

Camilli, A.; Beattie, D.T. and Mekalanos, J.J. (1994). Use of genetic recombination as a reporter of gene expression. Proc. Natl. Acad. Sci. USA. 91: 2634-2638.

Charlton, S.; Moir, A.J.; Baillie, L. and Moir, A. (1999). Characterization of the exosporium of *Bacillus cereus*. J. Appl. Microbiol. 87: 241-5.

Chung, M.-C.; Popova, T.G.; Millis, B.A.; Mukherjee, D.V.; Zhou, W.; Liotta, L.A.; Petricoin, E. F.; Chandhoke, V.; Bailey, C. and Popov S. G. (2006). Secreted neutral metalloproteases of *Bacillus anthracis* as candidate pathogenic factors. J. Biol. Chem. 281: 31408-31418.

Dalhammar, G. and Steiner, H. (1984). Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur. J. Biochem. 139: 247-252.

Edlund, T.; Siden, I. and Boman, H.G. (1976). Evidence for two immune inhibitors from *Bacillus thuringiensis* interfering with the humoral defense system of saturniid pupae. Infect. Immun. 14: 934-941.

Fedhila, S. Contribution des métalloprotéases InhA et des protéines de choc thermique Clp à la virulence de *Bacillus thuringiensis*. [Thèse]. Paris; 2002.

Fedhila, S.; Daou, N.; Lereclus, D. and Nielsen-Leroux, C. (2006). Identification of *Bacillus cereus* internalin and other candidate virulence genes specifically induced during oral infection in insects. Mol. Microbiol. 62: 339-55.

Fedhila, S.; Gohar, M.; Slamti, L.; Nel, P. and Lereclus, D. (2003a). The *Bacillus thuringiensis* PlcR-regulated gene inhA2 is necessary, but not sufficient, for virulence. J. Bacteriol. 185: 2820-5.

Fedhila, S.; Guillemet, E.; Nel, P. and Lereclus, D. (2004). Characterization of two *Bacillus thuringiensis* genes identified by *in vivo* screening of virulence factors. Appl. Environ. Microbiol. 70: 4784-4791.

Fedhila, S.; Msadek, T.; Nel, P. and Lereclus, D. (2003b). Distinct *clpP* genes control specific adaptive responses in *Bacillus thuringiensis*. J. Bacteriol. 184: 5554-62.

Fedhila, S.; Nel, P. and Lereclus, D. (2002). The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. J. Bacteriol. 184: 3296-3304.

Ghelardi, E.; Celandroni, F.; Salvetti, S.; Beecher, D.J.; Gominet, M.; Lereclus, D.; Wong, A.C. and Senesi S. (2002). Requirement of *flhA* for swarming differentiation, flagellin export and secretion of virulence-associated proteins in *Bacillus thuringiensis*. J. Bacteriol. 184: 6424-6433.

Gominet, M.; Slamti, L.; Gilois, N.; Rose, M. and Lereclus, D. (2001). Oligopeptide permease is required for expression of the *Bacillus thuringiensis plcR* regulon and for virulence. Mol. Microbiol. 40: 963-75.

Gottesman, S.; Maurizi, M.R. and Wickner, S. (1997a). Regulatory subunits of energy-dependent proteases. Cell 91: 435-8.

Gottesman, S.; Wickner, S. and Maurizi, M.R. (1997b). Protein quality control: triage by chaperones and proteases. Gen. Dev. 11: 815-23.

Granados, R.R.; Fu, Y.; Corsaro, B. and Hughes, P.R. (2001). Enhancement of *Bacillus thuringiensis* toxicity to Lepidopterous species with the enhancin from *Trichoplusia ni* Granulovirus. Biol. Control. 20: 153-159.

Grandvalet, C.; Gominet, M. and Lereclus, D. (2001). Identification of genes involved in the activation of the *Bacillus thuringiensis inhA* metalloprotease gene at the onset of sporulation. Microbiology. 147: 1805-13.

Ha, E.M.; Oh, C.T.; Ryu, J.H.; Bae, Y.S.; Kang, S.W. and Jang, I.H. (2005). An antioxidant system required for host protection against gut infection in *Drosophila*. Dev. Cell. 8: 125-132.

Hajaij-Ellouze, M.; Fedhila, S.; Lercelus, D. and Nielsen-Leroux, C. (2006). The enhancin-like metalloprotease from the *Bacillus cereus* group is regulated by the pleiotropic transcriptional activator PlcR but is not essential for larvicidal activity. FEMS. Microbiol. Lett. 260: 9-16.

Hansen, B.M. and Salamitou, S. (2000). Virulence of *Bacillus thuringiensis*. In: Charles, J.F.; Delécluse, A. and Nielson-Leroux, C. (eds). Entomopathogenic bacteria: from laboratory to field application. 41-63. Kluwer Academic Publishers.

Hardy, S.P.; Lund, T. and Granum, P.E. (2001). CytK toxin of *Bacillus cereus* forms pores in

planar lipid bilayers and is cytotoxic to intestinal epithelia. FEMS. Microbiol. Lett. 197: 47-51.

Harvie, D.R. and Ellar, D.J. (2005). A ferric dicitrate uptake system is required for the full virulence of *Bacillus cereus*. Curr. Microbiol. 50: 246-250.

Harvie, D.R.; Vilchez, S.; Steggles, J.R. and Ellar, D.J. (2005). *Bacillus cereus* Fur regulates iron metabolism and is required for full virulence. Microbiology. 151: 569-577.

Hensel, M.; Shea, J.E.; Gleeson, C.; Jones, M.D.; Dalton, E. and Holden, D.W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. Science. 269: 400-403.

Hilliard, N.J.; Schelonka, R.L. and Waites, K.B. (2003). *Bacillus cereus* bacteremia in a preterm neonate. J. Clin. Microbiol. 41: 3441-3444.

Hoffmaster, A.R.; Ravel, J.; Rasko, D.A.; Chapman, G.D.; Chute, M.D. and De Marston, C.K.B.K. (2004). Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc. Natl. Acad. Sci. USA. 101: 8449-8454.

Horsburgh, M.J.; Ingham, E. and Foster, S.J. (2001). In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J. Bacteriol. 183: 468-475.

Ivanova, N.; Sorokin, A.; Anderson, I.; Galleron, N.; Candelon, B.; Kaptral, V. Bhattacharyya, A.; Reznik, G.; Mikhailova, N.; Lapidus, A.; Chu, L.; Mazur, M.; Goltsman, E.; Larsen, N.; D'Souza, M.; Walunas, T.; Grechkin, Y.; Pusch, G.; Haselkorn, R.; Fonstein, M.;

Ehrlich, S. D.; Overbeek, R .and Kyrpides, N. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature. 423: 87-91.

Kotiranta, A.; Lounatmaa, K. and Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect.2: 189-98.

Lepore, L.S.; Roelvink, P.R. and Granados, R.R. (1996). Enhancin, granulosis virus protein that falicitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. J. Invertebr. Pathol. 68: 131-140.

Lereclus, D.; Agaisse, H.; Gominet, M. and Chaufaux, J. (1995). Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis spo0A* mutant. Bio/Technol. 13: 67-71.

Lereclus, D.; Agaisse, H.; Gominet, M.; Salamitou, S. and Sanchis, V. (1996). Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. J. Bacteriol. 178: 2749-2756.

Lereclus, D.; Agaisse, H.; Grandvalet, C.; Salamitou, S. and Gominet, M. (2000). Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. Int. J. Med. Microbiol. 290: 295-9.

Li, Q.; Li, L.; Moore, K.; Donly, C.; Theilmann, D.A. and Erlandson, M. (2003). Characterization of *Mamestra configurata* nucleopolyhedrovirus enhancin and its functional analysis via expression in an *Autographa californica* M nucleopolyhedrovirus recombinant. Gen. Virol. 84: 123-132.

Litwin, C.M. and Calderwood, S.B. (1993). Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. 6: 137-149.

Lövgren, A.; Zhang, M.; Engström, A.; Dalhammar, G. and Landén, R. (1990). Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*. Mol. Microbiol. 4: 2137-2146.

Lövgren, A.; Zhang, M.-Y.; Engström, A. and Landén, R. (1993). Identification of two expressed flagellin genes in the insect pathogen *Bacillus thuringiensis* subsp. *alesti*. J. Gen. Microbiol. 139: 21-30.

Lund, T.; De Buyser, M.L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol. Microbiol. 38: 254-61.

Lund, T. and Granum, P.E. (1997). Comparison of biological effect of the two different

enterotoxin complexes isolated from three different strains of *Bacillus cereus*. Microbiology. 143 (10): 3329-3336.

Mahan, M.J.; Slauch, J.M. and Mekalanos, J.J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. Science. 259: 686-688.

Meena, B.S.; Kapoor, K.N. and Agarwal, R.K. (2000). Occurrence of multi-drug resistant *Bacillus cereus* in foods. J. Food. Sci. Technol. 37: 289-291.

Miller, J.M.; Hair, J.G.; Hebert, M.; Hebert, L.; Roberts, F.J.Jr. and Weyant, R.S. (1997). Fulminating bacteremia and pneumonia due to *Bacillus cereus*. J. Clin. Microbiol. 35: 504-507.

Minamino, T. and Macnab, R.M. (1999). Components of the *Salmonella flagellar* export apparatus and classification of export substrates. J. Bacteriol. 181: 1388-1394.

Mock, M. and Fouet, A. (2001). Anthrax. Ann. Rev. Microbiol. 55: 647-71.

Osbourn, A.E.; Barber, C.E. and Daniels, M.J. (1987). Identification of plant-induced genes of the bacterial pathogen *Xanthomonas campestris* pathovar *campestris* using a promoter-probe plasmid. Embo J. 6: 23-28.

Parkhill, J.; Wren, B.W. and Thomson, N.R. (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature. 413: 523-527.

Popham, H.J.; Bischoff, D.S. and Slavicek, J.M. (2001). Both *Lymantria dispar* nucleopolyhedrovirus enhancin genes contribute to viral potency. J. Virol. 75: 8639-8648.

Ramarao, N. and Lereclus, D. (2005). The InhA1 metalloprotease allows spores of the *Bacillus cereus* group to escape macrophages. Cell. Microbiol. 7: 1357-64.

Ramarao, N. and Lereclus, D. (2006). Adhesion and cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to epithelial cells are FlhA and PlcR dependent, respectively. Microbes Infect.8: 1483-1491.

Rasko, D.A.; Rave, I.J.; Okstad, O.A.; Helgason, E.; Cer, R.Z.; Jiang, L. et al. (2004). The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids. Res. 32: 977-988.

Ratledge, C. and Dover, L.G. (2000). Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54: 881-941.

Read, T.D.; Peterson, S.N. and Tourasse, N. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature. 423: 81-86.

Rediers, H.; Rainey, P.B.; Vanderleyden, J. and De Mot, R. (2005). Unraveling the secret lives of bacteria: use of *in vivo* expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression. Microbiol. Mol. Biol. Rev. 69: 217-261.

Saenz, H.L. and Dehio, C. (2005). Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. Curr. Opinion. Microbiol. 8: 612-619.

Salamitou, S.; Ramisse, F.; Brehelin, M.; Bourguet, D.; Gilois, N.; Gominet, M. et al. (2000). The PlcR regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. Microbiology. 146: 2825-32.

Schnepf, E.; Crickmore, N.; Van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J. et al. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775-806.

Siden, I.; Dalhammar, G.; Telander, B.; Boman, H.G. and Somerville, H. (1979). Virulence factors in *Bacillus thuringiensis*: purification and properties of a protein inhibitor of immunity in insects. J. Gen. Microbiol. 114: 45-52.

Slamti, L. and Lereclus, D. (2002). A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. Embo J. 21: 4550-9.

Steggles, J.R.; Wang, J. and Ellar, D.J. (2006). Discovery of *Bacillus thuringiensis* virulence genes using signature-tagged mutagenesis in an insect model of septicaemia. Curr. Microbiol. 53: 303-10.

Strauss, R.; Mueller, A.; Wehler, M.; Neureiter, D.; Fischer, E.; Gramatzki, M. and Hahn, E.G. (2001). Pseudomembranous tracheobronchitis due to *Bacillus cereus*. Clin. Infect. Dis. 33: 39-41.

Touati, D. (2000). Iron and oxidative stress in bacteria. Arch. Biochem. Biophys. 373: 1-6.

Wang, P. and Granados, R.R. (1997). An intestinal mucin is the target substrate for a baculovirus enhancin. Proc. Natl. Acad. Sci. USA. 94: 6977-82.

Wassenaar, T.M. and Gaastra, W. (2001). Bacterial virulence: can we draw the line? FEMS Microbiol. Lett. 201: 1-7.

Wickner, S. and Maurizi, M.R. (1999). Here's the hook: similar substrate binding sites in the chaperone domains of Clp and Lon. Proc. Natl. Acad. Sci. USA. 96: 8318-20.

Yoshiga, T.; Georgieva, T.; Dunkov, B.C.; Harizanova, N.; Ralchev, K. and Law, J.H. (1999). *Drosophila melanogaster* transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. Eur. J. Biochem. 260: 414-20.

Zhang, M.Y.; Lövgren, A.; Low, M.G. and Landén, R. (1993). Characterization of an avirulent pleitropic mutant of the insect pathogen *Bacillus thuringiensis*: reduced expression of flagellin and phospholipases. Infect. Immun. 61: 4947-4954.

**BIOLOGIA TUNISIE**

# *Allium sativum* feeding induces apoptosis of germ cells through caspase-3 pathway

**Hammami Imen[1,2], Claire Mauduit[2], Amri Mohamed[3], Benahmed Mohamed[2], Michèle Véronique El May[1].**

1- Unité de recherche n° 01/UR/08-07, Faculté de Médecine, 15 rue Djebel Lakhdar, 1007 Tunis; Tunisie.
2- Inserm, U407, Oullins, F-69921, France ; Université de Lyon, Oullins, F-69921, France ; Université Lyon 1, Oullins, F-69921, France.
3- Nutrition Physiology Laboratory, Faculty of Sciences, Tunis 1006, Tunisia

## INTRODUCTION

Medicinal plants such as thyme, onion, blackseed, lemon balm and nettle are intensively used in traditional medicine, today. Garlic is among the most important of these plants. For this purpose, garlic has been extensively used worldwide for centuries, especially in the Far East. Garlic is reported to be a wonderful medicinal plant owing to its preventive characteristics in cardiovascular diseases, regulating blood pressure, lowering blood sugar and cholesterol levels, effective against bacterial, viral, fungal and parasitic infections, enhancing the immune system and having antitumoral and antioxidant features (Amagase H, 2006). The side effects, particularly on male reproduction, of such chronic treatments are poorly investigated. In the present study, we tried to identify the cellular and molecular targets of crude garlic administrated at various doses to adult male rats.

## MATERIALS AND METHODS

### Plant preparation

The type of *Allium sativum* (As) used in the present study was “spring garlic”; it was grown in Tunisia and purchased on a local market. Every day the garlic pellets were made by mixing peeled cloves of garlic with powdered standard rat pellet diet (Industrial Society of Food, Sfax; Tunisia) at four doses: 5%, 10%, 15% and 30%. Cloves were crushed in distilled

### Western Blotting analysis

Testicular tissues were incubated in 200 µl of ice-cold hypotonic buffer (25 mL Tris-HCl pH 7.4 containing 1% protease-inhibitor cocktail), homogenized with a potter. Protein concentration was determined by the bincinchoninic acid assay. Proteins (15 µg for c-IAP1, c-IAP2 and XIAP, 10 µg for Smac, 20 µg for caspase-6, 30 µg for caspase-3 and 40 µg for AIF and survivin) were resolved on a 10% (for c-IAP1, c-IAP2 and XIAP), 12% (for Smac, AIF and survivin) or 15% (caspase-6 and caspase-3) sodium dodecyl sulphate/polyacrylamide gel. Proteins were electrophoretically transferred to a nitrocellulose membrane using 25 mM Tris -185 mM glycine buffer (pH 8.3) containing 20% methanol at a constant voltage of 100 V for 1 h. After transfer, the membranes were incubated in a blocking buffer (TBS containing 1% of BSA and 0.1% Tween-20) for 2 h at RT. The membranes were rinsed three times with TBS/0.1%Tween-20 for 10 min each, and incubated with the first antibody (in TBS containing 1% of BSA) overnight at 4°C. The antibodies were diluted as follows: 1/400 for c-IAP1; 1/2,500 for c-IAP2; 1/6,000 for XIAP; 1/400 for survivin; 1/600 for Smac; 1/1,000 for caspase-6; 1/200 for caspase-3. The protein loading

water in order to minimize volatile compound loss. A similar volume of water was added to the other doses.

**Animal treatments**

A total number of 30 adult male rats of Wistar strain (Pasteur Institute of Tunis; Tunisia) were used for this study. The rats were randomly assigned into the different groups (of 6 animals each) using a hazard permutation table. Control animals received standard pellet diet (group 1). The other groups received diet supplemented with 5%, 10%, 15% and 30% of As, noted groups 2, 3, 4 and 5, respectively. After 30 days of treatment, all the rats were sacrificed by decapitation the same day from 9:00 to 11:00 a.m. Testis were weighed and dissected. For each animal, the first testis was frozen at -80°C for Western blotting analysis.

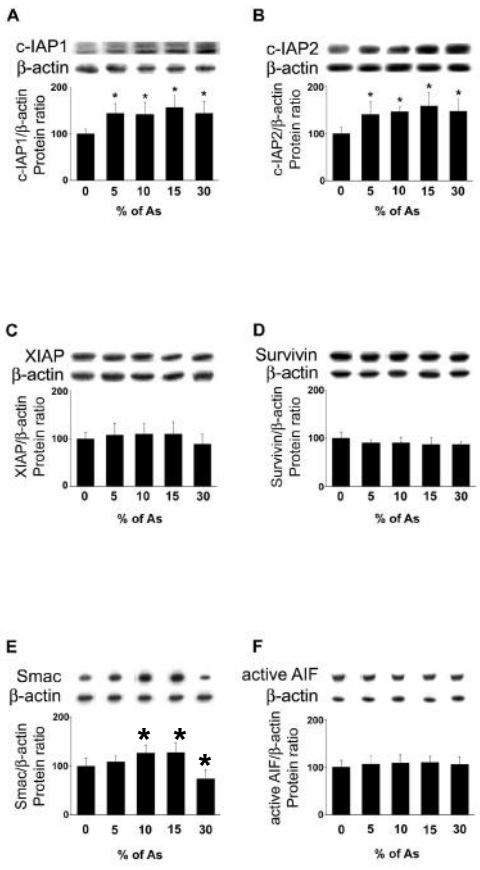
Similarly, the c-IAP2 protein levels were significantly increased after treatment with 5% (29%; $p$<0.002), 10% (31.5%; $p$<0.004), 15% (37%; $p$<0.0003) and 30% (32%; $p$<0.002) of As (Fig. 2B).

In contrast, As feeding did not modify the protein levels of XIAP (Fig. 2C) or survivin (Fig. 2D) at the different tested doses. We evaluated then the third partner of the executioner step of apoptosis, i.e. the IAP inhibitors such as Smac/DIABLO. The Smac/DIABLO protein levels were increased significantly at doses 10% (21%; $p$<0.02) and 15% (21.5%; $p$<0.02). In contrast, at dose 30% of As, Smac/DIABLO protein levels were decreased significantly (27%; $p$<0.04, Fig. 2E). The apoptotic cell death process may also be induced by a caspase-independent pathway represented by AIF. In the testicular tissue from As fed rats, the expression of active AIF protein (Fig. 2F) was unchanged at the different doses tested.

was checked by probing the blot with a rabbit IgG anti-actin antibody (1/20,000). The antigen-antibody complexes were detected with a chemiluminescent kit. The membranes were exposed onto Biomax MR films. The intensity of the bands was determined with the Optiquant software. The data were expressed as a target/actin protein ratio.

**RESULTS**

Cleaved caspase-3 expression was increased in a dose-dependant manner (Fig. 1A) and the increase was significant at doses 10% (26%, $p$=0.0003), 15% (29%, $p$<0.0001) and 30% of As (30%, $p$<0.0001). In contrast, As feeding did not modify the expression of cleaved caspase-6 (Fig. 1B). The c-IAP1 protein levels were significantly increased at doses 5% (30.5%; $p$<0.01), 10% (29%; $p$<0.02), 15% (36%; $p$<0.003) and 30% (30.5%; $p$<0.01) of As (Fig. 2A).



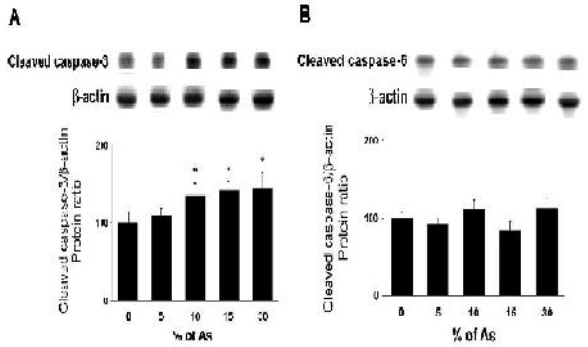



**Figure 1**: Quantitative effects of *Allium sativum* administration on apoptosis in the testis. Adult rats were untreated (0%) or fed with 5, 10, 15 and 30% As. (A) cleaved caspase-3 and (B) cleaved caspase -6 protein levels were analyzed through western blotting approach. The results are expressed as the mean ± SD.

**Figure 2**: Effects of *Allium sativum* administration on c-IAP1, c-IAP2, XIAP, Survivin, Smac and active AIF protein levels. Adult rats were untreated (0%) or fed with 5, 10, 15 and 30% As. A) c-IAP1, B) c-IAP2, C) XIAP, D) Survivin, E) Smac and F) active AIF protein levels were analyzed through western blotting approach. In the upper panels, representative autoradiograms are shown. The results are expressed as the mean ± SD.

## Discussion

The present study has been focused on the effects of *Allium sativum* (As) on testicular cells and on the mechanisms of action of crude garlic on testicular functions. We showed here that oral administration of crude garlic induced germ cell death via an apoptotic process. This germ cell apoptotic process could be related to an increase in the expression of active caspase-3. Indeed, active caspase-3 was immunodetected mainly in spermatocytes and spermatids cells. In contrast, the active effector caspase-6 seemed not to be involved in our model, while procaspase-6 is expressed in normal germ cells (Omezzine A and al., 2003). While it was previously reported that chronic administration of 50 mg of garlic powder induced an arrest of spermatogenesis (Dixit VP, Joshi S, 1982) our present study, for the first time, shows the involvement of an apoptotic process that targets testicular germ cells. Moreover, very few if no study showed an apoptotic effect of As on non-tumoral cells while the programmed cell death was described in tumoral C-IAP1 and c-IAP2, are highly expressed in rat testis (Holcik M et al., 2002) and c-IAP1 was detected in the nucleus of B spermatogonia, spermatocytes at different stages and somatic cells (Wang Y et al., 2005), while c-IAP2 was localized to the cytoplasmic cells (Yu R and al., 1998; Xiao D and al., 2004; Shukla Y and al., 2007). For example, administration of crude extract of garlic to a human colon cancer cell line induced apoptosis by increasing the levels of Bax, cytochrome c and caspase-3 activity while it decreased the mitochondrial membrane potential (Su CC and al., 2006). More specifically, allicin the major component present in freshly crushed garlic and the most biologically active compound of garlic, induced activation of caspases (-3, -8 and -9) and cleavage of poly (ADP-ribose) polymerase in several cancer cells (Oommen S and al., 2004).

Since the balance between pro-apoptotic molecules (active effector caspases, IAPs inhibitors) and anti-apoptotic molecules (IAPs) levels determines the fate of the cells towards the executioner step of the death process (Liston P and al., 2003), we have evaluated the expression of IAPs (cIAP1, cIAP2, XIAP and survivin) and Smac/DIABLO in our experimental model. c-IAP1 and c-IAP2 protein levels were increased after treatment with crude garlic.

-Holcik M. The IAP proteins. Trends Genet. 2002; 18(10): 537.
-Joza N, Susin SA, Daugas E, Stanford

compartment of spermatocytes, spermatids (round and elongated) and Leydig cells (Wang Y et al., 2005). In our study, cIAP1 and cIAP2 highly expressed in germ cells are unable to inhibit caspase-3 action.

A potential explanation is the high levels of Smac/DIABLO (in spermatocytes) by inhibiting the IAP action may favour the activity of caspase-3 in these germ cells that are mostly affected by apoptosis after As treatment. Interestingly, at 30% of As Smac/DIABLO expression decreased. Since this IAPs inhibitor is highly and mainly expressed in pachytene spermatocytes (El Chami N et al., 2005) a loss of these testicular germ cells is not excluded at this dose. For the spermatid apoptosis, the possibility exists that other IAPs inhibitors, such as HtrA2, are expressed and inhibit cIAP1 and 2.

Survivin, another member of IAPs family, was expressed in spermatocytes at first meiotic prophase, and highly in Leydig cells (Wang Y et al., 2004) or exclusively in Leydig cells (our unpublished data). X-IAP was also highly expressed in testis (Lagacé M et al., 2001), mainly in Sertoli cells (our unpublished data). Survivin and X-IAP levels were unchanged after As treatment, suggesting that Leydig (Survivin) and Sertoli (X-IAP) cells are not affected in terms of apoptosis by crude garlic administration even at the highest doses. DNA degradation might be triggered by caspase-dependent or a caspase-independent pathway through the action of the Apoptosis Inducing Factor (AIF). During the apoptotic process, AIF is released from mitochondria and translocated into the nucleus where its DNA binding activity mediates large-scale DNA fragmentation (Joza N et al., 2001; Candé C et al, 2002). According to the fact that As treatment, at the doses used in this study, had no effect on the expression of active AIF, we suggest, therefore, that the caspase-dependent pathway is mainly involved in our experimental model.

## REFERENCES

Amagase H. Clarifying the real bioactive constituents of garlic. J Nutr. 2006; 136(3 Suppl): 716S-725S.

-Candé C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G. WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zúñiga-Pflücker JC, Kroemer G, Penninger JM. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. 2001; 410(6828): 549-54.

Lagacé M, Xuan JY, Young SS, McRoberts C, Maier J, Rajcan-Separovic E, Korneluk RG. Genomic organization of the X-linked inhibitor of apoptosis and identification of a novel testis-specific transcript. Genomics. 2001; 77(3): 181-8.

-Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene. 2003; 22(53): 8568-80.

Omezzine A, Chater S, Mauduit C, Florin A, Tabone E, Chuzel F, Bars R, Benahmed M. Long-term apoptotic cell death process with increased expression and activation of caspase-3 and -6 in adult rat germ cells exposed in utero to flutamide. Endocrinology. 2003; 144(2): 648-61.

Oommen S, Anto RJ, Srinivas G, Karunagaran D. Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. Eur J Pharmacol. 2004; 485(1-3): 97-103.

Shukla Y, Kalra N. Cancer chemoprevention with garlic and its constituents. Cancer Lett. 2007; 247(2): 167-81.

Su CC, Chen GW, Tan TW, Lin JG, Chung JG. Crude extract of garlic induced caspase-3 gene expression leading to apoptosis in human colon cancer cells. In Vivo. 2006; 20(1): 85-90.

Wang Y, Suominen JS, Hakovirta H, Parvinen M, Martinand-Mari C, Toppari J, Robbins I. Survivin expression in rat testis is upregulated by stem-cell factor. Mol Cell Endocrinol. 2004; 218(1-2): 165-74.

Wang Y, Suominen JS, Parvinen M, Rivero-Muller A, Kiiveri S, Heikinheimo M, Robbins I, Toppari J. The regulated expression of c-IAP1 and c-IAP2 during the rat seminiferous epithelial cycle plays a

Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie. 2002; 84(2-3): 215-22.

-El Chami N, Ikhlef F, Kaszas K, Yakoub S, Tabone E, Siddeek B, Cunha S, Beaudoin C, Morel L, Benahmed M, Régnier DC. Androgen-dependent apoptosis in male germ cells is regulated through the proto-oncoprotein Cbl. J Cell Biol. 2005; 171(4): 651-61.

-Hansen TM, Smith DJ, Nagley P. Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome c. Cell Death Differ. 2006; 13(7):1181-90.

role in the protection of germ cells from Fas-mediated apoptosis. Mol Cell Endocrinol. 2005; 245(1-2): 111-20.

Wilkinson JC, Wilkinson AS, Scott FL, Csomos RA, Salvesen GS, Duckett CS. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. J Biol Chem. 2004; 279(49): 51082-90.

Xiao D, Choi S, Johnson DE, Vogel VG, Johnson CS, Trump DL, Lee YJ, Singh SV. Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. Oncogene. 2004; 23(33): 5594-606.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 85 – 88*

**BIOLOGIA TUNISIE**

# SCREENING OF ENZYMATIC ANTIOXIDANT ACTIVITIES IN ALLIUM SATIVUM L.

**Imen Hadji Sfaxi [1], Feten Belhadj [1] Ferid Limam [2] and M. Nejib Marzouki [1]**

1- National Institute of Applied Sciences and Technology, Tunis, Tunisia.
2- CBBC

M. Nejib Marzouki, National Institute of Applied Sciences and Technology, Department of Bioengineering, BP 676 – 1080 Tunis Cedex, Tunisia. E-mail: mnmarzouki@yahoo.fr, mn.marzouki@insat.rnu.tn

**Keywords:** *Allium sativum*, superoxide dismutase, antioxidant.

## ABSRACT

*Allium sativum* belongs to the genus Allium and family liliaceae, is one of the more commonly used health supplements in the world. Its most known pharmacological effect are due to molecules such as diallyl sulfide, S-allylmercaptocysteine and ajoene. Morover, some substances such as antioxidants are present in small quantities but they can confer to this plant a particular therapeutic physiognomy. Antioxidant enzymes could prevent tumor promotion and some processes that are associated with free radicals, such as cardiovascular diseases, aging and cancer.
This study investigated the screening of *Allium sativum* antioxidative activity in the different parts: bulb, leaves and roots. The results indicate that extracts from all plant organs exhibited enzymatic antioxidant activity: Superoxyde dismutases, peroxidases and catalases. Except the catalase activity was not detected in roots, however he highest antioxidant activity was observed in the bulbs.

## INTRODUCTION

Plants produce a large number of chemical substances of different structures which can have value as medicinal agents. Allium is the largest and most important representative genus of the Alliaceae family and comprises 450 species, widely distributed in the northern hemisphere. Allium species are a rich source of phytonutrients, useful for the treatment or prevention of a number of diseases, including cancer, coronary heart disease, obesity, hypercholesterolemia, diabetes type2, hypertension, cataract and disturbances of the gastrointestinal tract (e.g. colic pain, flatulent colic and dyspepsia) (Willet, 1999), ( Capasso et al., 2003), (Fattorusso et al., 2002), (Augusto, 1996), (Lawson et al., 1998). However, the elucidation of its mechanism for therapeutic action has proved to be related with its modulatory effects on Reactive oxygen species (ROS).
The aim of this work was designed to examine the antioxidant and free-radical scavenging activities of Allium specie: *Allium sativum*. This study reports the results concerning bulb, stalk and leaf antioxidant enzyme activities (superoxide dismutase, catalase, and guaiacol peroxidase).

## MATERIAL AND METHODS

### Plant material

Bulbs of *Allium sativum L*. purchased from the local market were homogenized with a mixer. Then stirred for 2 hours in a 50 mM Tris-HCl buffer pH 7.25 containing 0.1mM EDTA (Ethylenedi-ethylamine),0.1 mM PMSF (Phenylmethylsulfonylfluoride) and (0,5% v/v) Triton X-100. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10.000 rpm for 60 min. Soluble proteins from garlic bulbs homogenate were concentrated by ammonium sulfate precipitation at 80 % saturation. Then the obtained extract was used for further experiences.

**Superoxide dismutase activity**

SOD was examined in 10% acrylamides gels according the procedure of Laemelli 1970. The enzyme activity was revealed according the method described by Hajji et al., 2007. Measurement of SOD activity was assayed by monitoring the adrenochrome formation using modified epinephrine assay (Misra and Fridovich, 1972). One unit of SOD activity was defined as the amount of enzyme required to 50% inhibition of adrenochrome formation as monitored at 480 nm.

**Peroxidase activity**

The electrophoretic separation of acid POX was performed on native 10% acrylamide gels with pH 8.8 (El Ichi et al., 2008). The gels were then incubated in acetate buffer pH 4 in dark at room temperature for 30 minutes, and bands were revealed by using the peroxide in acetate buffer pH 5 (Guikema et al., 1980). Peroxidase activity was measured in a reaction medium containing 50 mM phosphate buffer (pH7), 9 mM guaiacol and 19 mM $H_2O_2$ according to the method of Lin and Kao (1999).

**Catalase activity**

Catalase activity was detected on nondenaturing acrylamide gels 8%, the gel was soaked in 5 mM $H_2O_2$ solution for 15 min. Then its is rinsed and stained in a mixture of 2% potassium ferricyanide and 2% ferric chloride (Jebara et al.,2005 ). Activity catalase was monitored by following the decrease in the absorbance at 240 nm of $H_2O_2$ (Abei et al., 1984).

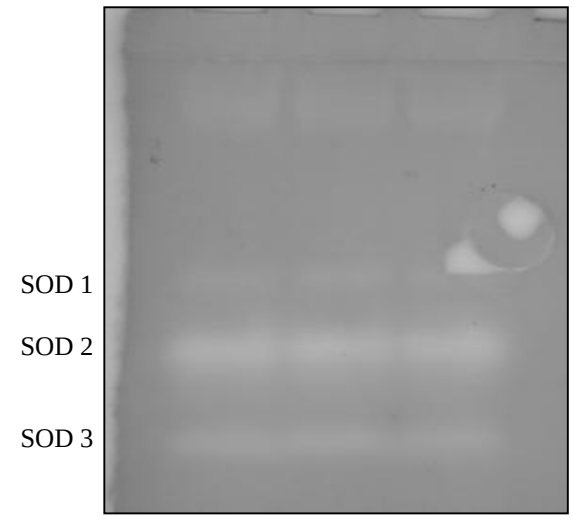
**Protein determination**

Protein content was determined using a Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976).

## RESULTS & DISCUSSION

Polyacrylamide gels for superoxide dismutase activity revealed three isoforms in the different parts of the plant: 2 major bands and one minor band of the same intensity (Figure 1).

**Figure 1:** Activity stain of Superoxide dismutase on native




gels (Polyacrylamide gel 10%) of bulbs leaves and roots. [B: bulb, L: leaves and R: roots.]

The specific activity of superoxide dismutase in bulb, leaves and roots is respectively about 50 U/mg, 48 U/mg and 43 U/mg (Table 1).

**Table1:** The quantification of the three antioxidant enzymes in the different organs.

| | Bulb | Leaves | Roots |
|---|---|---|---|
| Superoxide dismutase (U/mg) | 53 | 48 | 43 |
| Peroxidase (U/mg) | 40 | N.D | 27 |
| Catalase (U/mg) | 6.9 | 8 | N.D |

The presence of superoxide dismutase in all the compartments seems to be of equal amounts and almost certainly indispensable as a first line of defense against oxidative stress in most tissues. The specific identification of acidic peroxidases on native PAGE display three isoforms: P1, P2, P3 (Figure 2). The bulbs show the same isoenzymes but with a greater activity than in roots.

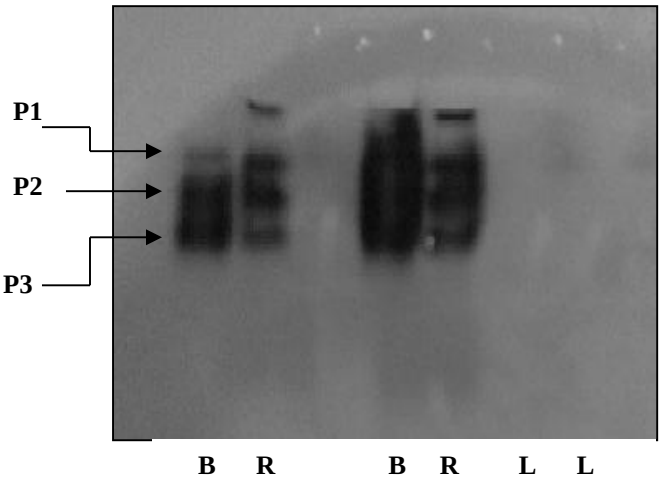
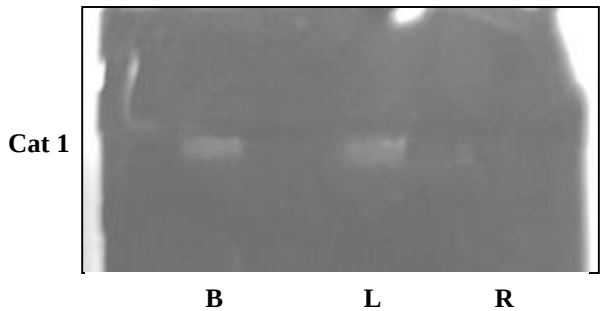



**Figure 2:** Nondenaturing gels (Polyacrylamide 7%) detected the acid Peroxidase activity. The Pox 1, 2 and 3 present different acidic POX isoforme. [B: bulb, L: leaves and R: roots.]

The leaves contain a very low peroxidase activity and display only a weak band P1. The catalase activity is represented by only one enzyme activity and it's present in leaves and bulbs (Figure 3). The roots did not display a catalase activity and this finding was similar to the result described by Stajner in 2006 (Stajner et al., 2006).

Figure 3: Catalase activity of different Allium sativum organs revealed on native gel (7%). [B: bulb, L: leaves and R: roots.]




## REFERENCES

Aebi, H. (1984). Catalase in Vitro. Methods Enzymol. 105 :121-126.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein dye binding. Anal. Biochem. 72: 241-254.

Capasso, F.; Gaginella, T.S.; Grandolini, G.; Izzo, A.A. (2003). Phytotherapy. A Quick Reference of Herbal Medicine, Springer-Verlag, Heidelberg, Germany,.

El Ichi S., Abdelghny A., Hajji I., Helali S., Limam F., and Marzouki M. N. (2008) A new Peroxidase from Allium sativum: Use in Hydrogen Peroxide Biosensing Biotechnology And Applied Biochemistry 51, issue 1, 33-41

Fattorusso**,** E.; Iorizzi, M.; Lanzotti, V.; Taglialatela-Scafati, O. (2002). Chemical composition of Shallot ( Allium ascalonicum Hort.). J. Agric. Food Chem. 50: 5686-5690.

Guikema, J.A. and Sherman, L.A. (1980) Electrophoretic profiles of cyanobacterial membrane polypeptides showing heme-dependant peroxidase activity. Biochem Biophys Acta 637 : 189-201.

Hajji I. , Marzouki M. N. , Ferraro D. , E. Fasano, Majdoub H. , Pani, G. and Limam F. (2007) Purification, Biochemical Characterization of a Garlic Cu,Zn-SOD. Effect on Reducing Anion Superoxide in Tumoral Cells Cultures
Applied Biochemistry and Biotechnology. 143, Issue 2 p 129-141

Jebara, S., Jebara, M., Limam, F., Aouni, M.E. (2005). Changes in Ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase activities in common bean (Phaseolus vulgaris) nodules under salin stress. Journal of Plant Physiology. 162 : 929-936.

K.T. Augusti, (1996). Ind. J. Exp. Biol. 34: 634.

Lammeli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lawson, L.D (1998). Effect of garlic on serum lipids. J.A.M.A. 280, 1568.

Lin, C.C. and Kao, C.H. (1999). NaCl induced changes in ionically bounds peroxidase activity in roots of rice seedlings. Plant Soil, 216: 147-153.

Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in autoxidation of epinephrine and a simple assay for SOD. J. Biol. Chem. 247: 3170-3175.

Stajner, D.; MiliÇ, N.; Canadanovic-Brunet, J.,; Kapor, A.; Stajner, M. and Popovic, B. M. (2006). Exploring Allium species as a Source of Potential Medicinal Agents. Phytother. Res. 20: 581-584.

Willet, W.C. (1999). Goals of nutrition in the year 2000. CA: Cancer. J. Clin. 49: 331-352.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 85 – 88*

**BIOLOGIA TUNISIE**

# SCREENING OF ENZYMATIC ANTIOXIDANT ACTIVITIES IN ALLIUM SATIVUM L.

**Imen Hadji Sfaxi [1], Feten Belhadj [1] Ferid Limam [2] and M. Nejib Marzouki [1]**

1- National Institute of Applied Sciences and Technology, Tunis, Tunisia.
2- CBBC

M. Nejib Marzouki, National Institute of Applied Sciences and Technology, Department of Bioengineering, BP 676 – 1080 Tunis Cedex, Tunisia. E-mail: mnmarzouki@yahoo.fr, mn.marzouki@insat.rnu.tn

**Keywords:** *Allium sativum*, superoxide dismutase, antioxidant.

## ABSRACT

*Allium sativum* belongs to the genus Allium and family liliaceae, is one of the more commonly used health supplements in the world. Its most known pharmacological effect are due to molecules such as diallyl sulfide, S-allylmercaptocysteine and ajoene. Morover, some substances such as antioxidants are present in small quantities but they can confer to this plant a particular therapeutic physiognomy. Antioxidant enzymes could prevent tumor promotion and some processes that are associated with free radicals, such as cardiovascular diseases, aging and cancer.
This study investigated the screening of *Allium sativum* antioxidative activity in the different parts: bulb, leaves and roots. The results indicate that extracts from all plant organs exhibited enzymatic antioxidant activity: Superoxyde dismutases, peroxidases and catalases. Except the catalase activity was not detected in roots, however he highest antioxidant activity was observed in the bulbs.

## INTRODUCTION

Plants produce a large number of chemical substances of different structures which can have value as medicinal agents. Allium is the largest and most important representative genus of the Alliaceae family and comprises 450 species, widely distributed in the northern hemisphere. Allium species are a rich source of phytonutrients, useful for the treatment or prevention of a number of diseases, including cancer, coronary heart disease, obesity, hypercholesterolemia, diabetes type2, hypertension, cataract and disturbances of the gastrointestinal tract (e.g. colic pain, flatulent colic and dyspepsia) (Willet, 1999), ( Capasso et al., 2003), (Fattorusso et al., 2002), (Augusto, 1996), (Lawson et al., 1998). However, the elucidation of its mechanism for therapeutic action has proved to be related with its modulatory effects on Reactive oxygen species (ROS).
The aim of this work was designed to examine the antioxidant and free-radical scavenging activities of Allium specie: *Allium sativum*. This study reports the results concerning bulb, stalk and leaf antioxidant enzyme activities (superoxide dismutase, catalase, and guaiacol peroxidase).

## MATERIAL AND METHODS

### Plant material

Bulbs of *Allium sativum L*. purchased from the local market were homogenized with a mixer. Then stirred for 2 hours in a 50 mM Tris-HCl buffer pH 7.25 containing 0.1mM EDTA (Ethylenedi-ethylamine),0.1 mM PMSF (Phenylmethylsulfonylfluoride) and (0,5% v/v) Triton X-100. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10.000 rpm for 60 min. Soluble proteins from garlic bulbs homogenate were concentrated by ammonium sulfate precipitation at 80 % saturation. Then the obtained extract was used for further experiences.

**Superoxide dismutase activity**

SOD was examined in 10% acrylamides gels according the procedure of Laemelli 1970. The enzyme activity was revealed according the method described by Hajji et al., 2007. Measurement of SOD activity was assayed by monitoring the adrenochrome formation using modified epinephrine assay (Misra and Fridovich, 1972). One unit of SOD activity was defined as the amount of enzyme required to 50% inhibition of adrenochrome formation as monitored at 480 nm.

**Peroxidase activity**

The electrophoretic separation of acid POX was performed on native 10% acrylamide gels with pH 8.8 (El Ichi et al., 2008). The gels were then incubated in acetate buffer pH 4 in dark at room temperature for 30 minutes, and bands were revealed by using the peroxide in acetate buffer pH 5 (Guikema et al., 1980). Peroxidase activity was measured in a reaction medium containing 50 mM phosphate buffer (pH7), 9 mM guaiacol and 19 mM $H_2O_2$ according to the method of Lin and Kao (1999).

**Catalase activity**

Catalase activity was detected on nondenaturing acrylamide gels 8%, the gel was soaked in 5 mM $H_2O_2$ solution for 15 min. Then its is rinsed and stained in a mixture of 2% potassium ferricyanide and 2% ferric chloride (Jebara et al.,2005 ). Activity catalase was monitored by following the decrease in the absorbance at 240 nm of $H_2O_2$ (Abei et al., 1984).

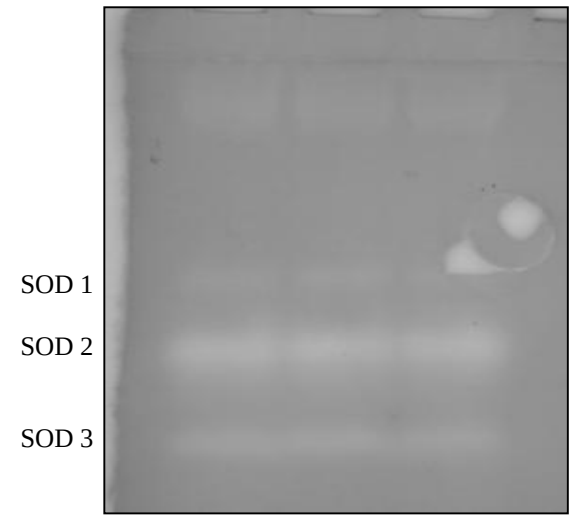
**Protein determination**

Protein content was determined using a Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976).

## RESULTS & DISCUSSION

Polyacrylamide gels for superoxide dismutase activity revealed three isoforms in the different parts of the plant: 2 major bands and one minor band of the same intensity (Figure 1).

**Figure 1:** Activity stain of Superoxide dismutase on native




gels (Polyacrylamide gel 10%) of bulbs leaves and roots. [B: bulb, L: leaves and R: roots.]

The specific activity of superoxide dismutase in bulb, leaves and roots is respectively about 50 U/mg, 48 U/mg and 43 U/mg (Table 1).

**Table1:** The quantification of the three antioxidant enzymes in the different organs.

| | Bulb | Leaves | Roots |
|---|---|---|---|
| Superoxide dismutase (U/mg) | 53 | 48 | 43 |
| Peroxidase (U/mg) | 40 | N.D | 27 |
| Catalase (U/mg) | 6.9 | 8 | N.D |

The presence of superoxide dismutase in all the compartments seems to be of equal amounts and almost certainly indispensable as a first line of defense against oxidative stress in most tissues. The specific identification of acidic peroxidases on native PAGE display three isoforms: P1, P2, P3 (Figure 2). The bulbs show the same isoenzymes but with a greater activity than in roots.

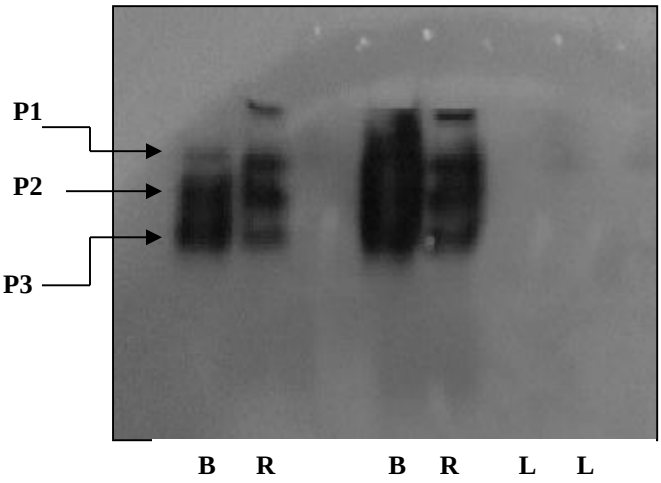
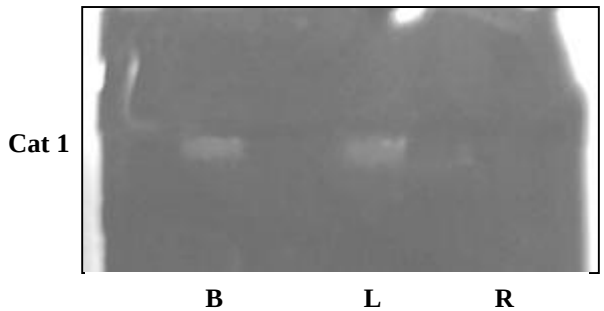



**Figure 2:** Nondenaturing gels (Polyacrylamide 7%) detected the acid Peroxidase activity. The Pox 1, 2 and 3 present different acidic POX isoforme. [B: bulb, L: leaves and R: roots.]

The leaves contain a very low peroxidase activity and display only a weak band P1. The catalase activity is represented by only one enzyme activity and it's present in leaves and bulbs (Figure 3). The roots did not display a catalase activity and this finding was similar to the result described by Stajner in 2006 (Stajner et al., 2006).

Figure 3: Catalase activity of different Allium sativum organs revealed on native gel (7%). [B: bulb, L: leaves and R: roots.]




## REFERENCES

Aebi, H. (1984). Catalase in Vitro. Methods Enzymol. 105 :121-126.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein dye binding. Anal. Biochem. 72: 241-254.

Capasso, F.; Gaginella, T.S.; Grandolini, G.; Izzo, A.A. (2003). Phytotherapy. A Quick Reference of Herbal Medicine, Springer-Verlag, Heidelberg, Germany,.

El Ichi S., Abdelghny A., Hajji I., Helali S., Limam F., and Marzouki M. N. (2008) A new Peroxidase from Allium sativum: Use in Hydrogen Peroxide Biosensing Biotechnology And Applied Biochemistry 51, issue 1, 33-41

Fattorusso**,** E.; Iorizzi, M.; Lanzotti, V.; Taglialatela-Scafati, O. (2002). Chemical composition of Shallot ( Allium ascalonicum Hort.). J. Agric. Food Chem. 50: 5686-5690.

Guikema, J.A. and Sherman, L.A. (1980) Electrophoretic profiles of cyanobacterial membrane polypeptides showing heme-dependant peroxidase activity. Biochem Biophys Acta 637 : 189-201.

Hajji I. , Marzouki M. N. , Ferraro D. , E. Fasano, Majdoub H. , Pani, G. and Limam F. (2007) Purification, Biochemical Characterization of a Garlic Cu,Zn-SOD. Effect on Reducing Anion Superoxide in Tumoral Cells Cultures
Applied Biochemistry and Biotechnology. 143, Issue 2 p 129-141

Jebara, S., Jebara, M., Limam, F., Aouni, M.E. (2005). Changes in Ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase activities in common bean (Phaseolus vulgaris) nodules under salin stress. Journal of Plant Physiology. 162 : 929-936.

K.T. Augusti, (1996). Ind. J. Exp. Biol. 34: 634.

Lammeli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lawson, L.D (1998). Effect of garlic on serum lipids. J.A.M.A. 280, 1568.

Lin, C.C. and Kao, C.H. (1999). NaCl induced changes in ionically bounds peroxidase activity in roots of rice seedlings. Plant Soil, 216: 147-153.

Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in autoxidation of epinephrine and a simple assay for SOD. J. Biol. Chem. 247: 3170-3175.

Stajner, D.; MiliÇ, N.; Canadanovic-Brunet, J.,; Kapor, A.; Stajner, M. and Popovic, B. M. (2006). Exploring Allium species as a Source of Potential Medicinal Agents. Phytother. Res. 20: 581-584.

Willet, W.C. (1999). Goals of nutrition in the year 2000. CA: Cancer. J. Clin. 49: 331-352.

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 89 – 95*

**BIOLOGIA TUNISIE**

# STUDY OF MOLECULAR POLYMORPHISM OF TUNISIAN POMEGRANATE (*Punica granatum* L.) CULTIVARS USING SSRs AND AFLPs MARKERS

**RANIA JBIR [1, 2], SALWA ZEHDI [1], NÉJIB HASNAOUI [1, 2], MOHAMED MARRAKCHI [1], MESSAOUD MARS [2] & MOKHTAR TRIFI [1*],**

1: Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences of Tunis, Campus Universitaire, 2092 El Manar, Tunis, Tunisia
2: U.R. Agrobiodiversity, High Agronomic Institute, 4042 Chott-Mariem, Sousse, Tunisia
*: Author to whom all correspondence should be addressed: Phone/Fax: +216 70 86 04 32;
e-mail: mokhtar.t@fst.rnu.tn; trifimokhtar@yahoo.fr

***Key words:*** *Tunisian pomegranate (Punica granatum L.), AFLP, SSR.*

**ABSTRACT**

Microsatellite markers (*SSRs*) were used in the molecular characterization of 18 Tunisian pomegranate cultivars. The DNA was analyzed using four pre-selected *SSR* primers that revealed 13 alleles, which allowed each genotype to be identified. An UPGMA dendrogram was generated using Cavalli-Sforza & Edwards genetic distances. This result was compared to the one based on AFLPs. For AFLPs study, four primers' combinations tested for their ability to generate dominant markers. A total of 192 bands were scored. A genetic distance matrix according to Nei and Li's formula was calculated to construct an UPGMA phylogram. In addition, correlation between distance matrices was assessed using the Mantel test. These results proved a high level of molecular polymorphism in the local pomegranate germplasm and the efficient of these molecular markers in the surveying or in the differentiation between cultivars.

**RESUME**

18 cultivars de grenadiers tunisiens sont caractérisés au moyen des marqueurs moléculaires SSRs. Au cours de cette technique, 4 amorces SSRs précédemment choisies ont permis la révélation de 13 allèles chez l'ensemble des cultivars étudiés. A partir du résultat ainsi obtenue, un dendrogramme UPGMA est construit selon la formule des distances génétiques Cavalli-Sforza & Edwards. La topologie de ce dernier phylogramme est par la suite comparer avec celle générée au moyen des marqueurs moléculaires AFLP. L'amplification des marqueurs AFLP était aussi réalisée par 4 couples d'amorces. Ensuite les distances génétiques étaient calculées selon les formules de Nei and Li. Les corrélations entre les analyses SSRs et AFLPs étaient réalisées par le test Mantel. L'ensemble des résultats a montré un niveau de polymorphisme assez élevé au niveau du germoplasme local de grenadier ainsi que l'efficacité des marqueurs moléculaires dans la sauvegarde et la différentiation entre les cultivars.

## INTRODUCTION

Pomegranate (*Punica granatum L.*) is an economically important fruit tree. This species is included in the family of Punicaceae, genus *Punica* with 2n = 16. This fruit tree is cultivated mainly in Central Asia and Mediterranean countries. Discrimination of varieties was based on morpho-phenological evaluation (Levin, 1995; Ozguven, 1996; Mars, 1996; Mars and Marrakchi, 1998). Genetic erosion was recorded for many of these local varieties. Nevertheless, some breeding program have been achieved to preserve these genetic resources and some selections may be found as results of these works. In Tunisia, many efforts of prospection were conducted to permit the collection of different ecotypes. For instance, more than 60 cultivars

denominated have been identified. These were collected from traditional plantations and *ex situ* conserved (Mars, 2000). But there is little known about the structure and the organization of genetic diversity of this local germplasm. Only studies have reported the use of morphometric parameters to examine the genetic variability in this crop (Mars and Marrakchi, 1998; 1999). In spite of their use to describe cultivars, these parameters are either limited in number or highly influenced by the environmental conditions. To overcome this inconvenience, we become interested in the development of molecular markers in order to provide markers reliable in the genetic diversity surveying and to molecularly characterise pomegranate cultivars. In fact, genetic diversity estimation based on DNA markers is more informative since these markers are distributed over the genome note that large number of methods mainly based on Polymerase Chain Reaction (PCR) has been reported in higher plants. As part of our work, we investigated two different methods namely:

*The Amplified Fragment Length Polymorphism (AFLP) that generate dominant markers (Vos *et al*., 1995) and successfully applied to a large number of fruit species such as apricot (Panaud *et al.*, 2002; Krichen *et al.*, 2008), figs (Cabrita *et al.*, 2001) and date-palms (El-Assar *et al.*, 2005; Rhouma *et al.*, 2007).

*The simple sequence Repeats known as powerful method to generate highly polymorphic co-dominant markers and to fingerprint cultivars (Liu *et al.*, 1996; Weber and May, 1989, Zehdi *et al.*, 2004).
The present study portrays possible relations between genetic distances based on the designed methods *SSRs* and *AFLPs*. Data are discussed in relation with the efficiency of each marker in the surveying and/or in the differentiation of cultivars.

## MATERIAL AND METHODS

### Plant material and DNA isolation

Eighteen Tunisian pomegranate accessions are used in this study. They were sampled from the pomegranate collection (Mars, 2000) (Table 1).

**Table 1:** Denomination, label and geographical origin of the 18 studied Tunisian pomegranate cultivars.

| Label | Cultivar | Origin |
|---|---|---|
| JB5 | Jebali | Mehrine |
| GB11 | Gabsi | Sedaghiane (Djerba) |
| ZH4 | Zehri | Sidi Bou Ali |
| ZH11 | Zehri | Sedaghiane (Djerba) |
| CH3 | Chelfi | Testour |
| CH4 | Chelfi | Sidi Bou Ali |
| CH7 | Chelfi | Srandi (Djerba) |
| CH8-2 | Chelfi | Sedaghiane (Djerba) |
| CH8-3 | Chelfi | Sedaghiane (Djerba) |
| CH9 | Chelfi | Sedaghiane (Djerba) |
| CH15 | Chelfi | Sedaghiane (Djerba) |
| CH17 | Chelfi | Srandi (Djerba) |
| TN2 | Tounsi | Testour |
| TN9-2 | Tounsi | Sedaghiane (Djerba) |
| TN10 | Tounsi | Srandi (Djerba) |
| NB1 | Nabli | Esslouguia |
| BY1 | Beyounsi | Sedaghiane (Djerba) |
| FP2 | Florepleno Panache | Tunis |

Total genomic DNA was isolated from young leaves of adult trees as described by Dellaporta *et al.*, (1983). DNA concentration was estimated on 0,8% agarose gel electrophoresis.

### Molecular analysis

*AFLP assays and data analysis*
The AFLP analysis was carried out using the AFLP Core Reagent Kit and AFLP Primer Kits (Invitrogen). Four primers combinations were randomly tested in this study. The final PCR products were electrophorised using a 6 % denaturing polyacrylamide gel and stained with the silver straining method (Chalhoub *et al.*, 1997). Genomic DNA was digested by *Eco*RI and *Mse*I restriction enzymes. After enzyme inactivation by heating, the generated DNA fragments were ligated to *Eco*RI and *Mse*I adapters to generate DNAs as template for PCR amplifications. PCR assays consisted of two consecutive reactions. The subsequent DNAs were firstly pre-amplified using appropriate AFLP primers 5' anchored with one selective nucleotide. The PCR products of the pre-amplification reaction were then used as template for selective amplification. Two AFLP primers, were 5' anchored with three selective nucleotides (Table2). In this study, only reproducible and well resolved fragments were considered for further analysis. Therefore, the resultant banding patterns were transformed into a binary matrix. A genetic distance matrix according to Nei and Li's formula (Nei and Li, 1979) was calculated using the Genedist program of the PHYLIP package. Cluster analysis using UPGMA method was carried out using the Neighbour program of the PHYLIP software version 3,5c. In addition, the ability of tested primers to differentiate cultivars was appreciated using the resolving power coefficient (Rp) (Prevost and Wilkinson, 1999). Rp was calculated according to the Gilbert *et al*. (1999) formula: $Rp = \sum Ib$, where I (Ib)

= (2 × |0.5 – *p*|), *p* is the proportion of accessions containing the I band. Ppb: rate of polymorphic bands *SSR assays and data analysis:*
All tested primers were designed from identified pomegranate *SSRs* markers, these microsatellites were previously screened from library genomic DNA (Hasnaoui *et al*., unpublished data). The pomegranate genotypes were analyzed using four pre-selected SSR primers
PCR assays were performed in a reaction mixture counting 20 to 30 ng of total cellular DNA. Amplifications were performed in a TECHNE Thermocycler (TC-512). SSR banding profiles were resolved on non denaturing polyacrylamide gels (10%) and revealed by ethidium bromide straining according to Sambrook *et al*. (1989).
The amplified fragments were scored according to the molecular size of each allele. The genetic diversity was estimated by calculating genotypes and allele's frequency. In order to establish hierarchical classifications, we calculated the shared allele distance using Cavalli-Sforza and Edwards (1967) with Populations 1.2.28 Software (Langella, 2002). The distance matrix obtained was used as input file to Neighbour program of the PHYLIP software version 3,5c. An UPGMA (Unweighted Pair Group Method using Arithmetic Average) dendrogram was constracted.

**Correlation test:**

All matrices were tested for pair-wise correlation, using Mantel's non-parametric test. Under the null-hypothesis of no correlation, the value of this score should not deviate significantly from the distribution of corresponding values obtained by repeatedly comparing one of the original matrices with 1000 randomly generated matrices. The null hypothesis of no correlation is rejected when Mantel statistic falls outside the 0,05 confidence level. Pearson's r-value was used to measure linear correlation between two matrices.

## RESULTS AND DISCUSSION

To characterize 18 Tunisian pomegranate cultivars two efficient methods were studied to generate *AFLPs* and *SSRs* markers. These techniques have permitted a large number of unambiguously polymorphic DNA bands (Figure 1 et 2).

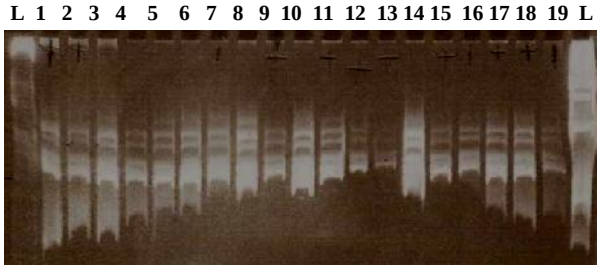



**Figure 1:** typical examples of SSR patterns resolved on non denaturing 10% polyacrylamide gel.

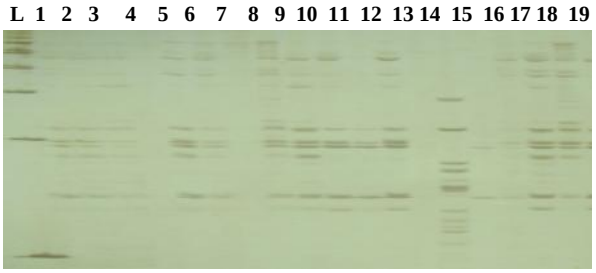



**Figure 2**: typical AFLP banding patterns generated from the primer combination and resolved on denaturing 10% polyacrylamide gel.

Starting from four *AFLP* primers' combinations, the banding patterns showed a total of 192 polymorphic *AFLPs* bands out of 228. This result was considered as parsimony informative *AFLP* markers among the 18 pomegranate cultivars analysed (Table2).

**Table 2:** Summary of AFLP data generated using four primers' combinations in 18 Tunisian pomegranate cultivars

| Primer combination | AFLP bands | | Ppb% | Rp |
|---|---|---|---|---|
| | Total | Polymorphic | | |
| $E_{AGC}/M_{CAA}$ | 63 | 57 | 90.5 | 28.11 |
| $E_{AAC}/M_{CAA}$ | 61 | 48 | 79 | 20.11 |
| $E_{ACA}/M_{CAG}$ | 55 | 45 | 82 | 19.00 |
| $E_{ACC}/M_{CTA}$ | 49 | 42 | 93.3 | 17.00 |
| Total | 228 | 192 | 86.2 | |

On average, each primer generated 56.25 fragments. In addition, different levels of polymorphisms were detected since the percentage of polymorphic bands (ppb) is ranging from 79 % for $E_{AAC}/M_{CAA}$ to 93.3 % for $E_{ACC}/M_{CTA}$ primers' combinations. In addition, the Rp values with a collective rate of 84.22 varied from 17.00 for the $E_{ACC}/M_{CTA}$ combination to 28.11 for the $E_{AGC}/M_{CAA}$ one. Furthermore, taking into account the highest Rp rate and ppb value the $E_{AGC}/M_{CAA}$ primers' combination seems to be the most powerful either to discriminate among cultivars or to assess the genetic diversity in this crop.

AFLP procedure was more efficient to generate a big number of markers than the SSR method. Using four pre-selected *SSR* primers, only 13 alleles have been revealed but the Ppb rate was 100% for all markers. The number of alleles per locus ranged from 2 to 5. The maximum number of alleles was generated by Pom006 primer's pairs (Table 3).

This result suggests that the AFLP procedure constitutes an attractive and efficient approach to examine the DNA polymorphisms in pomegranate. From the 18 studied pomegranate cultivars, 15 genotypes were differentiated with four *SSR* primers.

**Table 3:** Summery of SSR data using four primers' combinations in 18 Tunisian pomegranate cultivars

| Locus | Alleles | | | Genotypes |
|---|---|---|---|---|
| | Number | Length and allele's frequency | Ppb (%) | |
| Pom001 | 2 | 150pb: **0.66**<br>160pb: **0.34** | 100<br>100 | 2 |
| Pom004 | 3 | 112pb: **0.03**<br>116pb: **0.88**<br>120pb: **0.09** | 100<br>100<br>100 | 3 |
| Pom006 | 5 | 153pb: **0.11**<br>155pb: **0.59**<br>159pb: **0.08**<br>165pb: **0.13**<br>171pb: **0.08** | 100<br>100<br>100<br>100<br>100 | 7 |
| Pom010 | 3 | 234pb: **0.34**<br>236pb: **0.25**<br>248pb: **0.40** | 100<br>100<br>100 | 3 |
| Total | 13 | | 100 | 15 |

In order to illustrate the relatedness between tested genotypes, an UPGMA dendrogram based on Cavalli-Sforza and Edwards genetic distances was generated from *SSR* data (Figure 3).

Resulted dendrogram shows two main clusters. The first one (B) is composed by Jebali5, Gabsi11 and Tounsi2 cultivars; this group seems to be the most divergent.

The second cluster (A) shows many similar genotypes with distinct appellation, example the case of Tounsi10, Chelfi8-2, Chelfi9, Chelfi17 and Beyounsi1 cultivars. These lasted cultivars in the subgroup (a') divide the same alleles and genotypes and they are from the same geographical origin. However the *SSR* markers used in this study were not specific to a single *Punica granatum* genome.

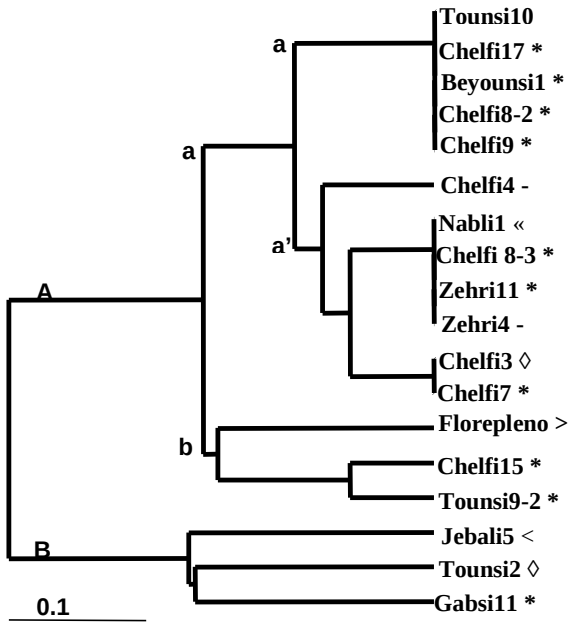



**Figure 3:** UPGMA phylogram of SSR analysis generated from genetic distances of Cavalli-Sforza & Edwards (1967). ). ***:** Djerba ; -: Sidi Bou Ali ; ◊**:** Testour ; <:Mehrine ; > : Tunis ; «: Esslouguia.

The derived typology is different to the UPGMA dendrogram generated from *AFLP* markers. This result is illustrated in figure 4.

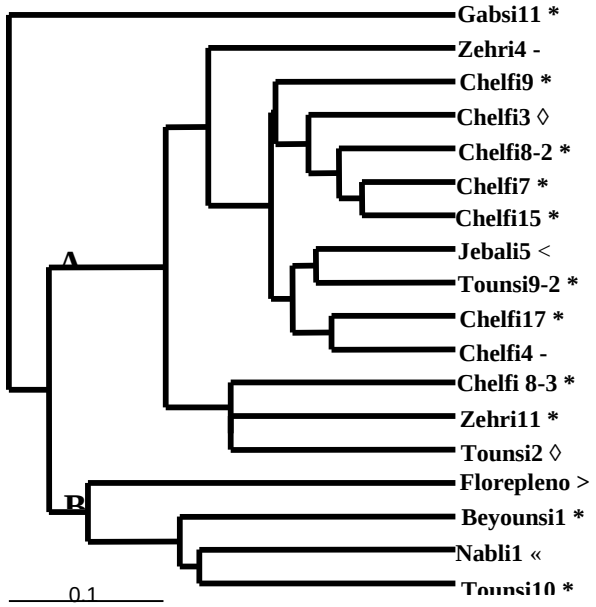



**Figure 4:** UPGMA dendrogram of 18 pomegranate accessions constructed according to Nei & Li's genetic distances. ***:** Djerba ; -: Sidi Bou Ali ; ◊**:** Testour ; <:Mehrine ; > : Tunis ; «: Esslouguia.

All tested cultivars are clustered into three main groups The first are monophyletic branch containing Gabsi11 cultivars and diverged to two groups labelled A and B. Taking into account the geographic origin of the cultivars, we assume that a typically continuous genetic diversity characterizes the local pomegranate germplasm. *AFLP* procedure was more efficient than *SSR* method. The high number of generated *AFLP* markers has permitted a global differentiation of pomegranate cultivars. And suggest that the studied cultivars constitute an important source of genetic diversity very useful in future breeding programs.

*AFLP* data show that there was a problem of false homonymy for some cultivars. Many cultivars could be differentiated without a significant correlation with their geographic origin as reported by Ben Nasr *et al.* (1996) and Mars and Marrakchi (1999). These authors could be reported that the phenotypic variation influenced by the ecosystem. Nevertheless, some varieties are considered multiclone regarding differences of morphometric traits (Mars, 2000).

Comparison of distance matrices generated from *AFLP* and *SSR* data showed no significant correlation (r(AB) 0,055, p-value 0,486 is higher to significant threshold $\alpha = 0,05$) following 1000 random permutations with Mantel's test. In our case, this lack of correlation can be attributed to the low number of generated *SSR* markers in comparison with *AFLP* one. Other limit in this comparison approach is the reduced number of studied cultivars. However, low correlations between matrices based on *AFLP* and *SSR* markers were also reported in others studies (Medini et al., 2005). This result can be interpreted by the difference of distribution of these markers in the pomegranate genome.

In addition, other studies have shown the large phenotypic diversity for pomegranate germplasm (Mars and Marrakchi, 1999), with emphasis on pomological traits (Mars and Sayadi, 1992; Melgarejo and Martínez, 1992; Mars and Marrakchi, 1998) and on technological properties (Al Kahtani, 1992) together with the evidenced molecular markers would be of a great interest to differentiate either cultivars or polyclonal varieties. Moreover, such strategy would provide molecular markers related with quantitative trait loci (QTLs) and therefore suitable to assist the selection and to rationally manage the conservation of this important phytogenetic resources as reported in other fruit crops such as apricot (Panaud *et al.*, 2002) and fig (Cabrita *et al.*, 2001). Work is currently in progress to enlarge either the number of cultivars or the primers' combinations in order to evidence a large number of *AFLPs*.

## CONCLUSION

The AFLP procedure permitted to generate a large number of molecular markers compared to the SSR analysis, these two results together suitable in the pomegranate genetic diversity surveying. All the tested combinations are efficient to evidence molecular polymorphism in this crop. *AFLP* and *SSR* data are not significantly correlated suggesting an evident difference via the type of targeted markers. The use of other *SSR* primer's pairs is recommended to fingerprint all Tunisian pomegranate cultivars. Studied cultivars constitute an important source of genetic diversity usable in future breeding programs. These results proved that a high level of molecular polymorphism characterizes the local pomegranate germplasm.

## AKNOWLEGMENTS

This work was partially supported by grants from the Tunisian "Ministère de l'Enseignement Supérieur, et de la Recherche Scientifique "Projet Lab B02.

## REFERENCES

Al Kahtani, H.A., 1992: Intercultivars differences in quality and postharvest life of pomegranate influenced by partial drying. Journal of the American Society for Horticultural Science. 117(1), 100-104.

Ben Nasr, C., Ayed, N., Metche, M., 1996. Quantitative determination of the polyphenolic content of pomegranate peel. Zeitschrift fur Lebensmittel Untershung und Forshung. 203(4), 374-378.

Cabrita, L.F., Aksoy, U., Hepaksoy, S., Leitao, J.M., 2001. Suitability of isozyme, *RAPD* and *AFLP* markers to assess genetic differences and relatedness among fig (*Ficus carica* L.) clones. Scientia Horticulturae. 87 (4), 261-273.

Cavalli-Sforza, L.L., Edwards, A.W.F., 1967. Phylogenetic analysis: models and estimation procedures. American Journal of Human Genetics. 19, 233-257

Chalhoub, B.A., Thibault, S., Laucou, V., Rameau, C., Hofte, H., Cousin, R., 1997. Silver staining and recovery of *AFLP*: TM amplification products on large denaturing polyacrylamide gels. Biotechniques. 22(2), 216-220.

Dellaporta, S.L., Wood, J., Hicks, J.B., 1984. Maize DNA miniprep. In: Molecular Biology of Plants. Cold Spring Harbor Laboratory, Cold Spring Harbor Press 36-38.

El-Assar, A., Krueger, R.R., Devanand, P.S., Chao, C.T., 2005. Genetic analysis of Egyptian date *Phoenix dactylifera* L. accessions using AFLP markers. Genetic Resources and Crop Evolution. 52, 601–607.

Gilbert, J.E., Lewis, R.V., Wilkinson, M.J., Galigari, P.D.S., 1999. Developing and appropriate strategy to assess genetic variability in plant germplasm collections. Theoretical and Applied Genetics. 98, 1125–1131.

Krichen, L., Martins, J.M.S., Lambert, P., Daaloul, A., Trifi-Farah, N., Marrakchi, M., Audergon, J.M., 2008. Using *AFLP* Markers for the Analysis of the Genetic Diversity of Apricot Cultivars in Tunisia. Journal American Society for Horticultural Science. 133(2), 204-212

Langella, O., 2002. Populations 1. 2. 28 Software. CNRS. UPR9034. France.

Levin, G.M., 1995. Aspects of pomegranate culture in Turkmenistan. Plant Genetic Resources Newsletter. 102, 29-31.

Liu, Z.W., Biyashev, R. M. and Saghai-Maroof, M. A., 1996. Development of simple sequence repeat DNA markers. Theoretical and Applied Genetics. 93, 869-876.

Mantel, N., 1967. The detection of disease clustering and generalized regression approach. Cancer Research. 27, 209-220.

Mars, M., Sayadi, S., 1992. Etude comparative de la qualité des fruits de cinq variétés de grenadier (*Punica granatum* L.). Revue des. Régions Arides. 4 (92), 45-57.

Mars, M., 1996. Pomegranate genetic resources in the Mediterranean regions. In: Proceeding. First MESFIN Plant Genetic Resources Meeting , Tenerife, Spain, 2-4 Oct. 1995. 345-354.

Mars, M., Marrakchi, M., 1998. Conservation et valorisation des ressources génétiques du grenadier (*Punica granatum* L.) en Tunisie. Plant Genetic Resources Newsletter. 114, 35-39.

Mars, M, Marrakchi, M., 1999. Diversity among pomegranate (*Punica granatum* L.) germplasm in Tunisia. Genetic resources and Crop Evolution. 46, 461-467.

Medini, M., Hamsa, S., Rebai, A., Baum, M., 2005. Analysis of genetic diversity in Tunisian durum wheat cultivars and related wild species by *SSR* and *AFLP* markers. Genetic Resources and Crop Evolution. 52, 21-31.

Melgarejo Moreno, P., Martínez Valero, R., 1992. El Granado. Mundi Prensa, Madrid, Spain.

Nei, M., Li, W.H., 1979. Mathematical models for studying genetic variation in terms of restriction endonucléases. Proceedings of the National Academy of Sciences, USA. 76, 5269-5273.

Ozguven, A.I., 1996. The genetic resources of pomegranate (*Punica granatum*) in Turkey. In:

Proceeding First MESFIN Plant Genetic Resources Meeting. Tenerife, Spain, 2-4 Oct. 1995, 269-284.

Panaud, O., Chaib, A., Sarr, A., 2002. Dynamic conservation of apricot *Prunus americana* in Saharian oases: use of AFLP markers to assess diversity in traditional orchards. Euphytica 128, 301-305.

Prevost, A., Wilkinson, M.J., 1999. A new system of comparing PCR primers applied to *ISSR* fingerprinting of potato cultivars. Theoretical and Applied Genetics. 98, 107–112.

Rhouma, S., Zehdi-Azouzi, S., Ould Mohamed Salem, A., Rhouma, A., Marrakchi, M. and Trifi, M., 2007. Genetic diversity in ecotypes of Tunisian date-palm (Phoenix dactylifera L.) assessed by *AFLP* markers. The Journal of Horticultural Science and Biotechnology. 82 (6), 929-933

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Weber, J.L., May, P., 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. American Journal of Human Genetics. 44, 388-396.

Vos, P., Hoger, R., Bleeker, M., Rejans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. *AFLP*: a new technique for DNA fingerprinting. Nucleic Acids Research. 23, 4407-4417.

Zehdi, S., Trifi, M., Billotte, N., Marrakchi, M., and Pintaud, J.K., 2004. Genetic diversity of Tunisian date palms (*Phoenix dactylifera* L.) revealed by nuclear microsatellite polymorphism. Hereditas. 141, 278-287.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7 ; 96 – 103*

**BIOLOGIA TUNISIE**

# MORPHOLOGICAL AND MOLECULAR EVALUATION OF THE GENETIC DIVERSITY OF TUNISIAN LOCAL BARLEY ACCESSIONS *(HORDEUM VULGARE L.)*

**KADRI. K [1,2], CHEIKH MED. H[2] ABDELLAWI. R [2], & BEN NACEUR. M [2]**

1: Centre Régional de la Recherche en Agriculture Oasienne, Laboratoire de Biotechnologie végétale et de Culture des Tissus, Degach 2260. TunisieTel / fax : 216 76 420 085.
2: Institut National de la recherche Agronomique de Tunisie, Laboratoire de Biotechnologie et Physiologie Végétale, Rue Hédi karray 2049, Ariana, Tunisie

*Corresponding author: Kadri karim, Tel / fax : 216 76 420 085, email : kadrikarim2001@yahoo.fr; kadri.karim@iresa.agrinet.tn

**Key words:** Barley accessions, PCA, morphological traits, RAPD markers, polymorphism

## ABSRACT

This research aim is to study the genetic diversity of barley *(Hordeum vulgare L.)* accessions all around the country. Morphological traits were studied and analyzed using principal components analysis (PCA) and clusters were constrained based on median joining distance. Further, molecular analysis was studied using RAPD markers. Both methods were used to compare how morphological traits and RAPD molecular markers described accessions relationship. Morphological PCA traits and cluster indicated geographic information. Indeed, they grouped barley accessions according to genetic criteria such as ear attitude, length of glum and its awn relative to grain. The molecular analysis showed the limits of the morphological approach. In fact ecotypes that were grown together in the same area and having similar morphological behaviour were found different using the RAPD markers. Distances obtained by each of the approaches were compared with special attention to the coincidences and divergences between the two methods. Comparison of morphological and molecular data using the Mantel test indicated a very low correlation (r = 0.14). Therefore both methods are not complementary but necessary and showed a high degree of variation among analyzed accessions, indicating an important source of genetic diversity that can be used in future breeding programs.

## INTRODUCTION

North Africa is considered as one of the main secondary cereal centers (Bouef, 1931). In deed, Tunisia constitutes an area of great cereal diversity. The local landraces are very adapted to stress conditions (drought and salt), since they contribute to genetic diversity and to new variety creations (Ben Naceur et *al*, 1998). However replacing native germoplasm by an improved and introduced material could lead to local phytogenetic resources erosion. Therefore, local resource conservation should be given more importance. Barley *(Hordeum vulgare L.)* was domesticated for 10,000 years ago front the wild barley *(Hordeum spontaneum C*. Koch) (Zohary and Hopf, 1998). It is used for human consumption, fodder, brewing and whiskey production. Barley has been used in human and animals nutrition. It still constitutes an important source of human food especially in Africa. It is a major cereal crop in Tunisia and is of great importance as forage species. It had been the

subject of intensive genome mapping and quantitative trait dissection efforts. Barley is raking fourth in the world after rice, wheat and maize (Forster et al., 2000). Local barley is of six rows ear and presenting a genome of 2n = 2x = 14. In Tunisia, since die begening of the cereal improvement program, we have registered only 15 varieties which make a narrow genetic diversity. In the opposite, we have more than 30 varieties of *durum* and bread wheat officially recorded (El Faleh, 1998). Genetic barley erosion could be avoided through die establishment of a local genotype ressources collection and conservation. Consequently, genetic diversity, identification and maintain of our local ressources should be achieved to be used in breeding programs.

In this paper, thirteen local barley accessions collected front different regions together with an improved variety "Martin" were described using some morphological traits. Analysis of principal components (PCA) was also dealt. Morphological distance clustering and phenologic RAPD clustering were compared to estimate correlation between morphological traits and RAPD markers.

## MATERIAL AND METHODS

### Plant material and experimental design

Twelve local winter barley accessions (*Hordeum vulgare*,L.) were collected from different Tunisian regions and named according to their origin. In addition, a cultivated variety 'Martin', 'Rihane' and 'Manel' were used as control. These accessions were obtained after prospection carried out in different Tunisian bioclimatic regions (Table 1). Seeds of each accession were sown in pots in three replications. The experiment was carried out at field capacity in the National Agronomic Research Institute of Tunisia (IINRAT). Morphological measurements of 16 traits were assigned (Table 2) using UPOV scale, which attributes ranges for non-quantitative characters. The growth habit stage and die presence of leaf sheaths hairiness of the lowest leaves were noticed at the tillering stage. The other characters were recorded after ear emergence and during ripening period. Analyses of principal components (PCA) were dealt and also were morphological distance clustering and phenologic RAPD markers tree.

**Table 1.** Accessions' origin, bioclimatic stage and rainfall (Monthly Bulletin of the National Meteorological Institute from 1975 to 2004).

| *Accessions* | *Origin* | *Bioclimatic stage* | Rainfall (mm) |
|---|---|---|---|
| **Tozeur 1** | Tozeur | Sahara | 150 |
| **Tozeur 2** | Tozeur | Sahara | 150 |
| **Kébilli 1** | Kébilli | Sahara | 150 |
| **Kébilli 2** | Kébilli | Sahara | 150 |
| **Kébilli 3** | Kébilli | Sahara | 150 |
| **Kasserine** | Kasserine | Arid-sup | 300 |
| **Sidi Bouzid** | Sidi Bouzid | Arid-sup | 300 |
| **Jendouba 1** | Jendouba 1 | Humid-inf | 800 |
| **Jendouba 2** | Jendouba 1 | Humid-inf | 800 |
| **Manel** | jendouba | Humid-inf | 800 |
| **Rihane** | Beja | Humid-inf | 800 |
| **Kalaâ** | k. landlos | Sub-humid | 600 |
| **Kélibia 1** | kélibia | Sub-humid | 600 |
| **Kélibia 2** | kélibia | Sub-humid | 600 |
| **Martin** | Introduced from Algeria (1931) | | |

### Extraction, purification and quantification of the DNA

The DNA was extracted and purified from 100 mg of fresh leaves, using a CTAB (Cetyl himethyl ammonium Bromide) method (Webb and Knapp, 1990) as modified by Ben Naceur *et al.* (1998). DNA was then quantified at 260 nm using a spectrophotometer (standard CECIL CE2501 sertes 2000/3000): 5 µL DNA samples was diluted in 995 µL of Tris-EDTA (TE) buffer and compared with a control containing 1000 µL of TE. The DNA concentration (C) was calculated as follows: C (µg µL') = $D0_{260}X10$. $DO_{260}/DO_{Z80}$ ratio was also calculated to determine DNA purity.

### PCR amplification and amplified product electrophoresis

Eighty Operon's primers were tested on DNA samples, DNA amplification was carried out in a final volume of 25 µL containing 12.5 µL of ready mix Promega (buffer with $MgC1_2$, dNTP and Taq polymerase), 20 pmol of Operon primer, 20 ng µL' of DNA and adjusted with distilled water. The program of amplification; using a thermocycler (Biometra UNO II); consisted of a pre-denaturation cycle of 3 min at 94°C, 40 cycles of a denaturation for 30 sec at 94°C, an hybridization for 45 sec at 38°C, an extension for 60 sec at 72°C followed by a post-

extension cycle for 10 min at 72°C. Amplification products of each primer were electrophoresed at 80V for 2 h in horizontal 1.5 % agarose gel prepared in IxTAE (TRIS Acetate EDTA) buffer containing 0.01 % of ethidium bromide. For each sample, 8 µL of the amplified product were mixed with 2 µL of 5 X loading dye (Biorad) and loaded in well gel.. Bands were visualized under UV waves on a Polaroid camera system. Only clear and reproducible bands were considered to make binary matrix, study similarity and discus polymorphism among accessions. RAPD clustering was compared to estimate correlation between morphological traits and RAPD markers.

**Table 2.** Morphological and phenological traits *in Hordeum rulgare L.*

| *Characters* | *Abbreviations* | Signification |
|---|---|---|
| C1 | GH | Growth habit |
| C2 | HLS | Lowest leaves: hairiness of leaf sheaths |
| C3 | TEE | Time of ear emergence |
| C4 | EAt | Ear: attitude |
| *C5* | PL | Plant: length cm (stem, car and awns) |
| C6 | ES | Ear: shape |
| C7 | ED | Ear: density |
| C8 | EL | Ear: length (excluding avens) |
| C9 | AwL | Awn: length (compared to car) |
| C10 | RLfs | Rachis: length of first segment |
| Cil | RCfs | Rachis: curvature of first segment |
| C12 | SSAt | Sterile spikelet: attitude (in mid-third of ear) |
| C13 | MSLG | Median spikelet: length of glume and its awn relative to grain |
| C14 | GRAT | Grain: rachilla hair type |
| C15 | HVF | Hairiness of ventral furrow |
| **C16** | GDL | Grain: disposition of lodicules |

## The primers used and the data analysis

Among eighty primers from Operon (kits A, B, D, F, H, and J) were used in RAPD analysis, only fifteen 10-mer oligonucleotides showing clear, reproducible and polymorphic bands were used. These primers were considered to make binary matrix, study dissimilarity and discos polymorphism between ecotypes (Table 3).

Data obtained were scored in a binary form as presence (1) or absence (0) of bands for each ecotypeand entered into a data matrix (Hou *et al.*, 2005). Genetic Similarity (GS) between ecotypes was calculated according to Nei and Li (1979) formula. Based on the Similarity matrix, a dendrogram showing the genetic relationships between ecotypes was constructedusing the Unweighted Pairgroup Method with Arithmetic Average (UPGMA) (Sneath and Sokal, 1973) by means of NTSYS 2.02 program (Rholf, 1993).

**Table 3**: RAPD primers' sequence, number of total band and number of polymorphic bands

| *primers* | *Primers sequences 5'---- 3'* | *Number of total bands* | Number of Polymorphic bands |
|---|---|---|---|
| **OPD02** | **GGACCCAACC** | 8 | 6 |
| **OPD10** | **GGTCTACACC** | 6 | 5 |
| **OPD18** | **GAGAGCCAAC** | 4 | 2 |
| **OPD20** | **ACCCGGTCAC** | 9 | 7 |
| **OPG12** | **CAGCTCACGA** | 8 | 6 |
| **OPG14** | **GGATGAGACC** | 5 | 2 |
| **OPG10** | **AGGGCCGTCT** | 8 | 3 |
| **OPJ10** | **AAGCCCGAGG** | 4 | 2 |
| **OPF03** | **CCTGATCACC** | 10 | 8 |
| **OPH13** | **GACGCCACAC** | 9 | 7 |
| **OPE03** | **CCAGATGCAC** | 4 | 3 |
| **OPE07** | **AGATGCAGCC** | 5 | 3 |
| **OPE12** | **TTATCGCCCC** | 8 | 5 |
| **OPB05** | **TGCGCCCTTC** | 7 | 6 |
| **OPB18** | **CCACAGCAGT** | 6 | 4 |
| **Total** | | 93 | 69 |
| **Average** | | 6.2 | 4.6 |

## Statistical analysis

The PCA was dealt and the genetic distances between the different accessions were calculated on the centered and standardized variates using measured data. Cluster analysis was conducted on the distances matrix with MVSP 3.13 software (Kovach, 1993). The relationship between the morphological distance matrix and the distance obtained with RAPD markers was analyzed. The comparison between the dendrograms is not fully adequate in some cases. Thus, the relationship was

performed according to the approach developed by Mantel (1967) using MxComp procedure from NTSYS 2.02 program (Rholf, 1993). The principle of this approach is to compare the observed Z - value or r-value with its permutational distribution according to a null hypothesis (no difference between the distance matrix, Z = 0). In This comparison, 5000 random permutations were made

## RESULTS

### Molecular study

Electrophoresis of the amplified DNA product for the 80 primers tested, showed only 15 primers, which were able to generate visible and reproducible band profile (Fig .1).

The size of bands varies from 250 to 3000 bp. A total of 93 bands were detected, among which 69 were polymorphic with the mean of 4.6 per primer (Table3). For each primer, the bands number ranged from 4 to 11, with an average of 6.2. The dendrogram of genetic distances (Fig. 2) was constructed based on UPGMA Method using midpoint-joining procedure of Nei and Li, (1979) similarity matrix. According to genetic distances dendrogram obtained by RAPD markers and referring to a similarity rate of 60 %, we distinguished five groups. The first group is composed of three accessions: ‘Kébilli 3’, ‘Martin’ and ‘Manel’, the similarity percentage between accessions of the first group, varies between 60 % and 65 %. The second is subdivided in three sub-group (‘Tozeur 2’ and ‘Kébilli 1’); (‘Kébilli 2’) and (‘Rihane’). Their similarities vary from 68 % to 78 %. Six accession composed the third group which were subdivided on three sub-group (‘Kasserine’,’Sidi Bouzid’ and ‘Jendouba 1); (‘Kalâa’ and ‘Kilibia 2’) and (‘Jendouba 2’). The similarity percentage between accessions of the first sub-group varies between 77 % and 83 %; but that of the second sub-group and the last sub-group was ranged between 67 % and 75%. Finally, the accessions 'Tozeur l' (62%), and 'Kélibia l'(64%) form, respectively, groups IV and V. Despite being collected from the same region, the accession 'Tozeur l' is distant from 'Tozeur 2'; 'Kébilli 3' is distant from 'Kébilli l' since they are classified in different groups. The same case is observed one the one hand for the accessions 'Kélibia l' and 'Kélibia 2' and on the other hand for ‘Jendouba 1’ and ‘Jendouba 2’. It's also to be remarked that the group consisted of ‘Tozeur 1’ is very far away from the others.

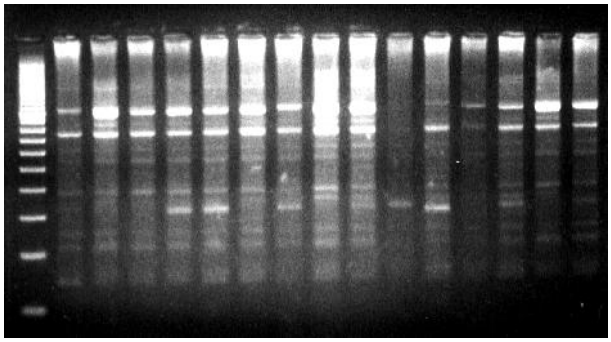


**Figure 1**: Typical examples of amplimeres obtained by RAPD tool using genomic DNA template of Tunisian barley accessions, in presence of OPD20 primer. M: marker (100 pb PCR Molecular Ruler, Biorad). Numbered wells correspond to the studied accessions. 1= Tozeur1, 2 = Tozeur2, 3 = Kebilli1, 4 = Kebilli2, 5 = Kebilli3, 6 = Sidi Bouzid, 7 = Kasserine, 8 = Jendouba1, 9 = Jendouba2, 10 = Martin, 11 = Kalâa, 12 = Klibia1, 13 = Klibia2, 14 = Rihane, 15 = Manel.

### Principal components analysis

Considering the Eigenvalue of 0.8 (0.77) as suggested by Tomassone et al. (1993), principal components analysis grouped variables in six components which explained up to 92.75% of the total variance. The two first axes were considered as they elucidate the maximum simple variance (respectively 27.44 and 20.25% of the total variation) with a cumulative variance of 47.57%.

The principal components assembly accessions into four groups. The first group consisted of 'Kébilli l', and 'Tozeur 2' that are positively correlated to both axis one and two. The second one formed by `Martin', 'Kébilli 2’ and ‘Kébilli 3’, which are negatively correlated to the second axis. The third set composed of 'Sidi Bouzid', 'Tozeur l’ and ’Jendouba1’, ‘Rihane’ and ‘Jendouba2’ are negatively correlated to axis one. Finally, the latest group is negatively correlated to the two axes and gathered 'Kalaâ', `Kasserine', 'Kélibia 1,’Kélibia 2’ and ‘Manel’, (Fig. 3).

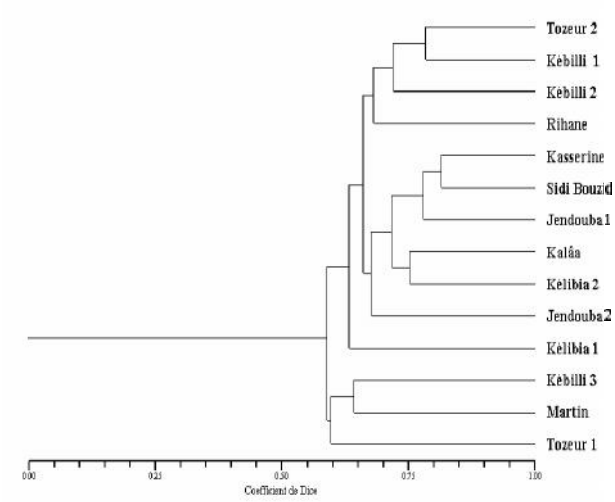



**Figure 2:** Phylogenetic simimlarity distance generated by RAPD markers using UPGMA procedure according to Nei and Li (1979) method.

Loadings variables, correlation coefficients between the original data (standardized matrix) and the PCA scores were illustrated (Fig. 4). Each principal axis was interpreted by its correlation with the original variables. Five groups of characters can be distinguished. The group is positively correlated to the first axis, it's composed of EAT and MSLG and is also positively correlated to the second one. The second group including RCfs, AwL, ED and HLS is negatively correlated to axis two. The third group is formed by GRAT and EL which are negatively correlated to the first axis. However, the fourth group composed of GH, ES, SSAT and is positively correlated to axis two, in contrast of variables, GDL, PL, RLfs, HVF and TEE which represent the fifth group negatively correlated to the second Axis. We used the percent similarity and median joining method to draw morphological distances (Fig. 5).

The Comparison of morphological characters using similarity percentage gave five accession groups (Fig. 4b).The first group consisted of 'Tozeur l', `Tozeur 2' and 'Kébilli l' with a percentage similarity that varies between 53% and 83 %. `Kébilli 2', 'Kébilli 3' and 'Kasserine' form the second with a similarity that oscillates between 73% and 91%. 'Sidi Bouzid' and 'Jendouba l', showing a very high similarity (92.5%), occupy the third position.

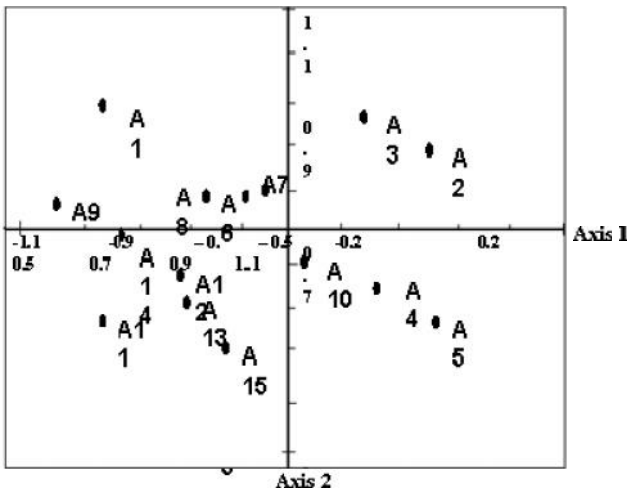



**Figure 3:** Principal Components Analysis of cultivars' morphological traits: V1: Tozeur 1, V2: Tozeur 2, V3: Kébilli 1, V4: Kébilli 2, V5: Kébillli 3, V6: Sidi Bouzid, V7: Kasserine, V8: Jendouba 1, V9: Jendouba 2, V10: Martin, V11 Kalaâ:, V12: Kélibia 1, V13: Kélibia 2, V14: Rihane and V15 : Manel.

The accessions 'Jendouba 2', `Rihane' and `Martin' constituted the fourth group; they are assembled with a percentage spread out between 81% and 86%. Nevertheless, 'Kalaâ', 'Kélibia l', 'Kélibia 2' and 'Manel' share the latest group with a high similarity situated between 75% and 90%. Referring to the dendrogram, we remark that accessions collected from the same origin have high similarity rates as examples, 'Kébilli 2/ Kébilli 3' with 93 %.

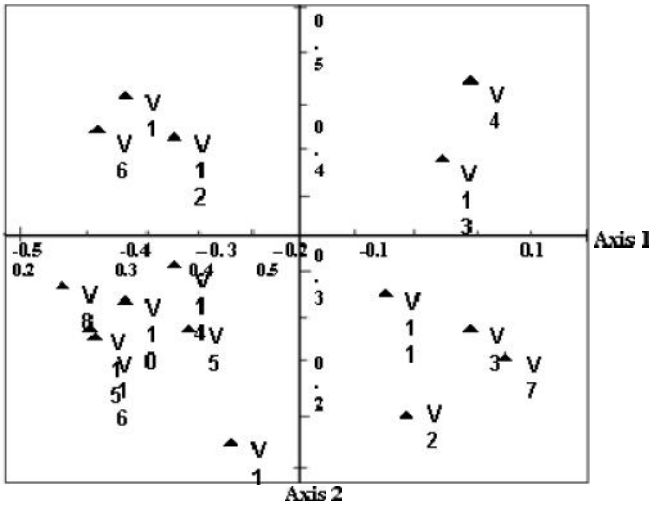



**Figure 4:** PCA diagram of variable loadings. C1: Growth habit ; C2: Lowest leaves: hairiness of leaf sheaths; C3: Time of ear emergence; C4: Ear attitude; C5: Plant length cm (stem, ear and awns); C6: Ear shape; C7: Ear density; C8: Ear length (excluding awns); C9: Awn length (compared to ear); C10: Rachis length of first segment;

C11: Rachis curvature of first segment; C12: Sterile spikelet attitude (in mid-third of ear); C13: Median spikelet (length of glume and its awn relative to grain); C14: Grain (rachilla hair type); C15: Hairiness of ventral furrow; C16: Grain disposition of lodicules.

Despite that the PCA of accessions showed four groups and the dendrogram clustering gave five groups, the most accessions gathered in the same group by PCA are also assembled in the same cluster. In fact, 'Tozeur 2' and 'Kébilli l' belong to the same group according to the two methods. The same remark is given for 'Kébilli 2' and 'Kébilli 3'. Also, we remark that 'Kalaâ', 'Kélibia 1' and 'Kélibia2' are included in the same group. The two methods also showed that 'Sidi Bouzid', 'Jendouba 1' and 'Jendouba2' are associated in the same group.

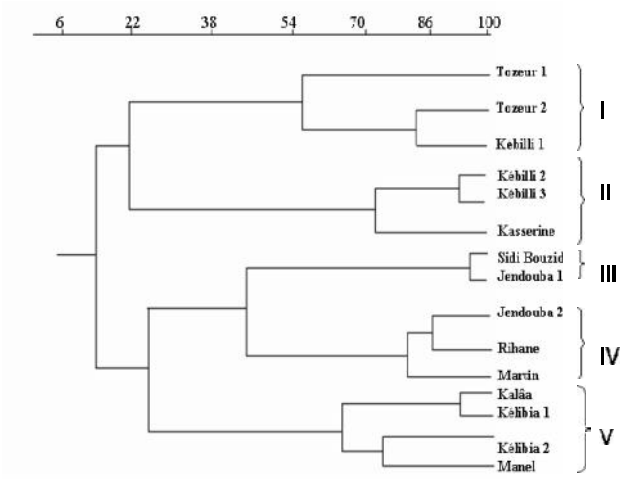



**Figure 5:** Morphological classification of the accessions based on median constrained distance using MVSP 3.131.

**Relationship between morphological distance and RAPD markers distance**

The relationship between morphological traits and the RAPD markers distances among studied accessions showed a very weak matrix correlation coefficient (r = 0.14) and a high value of global relationship (Z) with a probability p = 0.79. In fact, calculated distances between accessions by both methods are different. This large variation is clearly observed with the accession 'Tozeur 1' that showed a weak genetic structure similarity with the other accessions using RAPD markers.

## CONCLUSION & DISCUSSION

The PCA of accessions classify them according to their geographical origin (climatic stage). In fact, 'Kébilli 2', 'Kébilli 3', 'Martin', 'Tozeur 2' and 'Kébilli l' are positively correlated to the first component. Except the variety `Martin' which is widely cultivated by Tunisian farmers from the north to the south, these accessions seem to be adapted to desert climate. However, 'Jendouba l', 'Jendouba 2', 'Rihane', 'Manel', 'Kalaâ', 'Kélibia l', 'Kélibia 2', 'Kasserine', , 'Sidi Bouzid', and 'Tozeur l', are negatively correlated to the first component and form a second group. The first seven accessions belong to humid and sub-humid climatic stage. The accession 'Kasserine' was collected from a humid microclimate high land providing a sufficient argument for its belonging to the second group. The presence in this second environmental pool, of the accession 'Sidi Bouzid' collected from arid climate, also 'Tozeur l' that belongs to Sahara suggested that they derived from seeds mixture transferred from Nordic field to the center and southern region during seed storage and their distribution for farmers.

Several authors have shown that the geographic origin of the collected material was sufficient to obtain a reasonable structuration in groups (Julier et al., 1995). On the other hand, obtained classification depends on the material involved. In our study, the PCA variable loadings confirmed that characters, which are positively correlated to both axes, are related to genetic rather than geographic origin of the accessions studied. As a result, the geographic origin did not fit exactly with the dendrogram obtained. This could be due to high exchange of barley seeds between Tunisian cereal farmers of different regions and the spread of cultivated areas on different climatic stage. That is why; our clustering depends mainly on ear attitude (Eat), length of glumes and its awn relative to grain (MSLG). It is also common known that each accession has typical characteristics appreciated by farmers who conserve them every harvesting. The strong attachment of farmers to these accessions contributes to their conservation and consequently preserves the core of our plant germplasm.

Further more, farmers are involved in seed dynamic system transfer between local cultivated areas.

RAPD markers seemed to be proficient to discriminate local barleys defined as accessions or populations geographically based (Ben Hmida-Ben Salem, 2000). It is also a valuable tool for assessing genetic diversity levels. Also the dendrogram based on RAPD our dendrogram obtained by RAPD markers study showed that the structuration group depends on the geographical origin. This result is in agreement with that found using PCA variable loadings, PCA accession loadings and the dendrogram of morphological traits. In this study, distance matrices derived from RAPD markers and morphological data showed a low correlation (r=0.14). This result is in agreement with those of Chia-Szu and Hsiao (1999) working on *Lilium longiflorum* and reporting very low correlation *(r* = 0.035; *p* = 0.389) between RAPD markers and morphological characters. Furthermore, Marie et al. (2004); studying hexaploid wheat cultivars; obtained a non significant correlation between RAPD markers, morphological traits and coefficients of parentage. In the same way, Spooner et al. (2005) have obtained low correlation coefficient between potato genotypes by means of AFLP and morphologic characters. This weak correlation shows that there is no multilocus association between molecular and morphological traits in these accessions. Thus, RAPD markers were not efficient indicators of morphological divergence. In contrary to these authors, Roldân - Ruiz et al. (2001) working on perennial ryegrass found a correlation coefficient of 0.42 between STS markers and morphological traits methods. Moreover, Crochemore et al. (1998) working on 26 alfalfa population genetic structures found a global correlation coefficient of 0.51. While, Duarte et al. (1999) found a correlation of 0.89 between the genetic distances obtained with RAPD and the 'Mahalanobis' distances indicating that the markers provide similar estimates of genetic divergence to those obtained using morpho-agronomical data on bean cultivars.

PCA and dendrogram analysis of both morphological traits and RAPD markers showed an important genetic diversity of local Tunisian barleys. In fact, the accessions were distributed in different groups belonging to different bioclimatic location and on some genetic characteristics as ear attitude (Eat) and length of glumes and its awn relative to grain (MSLG).

As a conclusion, we believe that correlation between morphological and RAPD markers could be improved if there were more morphological traits analyzed as reported by other researchers (Martinez de Toda and Sancha, 1997) or more RAPD primers used. In fact, a combination of detailed molecular and morphological analyses proved to be a powerful tool for unravelling intraspecies taxonomy. When these two types of data are interpreted in concert, some of the problems encountered in traditional morphometric analysis can be avoided (Hedrick, 1986). Hence, important consideration should be given to collection and conservation of local material for breeding, in order to maintain and preserve local barley germplasm from genetic erosion.

## REFERENCES

Ben Hmida-Ben Salem W (2000) Contribution des marqueurs microsatellites et RAPD pour l'études du polymorphisme chez l'orge. Diplômes d'étude approfondie de génétique et biologie moléculaires. Faculté des Sciences de Tunis.

Ben Naceur M (1998) Development and assessment of new plant genotypes by molecular biology methods. PostDoctoral Report. Graduate School of Biotechnology Korea University, Korea.

Chia-szu W and Hsiao JY (1999) Genetic differentiation of *Lilium longiflorm* Thunb. var. Scabrum Masam (Liliacece) in Taiwan using Randon Amplified polymorphic DNAs and morphological characters. Botanical Bulletion of Academia Sinica 40: 65-71.

Crochemore ML, Huyghe C, Écalle C and Julier B (1998) Structuration of alafalfa. genetic diversity using agonimic and morphological chatacteristics. Relationship with RAPD markers. Agronomie 18: 79-94.

Duarte JM, dos Santos JB and Cunha Melo L (1999) Genetic divergence among common bean

cultivars from différent races based on RAPD markers. Genet. Mol. Biol. 3: 419-426.

El Faleh M (1998) Evaluation de 423 écotypes d'orge collectés en Tunisie (Centre et Sud). Utilisation en amélioration variétale pour la sélection d'idiotypes adaptés à différentes régions bioclimatiques. Thèse de Doctorat. Faculté des Sciences de Tunis.

Forster BP, Eus RP, Thomas WTB, Newton AC, Tuberosa R, This D, El-Enein RA, Bahri MH and BEN SALEM M (2000) The development and applications of molecular markers for abiotic stress tolerance in barley. J. Exp. Bot. 342: 19-27.

Bœuf F (1931) Le blé en Tunisie: la plante, le milieu physico-chimique. *Annales du Service Bota. et Agro*.

Hedrick PW (1986) Genetic polymorphisms in heterogeneous environments: a decade later. Annual Review of Ecology and Systematics 17: 535-566.

Julier B, Porcheron A, Écalle C and Guy P (1995) Genetic variability for morphlogy, growth and forage yield among perennial diploid and tetraploid Luceme populations *(Medicago sativa L.).* Agronomie 15: 295304.

Kovach WL (1993) MVSP (Multivariate Statistical Package), Kovach PLC.

Mantel NA (1967) The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209 - 220.

Marié S, Bolarié SJ, Martincic I P and Kozumplik V (2004) Genetic diversity of hexaploid wheat cultivars estimated by RAPD markers, morphological traits and coefficients of parentage. Plant Breeding 4: 366-369.

Martinez De Toda F and Sancha JC (1997). Ampelographical characterization of red *Vais vinifera L.* cultivars preserved in Rioja. Bulletin de l'OIV 70: 220-234.

Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321-4325.

Nei M and Li WH (1979) Mathematical models for studying genetic variation in ternis of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 52695273.

Rohlf FJ (1993) NTSYS-pc, nutnerical taxonomy and multivariate analysis system. Version 2.02 g. Exeter Software, Setauket, NY.

Roldân-Ruiz I, van Eeuwijk FA, Gilliland TJ, Dubreuil P, Dillmann C, Lallemand J, De Loose M and Baril CP (2001) A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass *(Lolium perenne L.)* varieties Theor. Appl. Genet. 103: 1138-1150.

Saghai-Maroof MF, Soliman KM, Jorjenson RA and Allarm RW (1984) Ribosomal DNA Spacer Polymorphisms in barley: Mendelian inheritence, chromosomal location, and poulation dynamics. Proc. Natl. Acad. Sci. USA 81: 8014-8018.

Spooner DM, McLean K, Ramsay G, Waugh and Bryan GJ (2005) A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. PNAS 41: 14694-14699.

Tomassone R, Dervin C and Masson JP (1993) Biométrie, Masson, Paris.

U.P.O.V. (1994) Guidelines for the conduct tests for distinctness, uniformity and stability. Revision of TG 19/7.

Webb DM and Knapp S.T (1990). DNA extraction from a previously recalcitrant plant genus. Plant Mol. Bio. Rep. 8: 180-185.

Zohary D and Hopf M (1998). Domesications of plants in the Old World. Oxford: University Press.

*Biologia Tunisie* ***Juillet 2012;*** *N°7 ; 104 – 112*

**BIOLOGIA TUNISIE**

# CARACTERISATION ET PURIFICATION D'UNE BACTERIOCINE PRODUITE PAR UNE SOUCHE LACTIQUE ISOLÉE A PARTIR D'UN DERIVE LAITIER ARTISANAL TUNISIEN

**REHAIEM Amel, GHRAIRI Taoufik, MANAI Mohamed***

Unité de Biochimie et Biologie Moléculaire, Département de Biologie, Faculté des Sciences de Tunis, Campus Universitaire 2092 El Manar, Tunis, Tunisie. * E-mail : Mohamed.Manai@fst.rnu.tn

**Mots clés:** Bactéries lactiques, bactériocines, anti-*Listéria*, SDS-PAGE , HPLC .

**ABSRACT**

A strain called MMR1 and identified as *Lacto. para. paracasei* was isolated from a Tunisian dairy beverage. This strain is proven out to be capable of inhibiting the growth of several indicator strains, particularly *Listeria.* The optimal antibacterial activity exhibiting by MMR1 was recovered after 10 to 12 hours of growth, at a temperature ranging from 30°C to 37°C and at 60% of ammonium sulfate saturation. The inhibitory agent was sensitive to the treatment with proteolytic enzymes, heat stable until 100°C, active over a wide pH range and present a bactericidal mode of action rather than bacteriostatical. The whole of this features where those of lactic acid bacteria bacteriocins. The result obtained after the partial purification of this peptide compound by reversed phase chromatography Sep pack C18 reveal that only the fraction eluted at 40% and 80% of acetonitrile was able to inhibit *Listeria ivanovii* BUG 496. Analytical reversed phase HPLC profiles of this active fractions shows several peaks which only the one corresponding to retention time of 37 min shown to be active against *Listeria.*The analysis by SDS-PAGE electrophoresis indicate that the apparent molecular weight of this bacteriocinogenic peptide is less than 6.5 KDa. The whole of these properties plead for a bacteriocin witch belongs to the classI or the classII.

**RESUME**

Une souche désignée MMR1 et identifiée comme étant un *Lacto. para. paracasei* a été isolée à partir d'un breuvage laitier artisanal tunisien. Elle s'est montrée capable d'inhiber plusieurs souches indicatrices, dont *Listéria*. le maximum d'activité inhibitrice mise en évidence dans le surnageant de culture de cette souche est enregistré après 10 à 12 heures de croissance, à des températures variant de 30°C à 37°C et à 60% de saturation en sulfate d'ammonium. L'agent inhibiteur est sensible à l'action des protéases, thermostable jusqu'à 100°C, actif à une large gamme de pH, et présente un mode d'action plutôt bactéricide que bactériostatique. L'ensemble de ces caractéristiques sont ceux des bactériocines produites par les bactéries lactiques. La purification partielle de ce composé peptidique par chromatographie en phase inverse sur colonne Sep pack C18 révèle que seules les fractions éluées à 40% et 80% d'acétonitrile sont actives contre *Listeria ivanovii* BUG 496. Le chromatogramme obtenu après analyse par HPLC de ces deux fractions actives présente plusieurs pics dont un seul ayant un temps de rétention de l'ordre de 37 min est doué d'une activité anti-*Listeria*. L'analyse par électrophorèse SDS-PAGE révèle que la masse moléculaire apparente du peptide bactériocinogénique est inférieur à 6.5 kDa. L'ensemble de ces caractéristiques physico-

chimiques et biologiques plaide en faveur d'une bactériocine appartenant à la classe I ou la classe II.

## INTRODUCTION

Parmi les technologies de conservation émergeantes, l'utilisation des bactéries lactiques en tant que bioconservateurs et biotransformateurs de nombreux produits alimentaires en particulier les dérivés laitiers, prend de plus en plus d'intérêt pratique (Paul Ross et al., 2002). En effet, dans plusieurs produits alimentaires ayant subit une fermentation lactique la croissance des microorganismes pathogènes est considérablement réduite. Le facteur à l'origine de ces inhibitions est principalement l'abaissement du pH, qui est dû essentiellement à l'accumulation d'acides organiques tels que l'acide acétique et l'acide lactique (Nes et al., 1996 ; Riley 1998). Cependant, il est connu que de nombreux autres processus sont, pour une partie, responsables des antagonismes observés en présence des bactéries lactiques, parmi les quels on cite particulièrement l'effet antagoniste des bactériocines (Garneau et al., 2002). Ces dernières sont de nature peptidiques, synthétisées par voie ribosomale et sont inhibitrices de bactéries Gram-positives taxonomiquement proches de la souche productrice. Celle-ci étant immunisée contre sa propre bactériocine (Tagg et al., 1976; Montville et Kaiser., 1993). Ces protéines sont également capables d'inhiber plusieurs microorganismes pathogènes tels que *Listeria* et *Enterococcus*. Elles présentent ainsi des capacités intéressantes non seulement dans la transformation et la conservation des aliments**,** mais aussi médicales. En effet, quelques bactériocines ont été récemment étudiées pour leur intérêt thérapeutique, par exemple la nisine qui est la bactériocine la plus étudiée actuellement et autorisée comme additif alimentaire (Hurst, 1981) a été aussi utilisée pour inhiber des bactéries responsables de plaques dentaires (Hoxell et al., 1993). La verotoxine1 s'est avérée active contre les cellules tumorales in vivo et in vitro (Farkas-Hinsley et al., 1995). Les bactériocines sont communément classées en quatre groupes (Klaenhammer 1993; Nes et al., 1996) en fonction de leurs natures (protéique ou peptidique), leurs tailles (faible ou haut poids moléculaire), leurs structures (présence ou non de modifications post-traductionnelles) ainsi que leurs spectres d'action (sélectif ou étendu) (Van Belkum et Stiles ; 2000 ; Héchard et al., 1992).

La Classe I : Comporte les Lantibiotiques de poids moléculaire inférieur à <5KDa et qui se caractérisent par la présence d'acides aminés modifiés telles que la lanthionine et la déhydroalanine. Cette classe se subdivise en deux sous classes:

Classe Ia: aomportant les Lantibiotiques de type A qui sont des molécules agissant par la formation de pores membranaires dans les cellules cibles. Parmi les plus connues de ces bactériocines on cite la nisine (Hurst, 1981 ; Harris et al ., 1992)

Classe Ib: ae sont des peptides capables de perturber certaines fonctions enzymatiques comme par exemple l'inhibition de la biosynthèse de la paroi cellulaire et appelés : les Lantibiotiques de type-B. On cite à titre d'exemple la mersacidine (Alténa et al., 2000).

La Classe II : appelée les non Lantibiotiques, ce sont des peptides de faible poids moléculaire (<10KDa) qui présentent une activité anti-*Listeria*. Elle se subdivise en quatre sous classes :

Classe IIa : comporte des bactériocines actives au niveau de la membrane de la cellule cible grâce à leur séquence caractéristique YGNGV à l'extrémité N-terminale. On peut citer à titre d'exemple : la mésentéricine Y105, la pediocine A-1 (Ennahar et al., 2000) et la Lactococcine MMFII ( Ferchichi et al., 2001).

Classe IIb : ce sont des bactériocines associant plusieurs peptides et capable de former des pores par action coopérative. On distingue la lactacine F (Muriana et al., 1991)

Classe IIc : c'est une classe qui comporte les bactériocines actives par la présence des thiols, par exemple la lactococcine B (Nes et al., 1996).

Classe IId : Cette classe englobe d'autres bactériocines diverses.

La Classe III : contenant des bactériocines thermosensibles de taille (> à 10KDa).

La Classe IV : Comporte des bactériocines complexes contenant une partie lipidique et/ou glucidique.

Dans le présent travail, nous nous sommes intéressés à l'identification, la caractérisation physico-chimique et la purification d'une bactériocine produite par une souche lactique que nous avons isolée à partir d'un breuvage laitier artisanal tunisien et désignée MMR1.

## MATERIEL ET METHODES

### Appareillages

Les centrifugations ont été réalisées dans une minie centrifugeuse de marque Denver Instrument et une centrifugeuse de marque SIGMA Bioblock Scientific 2-16.

Les cultures bactériennes ont été incubées dans une étuve Memmert à température réglable.

Le suivi de la densité optique des cultures bactériennes a été réalisé à l'aide d'un spectrophotomètre de marque JENWAY 6300 spectrophotometer.

L'électrophorèse de l'ADN plasmidique sur gel d'agarose a été faite dans une cuve horizontale de marque JENWAY. L'électrophorèse SDS-PAGE des protéines a été faite dans une mini cuve de marque BIOGEL II® munie d'un système de refroidissement par circulation d'eau.

Le lyophilisateur utilisé est un appareil de la firme FTS® Systems, type Flexy Dry.

L'appareil HPLC utilisé pour la purification des peptides est de type KNAUER équipé de deux pompes et un détecteur UV-visible.

### Produits chimiques

Les milieux de culture et les réactifs qui ont servi à la préparation des tampons utilisés pour l'extraction plasmidique, les électrophorèses et les chromatographies proviennent des firmes (Prolabo, SIGMA®, Merck et Fluka). Ils possèdent tous un degré de biologie moléculaire et une pureté supérieure à 90%.

### Isolement et identification des souches bacteriennes

L'isolement des souches bactériennes a été fait à partir de breuvages laitiers artisanaux tunisiens (raib, elben) préparés de façon traditionnelle et choisis chez des fermiers isolés dans des zones rurales. Un volume de 1ml de chaque échantillon de dérivé laitier a été pris aseptiquement et dilué dans du tampon tryptone-sel à 1%. Une série de dilution a été ensuite effectuée jusqu'a une dilution finale de $10^{-7}$. A partir des trois dernières dilutions, 100 µl ont été prélevés puis étalés sur des boites de Pétri contenant du milieu MRS ou M17. L'incubation a été poursuivie pendant 24 à 48 heures à une température de 30°C.

L'identification des souches isolées a été faite en se basant sur les caractéristiques morphologiques et biochimiques des bactéries lactiques décrites dans le « *Bergey's Mannual of Systematic Bactériology* » (Kandler et Weiss, 1986).

Une collection de souches de référence internationale a été utilisée pour déterminer le spectre d'inhibition des souches isolées (Tableau I). La culture des souches du genre *Lactococcus* a été réalisée en bouillon ou sur milieu solide M17. Les souches appartenant au genre *Listeria ivanovii* BUG 496 et *Serratia* ont été cultivées en bouillon ou sur milieu solide BHI.

### Caractérisation des souches

### Caractérisation morphologique

Les colonies de couleur blanche, ayant un contour régulier et de diamètre ne dépassant pas 2 mm ont été purifiées par repiquages successifs 2 à 3 fois sur leurs milieux sélectifs (MRS ou M17). Les souches pures ont été cultivées pendant 12 h. Les colonies apparues ont été colorées par la coloration de Gram suivie d'une observation microscopique, celles en forme de coques ou bacilles et à Gram-positif ont été gardées pour la caractérisation biochimique.

### Caractérisation biochimique

Test catalase

Pour mettre en évidence l'activité de la catalase, une goutte d'eau oxygénée à 10% a été déposée sur une lame de verre bien nettoyée puis une jeune colonie (âgée de 12h) est déposée sur cette goutte. L'existence d'une catalase se révèle par dégagement immédiat de bulles d'oxygènes.

Test API 50 CHL

L'API 50 CHL est un système d'identification permettant d'étudier le métabolisme fermentaire des souches lactiques vis-à-vis de 49 substrats déshydratés appartenant tous à la famille des hydrates de carbone et dérivés (hétérosides, polyalcools, acides uroniques). La souche a été

cultivée sur milieu M17 puis mise en suspension dans le milieu API 50 CHL qui a été réparti à l'aide d'une pipette stérile dans les 50 tubes de la galerie et par la suite incubée à 37°C. La lecture a été faite après 24 et 48 heures d'ïncubation, l'espèce a été identifiée par le logiciel Api Lab (Biomérieux, France).

**Caractérisation moléculaire**

Le profil plasmidique de la souche isolée a été identifié par la technique rapide KADO (Kado & Liu 1981). L'électrophorèse a été réalisée sur gel d'agarose à une concentration 0,8%. Les suspensions à analyser ont un volume final de 15 µl composé de 10 µl d'extrait plasmidique additionnés de 5 µl de tampon de charge (dilué 12X). Pour le marqueur de taille les 8 plasmides de la souche de référence *E.coli* K12-V517 de tailles : (53,7; 7,2; 5,4; 5; 4; 3; 2,6 et 2 Kb) ont été utilisés. Le résultat de l'électrophorèse a été visualisé sous une lumière UV incorporé à un appareil photo.

**Etude de l'antagonisme bactérien**

Détection de l'activité antibactérienne

La méthode adoptée a été celle de la diffusion en puits décrite par J.R Tagg et A.R Mc Given (1971). Une auréole d'inhibition du tapis bactérien a été observée autour des puits contenant une substance active, les diamètres des zones d'inhibition ont été à chaque fois mesurés.

Quantification de l'activité antibactérienne

L'activité bactériocinogénique a été dosée par la méthode de dilution critique. Elle a le même principe général que celui de la méthode de diffusion en puits, sauf qu'on a utilisé des dilutions de plus en plus grande du surnageant actif brut (1/2, 1/4, 1/8…..). L'activité anti-bactérienne est définie comme étant la réciproque de la plus grande dilution montrant une zone d'inhibition de la souche indicatrice. Cette activité bactériocinogénique est exprimée en unité arbitraire par millilitre de surnageant de la culture bactérienne (UA /ml). Les diamètres en millimètre des halos d'inhibition sont également mesurés.

**Purification de la bactériocine**

Le protocole de purification des peptides anti-microbiens développé dans notre laboratoire débute par une étape de précipitation au sulfate d'ammonium suivie d'une ou plusieurs chromatographies (Sep-pack C18, HPLC…) (Ghrairi et al., 2004.). Les fractions chromatographiques ont été ensuite testées pour leur activité bactériocinogénique contre *Listeria ivanovii* BUG 496 par la méthode de diffusion en puits.

**Analyse des protéines par électrophorèse SDS-PAGE**

L'électrophorèse est réalisée selon le système Laemmli (1970). Le gel d'acrylamide coulé entre deux plaques de verre est de 10 cm de hauteur et de 1.5 mim d'épaisseur. Les échantillons protéiques repris dans le tampon d'échantillon (Tris-HCl 0,5 M pH 6,8; glycérol 50%; 10% SDS; 0,2% β-mercaptoéthanol; 0,05% bleu de Bromophénol) sont chauffés pendant 4 minutes à 95°C puis centrifugés à 7000 rpm pendant 2 minutes. L'électrophorèse est réalisée à un ampérage constant de 25 mA. La migration à 125V se fait de la cathode vers l'anode pendant 8 heures. Après migration, les protéines sont fixées et colorées au bleu de Coomassie pendant 30 min dans une solution alcoolique contenant 50% de méthanol, 10% d'acide acétique et 0,1% de bleu de Coomassie G-250. Puis le gel est décoloré dans une solution de décoloration constituée de 50% méthanol et 10% d'acide acétique.

**Détection de l'activité bactériocinogénique après SDS-PAGE**

L'activité biologique des bactériocines purifiées peut être directement révélée sur le gel d'acrylamide. Après 12 heures d'immersion dans plusieurs bains d'eau bidistillée destinés à éliminer le SDS, le gel est placé dans une boite de Pétri stérile puis recouvert avec un milieu gélosé ensemencé à 1% avec *Listeria ivanovii* BUG 496. Après incubation à 30°C pendant 18 heures, le gel est examiné pour une éventuelle inhibition de la souche indicatrice.

**Caracterisation physico-chimique de la substance inhibitrice**

Action des hydrolases sur le compose inhibiteur

A fin de déterminer la nature de l'agent inhibiteur secrété, des volumes de 200 µl de surnageant actif ont été incubés pendant au moins 2 heures en présence des différentes protéases et enzymes telles que la protéinase K, la trypsine et la lysozyme a une concentration finale de 1mg/ml (Mitéva et al., 1998). L'activité bactériocinogénique résiduelle a été par la suite testée.

Effet de la température d'incubation de la culture bactérienne sur la production de l'agent inhibiteur

Pour établir les conditions de température optimale de production de l'agent inhibiteur, plusieurs cultures en bouillon de la souche inhibitrice ont été faites à différentes températures (28°C, 30°C, 37°C et 45°C) suivie par la détermination de l'activité inhibitrice résiduelle.

Stabilité thermique de l'agent inhibiteur

L'effet de la température sur l'agent inhibiteur a été déterminée en incubant le surnageant de culture bactérienne pendant 30 min à des températures allant de 60°C à 100°C. Après traitement, l'activité inhibitrice résiduelle a été déterminée en prenant comme témoin positif un surnageant actif non traité.

Stabilité de l'agent inhibiteur vis-à-vis des variations du pH

200 ml de surnageant actif ont été pris aseptiquement ensuite repartis sur des tubes falcons stériles (5 ml chacun). Quelques gouttes d'une solution de HCl (concentré 6 fois) ou de NaOH (3 fois normale) ont été ajoutées aux tubes de façon à obtenir une gamme de pH allant de 2 jusqu'à 12. Les mélanges ont été ensuite conservés pendant une nuit à 4°C puis le pH de chaque tube a été réajusté à un pH 6 avec les solutions de HCl et de NaOH. L'activité inhibitrice résiduelle est ensuite déterminée.

Précipitation de l'agent inhibiteur par le sulfate d'ammonium

A 200 ml de surnageant de la culture bactérienne, différentes saturations en sulfate d'ammonium sont ajoutées (Manai et Houari ; 1997). La solution est laissée sous faible agitation toute une nuit à 4°C, ensuite centrifugée à 12000 rpm pendant 20 mn. Le précipité obtenu après centrifugation a été récupéré puis dissout dans un volume minimum d'eau bidistillée stérile. L'activité bactériocinogénique est ensuite testée par la méthode de diffusion en puits contre *Listeria ivanovii* BUG 496.

**Cinetique de production de l'agent inhibiteur aux cours de la croissance bacterienne**

La croissance de la souche productrice de l'agent inhibiteur a été suivie depuis la phase de latence jusqu'à la phase stationnaire. Parallèlement la production de l'agent antibactérien a été estimée. Un volume de 200 ml de milieu de culture liquide a été ensemencé à 1% avec la souche inhibitrice. 2 ml de la culture bactérienne ont été pris aseptiquement toutes les 30mn : un ml sert pour mesurer la DO à 600 nm, l'autre ml est centrifugé à 12000 rpm pendant 15 mn. Le surnageant obtenu a été récupéré puis son activité inhibitrice a été déterminée.

**Mode d'action de l'agent inhibiteur vis a vis de la souche indicatrice**

L'étude de l'effet antimicrobien de l'agent inhibiteur vis-à-vis de la souche indicatrice a été réalisée par l'addition de différentes doses de l'agent inhibiteur exprimées en unité d'activité (0, 20, 40, 60 UA/ml de surnageant de culture bactérienne) à 200 ml d'une culture bactérienne de *Listeria ivanovii* BUG 496 en phase exponentielle. La DO à 600 nm ainsi que le UFC/ml (number of colony forming units) sont déterminés à différents intervals de temps.

## RESULTATS

**Isolement, identification morphologique et biochimique des souches productrices d'agents inhibiteurs**

Parmi les isolats testés pour leur antagonisme une souche isolée sur milieu M17 est capable d'inhiber la croissance de *Listeria ivanovii* BUG 496, on l'a désignée MMR1. L'observation microscopique après coloration Gram de la souche MMR1 montre des cellules à

Gram-positif qui se présentent sous forme de coques : isolées, regroupés en amas, en diplocoques ou en chaînettes (Figure 1).

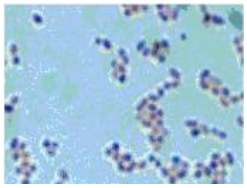


**Figure 1** : Observation microscopique de l'isolat MMR1 après coloration Gram

Le test catalase a révélé que la souche est catalase négative. L'étude de son profil fermentaire par l'analyse de 49 de ses caractères métaboliques à l'aide des plaques API 50 CHL a permis d'identifier une souche lactique d'espèce : *Lacto. para. paracasei* **.**

L'etude du profil plasmidique révèlent que la souche MMR1 présente deux plasmides de poids moléculaires respectifs 2.6 Kb et 4 Kb. (Figure 2).

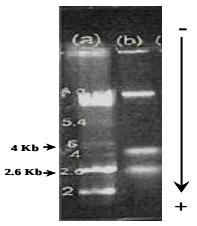



**Figure 2** : Profil plasmidique de la souche productrice de bactériocine

## Caractérisation physico-chimique de la substance inhibitrice

Le spectre d'inhibition de la souche MMR1 s'étend contre différentes bactéries, incluant des bactéries lactiques et des souches pathogènes et s'avère différent de celui de la souche *Lc lactis* ATCC 11454 productrice de la nisine A (Tableau I). L'activité inhibitrice du surnageant de culture de la souche MMRI reste intacte en présence de lysozyme, diminue faiblement en présence de trypsine alors qu'elle est totalement abolie en présence de protéinase K (Figure 3).

Cette activité antagoniste est stable jusqu'à 100°C ainsi qu'à une gamme de pH allant de pH 2 jusqu'à pH 11 (Figure 4).

**Tableau I :** Spectre d'inhibition de l'agent inhibiteur produit par la souche MMR1 et la nisine produite par Lc. lactis ATCC11454 sur différentes souches indicatrices

| **Souches** | **MMR1** | **Lc. lactis ATCC11454** |
|---|---|---|
| *Listeria ivanovii* BUG 496 | ++ | ++ |
| *Lactococcus lactis ssp. lactis* ATCC 11454[b] | - | - |
| *Lactococcus lactis ssp. Cremoris* ATCC 11603 | + | ++ |
| *Lactobacillus casei* DSM 20011 | ++ | - |
| *Lactobacillus delbrueckii* DSM 20081 | ++ | ++ |
| *Enterococcus faecium* ENSAIA 631 | ++ | + |
| *Enterococcus faecalis* JH-22 | ++ | ++ |
| *Serratia* sp. RF001[f] | - | + |

++ Forte activité inhibitrice ; + : présence d'activité inhibitrice ; -: absence d'activité

Cependant, elle est maximale à des valeurs de pH entre 5 et 7, à 60% de saturation en sel et à des températures de croissance bactériennes comprises entre 30°C et 37°C. Toutefois elle est très faible à 28°C et totalement aboli à 45°C. Cette activité apparaît assez tôt en phase exponentielle de croissance, environ après 4 heures d'incubation et reste maximale durant les 10 heures qui suivent la fin de la phase exponentielle (Figure 5).


**Figure 3 :** Test d'antagonisme sur boite de Pétri de la souche MMR1, **A** : test du surnageant de culture de la souche MMR1 ; **B** : test du surnageant de culture de la souche MMR1 traité à la protéinase K ; **C** : test du surnageant de culture de la souche MMR1 traité à 100°C pendant 30 mn ; **D** : test du surnageant de

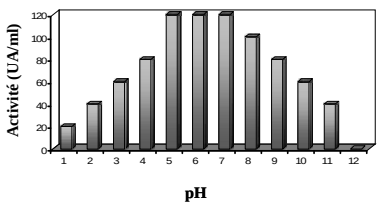



**Figure 4 :** Effet du pH sur l'activité bactériocinogénique de MMR1

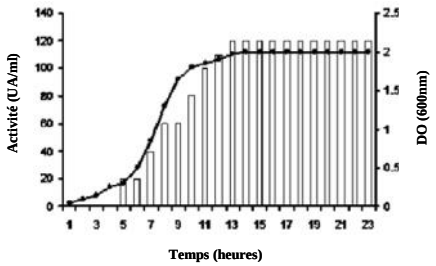



**Figure 5 :** Profil de l'activité bactériocinogénique en fonction du temps de croissance de la souche MMR1

## Mode d'action de l'agent inhibiteur

Les résultats obtenus (Figures 6 et 7) suggèrent que la bactériocine secrétée par l'isolat MMR1 possède un mode d'action bactéricide et non bactériostatique. En effet, une diminution considérable du nombre de cellules viables a pu être observée environ 1 heure après l'addition de l'agent antibactérien ainsi qu'une stabilité de la densité optique mesurée à 600 nm.

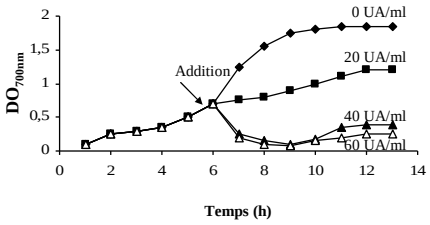



**Figure 6 :** Mesure de la densité optique en fonction du temps avant et après addition de différentes doses de bactériocines produite par MMR1 en UA/ ml

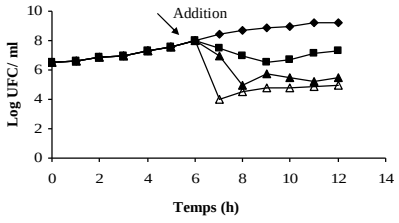



**Figure 7 :** Suivi du UFC en fonction du temps de croissance avant et après addition de différentes doses de bactériocine produite par MMR1 exprimées en UA/ ml

## Purification de l'agent inhibiteur

Le test d'antagonisme bactérien des différentes fractions obtenues par chromatographie sur colonne Sep-pack C18 a révélé la présence d'activité bactériocinogénique pour les fractions éluée à 40% et 80% d'acétonitrile.

Le chromatogramme obtenu (Figure 8) par la chromatographie liquide à haute performance en phase inversée pour ces fractions actives montre plusieurs pics qui ont été lyophilisés puis testés pour leur activité bactériocinogénique. Les résultats obtenus montrent que seul le pic avec un temps de rétention de l'ordre de 37 mn présente une activité anti-*Listeria*.

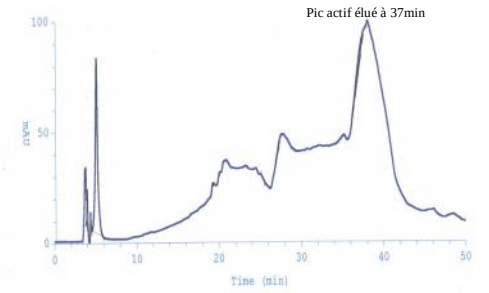



**Figure 8 :** Chromatogramme de l'élution HPLC en phase inverse des fractions 40% et 80% d'acetonitrile obtenues après Sep-pack

## Electrophorèse SDS-PAGE et détection de l'activité bactériocinogénique

L'analyse par électrophorèse SDS-PAGE des fractions révélées actives au cours de l'étape de purification montre que la substance inhibitrice sécrétée par MMR1 a une masse moléculaire apparente inférieure à 6,5 kDa. En effet, la bande d'inhibition obtenue suggère l'activité d'antagonisme manifestée par cette dernière (Figure 9).

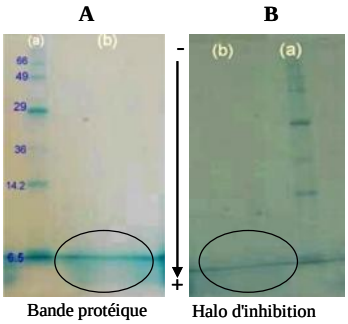



**Figure 9 : A** : Electrophorégramme des protéines analysées par SDS-PAGE, **B** : Détection directe de l'activité bactériocinogénique dans un gel de polyacrylamide en présence de SDS, (a) Marqueurs de poids moléculaire en kDa, (b) Surnageant concentré de la souche MMR1

## DISCUSSION ET CONCLUSIONS

Une cinquantaine d'échantillons issus à partir de breuvages laitiers artisanaux tunisiens (raib, elben) ont été analysés afin d'isoler des souches de bactéries lactiques. Les échantillons étudiés ont été collectés chez des petits fermiers qui n'utilisent pas de ferments commerciaux afin

que les souches découvertes soient d'origine tunisienne certaine. De plus, les manières de préparations peu rigoureuses sur le plan hygiénique, sont éventuellement favorables à enrichir le milieu en souches ayant des propriétés antagonistes. D'ailleurs jusqu'à présent très peu de souches antibactériennes isolées à partir de produits laitiers artisanaux tunisiens ont été décrites (Ferchichi et al., 2001 ; Ghrairi et al., 2004). Parmi les échantillons testés, l'isolat nommé MMR1 s'est avéré capable d'inhiber plusieurs souches indicatrices dont en particulier *Listeria ivanovii* BUG 496. Cette caractéristique est intéressante du fait que le facteur inhibiteur produit peut être actif à l'égard d'une bactérie pathogène telle *Listéria* et donc atteste que l'environnement rural où a été collecté l'échantillon représente un gisement vierge qui est susceptible de fournir des souches possédant des caractéristiques nouvelles et potentiellement apte à l'utilisation dans l'industrie alimentaire.

L'étude phénotypique ainsi que l'interprétation du logiciel API LAB ont permis d'identifier la souche MMR1 comme étant une bactérie lactique d'espèce *Lacto. para. paracasei.*

Toutefois, pour une identification authentique de l'espèce isolée, d'autres approches génotypique sont indispensables telles que l'analyse par RFLP-PCR de l' ADNr 16S ou la technique RAPD-PCR.

La caractérisation moléculaire de MMR1 qui révèle la présence de deux plasmides suggère que le déterminisme génétique responsable de la production de l'agent inhibiteur pourrait être de nature plasmidique. En effet la plupart des gènes codant la production des bactériocines chez les bactéries lactiques ont été très souvent portés par des plasmides (Jack et al., 1995).

La caractérisation physico-chimique de l'agent antibactérien révèle que le maximum d'expression de l'activité inhibitrice est enregistré après 12 heures de croissance bactérienne à des températures d'incubation variant de 30°C à 37°C. Cette activité est thermorésistante, stable à des pH extrêmes et sensible à l'action des protéases, ce qui suggère sa nature protéique. De plus, l'augmentation de l'activité antagoniste par précipitation et la détection directe de l'activité antagoniste sur gel de polyacrylamide à 15% confirme la nature protéique de cet agent antibactérien. Comme la stabilité vis-à-vis des variations du pH et la sensibilité aux protéases sont des critères clé dans la caractérisation d'inhibiteur de croissances bactériennes telles que les bactériocines des bactéries lactiques (Piard and Desmazeaud, 1992) et selon la définition donnée par Klaenhammer (1988), le facteur antibactérien produit par MMR1 qui est une substance de nature protéique avec un spectre d'activité relativement étroit et un mode d'action bactéricide, peut donc être considéré comme une bactériocine.

La purification de cette bactériocine a permis de prédire une masse moléculaire inférieur à 6.5 KDa. Cependant, l'obtention par HPLC d'un pic actif non symétrique suggère un mélange de composés différents et pour obtenir un peptide parfaitement pur des étapes supplémentaires de purification sont nécessaires.

L'ensemble de ces résultats suggère fortement que la substance inhibitrice secrétée par la souche MMR1 est une bactériocine qui appartient à la classe I ou la classe II différente de la nisine. La masse moléculaire exacte et la structure de cette bactériocine seront élucidées par d'autres approches : spectrométrie de masse, séquençage peptidique, synthèse chimique des peptides et modélisation moléculaire.

## REFERENCES

Altena K., Guder A., Cramer C. & Bierbaurn (2000). Biosynthesis of the lantibiotic mersacidine : organisation of a type B lantibiotic gene cluster. Appl. Environ. Microbiol. 66: 2565-2571.

Barritault D., Expert-Bezancon A., Guerin MF. & Hayes D. (1976). The use of acetone precipitation in the isolation of ribosomal proteins. Eur J Biochem. 16:131- 135.

Ennahar S., Sashihara T., Sonomoto K., Ishizaki A. (2000). Class IIa bacteriocins : Biosynthesis, structure and activity. FEMS Microbiology Reviews 24:85-106.

Farkas-Himsley H., Hill R., Rosen B., Arabs S & Lingwood CA. (1995). The bacteriol colicin active againts tumor cells in vitro and invivo is verotoxin1. Proc. Natl. Acad. Sci. U.S.A.. 15 : 6996-7000.

Ferchichi M., Frére J., Mabrouk k & Manai M.(2001). Lactoccoccine MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a tunisian dairy product. FEMS Microbiology letters 205: 49-55.

Garneau S., Martin IN., Vederas JC. (2002). Two- peptide bacteriocins produced by lactic acid bacteria. Biochimie 84: 577-592.

Ghrairi T. Manai M., Bergeaud JM., Frere J. (2004). Antilisterial activity of lactic acid bacteria isolated from rigouta, a traditional Tunisian cheese .J. Appl. Microbiol. 97: 621-628.

Héchard Y. Dérijard B., Lettellier F. & Cenatiempo Y. (1992). Characterization and purification of mesentericine Y105 and antibactériocine from Leuconostoc mesenteroides. J. Gen. Microbiol. 138: 2725-2731.

Hurst A. 1981.Nisine. Adv. App. Microbiol. 27 : 85-123.

Jack R W., Tagg J R., and Ray B. (1995). Bacteriocins of Gram-positive bacteria. Microbiological Reviews. 59: 171-200.

Klaenhammer TR. (1988). Bacteriocins of lactic acid bacteria. Biochimie 70, 337-349.

Klaenhammer TR. (1993).Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12: 39-85.

Kandler O. and Weiss N. (1986). Regular, non sporing Gram positive rods. In Bergey's manual of systematic bacteriology. Edité par Peter H.A. Sneath.

Kado CI. & Liu (1981).Rapid procedure for detection and isolation of large and small plasmids.J.Bacteriol. 145: 1365-1373.

Laemmli UK. (1970). Cleavageof structural proteins during the assembly of the head of bactériophage.T4. Nature (London). 227: 680-685.

Piard, J.C., Desmazeaud, M., (1992). Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. Lait 72: 113-142.

Tagg J.R & Mcgiven A.R**.** (1971). Assay system for bacteriocins. Appl. Microbiol**.** 21**,** 943.

Tagg JR., Dajani AS. & Wannamaker LW. (1976). Bacteriocins of gram positive bacteria. Bacteriol. Rev. 40: 722-756.

Manai M. & Haouari M. (1997). Etude et caractérisation biochimique des bactériocines de quelques souches du genre *Lactobacillus* isolées de quelques dérivés laitiers de fabrication artisanale. Bull. Soc. Sci. Nat. Tunisie. 26: 56-66.

Montville TJ., Kaiser A.L. (1993). Antimicrobial protéines : classification, nomenclature, diversity, and relation ship to bactériocins. In: Bacteriocins of lactic acid bacteria. Eds. Hoover D.G. & Steenson L.R., Academic press.

Miteva V., Ivanovi I., Budakov I., Pantev A., Stephanoa T., Danova S., Moncheva P., Mitev V., Dousset X & Boyaval P. (1998). Detection and characterization of a novel antimicrobial substance produced by a *Lactobacillus delbrueckii strain* 1043. Journal of Applied Microbiologie. 85: 603-614.

Muriana P. M. & Klaenhammer T.R., **(**1991). Clonning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by *Lactobacillu*s sp. J. Bacteriology. 173 : 1779- 1788.

Nes I.F., Diep D.B., Harvestein L.S., Burguers M.B., Einjsink V., Holo H. (1996). Biosynthese of bactériocines in lactic acid bacteria. Antonie Van Leeuwenhoeck. 70 : 113-128.

Paul Ross R., Morgan. S & Hill. C. (2002). Preservation and fermentation: past, present and future. International Journal of Food Microbiology. 79: 3-16.

Riley AM. (1998) Molecular mechanisms of bacteriocin evolution. Ann. Rev. Genet. 32**:** 255-278.

Van Belkum M J & Striles ME. (2000). Non lantibiotic antibactirial peptides from lactic acid bacteria. Nat. Prod- Rep. 17 : 323-335.

*Biologia Tunisie* ***Juillet 2009 ; N°7 ; 113 – 118***

**BIOLOGIA TUNISIE**

# OCTADECANEUROPEPTIDE, ODN, PROMOTES CELL SURVIVAL IN RAT CEREBELLAR GRANULE NEURONS

**Kaddour Hadhemi[a], Hamdi Yosra[a], Vaudry David[b-d], Tonon Marie-Christine[b-d], Amri Mohamed[*a] and Masmoudi-Kouki Olfa[a]**

Laboratory of Functional Neurophysiology and Pathology, Research Unit - 00/UR/08-01, Department of Biological Sciences, Faculty of Science of Tunis ; University Tunis El Manar, 2092 Tunis, Tunisia ; bInserm U982, Laboratory of Neuronal and Neuroendocrine Communication and Differentiation, Mont-Saint-Aignan, University of Rouen ; 6128 Mont-Saint-Aignan, France ; cInternational Associated Laboratory Samuel de Champlain ; dEuropean Institute for Peptide Research (IFRMP 23), Regional Platform for Cell Imaging (PRIMACEN), Rouen, France.
*Corresponding authors: ; Pr Amri Mohamed. Fax: (216) 216 71 87-16-66. ; E-mail address: mohamed.amri@fst.rnu.tn

**Keywords:** Cerebellar granule neurons ; cell death ; Octadecaneuropeptide ; Neurotoxine

## ABSRACT

High concentrations of diazepam-binding inhibitor (DBI) and its receptors have been detected in the rat cerebellum during ontogenesis. In particular, depletion of DBI expression in newborn rats affects the postnatal development and reduces the granular cell area in the neocerebellum, suggesting that DBI may act as a neurotrophic factor in the developing of rat cerebellum. In the present study, we have investigated the potential neuroprotective effect of a processing product of DBI, the octadecaneuropeptide ODN, on mature cerebellar granule cells in primary culture. In control conditions, cultured granule cells undergo programmed cell death when the extracellular level of K+ is lowered. Incubation of cultured cells with 1nM ODN for 72 h provoked a significant increase in the number of living cells. Exposure of cultured cells to 1-methyl-4-phenylpyridinium (MPP+), an ultimate toxic metabolite of a mitochondrial neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) inhibited neurite outgrowth and provoked apoptotic cell death. Incubation of granule cells with ODN prevents MPP+-induced apoptosis. Taken together, these data demonstrate for the first time that ODN is a potent neuroprotective agent that prevents cerebellar granule neurons from apoptotic cell death and suggests that ODN could have potential therapeutic value for the treatment of cerebral injuries.

## INTRODUCTION

The octadecaneuropeptide (ODN) has been originally isolated from the rat brain and characterized as an endogenous ligand of benzodiazepine receptors (Ferrero et al., 1986). ODN is generated by proteolytic cleavage of an 86-amino acid polypeptide precursor called diazepam-binding inhibitor (DBI) (Guidotti et al., 1983) whose gene is mainly expressed in glial cells in the central nervous system (CNS) (Alho et al., 1995; Burgi et al., 1999; Compère et al., 2006). The primary structure of ODN has been strongly preserved during evolution (Webb et al., 1987; Lihrmann et al., 1994; Chang and Tsai, 1996), indicating that this peptide plays important biological functions. Indeed, behavioral studies have shown that ODN induces anxiety, attenuates pentylenetetrazol-evoked convulsions, suppresses apomorphine-induced yawning, and inhibits food intake in rodents (Tonon et al., 2006). It was initially shown that

DBI and its processing product ODN interact with central-type benzodiazepine receptors (CBR) associated with the GABAA-receptor complex (Tonon et al., 2006). In addition, ODN may activate a non-benzodiazepinic membrane receptor coupled to phospholipase C (Gandolfo et al., 1997; Leprince et al., 2001; Marino et al., 2003; Compère et al., 2004).

1-methyl-4-phenilpyridinium (MPP+) is a well-known neurotoxin (Gibb et al., 1988) which causes a clinical syndrome similar to Parkinson's disease (PD) in human and laboratory animals, and is used as a suitable experimental model to study new therapeutic strategies in PD (Eberhardt and Schulz, 2003; Moore et al., 2005). Cerebellar granule cells are often used as models in the study of cellular and molecular mechanisms underlying neuronal death and apoptosis (Yalcin et al., 2003; Falluel-Morel et al., 2004). Accordingly, cerebellar granule cells die by apoptosis when cultured in physiological KCl concentrations (5mM; K5), and they survive in the presence of depolarizing concentrations of KCl (25mM; K25) (Xifro et al., 2006). Although the cerebellum has not been extensively studied as a prominent target for the MPP+ neurotoxicity, cerebellar granule cells are quite sensitive to the toxic effects of MPP+ in vitro (Gonzalez-Polo et al., 2001; Shang et al., 2004; Alvira et al., 2007) and in vivo (Takada et al., 1991).

It has been reported that, that intra-cerebral administration of benzodiazepines receptors agonists prevent neuronal death induced by ischemia or stroke (Schwartz-Bloom and Sah, 2001; Aerden et al., 2004; Corbett et al., 2008). On the other hand, the concentration of DBI in the cerebrospinal fluid and the expression of benzodiazepines receptors in brain are increased in diverse nervous system injuries, including Parkinson disease (Ferrarese et al., 1990; Veiga et al., 2007). Although the effect of ODN on cellular protection has never been investigated, these data suggest that ODN, acting as an endogenous ligand for benzodiazepine receptors, may exert a neuroprotective effect. In order to test the hypothesis of a protective action of ODN, we analyzed the ability of the peptide to protect cerebellar granule cells against cells death induced by neurotoxin MPP+ and serum and potassium deprivation.

## MATERIAL AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM), F12 culture medium, D(+)-glucose, L-glutamine, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), foetal bovine serum (FBS) and the antibiotic-antimycotic solution were obtained from Gibco (Invitrogen, Grand Island NY, USA). MPTP was purchased from Sigma Aldrich (St. Louis, MO, USA). Lactate dehydrogenase (LDH; EC 1.1.1.27) assay kit was commercialized by Bio-Maghreb (Ariana, Tunisia). Rat ODN (QATVGDVNTDRPGLLDLK) was synthesized by using the standard Fmoc procedure, as previously described (Leprince et al., 2001). All other reagents were of A grade purity.

### Primary culture of cerebellar granule cells (CGCs)

Cultures enriched in granule neurons were obtained from the cerebella of 8-day old rats as described previously (Gonzalez et al., 1992). Briefly, freshly dissected cerebella were dissociated in the presence of trypsin and DNase I and planted on poly-L-lysine coated dishes. Cells were seeded at a density of 3.5 x 106 cells/ml in basal medium Eagle supplemented with 10% FBS, 2 mM glutamine, 5 mM KCl, and 1 % antibiotic-antimycotic solution. Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO2 / 95% air.

### Measurement of cell cytotoxicity

Granule cells were incubated at 37°C with fresh serum-free medium in the absence or presence of test substances. At the end of the incubation, the cytotoxicity of MPP+ on neurons was determined by measurement of LDH activity in culture medium. The amount of LDH released into medium was measured by LDH assay kit (Bio-Maghreb) according to the manufacturer's instructions.

### Examination of cell morphology

Cultured cells were incubated at 37°C for 1 h with fresh serum-free culture medium in the absence or presence of MPP+ and/or ODN. At the end of the incubation period, culture media

were removed and cells were rinsed twice with culture medium. Cell morphology was assessed by observation of cultures under a Zeiss (Axiostar plus, Göttingen, Germany) inverted microscope equipped with a Canon Power Shot A640 photo camera.

**Statistical analysis**

Data are presented as the mean SEM from three independent experiments performed in quadruplicate or quintuplicate. Statistical analysis of the data was performed by using Student's t test and ANOVA, followed by Bonferroni's test.

## RESULTS

Effect of ODN on cultured granule cells survival

Cultured granule cells in K5 medium present a 2-fold increase in extracellular LDH activity when compared with control cultures. By contrast, CGCs survive when cultured in the presence of depolarizing concentrations of KCl (25 mM; K25). Treatment of GCs with 0.1nM ODN for 72 h significantly prevents the K5-induced cell death (Fig. 1A).

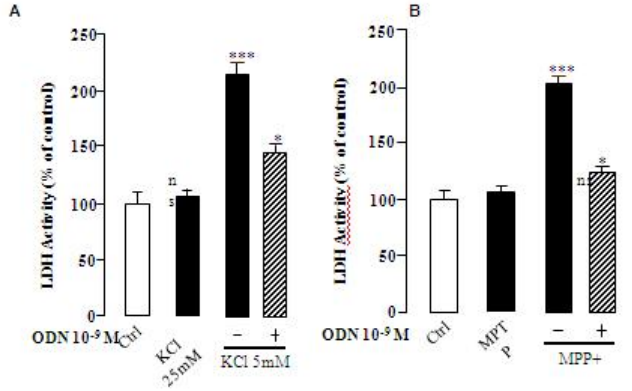



**Figure 1:** Effect of ODN on K5 medium- and MPP+ induced release of lactate dehydrogenase from cultured granule neurons. Cells were incubated for 72h in the absence or presence of medium or KCl solution (5mM and 25mM) or MPTP or MPP+ without or with 1 nM ODN. Each value is the mean (± SEM) calculated from at least 10 different wells from 3 independent cultures. ANOVA followed by the Bonferroni's test: *P< 0.05; ***P < 0.001; ns, not statistically different from control.

Incubation of granule cells with 100 µM MPTP for 72 h did not induce any significant modification in LDH release. As previously reported (Gonzalez-Polo et al., 2003; Gonzalez-Polo et al., 2004) CGCs are sensitive to the toxic properties of MPP+. Treatment with MPP+ caused a time-dependent increase in cell death. Addition of ODN (0.1 nM) to the culture medium markedly attenuated the effect of MPP+ on LDH release, indicating that ODN increase the number of surviving (Fig. 1B).

**Effect of ODN on cultured granule cells morphology**

Examination of cultures by phase-contrast microscopy revealed that ODN-treated cells displayed a flat polygonal morphology similar to that of untreated-CGCs (Fig. 2A and 2D). The neurotoxic effect of MPP+ was associated with morphological modifications of granule cells that were suggestive of apoptotic cell death, such as cell shrinkage and nuclear condensation (Fig. 2B). Coincubation of granule cells with MPP+ and ODN (10-9 M) restored the typical shape of differentiated neurons with bipolar fusiform cell bodies and long neurites (Fig. 2C). Similarly, cultured CGCs in K5 medium induces the appearance of floating masses composed of dead cells associated with the presence of many empty beaches (Fig. 2E) and the addition of ODN totally prevented the morphological changes provoked by K5 medium (Fig. 2F).

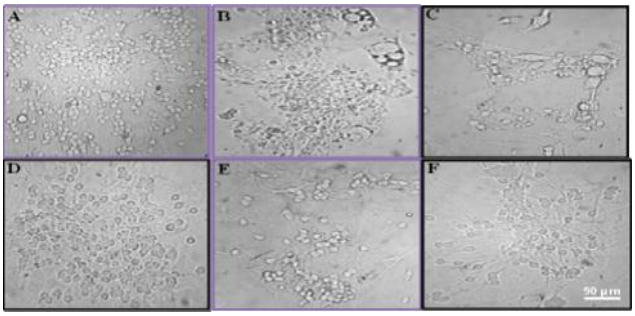



**Figure 2:** Phase-contrast images illustrating the effect of ODN on potassium deprivation- and MPP+- induced changes in morphology of cultured cerbellar granule cells. Cells were incubated in the absence (A) or presence 1 nM of ODN (D) or MPP+ without (B) or with ODN (E) or K5 without (C) or with ODN (F). Scale bar = 50 µm.

## DISCUSSION

It has been reported that the concentration of DBI in brain and in cerebrospinal fluid is increased in various pathological conditions including brain injury and neurodegenerative diseases (Ferrero et al., 1988; Ferrarese et al., 1990; Lacor et al., 1996), suggesting that DBI and its processing peptides may act as

neurotrophic factors regulating proliferation and/or survival of nerve cells. In the present study we demonstrate that ODN can protect cultured granule neurons from apoptosis induced by potassium deprivation or exposure to neurotoxin MPP+.In agreement with previous reports performed on granule cells from 8-day-old rat, we observed that CGCs undergo apoptosis when switched from a medium containing depolarizing levels of KCl (K25) to medium containing lower levels of KCl (K5) (D'Mello et al., 1993; Zhong et al., 2004). Coincubation of K5-treated granule cells with the neuropeptide ODN increase the number of surviving neurons, indicating that ODN can protect neurons against the deleterious effects of potassium deprivation. At present, it is poorly known which mechanisms are involved in the neuroprotective effect of ODN or K25. Several evidences have shown that calcium influx, through voltage operated L-type calcium channels, is necessary for the antiapoptotic effect of depolarizing agents (Balazs et al., 1988; Xifro et al., 2006). Since ODN induces activation of phospholipase C and calcium entry into astroglial cells (Leprince), these observations suggest that the effect of ODN on CGCs survival could be mediated by calcium influx into granule cells. We next determined the ability of ODN to counteract the neurotoxic effects of MPP+ on CGCs. MPP+,1-methyl-4-phenylpyridinium, is an ultimate toxic metabolite of a mitochondrial neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), that causes Parkinson-like symptoms in experimental animals and humans. As previously shown ((Marini et al., 1989; Nicotra and Parvez, 2002), we indicate that a 72-h exposure to 100 µM MPTP did not affect viability of CGCs when cultured in K25. The insensitivity of CGCs to MPTP may be ascribed to their lack of monoamine oxidase B, enzyme responsible for conversion of MPTP to its neurotoxic metabolite MPP+ (Nicotra and Parvez, 2002). Thus, treatment of CGCs with MPP+ for 72h in K25 medium provokes neuron cells death. The MPP+-induced neuronal death exhibited the characteristic features of apoptosis including cell shrinkage and nuclear condensation. Coincubation of MPP+-treated granule cells with the ODN increased the number of surviving neurons, indicating that ODN can protect neurons upon MPP+ injury.

In conclusion, the present study has demonstrated that ODN exerts a potent neuroprotective effect against potassium deprivation- and MPP+-induced cell death. These data suggest that ODN could have a therapeutic value for the treatment Parkinson disease and other age-related chronic neurological disorders.

## Acknowledgments

Y.H., H.K. and R.H. were recipients of fellowships from the University of Tunis El Manar and a France-Tunisia exchange program CMCU-Utique. This study was supported by the Research Unit 00-UR-08-01 and the CMCU-Utique program (to MA and MCT).

## REFERENCES

Aerden, L.A., Kessels, F.A., Rutten, B.P., Lodder, J., Steinbusch, H.W., 2004. Diazepam reduces brain lesion size in a photothrombotic model of focal ischemia in rats. Neurosci Lett. 367, 76-8.

Alho, H., Kolmer, M., Harjuntausta, T., Helen, P., 1995. Increased expression of diazepam binding inhibitor in human brain tumors. Cell Growth Differ. 6, 309–14.

Alvira, D., Yeste-Velasco, M., Folch, J., Verdaguer, E., Canudas, A.M., Pallas, M., Camins, A., 2007. Comparative analysis of the effects of resveratrol in two apoptotic models: inhibition of complex I and potassium deprivation in cerebellar neurons. Neuroscience. 147, 746-56.

Balazs, R., Gallo, V., Kingsbury, A., 1988. Effect of depolarization on the maturation of cerebellar granule cells in culture. Brain Res. 468, 269-76. Burgi, B., Lichtensteiger, W., Lauber, M.E., Schlumpf, M., 1999. Ontogeny of diazepam binding inhibitor/acyl-CoA binding protein mRNA and peripheral benzodiazepine receptor mRNA expression in the rat. J. Neuroendocrinol. 11, 85–100.

Chang, J.L., Tsai, H.J., 1996. Carp cDNA sequence encoding a putative diazepam-binding inhibitor/endozepine/acyl-CoA-binding protein. Biochim Biophys Acta. 1298, 9-11.

Compère, V., Li, S., Leprince, J., Tonon, M.C., Vaudry, H., Pelletier, G., 2004. In vivo action of a new octadecaneuropeptide (ODN) antagonist on gonadotropin-releasing hormone gene expression in the male rat brain. Neuroscience. 125, 411–5.

Compère, V., Ouellet, J., Luu-The, V., Dureuil, B., Tonon, M.C., Vaudry, H., Labrie, F., Pelletier, G.,

2006. Role of androgens and glucocorticoids in the regulation of diazepam-binding inhibitor mRNA levels in male mouse hypothalamus. Brain Res. 1119, 50–7.

Corbett, D., Larsen, J., Langdon, K.D., 2008. Diazepam delays the death of hippocampal CA1 neurons following global ischemia. Exp. Neurol. 214, 309–14.

D'Mello, S.R., Galli, C., Ciotti, T., Calissano, P., 1993. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci U S A. 90, 10989-93.

Eberhardt, O., Schulz, J.B., 2003. Apoptotic mechanisms and antiapoptotic therapy in the MPTP model of Parkinson's disease. Toxicol Lett. 139, 135-51.

Falluel-Morel, A., Aubert, N., Vaudry, D., Basille, M., Fontaine, M., Fournier, A., Vaudry, H., Gonzalez, B.J., 2004. Opposite regulation of the mitochondrial apoptotic pathway by C2-ceramide and PACAP through a MAP-kinase-dependent mechanism in cerebellar granule cells. J Neurochem. 91, 1231-43.

Ferrarese, C., Appollonio, I., Frigo, M., Meregalli, S., Piolti, R., Tamma, F., Frattola, L., 1990. Cerebrospinal fluid levels of diazepam-binding inhibitor in neurodegenerative disorders with dementia. Neurology. 40, 632–5.

Ferrero, P., Santi, M.R., Conti-Tronconi, B., Costa, E., Guidotti, A., 1986. Study of an octadecaneuropeptide derived from diazepam binding inhibitor (DBI): biological activity and presence in rat brain. Proc. Natl. Acad. Sci. U.S.A. 83, 827–31.

Ferrero, P., Benna, P., Costa, P., Tarenzi, L., Baggio, G., Bergamasco, B., Bergamini, L., 1988. Diazepam binding inhibitor-like immunoreactivity (DBI-LI) in human CSF. Correlations with neurological disorders. J Neurol Sci. 87, 327-49.

Gandolfo, P., Patte, C., Leprince, J., Thoumas, J.L., Vaudry, H., Tonon, M.C., 1997. The stimulatory effect of the octadecaneuropeptide (ODN) on cytosolic Ca2+ in rat astrocytes is not mediated through classical benzodiazepine receptors. Eur. J. Pharmacol. 322, 275–81.

Gibb, W.R., Costall, B., Domeney, A.M., Kelly, M.E., Naylor, R.J., 1988. The histological effects of intracerebral injection or infusion of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and MPP+ (1-methyl-4-phenylpyridinium) in rat and mouse. Brain Res. 461, 361-6.

Gonzalez-Polo, R.A., Mora, A., Clemente, N., Sabio, G., Centeno, F., Soler, G., Fuentes, J.M., 2001. Mechanisms of MPP(+) incorporation into cerebellar granule cells. Brain Res Bull. 56, 119-23.

Gonzalez-Polo, R.A., Soler, G., Alonso, J.C., Rodriguez-Martin, A., Fuentes, J.M., 2003. MPP(+) causes inhibition of cellular energy supply in cerebellar granule cells. Neurotoxicology. 24, 219-25.

Gonzalez-Polo, R.A., Soler, G., Fuentes, J.M., 2004. MPP+: mechanism for its toxicity in cerebellar granule cells. Mol Neurobiol. 30, 253-64.

Gonzalez, B., Leroux, P., Lamacz, M., Bodenant, C., Balazs, R., Vaudry, H., 1992. Somatostatin receptors are expressed by immature cerebellar granule cells: evidence for a direct inhibitory effect of somatostatin on neuroblast activity. Proc Natl Acad Sci U S A. 89, 9627-31.

Guidotti, A., Forchetti, C.M., Corda, M.G., Konkel, D., Bennett, C.D., Costa, E., 1983. Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Proc. Natl. Acad. Sci. USA. 80, 3531–5.

Lacor, P., Benavides, J., Ferzaz, B., 1996. Enhanced expression of the peripheral benzodiazepine receptor (PBR) and its endogenous ligand octadecaneuropeptide (ODN) in the regenerating adult rat sciatic nerve. Neurosci Lett. 220, 61-5.

Leprince, J., Oulyadi, H., Vaudry, D., Masmoudi, O., Gandolfo, P., Patte, C., Costentin, J., Fauchère, J.L., Davoust, D., Vaudry, H., Tonon, M.C., 2001. Synthesis, conformational analysis and biological activity of cyclic analogs of the octadecaneuropeptide ODN. Design of a potent endozepine antagonist. Eur. J. Biochem. 268, 6045–6057.

Lihrmann, I., Plaquevent, J.C., Tostivint, H., Raijmakers, R., Tonon, M.C., Conlon, J.M., Vaudry, H., 1994. Frog diazepam-binding inhibitor: peptide sequence, cDNA cloning, and expression in the brain. Proc Natl Acad Sci U S A. 91, 6899-903.

Marini, A.M., Schwartz, J.P., Kopin, I.J., 1989. The neurotoxicity of 1-methyl-4-phenylpyridinium in cultured cerebellar granule cells. J Neurosci. 9, 3665-72.

Marino, F., Cosentino, M., Fietta, A.M., Ferrari, M., Cattaneo, S., Frigo, G., Lecchini, S., Frigo, G.M., 2003. Interleukin-8 production induced by the endozepine triakontatetraneuropeptide in human neutrophils: role of calcium and pharmacological investigation of signal transduction pathways. Cell Signal. 15, 511–7.

Moore, D.J., West, A.B., Dawson, V.L., Dawson, T.M., 2005. Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci. 28, 57-87.

Nicotra, A., Parvez, S., 2002. Apoptotic molecules and MPTP-induced cell death. Neurotoxicol Teratol. 24, 599-605.

Schwartz-Bloom, R.D., Sah, R., 2001. gamma-Aminobutyric acid(A) neurotransmission and cerebral ischemia. J Neurochem. 77, 353-71.

Shang, T., Kotamraju, S., Kalivendi, S.V., Hillard, C.J., Kalyanaraman, B., 2004. 1-Methyl-4-phenylpyridinium-induced apoptosis in cerebellar granule neurons is mediated by transferrin receptor iron-dependent depletion of tetrahydrobiopterin and neuronal nitric-oxide synthase-derived superoxide. J Biol Chem. 279, 19099-112.

Takada, M., Campbell, K.J., Hattori, T., 1991. Regional localization of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) uptake: mismatch between its uptake and neurotoxic sites. Neurosci Lett. 133, 137-40.

Tonon, M.C., Leprince, J., Gandolfo, P., Compère, V., Pelletier, G., Malagon, M.M., Vaudry, H., 2006. Endozepines. In Kastin AJ, editor. Handbook of Biologically Active Peptides New York: Elsevier-813–819.

Veiga, S., Carrero, P., Pernia, O., Azcoitia, I., Garcia-Segura, L.M., 2007. Translocator protein 18 kDa is involved in the regulation of reactive gliosis. Glia. 55, 1426–36.

Webb, N.R., Rose, T.M., Malik, N., Marquardt, H., Shoyab, M., Todaro, G.J., Lee, D.C., 1987. Bovine and human cDNA sequences encoding a putative benzodiazepine receptor ligand. DNA. 6, 71-9.

Xifro, X., Falluel-Morel, A., Minano, A., Aubert, N., Fado, R., Malagelada, C., Vaudry, D., Vaudry, H., Gonzalez, B., Rodriguez-Alvarez, J., 2006. N-methyl-D-aspartate blocks activation of JNK and mitochondrial apoptotic pathway induced by potassium deprivation in cerebellar granule cells. J Biol Chem. 281, 6801-12.

Yalcin, A., Koulich, E., Mohamed, S., Liu, L., D'Mello, S.R., 2003. Apoptosis in cerebellar granule neurons is associated with reduced interaction between CREB-binding protein and NF-kappaB. J Neurochem. 84, 397-408.

Zhong, J., Deng, J., Huang, S., Yang, X., Lee, W.H., 2004. High K+ and IGF-1 protect cerebellar granule neurons via distinct signaling pathways. J Neurosci Res. 75, 794-806.

*Biologia Tunisie* ***Juillet 2012 ;*** *N°7; 119– 122*

**BIOLOGIA TUNISIE**

# ANTIOXIDANT ACTIVIY OF PULP AND PEEL POLYPHENOLIC EXTRACTS FROM TUNISIAN AZEROLE (*CRATAEGUS AZAROLUS*) FRUIT

**Manel BELKHIR[1-2*], Olfa Rebai[1-2], Mohamed AMRI [1] & Sami FATTOUCH [2]**

[1] Laboratoire de Neurophysiologie Fonctionnelle et Pathologies, Faculté des Sciences de Tunis, El Manar II, Tunis,; [2]Tunisia National Institute of Applied Sciences and Technology (INSAT), Zone Urbaine Nord, 1080, Tunis, Tunisia.*E-mail: manel.belkhir@yahoo.fr

***Keywords:*** Antioxidant; *Crataegus azarolus*; polyphenols; RP-HPLC.

**ABSRACT**

The species Crataegus azarolus, azerole (known as "Zaarour" in Tunisia) is considered as one of the oldest medicinal plants of the western word. The peel and pulp aqueous acetonic extracts prepared from Tunisian C. azarolus fruits were investigated. The contribution of the extracts to the free radical-scavenging activity was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) method in comparison to that of Trolox, a synthetic antioxidant. The stronger antioxidant properties corresponded to those obtained from peel material with a 70.66 % inhibitory effect on DPPH radicals which correspond to 1,607 mmol Trolox equivalent per gram of fresh weight. In addition, the polyphenolic compounds were identified and quantified using RP-HPLC-DAD. The total phenolic contents of the pulp and peel parts were found 118 ±8 and 380 ±15 mg/100g of fresh weight, respectively. Hyperosid (quercetin 3-O-rutinoside) and epicatechin were the most abundant compounds in the peel (72.01 %) and the pulp (68.41%), respectively.

**INTRODUCTION**

The genus Crataegus (Rosacae) was used by the Romans and is considered as one of the oldest medicinal plants of the western word (Bahorun et al., 2003). Azerole (Crataegus azarolus L.) tree is a deciduous tree growing up to 3–4 m high and cultivated for centuries in the Mediterranean area, particularly in North Tunisia. It is in flower in April and May. The plant can grow in light, medium and heavy soils. It requires moist or wet soil and can tolerate drought. Its fruits, up to 25 mm in diameter, are very variable in size and colour (Koyuncu et al., 2006). Azerole fruits, known also as hawthorn, are extensively consumed not only fresh and dried but also used to produce jam, marmalade and syrup (Koyuncu et al., 2006; Bignami et al., 2000; Ljubuncic et al., 2005). Fruits and flowers are also used for medicinal purposes (Svedstroma et al., 2006) including their sedative action (Vierling et al., 2008; Chevallier and Crouzet-Segarra 2004).
The aim of this study is to determine the total phenols contents and to evaluate the antioxidative activity of the aqueous acetonic extracts prepared from Tunisian C. azarolus fruits.

**MATERIAL AND METHODS:**

***2.1. Chemicals.*** 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8 tetramethyl chroman-2 carboxylic acid (Trolox) and Folin–Ciocalteu reagent were purchased from Sigma (France). Methanol and Formic acid were from Fluka (France). All reagents and solvents were of analytical grade.

***2.2. Sample preparation.*** Fruit samples (*Crataegus azarolus L*) were harvested at maturity from the North of Tunisia and frozen at -20 °C until used.

***2.3. Extraction procedure.*** Each sample (1 g) was thoroughly mixed with 7 mL of cold acetone/water (3:1 v/v). The mixture was sonicated, filtered and evaporated to final 3 mL of volume (aqueous phase). Following a centrifugation at 10000 rpm for 15 min at room temperature, the supernatant was collected and conserved at -20°C until analysis or used directly for the next experimental steps.

***2.4. Total phenolics.*** The total phenolic content of the extracts was measured by the colorimetric oxidation/reduction reaction using the Folin–Ciocalteu reagent according to Singleton *et al.* (1999). To 100 µL of diluted extract, 400 µL of Folin–Ciocalteu reagent (10%) was added. After

incubation for 10 min, 500 µL of Na2CO3 (75 g/L) were added and the reaction was incubated during 60 min. For the control sample, 100 µL of distilled water (solvent) was used. The absorbance was measured at 725 nm. Catechol (Cat) was employed as a calibration standard and results were expressed as Catechol equivalents (mg Cat eq/100 g of fw).

***2.5. Antioxidant activity.*** The hydrogen atoms or electron-donation ability of the corresponding extracts was measured from the bleaching of a purple-coloured methanol solution of DPPH. This spectrophotometric assay

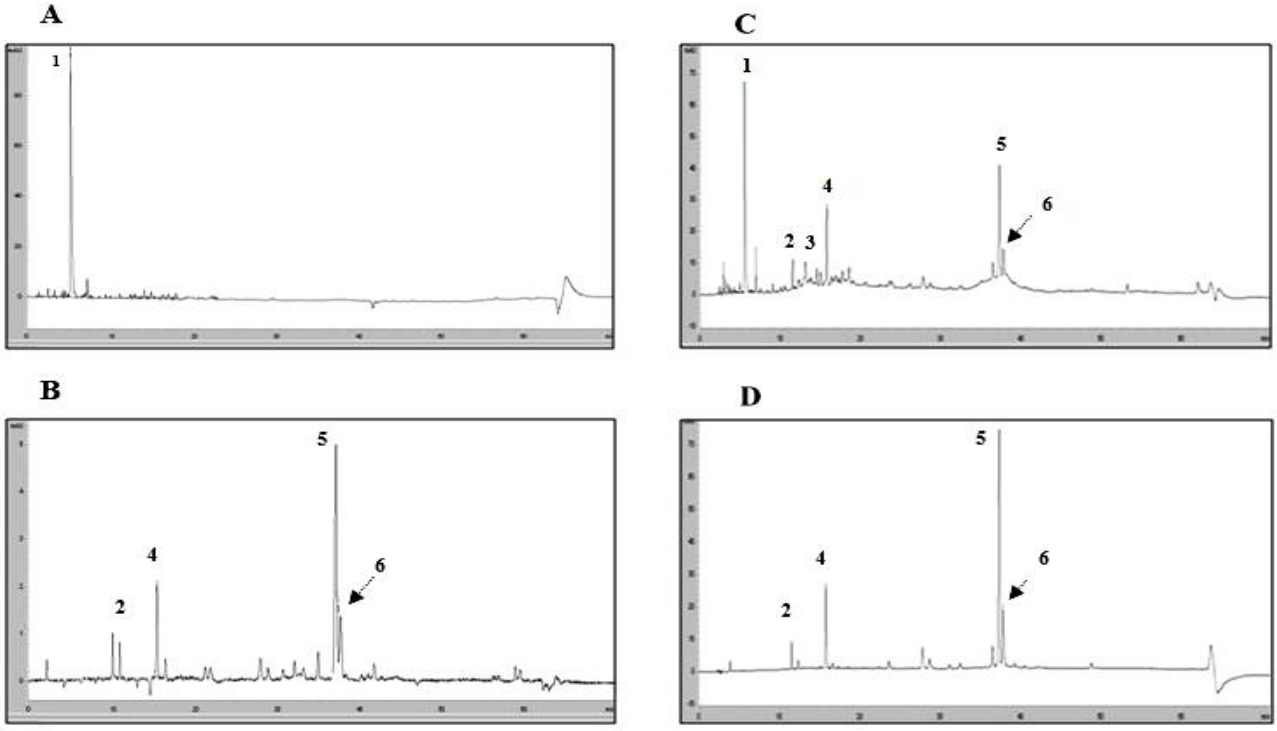



**Figure 1**: RP-HPLC Chromatographic profiles of pulp (A, B) and peel (C, D) aqueous-acetone extracts with detection at 280 nm (A, C) and 350 nm (B, D). The peaks correspond to: 1, (-) Epiatechin ; 2, Neochlorogenic acid (3-*O*-caffeoylquinic acid); 3, Cryptochlorogenic acid (4-*O*-caffeoylquinic acid); 4, Chlorogenic acid (5-*O*-caffeoylquinic acid); 5, Hyperosid (quercetin-3-*O*-galactoside); 6, Isoquercetin (quercetin-3-*O*-glucoside).

uses the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH•) radical as previously reported (Fattouch *et al.*, 2008; Tuba *et al.*, 2008). An aliquot (25 µL) of sample solution was added to 1 mL of 40 µM DPPH in methanol. The mixture was shaken vigorously and left for 60 min at room temperature in the dark. The absorbance was determined against a blank at 517 nm. Controls or blanks were prepared without the sample solution. The antiradical activity was expressed as the inhibition percentage and was calculated using the following formula:

*% Inhibition = (1 - sample OD/control OD) ×100*

The Trolox equivalent antioxidant capacity (TEAC) values were calculated from the equation determined from linear regression after plotting known solutions of Trolox with different concentrations (0.02-0.8 mM). Results are expressed as mmol Trolox equivalents per gram of fresh weight (Bahorun *et al.*, 2003; Fattouch *et al.*, 2007).

***2.6. RP-HPLC analysis.*** Phenolic compounds were separated using a reversed-phase column Discovery, RP C18 (Supelco, 250 × 4.6 mm; 5µm, particle size). Components were detected at 280 and 350 nm with a U.V. VIS detector (Beckman, Fullerton, CA, USA). Total phenolic content of the azerole extract, expressed in mg $mL^{-1}$, was estimated from HPLC analysis using the calibration curves of authentic standards. The HPLC separation was performed using the gradient elution described by Fattouch *et al.*, (2007). The mobile phase of the optimized chromatographic method consisted of solvent A (water -formic acid) and solvent B (methanol). The elution profile was: 0 min 95% A and 5% B, reaching 75% A at 10 min, 65% A at 30min, 55% A at 35 min, 55% A at 40 min, 50% A at 45 min, 45% A at 50 min, 30% A at 53 min, 25% A at 56 min, and 20% A at 60 min, followed by a post-time isocratic plateau for 10 min at 95% A before the next injection. The flow rate was 1 mL/min, and the injection volume

was 20 µL. The identification of each compound was based on a combination of retention time and spectral matching.

***2.7. Statistical Analysis.*** All tests and analyses were run in triplicate and averaged. Quantitative presented data are means ± standard deviations (SD).

## 3. RESULT

***3.1. Identification and quantification of phenolic compounds.*** Using the Folin-Ciocalteu method, the highest phenolic content was found in the peel extracts (380±15 mg Cat eq/100g fw), whereas in the pulp extracts (118±8 mg Cat eq/100g fw). The phenolic compounds of the peel and the pulp extracts were then fractionated and analyzed by reversed phase HPLC, and the corresponding chromatograms obtained at 280 and 350 nm are presented in Figure1.

The repeatability of the method was high, with respect to the retention times and peak areas. Compounds were identified by comparison of the retention times and UV/vis spectra with the available standards corresponding peaks (Table 1). Six obtained peaks matched with the standard compounds used in this work, namely, peaks 1-6 attributed to (-)Epicatechin, Neochlorogenic acid (3-*O*-caffeoylquinic acid), Cryptochlorogenic acid (4-*O*-caffeoylquinic acid), Chlorogenic acid (5-*O*-caffeoylquinic acid), Hyperoside (quercetin-3-*O*-galactoside); Isoquer-cetin (quercetine-3-*O*-glucosid) respectively.

The obtained results demonstrated that the differences in peel and pulp extracts phenolic composition were significantly more quantitative than qualitative.

***3.2. Antioxidant Activity.*** The antioxidant activity of the azerole peel and pulp phenolic extracts against DPPH radical was shown in Figure 2. Percent DPPH scavenging activities of the extracts were found to be dose-dependent. We compared the observed antioxidant activities with those of the Trolox, a synthetic antioxidant, and found that cuticle and pulp phenolic extracts exhibited 0.507±0.023 and 0.25± 0.013 mM TEAC respectively.

## 4. DISCUSSION

The aim of the present study was to study the aqueous-acetonic peel and pulp extracts prepared from the azerole fruit and to find out the relationship between their antioxidant potential and polyphenolic content as well as phenolic profiles.

Using the RP-HPLC-DAD and authentic standards, total polyphenols quantification was determined as the sum of individual identified compounds. According to Table 1, we estimated the polyphenol contents to 142.45 and 26.32 mg/100g of azerole peel and pulp fresh weight, respectively. The peel extract has about five time higher amount of phenolics than that of the pulp and present the major polyphenols. Whereas for the pulp, (-) epicatechin

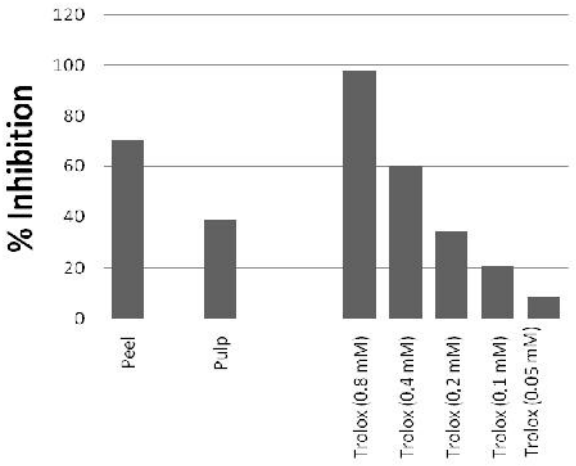



**Figure 2**: radical scavenging activity (%) of Trolox and acetone extracts.

was the predominant polyphenol with 68.41% of the total phenolic compounds. These results are in agreement with the studies achieved by Zhong *et al.* (2006) who showed that epicatechin and the hyperoside are among the major polyphenolics compounds present in the fruit of the hawthorn.

In this work, we assumed that major polyphenols in the Tunisian azerole fruit are hydroxycinnamic acids, principally chlorogenic acid, and flavonoids especially hyperoside and isoquercetin. These results are proven by the works of Svedstroma *et al.* (2006), Chang *et al.* (2001) and Zhong *et al.* (2006).

This fruit should have a very big interest in the medical domain because of the predominance of the hydroxycinnamic acids and flavonoids that can be used in the treatment of several cardiovascular illnesses, including heart weakness, hypertension and arteriosclerosis (Chang *et al.*, 2002; Ljubuncic *et al.*, 2005).

The DPPH scavenging activity of the azerole peel and pulp extracts was found proportional to the polyphenols concentrations**,** suggesting that these compounds are the major contributors to the extracts observed antioxidant potentials.

**Table1:** HPLC-RP analysis of main phenolics in Tunisian azerole pulp and peel extracts

| Peak Number | Rt[a] (min) | HPLC-DAD | Identity | Concentration (mg/100g fw)[b] | |
|---|---|---|---|---|---|
| | | | | Peel | Pulp |
| 1 | 5.68 | 279 | (-)- Epicatechin | 12.24 | 18.00 |
| 2 | 11.44 | 325 | Neochlorogenique acid (3-*O*-caffeoylquinic | 0.09 | 0.01 |
| 3 | 13.07 | 324 | Cryptochlorogenic acid (4-*O*-caffeoylquinic | 0.08 | ND |
| 4 | 15.70 | 326 | Chlorogenic acid (5-*O*-caffeoylquinic acid) | 0.24 | 0.11 |
| 5 | 37.26 | 355 | Hyperoside (quercetin-3-*O*-galactoside) | 102.60 | 6.84 |
| 6 | 37.78 | 354 | Isoquercitrin (quercetin-3-*O*-glucoside) | 27.20 | 1.36 |
| Total | | | | 142.45 | 26.32 |

[a] RP-HPLC retention time. [b] On the basis of fresh weight. ND : not detected.

The phenolic extracts of the Tunisian azerole were shown as strong antioxidants with 1.607±0.054 and 0.885 ± 0.062 mmol Trolox equivalent /g fw of peel and pulp, respectively, what confirms a protective role against oxidative stress (Manach *et al.*, 2004).
The present work could exalt the positive effect that azerole fruit may have on human health. Here, we demonstrate that prepared extracts from Tunisian azerole peel showed higher bioactive compounds content than pulp extracts. Thus, we must underline the importance of using whole fruit to the benefit of its total bioactive compounds. The extracts evaluated here present the advantage of being simply prepared from naturally occurring material, which could be marketed as nutraceuticals, and might be industrially exploited.

## References


Bahorun, T., Aumjaud, E., Ramphul, H., Rycha, M., Luximon-Ramma, A., Trotin, F. and Aruoma, O.I. (2003). Phenolic constituents and antioxidant capacities of *Crataegus Monogyna* (Hawthorn) callus extracts. *Nahrung*, 47: 191-198.

Bignami, C., Poalocci, A., Scossa, A. and Bertazza, G. (2000). Preliminary evaluation of nutritional and medicinal components of *Crataegus azarolus* fruits. *Journal of Food Enginnering*, 5: 241-247.

Chang, Q., Min, Z., Zhong Z., Moses C., Walter K. and Ho. K. 2001. High-performance liquid chromatographic method for simultaneous determination of hawthorn active components in rat plasma. *Journal of Chromatography B*, 760: 227-235.

Chang, Q., Zuo, Z., Harrison, F. and Chow, M.S.S. (2002). Hawthorn. *Journal of Clinical Pharmacology*, 42: 605-612.

Chevallier, L. and Crouzet-Segarra, C. (2004). Médicaments à base des plantes. Masson (Ed). Paris, France. p354.

Fattouch. S., Caboni, P., Coroneo V., Tuberoso, I.G.C., Angioni, A., Dessi, S., Marzouki, N. and Cabras, P. (2007). Antimicrobial Activity of Tunisian Quince (*Cydonia oblonga* Miller) Pulp and Peel Polyphenolic Extracts. *Journal of Agricultural and Food Chemistry*, 55: 963-969.

Fattouch, S., Sadok, S., Raboudi-Fattouch, F. and Ben Slama, M.(2008). Damage inhibition during refrigerated storage of mackerel (*Scomber scombrus*) fillets by a presoaking in quince (*Cydonia oblonga*) polyphenolic extract. *International Journal of Food Science and Technology*, 43: 2056-2064.

Koyuncu, T., Pinar, Y. and Lule, F., (2006). Convective drying characteristics of azarole red (*Crataegus monogyna* Jacq.) and yellow (*Crataegus aronia* Bosc.) Fruits. *Journal of Food Engineering*, 78: 1471-1475.

Ljubuncic, P., Portnaya, I. Cogan, U., Azaizeh, H. and Bomzon, A., (2005). Antioxidant activity of *Crataegus aronia* aqueous extract used in traditional Arab medicine in Israel. *Journal of Ethnopharmacology*, 101: 153-161.
Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L. (2004). Polyphenols: food sources and bioavailability, *American Journal of Clinical Nutrition*, 79: 727-747.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods* in *Enzymology*, 299: 152-178.

Svedstroma, U.,Vuorela, H., Kostiainen, R., Laakso, I. and Hiltunen, R. (2006). Fractionation of polyphenols in hawthorn into polymeric procyanidins, phenolic acids and flavonoids prior to high-performance liquid chromatographic analysis. *Journal of Chromatography A*, 11: 103-111.

Tuba, A.K. and Ilhami, G. (2008). Antioxydant and radical scavenging properties of curcumin. *Chemical Biological Interactions*, 174: 27-37.

Vierling, W., Brand, N., Gaedcke, F., Sensch, K.H., Schneider, E. and Scholz, M. (2003). Investigation of the pharmaceutical and pharmacological equivalence of different Hawthorn extracts. *Phytomedicine*, 10: 8-16.

Zhong, Z., Li, Z., Limin, Z., Qi, C. and Moses, C. (2006). Intestinal absorption of hawthorn flavonoids – *in vitro*, *in situ* and *in vivo* correlations. *Life Sciences*, 79: 2455-2462.

# Instructions aux auteurs

Biologia Tunisie est une revue tunisienne éditée par l'ATSB : Association Tunisienne des Sciences Biologiques. Elle publie des articles de recherche, des articles de synthèse et des communications en Anglais et en Français.

## SOUMISSION DU MANUSCRIT

Les manuscrits soumis pour publication et toute la correspondance concernant la rédaction doivent être envoyés au secrétariat de rédaction. Le manuscrit est adressé [(1)] sur disquette ou par e-mail et en quatre exemplaires (Trois originaux et une photocopie anonymes). Il doit être accompagné d'une lettre comportant le prénom, le nom, l'adresse, le numéro de téléphone, le numéro de télécopieur et l'adresse électronique de l'auteur avec lequel la rédaction communiquera. Dans sa lettre, l'auteur doit préciser que le texte n'est pas soumis simultanément à une autre revue scientifique en vue de publication.

Un formulaire de transfert de droits sera joint à l'accusé de réception de l'article soumis. Il sera dûment complété par l'auteur principal et cosigné par tous les auteurs de l'article, puis retourné à la rédaction dans les plus brefs délais.

Dès l'acceptation définitive, l'auteur est prié d'envoyer la version finale de son manuscrit, accompagnée du fichier correspondant sur disquette PC ou Macintosh.

Les documents ne seront pas restitués aux auteurs.

## PRÉSENTATION DU MANUSCRIT

Le manuscrit doit être dactylographié en double interligne, avec une marge de 4 cm à gauche, au recto seul de feuilles de format A4 (210 x 297 mm). Un feuillet A4 comprend environ 250 mots ou 1 500 signes. Le texte comporte, dans l'ordre: la page de titre ; les résumés français et anglais avec le titre de l'article en anglais, les mots clés français et anglais en ordre alphabétique ; le texte proprement dit ; les références bibliographiques ; les tableaux avec titre et légendes ; les figures ; les légendes des figures.

Pour l'envoi du manuscrit sur disquette, quelques règles doivent être respectées:

- étiqueter la disquette en indiquant le nom du fichier, le système d'exploitation (Windows 98, par exemple) et le logiciel utilisé (Word 97, par exemple)

- la disquette ne doit contenir que le(s) fichier(s) de l'article retenu pour publication, et tout le texte (sans les images) doit être inclus dans un seul fichier ;

- pour prévenir les risques d'incompatibilité entre logiciels, veiller à joindre sur la disquette un second fichier de l'article enregistré dans le format "RTF'

- lors de la saisie, ne jamais utiliser les fonctions avancées du logiciel telles que les insertions automatiques de caractères, de champs, de notes de bas de page ou d'images ;

- les figures fournies sur disquette, accompagnées impérativement dune copie papier de bonne qualité, doivent être enregistrées sur des fichiers séparés, dans le format "JPG" ou "TIF".

### Page de titre

La page de titre comporte les mentions suivantes :

- le titre, concis, qui indique de manière précise le sujet du travail et ne comporte pas d'abréviations ; la rédaction se réserve le droit de le modifier dans le sens de la concision ;

- le prénom usuel et le nom du ou des auteur(s) en lettres minuscules avec une majuscule à l'initiale ; quand les auteurs appartiennent à des services différents, leurs noms sont suivis de chiffres en exposant qui renvoient aux services ou adresses correspondants ;

- les intitulés et adresses complètes des services, laboratoires ou organismes d'origine des auteurs ; préciser les numéros de téléphone et de télécopie, ainsi que l'adresse électronique de l'auteur auquel doivent être envoyés la correspondance concernant l'article, le bon à tirer et la commande de tirés à part.

---

[(1)] A Monsieur M. Amri (FST) ou M.N. Marzouki (INSAT)

- le cas échéant, la date et le lieu de la réunion où le travail a été présenté. La mention de l'origine des sources de financement ou du contrat de recherche, les remerciements pour l'aide technique à la réalisation et à la dactylographie du travail ;

- le titre courant (40 signes au maximum, lettres et espaces compris), destiné à figurer en haut de chaque page de l'article imprimé.

Résumés et mots clés

Chaque article comporte un résumé en français et en anglais (summary), sans abréviation ni référence, de 250 mots au maximum pour les articles originaux, avec au plus cinq mots clés également en français et en anglais. Le summary doit être accompagné du titre de l'article en anglais.

Pour les articles originaux, il est demandé que les résumés soient structurés de la façon suivante : objectifs (objective) ; patients ou matériel et méthodes (patients or materials and methods) ; résultats (results) ; conclusions (conclusions).

Les mots clés, sans reprendre les termes du titre de l'article, doivent être pertinents ou descripteurs, et seront revus par la rédaction pour permettre l'indexation de l'article et être conformes à la liste du Medical Subject Headings de l'Index Medicus, renouvelée chaque année.

Texte

Rédaction. Le texte est rédigé dans un style clair, concis et précis. Il doit être compréhensible pour un lecteur qui n'appartient pas à la spécialité. L'emploi de la première personne n'est pas d'usage dans un document scientifique. Les retours à la ligne trop fréquents sont à éviter. En cas de mention de noms d'auteurs dans le texte, s'ils ne sont que deux, les deux sont cités ; s'ils sont plus de deux, le nom du premier auteur est suivi de la mention" et al."

Les titres et intertitres de même valeur sont signalés de façon identique. Les locutions et abréviations latines usuelles en français s'écrivent en caractères romains, et non en italique (et al., a priori, in vitro...).

Abréviations. Seules les abréviations courantes sont acceptées, en nombre réduit. Le terme en entier doit précéder l'abréviation, entre parenthèses, lors de sa première apparition dans le texte et dans le résumé, sauf s'il s'agit d'une unité de mesure internationale. L'emploi des abréviations est proscrit dans le titre et, si possible, évité dans les résumés.

Chiffres, unités, symboles et noms commerciaux. Les symboles et unités scientifiques doivent être cohérents et conformes aux normes internationales. Les médicaments sont identifiés par leur nom générique (dénomination commune internationale), sans majuscule à l'initiale.

Références bibliographiques

Les références doivent être présentées par ordre alphabétique et par ordre chronologique pour un même auteur. Si l'auteur principal présente deux et plus de références par année, indiquer par a, b, c l'année de parution.

Exemples :

Pinck, L. 1990[a]. ; Pinck, L. and Ritzenthaler, C. 1990[b]

Les références bibliographiques doivent être indiquées comme suit :

Nom, Prénom en abréviation. (Année). Titre complet de la référence. Nom du journal. Volume (souligné) : pages.

Exemples :

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34 : 475-483.

Exemple : pour les articles publiés dans les livres, préciser en plus le nom de la maison d'édition

Smith, E. L.; Austen, B. M.; Blumenthal, K. M. and Nyce, J. F. (1975). Glutamate deshydrogenases. In: Boyer PD (ed.) The enzymes. 11 :293-367. Academic Press, New York.

Toutes les références citées dans le texte figurent dans la liste des références et vice versa.

Les articles acceptés mais non encore publiés peuvent être référencés en indiquant la revue, l'année et le volume avec la mention (à paraître) ou (in press).

*Thèse*
Ben Salah A. Contribution à l'étude des syndromes d'obstruction de la région ...[thèse]. Tunis ; 1975.

Tableaux

Chaque tableau est dactylographié en double interligne sur une page ; il comporte, en haut, son numéro d'appel (en chiffres romains) et son titre et, en bas, les notes explicatives éventuelles (abréviations, sigles). Les unités sont données en tête de ligne ou de colonne. Les tableaux ne font pas double emploi avec les figures.

Figures

Les auteurs doivent impérativement fournir une sortie papier d'excellente qualité des figures, même si elles sont aussi transmises sur fichier informatique.

Les figures sont numérotées en chiffres arabes, le haut de la figure étant indiqué par une flèche. Les radiographies sont présentées sous forme de photographies bien contrastées.

Les légendes des figures sont regroupées sur une feuille à part. Une figure et sa légende forment un tout qui doit être compris sans recours au texte.

Il n'y a pas de frais de publication pour les textes et les figures en noir et blanc, seule la reproduction de figures en couleur est à la charge des auteurs.

En cas de reproduction partielle ou totale d'un document déjà publié, l'auteur doit impérativement fournir l'autorisation écrite de l'éditeur et des auteurs du document.

ANALYSE DES MANUSCRITS

Tout manuscrit proposé est soumis anonymement à l'avis de lecteurs dont l'approbation, après modifications éventuelles, est nécessaire pour sa publication.

CORRECTION D'ÉPREUVES, TIRÉS À PART ET DROITS DE REPRODUCTION

L'auteur principal recevra avec les épreuves de son article des tirés à part, les 15 premiers étant fournis gracieusement. Les modifications portant sur le fond ne sont pas acceptées sur les épreuves d'imprimerie. Les corrections se limiteront à la typographie. Les auteurs feront le nécessaire pour retourner à l'éditeur les épreuves revêtues de la mention "bon à tirer", dans les 48 heures suivant leur réception et ce à toute période de l'année. En cas de retard, l'éditeur se réserve le droit de procéder à l'impression sans le bon à tirer de l'auteur.

Dès parution, les demandes de reproduction devront être adressées au rédacteur de la revue BIOLOGIA TUNISIE.