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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Bacterial species identification getting easier

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The traditional methods of bacterial identification are based on observation of either the morphology of single cells or colony characteristics. However, the adoption of newer and automated methods offers advantage in terms of rapid and reliable identification of bacterial species. The review provides a comprehensive appreciation of new and improved technologies such fatty acid profiling, sequence analysis of the 16S rRNA gene, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), metabolic finger profiling using BIOLOG, ribotyping, together with the computational tools employed for querying the databases that are associated with these identification tools and high-throughput genomic sequencing in bacterial identification. It is evident that with the increase in the adoption of new technologies bacterial identification is becoming easier.

Key words: Bacteria, Biolog, computational tools, fatty acids, Gram staining, identification, metagenomics, morphology, MALDI-TOF MS, RiboPrinter, 16S rRNA gene.

INTRODUCTION

Bacteria are primarily grouped according to their morphological characteristics (shape, presence or absence of flagella, and arrangement of flagella), substrate utilisation and Gram staining. Another important trait is their pattern of growth on solid media as different species can produce very diverse colony structures (Christopher and Bruno, 2003). The traditional methods that employ observation of either the morphology of single cells or colony characteristics remain reliable parameters for bacterial species identification. However, these traditional techniques have some disadvantages. Firstly, they are time-consuming and laborious. Secondly, variability of culture due to different environmental conditions may lead to ambiguous results. Thirdly, a pure culture is required to undertake identification, making the identification of fastidious and unculturable bacteria difficult and sometimes impossible. To evade these problems, newer and automated methods which rapidly and reliably identify bacteria have been adopted by many laboratories worldwide. At least one of these methods, namely analysis of the 16S rRNA gene, does not require a pure culture. Combining these automated systems with the traditional methods provides workers with a higher level of confidence for bacterial identification. This review serves as a comprehensive appreciation of these new technologies. The methods we discuss are fatty acid profiling, sequence analysis of the 16S rRNA gene, protein profiling using matrix-assisted laser desorption/-ionization time-of-flight (MALDI-TOF), metabolic finger profiling using BIOLOG, and ribotyping, together with the computational tools employed for querying the databases that are associated with these iden-tification methods. We further discuss the role of high-throughput genomic sequencing in bacterial identification. Unfortunately, labo-

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Abbreviations: MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; **TFA**, trifluoroacetic acid; **SEM**, scanning electron microscopy; ISH, *in situ* hybridization; **GC,** gas chromatography; **CFA,** cellular fatty acid; **FAME,** fatty acid methyl ester analysis; **PCR,** polymerase chain reaction; **RFLPs,** restriction fragment length polymorphisms; **HTS,** high-throughput sequencing.

ratories in poor countries cannot afford some of these new systems. With increased access to these technologies, workers in many laboratories will find the identification of bacterial species easier.

THE MORPHOLOGICAL IDENTIFICATION OF BACTERIA

As it has always been the desire of humankind to understand the environment, the classification and identification of organisms has always been among the priorities of the early scientists. Unlike zoologists and botanists who have a plethora of morphological traits with which to identify animals and plants, the morphological characters for identifying bacteria are few and limiting. This not only provided a challenge, but also an opportunity for creativity. Gram staining was a result of the creative insight of Hans Christian Joachim Gram (1850-1938) to classify bacteria based on the structural properties of their cell walls. It was based on Gram staining that bacteria could be differentially classified as either Gram positive or Gram negative, a convenient identification and classification tool that remains useful today. Although there are few morphological traits, and little variation in those traits, identification based on morphology still has significant taxonomic value. When identifying bacteria, much attention is paid to how they grow on the media in order to identify their cultural characteristics, since different species can produce very different colonies (Christopher and Bruno, 2003). Each colony has characteristics that may be unique to it and this may be useful in the preliminary identification of a bacterial species. Colonies with a markedly different appearance can be assumed to be either a mixed culture or a result of the influence of the environment on a bacterial culture which normally produces known colony characteristics or a newly discovered species.

The features of the colonies on solid agar media include their shape (circular, irregular or rhizoid), size (the diameter of the colony: small, medium, large), elevation (the side view of a colony: elevated, convex, concave, umbonate/umbilicate), surface (how the surface of the colony appears: smooth, wavy, rough, granular, papillate or glistening), margin/border (the edge of a colony: entire, undulate, crenated, fimbriate or curled), colour (pigmentation: yellow, green among others), structure/-opacity (opaque, translucent or transparent), degree of growth (scanty, moderate or profuse) and nature (discrete or confluent, filiform, spreading or rhizoid). Cell shape has also been used in the description and classification of bacterial species (Cabeen and Jacobs-Wagner, 2005). The most common shapes of bacteria are cocci (round in shape), bacilli (rod-shaped) and spirilli (spiral-shaped) (Cambray, 2006).

Observations of bacterial morphologies are done by light microscopy, which is aided by the use of stains (Bergmans et al., 2005). Dutch microbiologist Antonie van Leeuwenhoek (1632-1723) was the first person to observe bacteria under a microscope. Without staining, bacteria are colourless, transparent and not clearly visible and the stain serves to distinguish cellular structure for a more detailed study. The Gram stain is a differential stain with which to categorise bacteria as either Gram positive or Gram negative. Observing bacterial morphologies and the Gram reaction usually constitutes the first stage of identification. Specialised staining for flagella reveals that bacteria either have or do not have flagella and the arrangement of the flagella differs between bacterial species. This serves as a good and reliable morphological feature for identifying and classifying bacterial species.

Light microscopy was traditionally used for identifying colonies of bacteria and morphologies of individual bacteria. The limitation of the light microscope was its often insufficient resolution to project bacterial images for clarity of identification. Scanning electron microscopy (SEM) coupled with high-resolution back-scattered electron imaging is one of the techniques used to detect and identify morphological features of bacteria (Davis and Brlansky, 1991). SEM has been widely used in identifying bacterial morphology by characterizing their surface structure and measure cell attachment and morphological changes (Kenzata and Tani, 2012). A combination of morphological identification with SEM and *in situ* hybridization (ISH) techniques (SEM-ISH) clarified the better understanding of the spatial distribution of target cells on various materials. This method has been developed in order to obtain the phylogenetic and morphological information about bacterial species to be identified using *in situ* hybridization with rRNA-targeted oligonucleotide probes (Kenzata and Tani, 2012).

These morphological identification techniques were improved in order to better identify poorly described, rarely isolated, or phenotypically irregular strains. An improved method was brought up for the bacterial cell characterization based on their different characteristics by segmenting digital bacterial cell images and extracting geometric shape features for cell morphology. The classification techniques, namely, 3σ and K-NN classifiers are used to identify the bacterial cells based on their morphological characteristics (Hiremath et al., 2013).

In addition to microscopy, several other tools for bacterial identification are useful to confirm identities based on morphology, thereby increasing the level of confidence of identity. Among these tools is the analysis of fatty acid profiles which will be discussed.

FATTY ACID ANALYSIS

Fatty acids are organic compounds commonly found in living organisms. They are abundant in the phospholipid

bilayer of bacterial membranes. Their diverse chemical and physical properties determine the variety of their biochemical functions. This diversity, which is found in unique combinations in various bacterial species, makes fatty acid profiling a useful identification tool.

The fatty acid profiles of bacteria have been used extensively for the identification of bacterial species (Purcaro et al., 2010). Fatty acid profiles are determined using gas chromatography (GC), which distinguishes bacteria based on their physical properties (Núñez-Cardona, 2012).

Reagents to cleave the fatty acids are required for saponification (45 g sodium hydroxide, 150 ml methanol and 150 ml distilled water), methylation (325 ml certified 6.0 N hydrochloric acid and 275 ml methyl alcohol), extraction (200 ml hexane and 200 ml methyl tert-butyl ether) and sample clean-up (10.8 g sodium hydroxide dissolved in 900 ml distilled water). Information on the fatty acid composition of purple and green photosynthetic sulphur bacteria includes fatty acid nomenclature, the distribution of fatty acids in prokaryotic cells, and published information on the fatty acids of photosynthetic purple and green sulphur bacteria (Núñez-Cardona, 2012). This information also describes a standardised gas chromatography technique for the fatty acid analysis of these photosynthetic bacteria using a known collection and wild strains.

The cellular fatty acid analysis for bacterial identification is based on the specific fatty acid composition of the cell wall. The fatty acids are extracted from cultured samples and are separated using gas chromatography. A computer generated, unique profile pattern of the extracted fatty acids is compared through pattern recognition programs, to the existing microbial databases. These databases include fatty acid profiles coupled with an assigned statistical probability values indicating the confidence level of the match. This has become very common in biotechnology.

The fatty acid analysis for bacterial identification using gas-chromatography became simpler with the available computer-controlled chromatography and data analysis (Welch, 1991). The fatty acid analysis method uses electronic signal from the gas chromatographic detector and pass it to the computer where the integration of peaks is performed (Sasser, 2011). The whole cellular fatty acid methyl esters content is a stable tool of bacterial profile in identification because the analysis is rapid, cheap, simple to perform and highly automated (Giacomini et at., 2000). In addition, bacterial identification can be done at or below the species level.

Adams et al. (2004) determined the composition of the cellular fatty acid (CFA) of *Bacillus thuringiensis* var. *kurstaki* using the MIDI Sherlock microbial identification system on a Hewlett-Packard 5890 gas chromatograph. This study revealed the capability to detect the strain variation in the bacterial species *B. thuringiensis* var. *kurstaki* and to clearly differentiate strain variants on the basis of qualitative and quantitative differences in hydrolysable whole CFA compositions in the preparations examined. Since this technology was used to resolve strain differences within a species, we can easily assume that the differentiation of species is done more accurately when fatty acid profiling is used. Kloepper et al. (1991) isolated and identified bacteria from the geocarposphere, rhizosphere, and root-free soil of field-grown peanut at three sample dates, using the analysis of fatty acid methyl-esters to determine if qualitative differences exist between the bacterial microflora of these zones. The dominant genera across all three samples were *Flavobacterium* for pods, *Pseudomonas* for roots, and *Bacillus* for root-free soil. Heyrman et al. (1999) isolated 428 bacterial strains, of which 385 were characterised by fatty acid methyl ester analysis (FAME). The majority (94%) of the isolates comprised Gram-positive bacteria and the main clusters were identified as *Bacillus* sp., *Paenibacillus* sp., *Micrococcus* sp., *Arthrobacter* sp. and *Staphylococcus* sp. Other clusters contained nocardioform actinomycetes and Gram-negative bacteria, respectively. A cluster of the latter contained extreme halotolerant bacteria isolated in Herberstein (Heyrman et al., 1999). At present, no bacterial identification method is guaranteed to provide absolute identity to all presently known bacterial species and therefore a number of methods are employed for a single identification procedure. Another method that is widely used for bacterial identification is sequence analysis of the 16S rRNA gene.

SEQUENCE ANALYSIS OF THE 16S rRNA GENE

Ribosomal RNA genes are a critical part of the protein synthesis machinery. They are omnipresent and therefore classification based on the analysis of ribosomal RNA genes does not leave out any of the known bacteria. For this reason, analysis of ribosomal RNA genes is a suitable tool for bacterial species identification and taxonomic categorisation. Moreover, ribosomal RNA genes are conserved but have sufficient variation to distinguish between taxa (Woese, 1987). In prokaryotes, ribosomal RNA genes occur in copies of three or four in a single genome (Fogel et al., 1999). The 16S rRNA gene has become a reliable tool for identifying and classifying bacteria. Over time, the 16S rRNA gene has shown functional consistency with a relatively good clocklike behaviour (Chanama, 1999) and its length of approximately 1,500 bp is sufficient for bioinformatic analysis (Janda and Abbott, 2007).

Analysis of the 16S rRNA gene requires that this gene be amplified by polymerase chain reaction (PCR) and the resultant PCR product sequenced. The gene sequence can then be matched with previously obtained sequences obtainable from various DNA databases. This method has been so widely adopted that DNA sequence database

databases are flooded with sequences of the 16S rRNA gene. Almost all new sequences deposited for query have matches and any 16S rRNA gene copy which does not match any known bacterial species is believed to be new (Chanama, 1999). In certain instances there is no requirement for pure colony amplification of the 16S rRNA gene, which makes this method suitable for studies of fastidious and unculturable bacteria and a good tool for the metagenomic analysis of environmental samples. Petrosino et al. (2009) defined metagenomics as "culture-independent studies of the collective set of genomes of mixed microbial communities, (which may) be applied to the exploration of all microbial genomes in consortia that reside in environmental niches, in plants or in animal hosts".

With the advent of metagenomic analyses of gross DNA samples, analysis of the 16S rRNA gene is proving its worth. In 16S rRNA-based metagenomics, gene sequencing has been widely used for probing the species structure of various bacteria in the environment (Shah et al., 2010). The 16S rRNA gene sequence is used to detect bacterial species in natural specimens and to establish phylogenetic relationships between them (Eren et al., 2011). This is made possible by the fact that all bacterial species contain the 16S rRNA gene, which has highly conserved regions on which to design universal primers, as well as hypervariable regions that are useful in distinguishing species.

The 16S rRNA gene has hypervariable regions which are an indication of divergence over evolutionary time. The 16S rRNA genes of bacteria possess nine hypervariable regions (V1 - V9) that display considerable sequence diversity in different species of bacteria (Chakravorty et al., 2007). These regions are flanked by conserved regions on which universal primers can be designed for their amplification. Based on the fact that the variation of the hypervariable regions is correlated with the identity of taxa, it is often of no use to analyse the whole 16S rRNA gene when identifying species. This adds to the convenience of using the 16S rRNA gene for identifying bacterial species. Since high-throughput sequencing platforms sequence short segments of DNA, analysis of only these hypervariable regions, which are a few hundred bases long, falls within the scale of massive parallel sequencing. This has accelerated the generation of 16S rRNA sequences and their entry into public databases.

It is easy for sequence analysis of the 16S rRNA gene to be adopted by many laboratories because it generally requires only PCR and sequencing, which are widely used techniques for many other applications. As a result, there are many studies that have employed sequence analysis of the 16S rRNA gene in taxonomic classify-cation. The computational tools have been employed to identify a wide range of bacteria through the sequence analysis of their 16S rRNA genes. Using this method, the 16S rRNA gene fragments are amplified using PCR method, and bacteria are identified based on 16S rRNA gene sequence similarity based method on the existing microbial databases.

According to Barghoutti (2011), when pure PCR products of the 16S gene are obtained, sequenced, and aligned against bacterial DNA data base, then the bacterium can be identified. For bacterial identification, the 16S rRNA gene is regarded as the most widely accepted gene (Song et al., 2003). Signature nucleotides of 16S rRNA genes allow classification and identification of bacterial species even if a particular sequence has no match in the database. The distinctive approach when identifying bacterial species using this method is to perform high-throughout sequencing of 16S rRNA genes, which are then taxonomically classified based on their similarity to known sequences in existing databases (Mizrahi-Man et al., 2013).

Kumrapich et al. (2011) examined the endophytic bacteria in the internal tissues of sugarcane leaves and stems using molecular methods. They used a nutrient agar medium to cultivate the endophytes, whereupon 107 isolates of bacteria in the internal tissues of sugarcane leaves and stems were selected for analysis and 23 species of bacteria were identified and divided into three groups, based on the 16S rRNA sequences and phylogenetic analysis. The taxa identified were *Sphingobacterium, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus megaterium, Bacillus pumilus, Bacillus subtilis*, *Agrobacterium larrymoorei*, *Burkholderia cepacia, Chromobacterium violaceum*, *Acinetobacter* (one strain), *Enterobacter* (three strains), *Klebsiella* (one strain), *Serratia* (one strain), *Pantoea* (three strains), and *Pseudomonas* (two strains).

Based on the amplified 16S rRNA gene sequencing, Bhore et al. (2010) identified bacterial isolates from the leaves of *Gaultheria procumbens* (eastern teaberry, checkerberry, boxberry, or American wintergreen) as *Pseudomonas resinovorans, Paenibacillus polymaxa*, and *Acinetobacter calcoaceticus*. Muzzamal et al. (2012) isolated and identified an array of 76 endophytic bacteria from the roots, stems, and fresh and wilted leaves of various plants in Pakistan. The morphological, biochemical and physiological characterisation and 16S rRNA gene sequence analysis of the selected endophytic isolates led to the identification of different bacterial species belon-ging to the genera *Bacillus, Pseudomonas, Serratia, Stenotrophomonas* and *Micromonospora*.

Although sequence analysis of the 16S rRNA gene has been by far the most common, reliable and convenient method of bacterial species identification, this technique has some shortfalls. Firstly, with this method it is not possible to differentiate between species that share the same sequence of this gene. Identification of bacterial species based on sequence analysis of the 16S rRNA gene relies on matching the obtained sequence with the existing sequence. Matching with a sequence that was incorrectly identified leads to incorrect identification.

Other problems associated with using the 16S rRNA gene are sequencing artefacts and problems with the purity of bacterial isolates which may lead to incorrect identification. Problems associated with sequence analysis of the 16S rRNA gene when identifying bacterial species argue for the use of alternative methods to confirm findings. Among these alternative methods is matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) which relies on exploiting differences in bacterial protein profiles.

MATRIX-ASSISTED LASER DESORPTION/ IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

A rapid, high-throughput identification method, MALDI-TOF MS, has been introduced in bacterial taxonomy. This system has brought reliability, simplicity and convenience. MALDI-TOF is the only polypeptide fingerprinting-based methods even to be used for bacterial identification. The first studies regarding the identification of bacteria by MALDI-TOF were conducted towards the end of the 1990s and technology was made available as a research tool. It was commercialised for use in private and public laboratories in 2008 and the delay was in commercialising MALDI-TOF was because of the lack of robust information tools and efficient databases. The MALDI-TOF MS technique offers easily determinable peptide/protein fingerprints for the identification of bacterial species. This technique has the ability to measure peptides and other compounds in the presence of salts and to analyse complex peptide mixtures, making it an ideal method for measuring non-purified extracts and intact bacterial cells.

Bacterial cultures to be queried are spotted on the MALDI-TOF plate which is placed in the time-of-flight (TOF) chamber. Each sample is spotted at least in duplicate, to verify reproducibility. A control specimen of known identity is included to ensure correct identity. The samples are allowed to air-dry at room temperature, inserted into the mass spectrometer and subjected to MALDI-TOF MS analysis. In addition to the cell-smear and cell-extract methods, additional sample preparation methods, as described previously (Smole et al., 2002), are used on a small number of strains. These include heat treatment (15 min at 95°C) of the cell extracts and cell smears, sonication (30 s, 0.3 MHz) of intact cells and the so-called sandwich method (Williams et al., 2003).

MALDI-TOF MS has been successfully applied to a number of taxa of *Listeria* species (Barbuddhe et al., 2008), *Campylobacter* spp. (Fagerquist et al., 2007; Grosse-Herrenthey et al., 2008), *Streptococcus pyogenes* (Moura et al., 2008), the *Burkholderia cepacia* complex (Vanlaere et al., 2006), *Arthrobacter* (Vargha et al., 2006), *Leuconostoc* spp., *Fructobacillus* spp., and *Lactococcus* spp. (De Bruyne et al., 2011). According to De Bruyne et al. (2011), different experimental factors, including sample preparation, the cell lysis method, matrix solutions and organic solvents may affect the quality and reproducibility of bacterial MALDI-TOF MS fingerprints and this warrants the use of alternative methods to guarantee correct identification. Computational tools for MALDI -TOF are used according to the tasks they perform: Firstly, pre-processing of spectra, then unsupervised data mining methods which can be used for preliminary data examination, then supervised classification applied for example, in biomarker discovery.

A MALDI-TOF dataset represents a set of mass spectra with two spatial coordinates *x* and *y* assigned to each spectrum. Unsupervised data mining, unsupervised methods are used for data mining, can be applied without any prior knowledge, and aim at revealing general data structure.

Supervised methods (mainly classification) require specifying at least two groups of spectra which need to be differentiated, for example, by finding *m/z*-values differentiating spectra of tumor regions from spectra of control regions (Alexandrov, 2012). For isolates requiring identification to the species level (*n* _986), correct species identifications is done by the Biotyperand Vitek MS systems and the Saramis database.

BIOLOG

Different methods have traditionally been used to identify bacteria based on biochemical activity. These methods include the oxidase test and the catalase test. The Biolog OmniLog Identification System [or simply “Biolog” (Biolog Inc, Hayward, California)], a system that utilises automated biochemical methodologies, as an instrument (Miller and Rhoden, 1991; Holmes et al., 1994; Morgan et al., 2009) that tests a microorganism's ability to utilise or oxidise a panel of 95 carbon sources.

Tetrazolium violet is incorporated in each of the substrates contained in a 96-well microtitre plate. Biolog's patented technology uses each microbe's ability to use particular carbon sources, and uses chemical sensitivity assays to produce a unique pattern or "phenotypic fingerprint" for each bacterial species tested. As a bacterium begins to use the carbon sources in certain wells of the microplate, it respires. With bacteria, this respiration process reduces a tetrazolium redox dye and those wells change colour to purple. The end result is a pattern of coloured wells on the microplate that is characteristic of that bacterial species.

A unique biochemical pattern or “fingerprint” is then produced when the results are surveyed. The fingerprint data are analysed, compared to a database, and identification is generated. The Biolog system was originally created for the identification of Gram-negative bacteria, but since the introduction of this system in 1989, the identification capability of the instrument has broadened to

include Gram-positive bacteria (Stager and Davis, 1992).

According to Morgan et al. (2009) isolates are prepared according to the manufacturer's instructions in the OmniLog ID System User Guide (Biolog, Hayward, CA). All isolates, except the *Bacillus* species, are cultured at 35°C on a Biolog Universal Growth (BUG) agar plate with 5% sheep blood. After an incubation period of 18 to 24 h, the bacterial growths are emulsified to a specified density in the inoculating fluid (0.40% sodium chloride, 0.03% Pluronic F-68, and 0.02% gellan gum). *Bacillus* species require a special “dry-tube method” preparation as described by the manufacturer. Colonies are picked with a sterile wooden Biolog Streakerz™ stick and rubbed around the walls of an empty, sterile, glass tube. Inoculating fluid (5 ml) is added to suspend the bacterial film. The suspension is subsequently used to inoculate culture wells of Gram-positive microplates (Biolog, Hayward, CA).

For all isolates, each well of the Gram-positive or Gram-negative microplate is inoculated with 150 µL of the bacterial suspension. Depending on the type of organism, the microplates are incubated at 30 or 35°C for 4 to 24 h. If bacterial identification has not occurred after 22 h, a reading of “no ID” is given. Each metabolic profile is compared with the appropriate GNor GPOmnilog Biolog database (Biolog, Hayward, CA), which contains biochemical fingerprints of hundreds of gram negative and gram positive species (Morgan et al., 2009). Biolog has been applied successfully to a number of taxa such as *Paenibacillus azotofixans* (Pires and Seldin, 1997), *Xanthomonas campestris* pv. *campestris* (Massomo et al., 2003) and *Glycine* spp. (Hung and Annapurna, 2004).

Computational tools such as standard multivariate analysis tools which include cluster analysis, principal component analysis and principal coordinate analysis are available for simple set summurization of numerical taxonomikc traits. Another tool is the co-inertia analysis which is a multivariate statistical method that perform a joint analysis on two data tables and assign egual consideration of both of them. These method is a two table ordination method that facilitate establishment of connections between tables with data domains that contain the same or even different numbers of variable method allow are to connect various standard single-table coordination methods such as principal component analysis and correspondence analysis.

Mantel test is a regression procedure in which variables themselves are either distance or dis-similarity matrices, summarising pair similarities among objects. Computations and graphic displays of Mantel test and the co-inertia analysis are obtained using ADE-4 package (Thiolouse et al., 1997). The documentation and downloading of this programme is available in the internet.

The Biolog method indicates potential, but not actual, catabolic activity of a community. Glimm et al. (1997) noticed that an assortment of substrates does not necessarily reflect substrates which are available to bacteria in in the soil environment, so one can suspect that some microbial species are incapable of growing on plates because of the lack of proper substrates.

According to Morgan et al. (2009) the Biolog system requires pure cultures and the subsequent growth of the bacteria - and pure culture and growth are frequently problematic when it comes to slow-growing, fastidious, unusual, nonviable, or non-culturable bacteria. The turn-around time required for identifying bacterial isolates can be several days to several weeks. The Biolog system is better at identifying fermentative organisms than nonfermenters. However, it should be noted that biochemically active nonfermenters do achieve high identification rates (88%) in the Biolog system, so a different product may be more suitable for inactive nonfermenters. Due to its disadvantages, other bacterial species identification procedures are required.

RIBOTYPING

The identification of bacterial species based on ribotyping exploits sequence differences in rRNA. DNA is extracted from a sample and is digested with restriction enzymes to generate a unique combination of discrete-sized fragments (ribotyping fingerprint) for a particular bacterial species. This pattern is queried in a database containing numerous patterns of different bacterial species. Before a ribotyping fingerprint database had been developed, rRNA fragments produced from restriction digestion would be probed with a known DNA probe for bacterial species identification.

A known ribotyping system, RiboPrinter®, is an automated system used for characterising bacterial samples and is a well regarded method of genotyping pure culture isolates which is often used in epidemiological studies. The basis of ribotyping is the use of rRNA as a probe to detect chromosomal restriction fragment length polymorphisms (RFLPs). The whole DNA of a pure culture is extracted and cleaved into various lengths of fragments using many endonucleases. The resultant fragments are separated by gel chromatography, then probed with labelled rRNA oligonucleotides.

Kivanç et al. (2011) used the RiboPrinter® to identify a total of 45 lactic acid bacteria from 10 different boza (a malt drink) samples in Turkey. In a study by Inglis et al. (2002) an automated ribotyping device was used to determine the ribotypes of a collection of *Burkholderia pseudomallei* isolates, and the comparison of automated ribotyping with DNA macrorestriction analysis showed that an *Eco*RI ribotyping protocol can be used to obtain discriminating molecular typing data on all isolates analysed. Optimal discrimination was obtained by analysing gel images of automated *Eco*RI ribotype patterns obtained with BioNumerics software in combination with the results of DNA macrorestriction analysis.

HIGH-THROUGHPUT SEQUENCING TECHNIQUES

There are four sequencing technologies available (capillary sequencing, pyrosequencing, reversible terminator chemistry, sequence-by litigation). The Sanger capillary sequencing is still based on the same general scheme applied in 1977 for the φX174 genome. Roche/454 GS FLX Titanium sequencer was the first of the new high-throughput sequencing platforms on the market and it was released in 2005. It is based on the pyrosequencing approach. Compared to Sanger sequencing, it is based on iteratively complementing single strands and simultaneously reading out the signal emitted from the nucleotide being incorporated. Illumine Genome Analyzer II/IIx is a reversible terminator techno-logy and employs a sequencing-by-synthesis concept that is similar to that used in Sanger sequencing, however the Illumina sequencing requires protocol the sequence to be determined are converted in to special sequencing library, which allows them to be amplified and immobilised for sequencing (Bentley et al., 2008). The SOLiD sequence platform (sequencing-by-litigation) is very different from the rest discussed thus far and the sequence extension reaction is not carried out by polymerases but rather by ligases (Shendure et al., 2005). The Sanger capillary sequencing is a low- throughput method and the sequencing error observed for Sanger sequencing is mainly due to errors in the amplification step (a low rate when done *in vivo*), natural variance, and contamination in the sample used, as well as polymerase slippage at low complexity sequences like simple repeats (short variable number tandem repeats) and homopolymers (stretches of the same nucleotide). The the high-throughput techniques (pyrosequencing, reversible terminator chemistry, sequence-by litigation) makes bacterial identification easier and even possible for even single research groups to generate large amounts of sequence data very rapidly and at substantially lower costs than traditional Sanger sequencing.

Novel DNA sequencing technologies called high-throughput sequencing (HTS) techniques are capable of generating massive amounts of genetic information with increased speed, accuracy and efficiency. High-throughput genome sequencing provides a more detailed real-time assessment of the genetic traits of bacteria than could be achieved with routine subtyping methods. HTS technologies are used for studying diversity and genetic variations and solving genomic complexities. Approximately 300 complete bacterial genomes had been sequenced by 2010. This has aided and sped identification of bacterial species and these HTS technologies remain useful especially for identification of bacterial species that constitute a population in a sample.

CONCLUSION

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. The more traditional methods whereby bacteria have been identified based on their physical properties, are compound light microscopy in combination with histological staining and electron microscopy. The later is the conventional scanning microscope which generally offers unique advantages such as high resolution and great depth of field. The fatty acid profiles of bacteria, which are determined with the aid of gas chromatography, have also been used extensively for the identification of bacterial species. Bacterial phylogeny and taxonomy have further benefited greatly from the use of the sequence analysis of 16S ribosomal RNA, which makes the identification of rarely isolated, phenotypically anomalous strains possible. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. The 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains can be routinely used for identification of mycobacteria and can lead to the recognition of novel pathogens and noncultured bacteria.

Cutting-edge techno-logies such as MALDI-TOF MS, Biolog and the RiboPrinter® has facilitated bacteriological identification even further. The MALDI-TOF MS technique offers easily determinable peptide or protein finger printing for the identification, typing and characterisation of various strains. Biolog has been used to identify various lactic acid bacteria strains. Biolog tests a microorganism's ability to utilise or oxidase a panel of carbon sources and this method is used when characterising bacterial samples within a fixed degree of similarities. The computational tools have been developed for quering the relevant microbial databases that are associated with the bacterial identification methods. From the current review, it is evident that with the increase in the adoption of new technologies and high-throughput sequencing techniques, bacterial identification is becoming easier.

REFERENCES

Adams DJ, Gurr S, Hogge J (2004). Cellular Fatty-Acid Analysis of *Bacillus thuringiensis* Var. *kurstaki* Commercial Preparations. J. Agric. Food Chem., 53(3):512-517.

Alexandrov T (2012). MALDI imaging mass spectrometry:statistical data analysis and current computational challenges. BMC Bioinformatics 13 (Suppl 16):S11.

Barbuddhe SB, Maier T, Schwarz G, Kostrzewa M, Hof H, Domann E, Chakraborty T, Hain T (2008). Rapid identification and typing of listeria species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. 74(17):5402-5407.

Barghoutti SA (2011). A Universal Method for the Identification of Bacteria Based on General PCR Primers. Indian J Microbiol. 51(4):430- 444.

Bentley DR, Balasubramanian S, Swerdlow HP, et al. (2008). Accuratewhole human genome sequencing using reversible terminator chemistry. Nature 456:53-59.

Bergmans L, Moisiadis P, Van Meerbeek B, Quirynen M, Lambrecht P (2005). Microscopic observation of bacteria:review highlighting the use of environmental SEM. Int. Endod. J. 38:775-788.

Bhore SJ, Nythia R, Loh CY (2010). Screening of endophytic bacteria G isolated from leaves of sambung nyawa [*Gynura procumbens* (Lour.)

Merr.] for cytokinin-like compounds. Bioinformation 5(5):191-197.
Cabeen MT, Jacobs-Wagner C (2005). Bacterial cell shape. Nat. Rev. Microbiol. 3(8):601-610.
Cambray G (2006). Basic and applied microbiology. Eds., Cloete TE, Atlas RM Van Schaik Publishers.
Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods 69(2):330-339.
Chanama S (1999). Comparative 16S rRNA sequence analysis. Warasan Wichai Witthayasat Kanphaet 13:107-117.
Christopher K, Bruno E (2003). Identification of bacterial species. In Tested studies for laboratory teaching. Ed., O'Donnell MA. Proceedings of the 24th Workshop/Conference of the Association for Biology Laboratory Education 24:103-130.
Davis CL, Brlansky RH (1991). Use of Immunogold Labelling with Scanning Electron Microscopy To Identify Phytopathogenic Bacteria on Leaf Surfaces. Appl. Environ. Microbiol. 57(10):3052-3055.
De Bruyne K, Slabbincka B, Waegeman W, Vauterin P, De Baets B, Vandamme P (2011). Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. Syst. Appl. Microbiol. 34:20-29.
Eren AM, Ferris MJ, Taylor CM (2011). A framework for analysis of metagenomic sequencing data. Pac. Symp. Biocomput.131-141.
Fagerquist CK, Yee E, Miller WG (2007). Composite sequence proteomic analysis of protein biomarkers of *Campylobacter coli*, *C. lari* and *C. concisus* for bacterial identification. Analyst 132(10):1010-1023.
Fogel GB, Collins CR, Li J, Brunk CF (1999). Prokaryotic genome size and SSU rDNA copy number:estimation of microbial relative abundance from a mixed population. Microb. Ecol. 38:93-113.
Giacomini M, Ruggiero C, Calegari L, Bertone S (2000). Artificial neural network based identification of environmental bacteria by gas-chromatographic and electrophoretic data. J. Microbiol Methods 43(1):45-54.
Glimm E, Heuerh, Engelen B, Smalla K, Backhaus H (1997). Statistical comparisons of community catabolic profiles. J. Microbiol. Methods 30:71.
Grosse-Herrenthey A, Maier T, Gessler F, Schaumann R, Böhnel H, Kostrzewa M, Krüger M (2008). Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Anaerobe 14(4):242-249.
Heyrman J, Mergaert J, Denys R, Swings J (1999). The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. FEMS Microbiol. Lett. 181:55-62.
Hiremath PS, Bannigidad P, Yelgond SS (2013). An Improved Automated Method for Identification of Bacterial Cell Morphological Characteristics. International Journal of Advanced Trends in Computer Science and Engineering, Vol. 2, No. 1:11-16 2 (1):11-16.
Holmes B, Costas M, Ganner M, On SL, Stevens M (1994). Evaluation of Biolog system for identification of some Gram-negative bacteria of clinical importance. J. Clin. Microbiol. 32:1970-1975.
Hung PQ, Annapurna K (2004). Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). Omonrice, 12:92-101.
Inglis TJJ, O'Reilly L, Foster N, Adele CA, Sampson J (2002). Comparison of rapid, automated ribotyping and DNA macro restriction analysis of *Burkholderia pseudomallei*. J. Clin. Microbiol., 40(9):3198-3203.
Janda JM, Abbot SL (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J. Clin. Microbiol., 45(9):2761-2764.
Kenzaka T, Tani K (2012). Scanning Electron Microscopy Imaging of Bacteria Based on Nucleic Acid Sequences, Scanning Electron Microscopy, Dr. Viacheslav Kazmiruk (Ed.), ISBN: 978-953-51- 0092-8, InTech, Available from: http://www.intechopen.com/books/scanning-electron-microscopy/scanningelectron-microscope-imaging-of-bacteria-based-on-nucleic-acid-sequences.
Kivanç M, Vilmaz M, Cakir E (2011). Isolation and identification of lactic acid bacteria from boza, and their microbial activity against several reporter strains. Turk. J. Biol. 35:313-324.
Kloepper JW, McInroy JA, Bowen KL (1992). Comparative identification by fatty acid analysis of soil, rhizosphere, and geocarposphere bacteria of peanut *(Arachis hypogaea* L.). Plant Soil 139:85-90.
Kumrapich B, Klayraung S, Wongkattiya N, Topoonyanont N (2011). Diversity of bacteria isolated from leaves and stems of sugarcane (Saccharum sp. Var. LK92-11). 37th Congress on Science and Technology of Thailand.
Massomo SMS, Nielsen H, Mabagala RB, Mansfeld-Giese K, Hockenhull J, Mortensen CN (2003). Identification and characterisation of *Xanthomonas campestris* pv. *campestris* strains from Tanzania by pathogenicity tests, Biolog, rep-PCR and fatty acid methyl ester analysis. Eur. J. Plant Pathol. 109:775-789.
Miller JM, Rhoden DL (1991). Preliminary evaluation of Biolog, a carbon source utilization method for bacterial identification. J. Clin. Microbiol., 29, 1143-1147.
Mizrahi-Man O, Davenport ER, Gilad Y (2013). Taxonomic Classification of Bacterial 16S rRNA Genes Using Short Sequencing Reads: Evaluation of Effective Study Designs. PLoS ONE 8(1).
Morgan MC, Boyette M, Goforth C, Sperry KV, Greene SR (2009). Comparison of the Biolog OmniLog Identification System and 16S ribosomal RNA gene sequencing for accuracy in identification of atypical bacteria of clinical origin. J. Microbiol. Methods, 79:336-343.
Moura H, Woolfitt, AR, Carvalho MG, Pavlopoulos A, Teixeira LM, Satten GA, Barr JR (2008). MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive *Streptococcus pyogenes* isolates. FEMS Immunol. Med. Microbiol. 53(3):333-342.
Muzzamal H, Sarwar R, Sajid I, Hasnain S (2012). Isolation, identification and screening of endophytic bacteria antagonistic to biofilm formers. Pakistan J. Zool., 44(1):249-257.
Núñez-Cardona MT (2012). Fatty acids analysis of photosynthetic sulfur bacteria by gas chromatography, gas chromatography - biochemicals, narcotics and essential oils. Ed. Salih B. InTech.
Petrosino JF, Highlander S, Luna RA, Gibbs RA, Versalovic J (2009). Metagenomic pyrosequencing and microbial identification. Clin. Chem., 55(5):856-866.
Pires MN, Seldin L (1997). Evaluation of Biolog system for identification of strains of *Paenibacillus azotofixans*. Antonie Leeuwenhoek, 71:195-200.
Purcaro G, Tranchida PQ, Dugo P, La Camera E, Bisignano G, Conte L, Mondello L (2010). Characterization of bacterial lipid profiles by using rapid sample preparation and fast comprehensive two-dimensional gas chromatography in combination with mass spectrometry. J. Sep. Sci., 33(15):2334-2340.
Sasser M (2011). Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note #101.
Shah N, Tang H, Doak TG, Ye H (2010). Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics. Pac. Symp. Biocomput., 2011:165-176.
Shendure J, Porreca GJ, Reppas (2005). Accurate multiplex polony sequencingof an evolved bacterial genome. Science 309:1728-1732.
Smole SC, King LA, Leopold PE, Arbeit RD (2002). Sample preparation of Gram-positive bacteria for identification by matrix assisted laser desorption/ionization time-of-flight. J. Microbiol. Methods 48(2-3):107-115.
Song Y, Liu C, McTeague M, Finegold SM (2003). 16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci. J Clin Microbiol., 41(4):1363-1369.
Stager CE, Davis JR (1992). Automated systems for identification of microorganisms. Clin. Microbiol. Rev. 5:302-327.
Vanlaere E, Sergeant K, Dawyndt P, Devreese B, Vandamme P (2006). Identification of *Burkholderia cepacia* complex using MALDI-TOF mass spectrometry. J. Cyst. Fibr. 5(Suppl. 1):S34-S134.
Vargha M, Takats Z, Konopka A, Nakatsu CH (2006). Optimization of MALDI-TOF MS for strain level differentiation of Arthrobacter isolates. J. Microbiol. Methods, 66:399-409.
Welch DF (1991). Application of cellular fatty acid analysis. Clin Microbiol. Rev. 4(4):422-438.
Williams TL, Andrzejewski D, Lay JO, Musser SM (2003). Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. J. Am. Soc. Mass Spectrom. 14(4):342-351.
Woese CR (1987). Bacterial evolution. Microbiol. Rev. 51(2):221-271.

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Full Length Research Paper

Genetic stability of mulberry germplasm after cryopreservation by two-step freezing technique

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Genetic stability of *Morus alba*, *Morus indica*, *Morus laevigata* (indigenous collection) and *Morus* species (exotic collection) have been studied in *in vitro* regenerated plants of mulberry (fresh, before and after cryopreservation) using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. This study examined the genetic stability of cryopreserved dormant buds of *Morus* germplasm that were stored in liquid nitrogen using two-step freezing, then rewarmed and regrown. Dormant buds of mulberry collected during winter period were found suitable for the cryopreservation in liquid nitrogen. In the present study, the plants were regenerated directly from dormant buds (before and after cryopreservation) without intermediary callus phase. These regenerants thus bear low risk of genetic instability. Both the single primer amplification reaction (SPAR) markers showed reproducible and well resolved banding patterns in mulberry germplasm, in which RAPD marker generated a total of 201 bands based on 15 primers; however, ISSR markers were given 145 bands using 11 primers. Both markers showed monomorphic banding patterns and did not reveal any polymorphism among the mother plant and *in vitro* regenerants before and after cryopreservation, suggesting that cryopreservation, using two-step freezing, does not affect genetic stability of mulberry germplasm. The transitory nature of these polymorphisms should be carefully considered when monitoring for genetic stability.

Key words: Cryopreservation, Genetic stability, *in vitro* culture, ISSR, mulberry, RAPD.

INTRODUCTION

Mulberry (*Morus* spp.) belongs to the family 'Moraceae', a family of deciduous or evergreen trees and shrubs, mostly of pantropical distribution and characterized by milky sap. The origins of most cultivated mulberry varieties are believed to be in the Himalayan foothills by the evidences gathered from fossils (Collinson, 1989), morphology, anatomy (Benavides et al., 1994; Hou, 1994) and molecular biological (Zerega et al., 2005) covering both temperate and sub-tropical regions of Northern hemisphere (Anonymous, 2006) and later spread to major continents including Asia, Europe, North and South America, and Africa (Machii et al., 1999). Sanjappa (1989) recognized 68 species within the genus *Morus*, out of which, *M. alba*, *M. indica*, *M. nigra*, *M. latifolia*, *M. multiculis* are cultivated for silkworm rearing, *M. rubra* and *M. nigra* for fruits (Yaltirik, 1982) and *M. laevigata* and *M. serrata* for timber (Tikader and Vijayan, 2010). Cultivation of mulberry and silkworm rearing started in China before 2200 BC (FAO, 1990) and currently mulberry is cultivated in almost all Asian countries (Vijayan et al., 2011). In India, four main species of *Morus*, namely, *M. alba*, *M. indica*, *M. laevigata* and *M. serrata* have been reported (Hooker, 1885; Tikader and Dandin, 2005; Vijayan, 2010).

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Table 1. List of samples of *Morus* species used for genetic stability.

S/N	Sample no.	Collector no.	Species
1	M1-A*, M1-B**, M1-C***	MI-0583	*Morus laevigata*
2	M2-A*, M2-B**, M2-C***	ME-0051	*Morus* sp.
3	M3-A*, M3-B**, M3-C***	MI-0622	*Morus indica*
4	M4-A*, M4-B**, M4-C***	RC-03	*Morus indica*
5	M5-A*, M5-B**, M5-C***	RC-02	*Morus laevigata*
6	M6-A*, M6-B**, M6-C***	ME-0087	*Morus* sp.
7	M7-A*, M7-B**, M7-C***	MI-0698	*Morus alba*
8	M8-A*, M8-B**, M8-C***	RC-01	*Morus alba*
9	M9-A*, M9-B**, M9-C***	ME-0099	*Morus* sp.
10	M10-A*, M10-B**, M10-C***	MI-0678	*Morus alba*

*Fresh leaf samples, ***in vitro* regenerated samples before cryopreservation and *** after cryopreservation

Tissue culture with its distinct advantages is used for short-term preservation (Withers and Engelmann, 1997) but it does not serve for long-term preservation. Hence, cryopreservation only economically viable method is adopted for long-term preservation. Under cryopreservation, plant materials are stored at ultra-low temperatures in liquid nitrogen (-196°C). At this temperature, cell division and metabolic activities remain suspended and the material remains unchanged for a long period. Thus, cryopreservation ensures genetic stability of the mulberry germplasm besides requiring only limited space and protecting material from contamination. In mulberry, the most appropriate material for cryopreservation is the winter bud (Fukui et al., 2011; Rao et al., 2009), *in vitro*-grown shoot apices in *Morus* species (Gupta, 2011; Padro et al., 2012) and *M. bombicys* (Yakuwa and Oka, 1988), though embryonic axes, pollen, synthetic seeds can also be used (Niino, 1995).

The survival rates of winter buds stored in liquid nitrogen up to three to five years did not change significantly (Rao et al., 2009). Either prefreezing at -10 or -20°C along with rapid thawing at 37°C or pre-freezing at -20 or -30°C along with slow thawing at 0°C was a suitable treatment for high percentages of survival and shoot regeneration (Rao et al., 2007). It is desirable to assess the genetic integrity of the germplasm of micropropagated plantlets with that of field plants and plants regenerated after surviving cryogenic (-196°C) storage to determine if they are true-to-type after cryopreservation. The most commonly used marker systems for genetic stability study are Random Amplified Polymorphic DNA (RAPD) (Srivastava et al., 2004), amplified fragment length polymorphism (AFLP) (Wang and Yu, 2001), and inter-simple sequence repeat (ISSR) (Vijayan et al., 2005, 2006; Zhao et al., 2006). The application of RAPD and ISSR for the characterization of genetic stability has been well documented in *Morus* species (Rao et al., 2007, 2009; Vijayan, 2004; Vijayan and Chaterjee, 2003; Vijayan et al., 2004, 2005).

In the present study, the genetic stability of the *in vitro* regenerated plants of mulberry (fresh, before and after cryopreservation) was analysed through the RAPD and ISSR markers using 10 mulberry germplasm.

MATERIALS AND METHODS

Dormant buds of different *Morus* species were collected from the field genebank of Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, Tamil Nadu (Table 1). Three accessions were collected from bio-diversity garden of National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Buds attached to twigs wrapped in cotton bags were air lifted to cryolab at NBPGR reaching within 48 h of harvesting. After receipt, the twigs were wrapped in polyethene bags and kept in refrigerator at 10 to 15°C temperature and used for experimentation within 25 days of harvest.

Cryopreservation using two-step freezing

Descaled buds of mulberry were tied in muslin cloth and put in charged silica gel for 4 to 7 h at room temperature for desiccation. The desiccated buds were packed in 1.0 ml polypropylene cryovials. These vials were shifted sequentially at 5, -5, -10, -15, -20, -25 and -30°C keeping at each of the temperatures for a minimum of 24 h. The cryovials were held at -30°C for 48 h and then directly plunged in the liquid nitrogen at -196°C. Cryopreserved buds were thawed by slow thawing and transferred in sterile moist moss for rehydration. The viability of fresh, desiccated and cryopreserved dormant buds of *Morus* species was tested *in vitro* culturing method. For recovery growth of the cryostored dormant buds, 1 to 2 outer scales of the rehydrated buds were further removed followed by washing with Tween 20 for 15 min. Tween 20 was rinsed off with running tap water. These buds were then surface sterilized with 0.1% mercuric chloride for 9 min, rinsed three times with sterile water washes using autoclaved distilled water (5 min each). The sterilized buds were cultured on basal MS medium (Murashige and Skoog, 1962) with 3% sucrose (w/v) and solidified with 0.8% agar. MS medium was supplemented with 1 mgl^{-1} BAP initially for bud sprouting. The cultures raised from cryopreserved buds were maintained in culture room in dark for seven days.

After dark incubation, these cultures were shifted in diffused light for 3 days. After 10 days of culturing, these cultures were exposed

to normal culture room light intensity (3000 lux/ 36 µ mol $^{-1}$ sec^{2}). The sprouted buds were sub-cultured on the MS medium supplemented with 1.0 mgl^{-1} BAP and 0.2 mgl^{-1} GA_3 for elongation. The elongated plants were further sub-cultured and transferred to multiplication medium (0.5 mgl^{-1} BAP + 0.5 mgl^{-1} Kn + 0.1 mgl^{-1} IAA) and finally transferred to rooting medium (half MS + 0.5 mgl^{-1} IBA). Fresh leaf tissues of these mulberry samples were cleaned with water, air dried and stored in -80°C refrigerator for further experiments (Table 1).

DNA isolation

Total genomic DNA was isolated from leaf tissues using the cetyl trimethyl ammonium bromide method with few modifications (Doyle and Doyle, 1990). DNA concentration was determined spectrophotometrically at 260 nm. Quality of genomic DNA was determined through electrophoresis on 0.8% agarose gel.

RAPD-PCR amplification

The RAPD primers of Operon Technologies Alameda, CA, USA were used for molecular analysis. A total of 40 primers were screened in *Morus* species, of which 15 primers were selected for final profiling based on banding patterns and reproducibility. The basic protocol of RAPD-PCR reported by William et al. (1990) was followed for PCR amplification in a total reaction volume of 15 ml, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase (Life Tech, India), 0.2 mM of dNTP each, 10 pmoles of RAPD primer and 20 ng of DNA template. DNA amplification was carried out in a PTC-200 TM thermocycler and the thermal cycler conditions for PCR reactions were an initial denaturation cycle for 3 min at 94°C followed by 40 cycles comprising 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. An additional cycle of 5 min at 72°C was used for final extension. Amplification products were separated by electrophoresis in 1.8% agarose gels and stained in ethidium bromide. A photographic record was taken under UV gel doc system (Alpha Innotech, USA).

ISSR-PCR amplification

A total of hundred primers of University of British Columbia (UBC) procured from Geno Biosciences Pvt. Ltd. were used for ISSR-PCR optimization trials. Eleven primers, which gave the best amplification results with the sample DNA, were selected for final ISSR-PCR analysis. PCR-amplification was carried out in 25 µl reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.0 to 2.5 mM $MgCl_2$, 0.2 mM dNTP each, 1.0 U *Taq* DNA polymerase (Bangalore Genie, India), 0.2 µM primer and 20 ng genomic DNA. The amplification was performed in a PTC-200 thermocycler (MJ Research, Massachusetts, USA), with reaction conditions programmed as initial pre-denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min and final extension at 72°C for 7 min. Amplification products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide, and bands were visualized and documented in UV gel doc system (Alpha Innotech Corporation).

Data analysis

Amplified bands were scored as present (1) or absent (0) homologous bands across all the accessions studied. Molecular weight of the amplified bands was estimated using 1 kb DNA ladder (Gibco BRL Life Technologies, NY, USA) as a standard. A pairwise similarity matrix of all the accessions was estimated based on Jaccard's coefficient (Jaccard, 1908) and a dendrogram was generated based on the unweighted pair-group method for arithmetic mean (UPGMA) using the software NTSYS version 2.10e (Rohlf, 2000). Principal component analysis was also carried out to study relationships among accessions using the same software. The test for association was conducted based on two-way Mantel test (Mantel, 1967).

RESULTS

The genetic stability of the fresh, *in vitro* raised plants before and after cryopreservation was studied through the ISSR and RAPD markers using 10 mulberry accessions comprising four different species of *Morus* (Table 1). The PCR amplification products of control (unfrozen) *in vitro* and cryopreserved (frozen in liquid nitrogen) samples were plotted together for comparison.

Genetic stability analysis in mulberry

RAPD analysis

Fifteen (15) primers were selected for the RAPD analysis based on the reproducibility and banding patterns. A total of 201 bands were generated from 15 RAPD primers, of which 169 bands were polymorphic (84.08%) with an average of 11.27 polymorphic bands per primer. The fragment size ranged from 200 to 3000 bp (Table 2). A representative gel profiles generated using primers OPA-02 and OPA-04 are shown in Figure 1. Each primer amplified at a range of 4 to 20 bands with an average of 13.40 bands per primer. OPA-02 primer amplified the maximum number of 20 bands, whereas OPA-10 primer generated the minimum of 4 bands. The polymorphism percentage ranged from 53.85 (primer OPE-03) to 100% (OPA-06 and OPA-11) with an average of 84.08% polymorphism (Table 2). Pattern of distribution of amplified bands across all the accessions revealed that some primers generated unique bands, namely OPA-02 in M1 (400 bp in *M. laevigata*) or M4 (2200 bp in *M. indica*), OPA-04 in M1 (870 bp in *M. laevigata*), OPA-06 in M1 (750 bp in *M. laevigata*), OPA-13 in M4 (650 bp in *M. indica*), OPA-17 in M1 (250 bp in *M. laevigata*), OPA-18 in M9 (870 bp in *Morus* sp), and OPE-04 amplified a single fragment in M9 (2000 bp in *Morus* sp).

A pairwise Jaccard's similarity values ranged from 0.37 to 0.83 (average 0.60) among the 10 accessions of mulberry (Table 3). A maximum similarity value of 0.83 was observed between M1 and M5 samples (both belong to *M. laevigata*), whereas M2 and M9 (*Morus* sp) showed least similarity coefficient of 0.37. All the three samples (fresh, *in vitro* raised before and after cryopreservation) of each *Morus* species showed 100% similarity among the treatments.

A dendrogram generated based on UPGMA method grouped all the 10 accessions into two major clusters

Table 2. Details of the RAPD and ISSR primers, their sequence and number of amplified bands used in analysis of *Morus species.*

Primer	Sequence (5'-3')	Total no. of bands	PB[a]	PPB[b]	Unique bands	Range of fragment size (bp)
OPA01	CAGGCCCTTC	12	10	83.33	0	550-1500
OPA02	TGCCGAGCTG	20	16	80.00	2	400-2500
OPA04	AATGGGGCTG	18	15	83.33	1	500-3000
OPA06	GGTCCCTGAC	08	08	100.00	1	650-920
OPA08	GTGACGTAGG	12	11	91.67	0	450-2000
OPA09	GGGTAACGCC	13	13	100.00	0	500-2000
OPA10	GTGATCGCAG	04	03	75.00	0	450-950
OPA11	CAAATCGCCGT	09	09	100.00	0	570-1000
OPA13	CAGCACCCAC	17	15	88.23	1	250-1400
OPA17	GACCGCTTGT	13	12	92.31	1	250-1400
OPA18	AGGTGACCGT	15	12	80.00	1	200-2500
OPC02	GTGAGGCGTC	17	14	82.35	0	300-2500
OPE03	CCAGATGCAC	13	07	53.85	0	350-2000
OPE04	GTGACATGCC	15	11	73.33	1	350-2000
OPE20	AACGGTGACC	15	13	86.67	0	350-2500
		201	**169**	**84.08**	**8**	
	ISSR					
UBC-807	AGAGAGAGAGAGAGAGT	16	16	100.00	1	200-1100
UBC-808	AGAGAGAGAGAGAGG	12	12	100.00	1	350-990
UBC-810	GAGAGAGAGAGAGAT	14	11	78.57	1	200-1000
UBC-811	GAGAGAGAGAGAGAC	11	07	63.63	1	370-960
UBC-812	GAGAGAGAGAGAGAA	15	14	93.33	1	300-1140
UBC-825	ACACACACACACACT	12	11	91.67	0	375-2000
UBC-827	ACACACACACACACG	14	14	100.00	0	400-1100
UBC-841	GAGAGAGAGAGAGAYC	15	14	93.33	0	300-2000
UBC-855	ACACACACACACACYT	15	14	93.33	1	250-1100
UBC-858	TGTGTGTGTGTGTGRT	11	11	100.00	2	300-1000
UBC-864	ATGATGATGATGATG	11	10	90.90	0	200-1100
		146	**134**	**91.78**	**8**	

[a] Total Polymorphic Bands; [b] Percentage of polymorphic bands

(Figure 2A). First cluster was the largest one and divided into two sub-clusters (II-a and II-b). Sub-cluster II-a, again sub-divided into II-a1 and II-a2 group. II-a1 group comprising 03 samples, namely, M1 (*M. laevigata*), M5 (*M. laevigata*) and M6 (*Morus* sp.). Within this cluster, the M1 and M5 showed 83% genetic similarity. Group II-a2 comprised of three samples naming M2 (*Morus* sp.), M3 (*M. indica*) and M4 (*M. indica*) in which, M3 and M4 were closely related with similarity value of 0.81. Sub-cluster II-b comprised of three samples namely, M7, M8 and M10 (all belong to *M. alba*) in which, M7 and M8 were closely related with similarity value of 0.75. Second cluster consisted only one sample M9 (*Morus* sp.) which was distinct from all other samples with similarity value of 0.46. Based on Mantel Z-statistics (Mantel, 1967), the correlation coefficient (r) was estimated to be 0.77. 2-D (Figure 2B) generated from PCOA of RAPD data was also in coherence with the clustering pattern of UPGMA dendrogram. First and second principal components accounted for 27.93 and 17.26%, respectively of the total variation.

ISSR analysis

Eleven primers were selected for the ISSR analysis based on the reproducibility and banding patterns. A total of 146 bands were generated, of which 134 bands were polymorphic (91.78%). Each primer amplified 7 to 16 polymorphic bands with an average of 12.18 bands per primer (Table 2). UBC-807 primer amplified the maximum number of 16 bands, whereas UBC-811 amplified the lowest number of polymorphic bands 7. The polymerphism percentage ranged from 63.63 (primer UBC-811) to 100% (UBC-807, UBC-808, UBC-827 and UBC-858). Average polymorphism across all the 10 accessions was

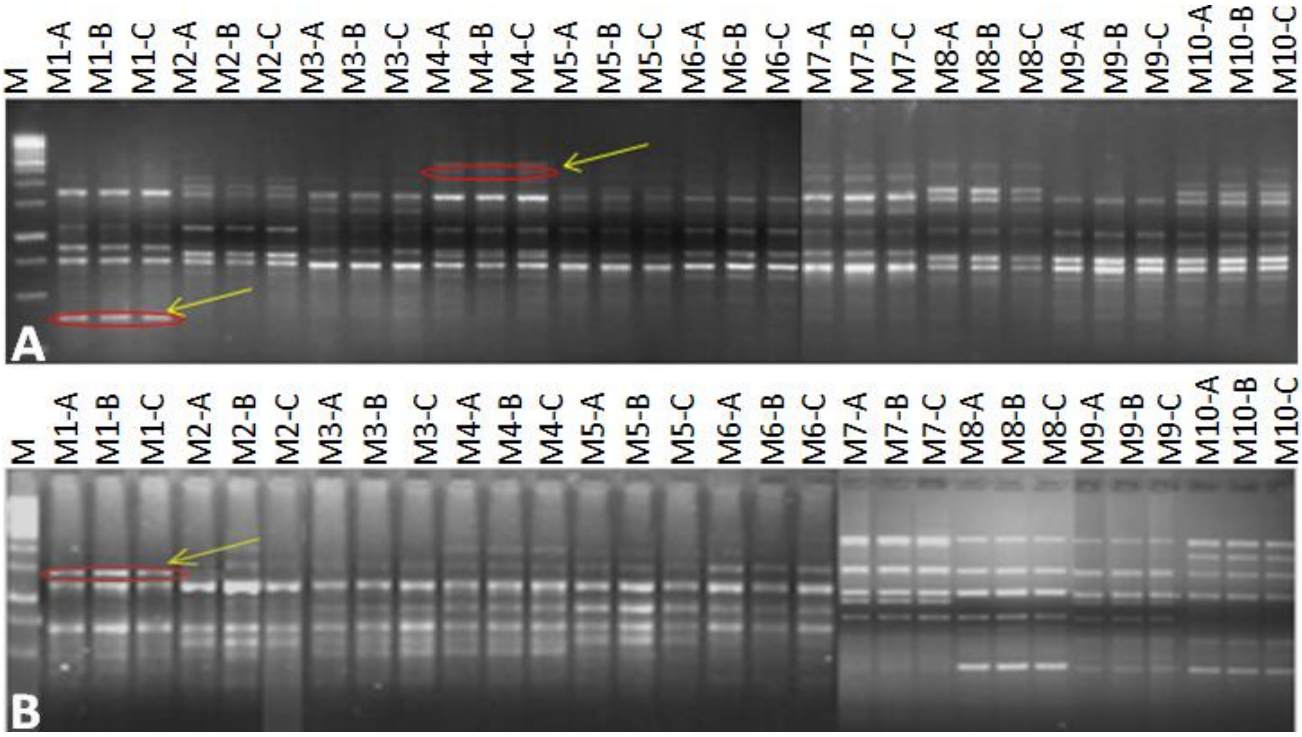

Figure 1. Gel profiles of the 10 mulberry accessions (ten control and ten *in vitro* regenerated samples before and after cryopreservation) generated with the RAPD primers: [A] OPA-2 and [B] OPA-4. M is the λ DNA marker. Arrows shows the unique band in red circle.

91.78%. Overall size of the PCR amplified fragments ranged from 200 to 2000 bp (Table 2). Pattern of distribution of bands across all accessions of mulberry revealed that the primer UBC-807 (1100 bp for *M. alba*), UBC-808 (990 bp for *M. laevigata*), UBC-810 (1000 bp *M. alba*), UBC-811 (960 bp for *M. laevigata*), UBC-812 (1000 bp for *M. laevigata*), UBC-855 (1100 bp for *M. alba*) and UBC-858 (300 bp for *M. laevigata* and 1000 bp for *Morus* sp.) amplified a unique DNA fragment which distinguished one species from the others (Figure 3). All cryopreserved and fresh samples showed 100% similarity among the treatments (fresh, *in vitro* raised before and after cryopreservation). A pairwise similarity values among all the 10 accessions of mulberry ranged from 0.41 to 0.97 (Table 4). The maximum similarity of 0.97 was observed between M3 (*M. indica*) and M4 (*M*. *indica*) accessions and showed close genetic similarity, whereas M9 (*Morus* sp.) showed least similarity coefficient of 0.41 with M1 and M5 (*M. laevigata*). Average similarity across all the cultivars was 0.69.

In the dendrogram, all the 10 accessions were grouped into three major clusters (Figure 4A). First cluster comprised of two accessions, namely M1 and M5 (both are *M. laevigata*) which were closely related with similarity value of 0.95. Second cluster was again divided into two sub-clusters (II-a and II-b). Sub-cluster II-a was the biggest comprising of the 4 accessions namely M2, M3, M4 and M6, in which M3 and M4 (both *M. indica*) were genetically most similar showing 97% similarity. The sub-cluster II-b comprised of three accessions that is, M7, M8 and M10 (all *M. alba*), in which, M7 and M10 were showing 98% genetic similarity to each other. In the third cluster, while M9 (*Morus* sp.) was diverse from other samples of this cluster with similarity value of 0.49. Based on Mantel Z-statistics (Mantel, 1967), the correlation coefficient (r) was estimated to be 0.93. 2-D plot generated from PCOA of ISSR data also in coherence with the clustering pattern of UPGMA dendrogram. The first five principal components accounted for 91.14% of the total variation and the first three accounted for 70.74% of the variation, in which maximum variation was contri-buted by first component (30.86%) followed by second component (23.91%), and third component (15.97%). 2-D plot generated through PCOA also showed the same grouping pattern as the UPGMA dendrogram (Figure 4B).

DISCUSSION

The aim of this study was to develop a simple and rapid technique to assist post-cryo assessment of genetic stability in vegetatively propagated germplasm. The results clearly demonstrate the application of molecular

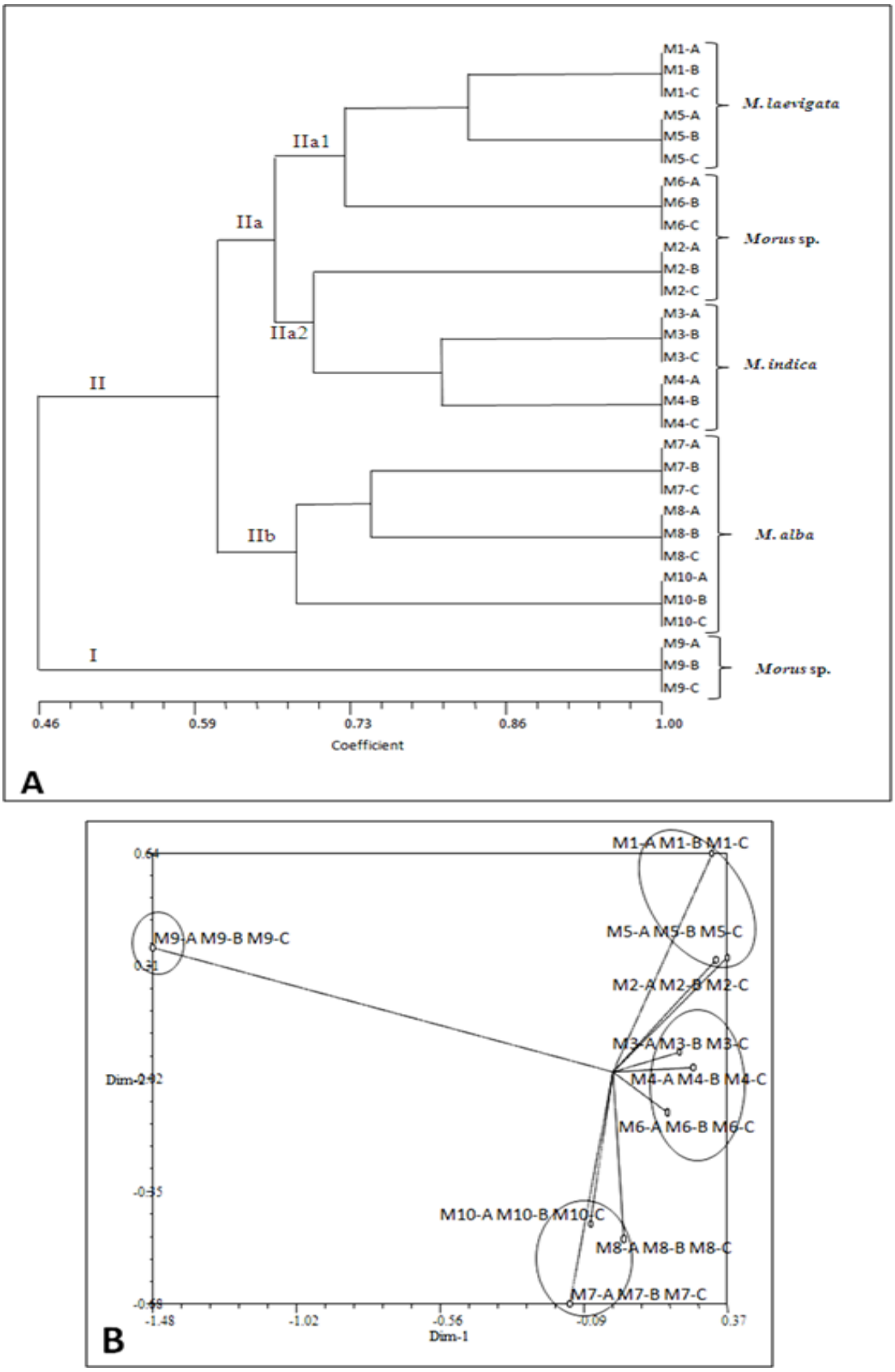

Figure 2. Mulberry [A] UPGMA dendrogram and [B] 2-D plot of 10 mulberry cultivars (ten control and 10 *in vitro* regenerated samples before and after cryopreservation) generated based on RAPD data.

Table 3. A pairwise similarity matrix of 10 samples (fresh, *in vitro* raised before and after cryopreservation) of different *Morus* species based on RAPD data.

	M1-A	M1-B	M1-C	M2-A	M2-B	M2-C	M3-A	M3-B	M3-C	M4-A	M4-B	M4-C	M5-A	M5-B	M5-C	M6-A	M6-B	M6-C	M7-A	M7-B	M7-C	M8-A	M8-B	M8-C	M9-A	M9-B	M9-C	M10-A	M10-B	M10-C
M1-A	1.00																													
M1-B	1.00	1.00																												
M1-C	1.00	1.00	1.00																											
M2-A	0.65	0.65	0.65	1.00																										
M2-B	0.65	0.65	0.65	1.00	1.00																									
M2-C	0.65	0.65	0.65	1.00	1.00	1.00																								
M3-A	0.66	0.66	0.66	0.70	0.70	0.70	1.00																							
M3-B	0.66	0.66	0.66	0.70	0.70	0.70	1.00	1.00																						
M3-C	0.66	0.66	0.66	0.70	0.70	0.70	1.00	1.00	1.00																					
M4-A	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00																				
M4-B	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00	1.00																			
M4-C	0.62	0.62	0.62	0.69	0.69	0.69	0.81	0.81	0.81	1.00	1.00	1.00																		
M5-A	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00																	
M5-B	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00	1.00																
M5-C	0.83	0.83	0.83	0.62	0.62	0.62	0.70	0.70	0.70	0.71	0.71	0.71	1.00	1.00	1.00															
M6-A	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00														
M6-B	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00	1.00													
M6-C	0.69	0.69	0.69	0.61	0.61	0.61	0.69	0.69	0.69	0.72	0.72	0.72	0.76	0.76	0.76	1.00	1.00	1.00												
M7-A	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00											
M7-B	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00	1.00										
M7-C	0.52	0.52	0.52	0.50	0.50	0.50	0.65	0.65	0.65	0.63	0.63	0.63	0.58	0.58	0.58	0.68	0.68	0.68	1.00	1.00	1.00									
M8-A	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00								
M8-B	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00	1.00							
M8-C	0.56	0.56	0.56	0.62	0.62	0.62	0.63	0.63	0.63	0.68	0.68	0.68	0.65	0.65	0.65	0.73	0.73	0.73	0.75	0.75	0.75	1.00	1.00	1.00						
M9-A	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00					
M9-B	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00	1.00				
M9-C	0.42	0.42	0.42	0.37	0.37	0.37	0.47	0.47	0.47	0.45	0.45	0.45	0.42	0.42	0.42	0.47	0.47	0.47	0.52	0.52	0.52	0.50	0.50	0.50	1.00	1.00	1.00			
M10-A	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00		
M10-B	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00	1.00	
M10-C	0.59	0.59	0.59	0.54	0.54	0.54	0.63	0.63	0.63	0.62	0.62	0.62	0.62	0.62	0.62	0.67	0.67	0.67	0.69	0.69	0.69	0.68	0.68	0.68	0.51	0.51	0.51	1.00	1.00	1.00

Table 4. A pairwise similarity matrix of 10 samples (fresh, *in vitro* raised before and after cryopreservation) of different *Morus* species based on ISSR data.

	M1-A	M1-B	M1-C	M2-A	M2-B	M2-C	M3-A	M3-B	M3-C	M4-A	M4-B	M4-C	M5-A	M5-B	M5-C	M6-A	M6-B	M6-C	M7-A	M7-B	M7-C	M8-A	M8-B	M8-C	M9-A	M9-B	M9-C	M10-A	M10-B	M10-C
M1-A	1.00																													
M1-B	1.00	1.00																												
M1-C	1.00	1.00	1.00																											
M2-A	0.52	0.52	0.52	1.00																										
M2-B	0.52	0.52	0.52	1.00	1.00																									
M2-C	0.52	0.52	0.52	1.00	1.00	1.00																								
M3-A	0.58	0.58	0.58	0.67	0.67	0.67	1.00																							
M3-B	0.58	0.58	0.58	0.67	0.67	0.67	1.00	1.00																						
M3-C	0.58	0.58	0.58	0.67	0.67	0.67	1.00	1.00	1.00																					
M4-A	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00																				
M4-B	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00	1.00																			
M4-C	0.59	0.59	0.59	0.66	0.66	0.66	0.97	0.97	0.97	1.00	1.00	1.00																		
M5-A	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00																	
M5-B	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00	1.00																
M5-C	0.95	0.95	0.95	0.51	0.51	0.51	0.59	0.59	0.59	0.60	0.60	0.60	1.00	1.00	1.00															
M6-A	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00														
M6-B	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00	1.00													
M6-C	0.52	0.52	0.52	0.73	0.73	0.73	0.76	0.76	0.76	0.76	0.76	0.76	0.51	0.51	0.51	1.00	1.00	1.00												
M7-A	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00											
M7-B	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00	1.00										
M7-C	0.60	0.60	0.60	0.62	0.62	0.62	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.58	0.58	0.58	1.00	1.00	1.00									
M8-A	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00								
M8-B	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00	1.00							
M8-C	0.56	0.56	0.56	0.60	0.60	0.60	0.64	0.64	0.64	0.63	0.63	0.63	0.54	0.54	0.54	0.57	0.57	0.57	0.89	0.89	0.89	1.00	1.00	1.00						
M9-A	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00					
M9-B	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00	1.00				
M9-C	0.41	0.41	0.41	0.66	0.66	0.66	0.63	0.63	0.63	0.64	0.64	0.64	0.41	0.41	0.41	0.61	0.61	0.61	0.61	0.61	0.61	0.57	0.57	0.57	1.00	1.00	1.00			
M10-A	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00		
M10-B	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00	1.00	
M10-C	0.60	0.60	0.60	0.61	0.61	0.61	0.65	0.65	0.65	0.67	0.67	0.67	0.58	0.58	0.58	0.56	0.56	0.56	0.99	0.99	0.99	0.89	0.89	0.89	0.62	0.62	0.62	1.00	1.00	1.00

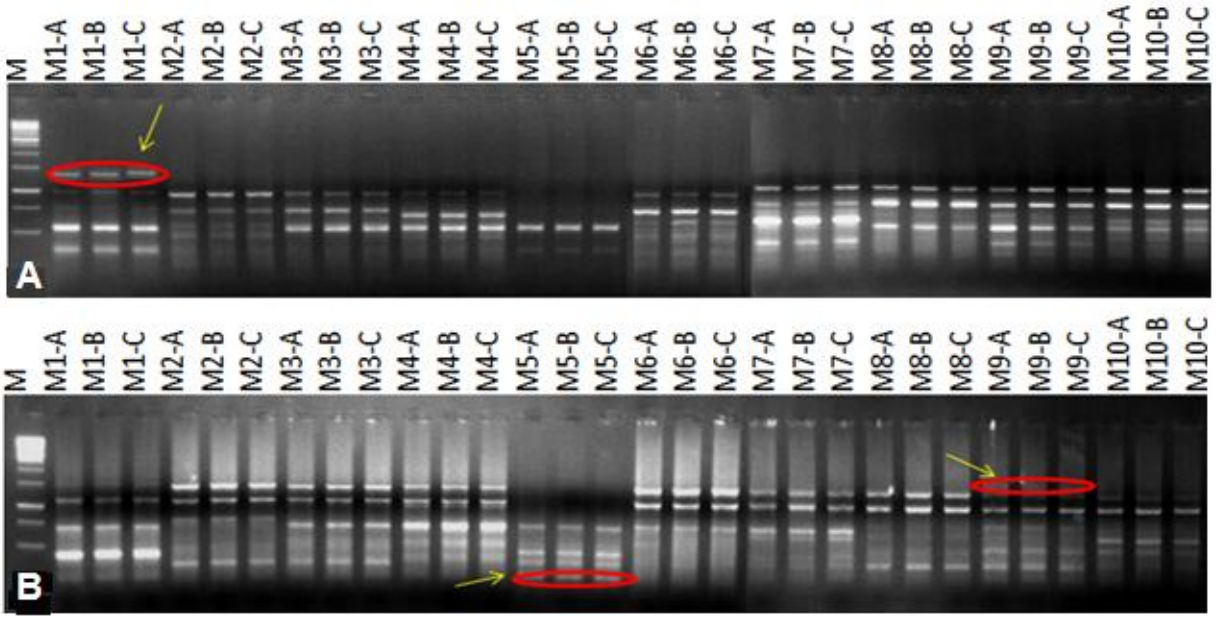

Figure 3. Gel profile of 10 mulberry accessions (ten controls, ten *in vitro* regenerated samples before and after cryopreservation) generated by ISSR primers: [A] UBC- 808 and [E] UBC-858. M is the λ DNA marker. Red circles showing the unique bands.

techniques to examine genetic stability in plants regenerated from cryopreserved dormant buds of mulberry germplasm. Genetic stability is the norm in most studies of possible plant genetic variation following cryopreservation (Harding, 2004). In the present studies, no differences were observed in mulberry between mother plants and *in vitro* regenerated before and after cryopreserved plants using RAPD and ISSR primers. This lack of variation suggests that there were no changes in the genetic fidelity of the plants due to cryopreservation. Cryopreserved yam (*Dioscorea*) shoots were genetically stable when compared to the original *in vitro* cultures (Mandal et al., 2008), and similar results were seen for apple shoot cultures (Liu et al., 2008). In the present study, the RAPD method of assessing genetic stability appeared simple and the reproducible results. However, there is little documentation on the effects of cryopreservation on the genetic stability and agronomic and/-genetic stability of plants regenerated from frozen explants.

RAPD analyses of cryopreserved *in vitro* grown shoot tips of *Prunus* and potato have shown no polymorphism between different amplified DNA patterns (Helliot, 1998; Hirai and Sakai, 2000). Similar results were found in the present study with mulberry dormant buds. The RAPD profiles were reproducible and no differences were found between the DNA patterns obtained with plantlets regenerated from control and cryopreserved plantlets. The RAPD technique therefore appears to be a fast, simple and efficient method for evaluating genetic stability of cryopreserved material, which can be used rapidly after the completion of a freezing experiment and will efficiently complement other genetic stability evaluation methods. Similar results were observed by Zhai et al. (2003) in grape and kiwi cryopreserved plants and found highly reproducible DNA pattern obtained with plantlets regenerated from control and cryopreserved plantlets. Condello et al. (2009) also found similar results after cryopreservation of pear germplasm using RAPD. ISSR markers were successfully applied for detection of genetic similarities or dissimilarities (Vijayan et al., 2006; Lakshmanan et al., 2007). The eleven ISSR primers generated high level of genetic diversity (91.78% polymorphism) in mulberry. Similar results were found in their studies by several researchers (Vijayan, 2004; Vijayan and Chatteerjee, 2003; Naik and Dandin, 2005; Vijayan et al., 2006; Rao et al., 2007, 2009).

Maintenance of genetic stability of cryopreserved germplasm has been reported in Melia (Scocchi et al., 2004); Dioscorea (Dixit et al., 2003); Grape and Kiwi (Zhai et al., 2003). Similarly in our study, plants regenerated from cryopreserved dormant buds were 100% genetically similar. Any accumulative DNA polymorphism may not be induced by cryopreservation (Harding, 2004). Maintenance of true-to-type clonal fidelity is one of the important aspects to be looked into in conservation activities of vegetatively propagated species.

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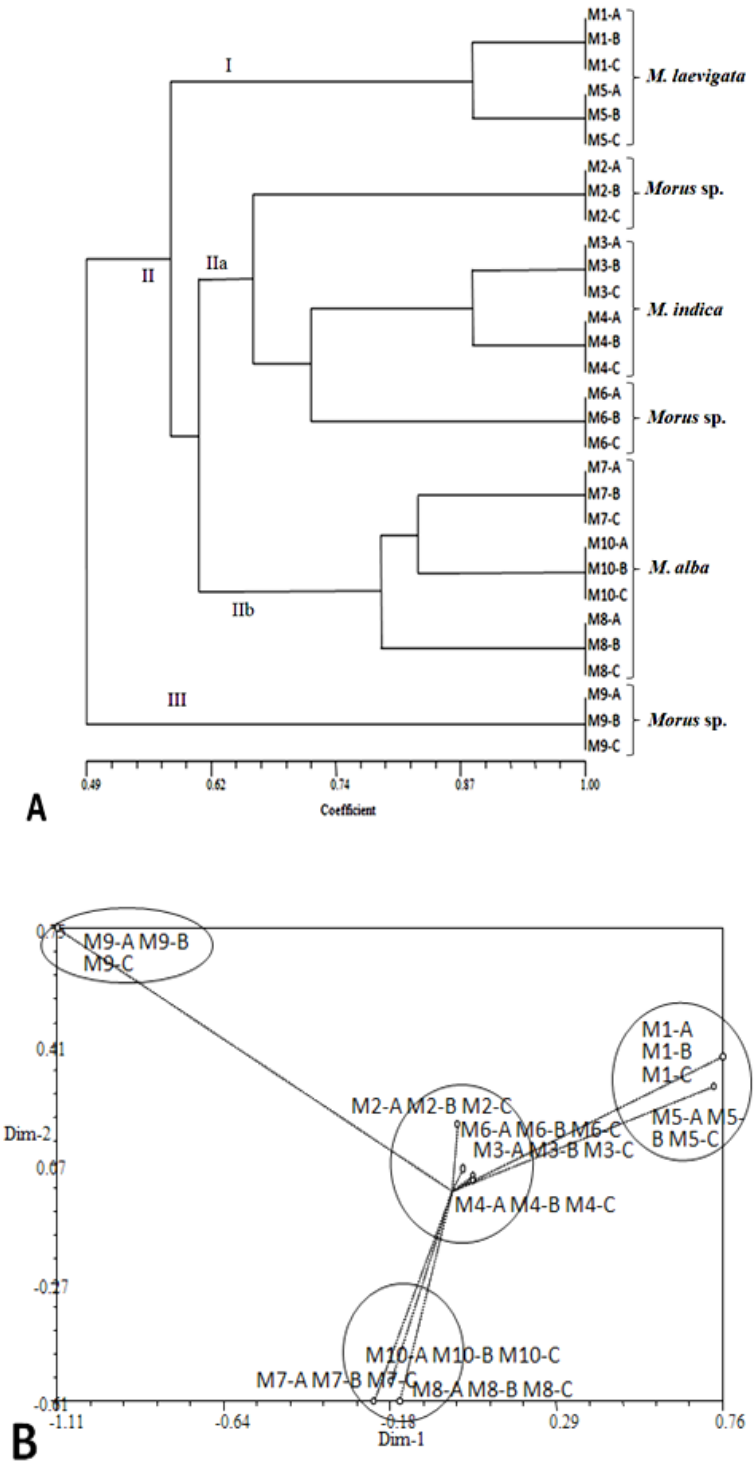

Figure 4. Mulberry [A] UPGMA dendrogram and [B] 2-D plot of 10 mulberry cultivars (ten control and ten *in vitro* regenerated samples before and after cryopreservation) generated based on ISSR data.

REFERENCES

Benavides JE, Lachaux M, Fuentes M (1994). Effect of goat manure application in soil on biomass production and quality of mulberry (*Morus* sp.). In: Benavides, J.E. (Ed.), Trees and Shrubs in Central America, Volume II. CATIE, Turrialba, pp. 495-514.

Collinson ME (1989). The fossil history of the *Moraceae*, *Urticaceae* (including *Cecropiaceae*), and *Cannabaceae*. In: Crane PR.,

Blackmore, S. (Eds), Evolution, Systematics, and Fossil History of the *Hamamelidae*, Vol 2: 'Higher' *Hamamelidae*. Clarendon Press, Oxford. pp. 319-339.
Condello E, Palombi MA, Tonelli MG, Damiano C, Caboni E (2009). Genetic stability of wild pear (*Pyrus pyraster*, Burgsd) after cryopreservation by encapsulation dehydration. Agri. Food Sci.18:136-143.
Dixit S, Mandal BB, Ahuja S, Srivastav PS (2003).Genetic stability assessment of plants regenerated from cryopreserved embryogenic tissue of *Dioscorea bulbifera* L. using RAPD, biochemical and morphological analysis. CryoLett. 24:77-84.
Doyle JJ, Doyle JL (1990). A rapid total DNA preparation procedure for fresh plant tissue. Focus 12:13-15.
FAO (1990). Sericulture training manual. Agricultural Services Bulletin No. 80. FAO, Rome.
Fukui K, Shirata K, Niino T (2011). Cryopreservation of Mulberry Winter Buds in Japan. (International Symposium; 1st, Cryopreservation in horticultural species; Proceedings) Acta Hort. 908:483-488.
Gupta S (2011).Cryopreservation of *in vitro*-grown shoot tips of *Morus* spp. and *Pyrus cossonii* Rehder by encapsulation-dehydration. Acta Hort. 908: 309-318.
Harding K (2004). Genetic integrity of cryopreserved plant cells: A review. CryoLett.25:3-22.
Helliot B (1998). Croissance et Stabilite genetique des Vitroplants de Prunier Ferlenain Plumina (R) apres cryoconservation des meristemes. PhD thesis, no. 2014, University of Bordeaux 1, France.
Hirai D, Sakai A (2000). Cryopreservation techniques. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. Japan Int. Res. Cent. Agri. Sci. 8: 205-211.
Hooker JD (1885). The flora of British India, vol. IV, Secretary of State for India Council, London. p. 185.
Hou YJ (1994). Mulberry Breeding. Sericulture Department, Zhejiang Agriculture University, Hangzhou, p. 4.
Jaccard P (1908). Nouvelles recherches sur la distribution florale. Bulletin de la Societe Vaudoise des Sciences Naturelles 44: 223-270.
Lakshmanan V, Venkataramareddy SR, Neelwarne B (2007). Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electronic J. Biotech.10: 1-8.
Liu YG, Liu LX, Wang L, Gao AY (2008). Determination of genetic stability in surviving apple shoots following cryopreservation by vitrification. CryoLett. 29: 7-14.
Machii H, Koyama A, Yamanouchi H (1999): A list of genetic mulberry resources maintained at National Institute of Sericultural and Entomological Science. Miscellaneous Publication Nat. Seri. Entomol. Sci. 26:1-77. (in Japanese)
Mandal BB, Ahuja-Ghosh S, Srivastava PS (2008). Cryopreservation of *Discorea rotundata* Poir. A comparative study with two cryogenic procedures and assessment of true-to-type of regenerants by RAPD analysis. CryoLett. 29: 399-408.
Mantel NA (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209-220.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco cultures. Physiol. Plant. 15: 473-497.
Naik GV, Dandin SB (2005). Molecular characterization of some improved and promising mulberry varieties (*Morus* spp.) of India by RAPD and ISSR Markers. Ind. J. Seri. 441:59-68.
Niino T (1995).Cryopreservation of germplasm of mulberry (*Morus* spp.). In: Bajaj,Y.P.S.(Ed.):Biotechnology in Agriculture and Forestry. Springer-Verlag, Berlin, pp. 102-113.
Padró MDA, Frattarelli A, Agueglia A, Condello E, Damiano C, Caboni E (2012). Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. Plant Cell, Tiss Org. Cult. 108: 167-172.
Rao AA, Chaudhury R, Kumar S, Velu D, Saraswat RP, Kamble CK (2007). Cryopreservation of mulberry germplasm core collection and assessment of genetic stability through ISSR markers. Int. J. Indust. Entomo. 15: 23-33.
Rao AA, Chaudhury R, Malik S, Kumar S, Ramachandran R, Qadri SMH (2009). Mulberry biodiversity conservation through cryopreservation. *In Vitro* Cell. Develop. Biol.- Pl. 45:639-649**.**
Rohlf FJ (2000). NTSYS-pc: numerical taxonomy and multivariate analysis system, ver. 2.1. Exeter software, Setauket.
Sanjappa M (1989). Genetic resources of mulberry and utilization. In: Sengupta, K., Dandin, S.B. (Eds.) Geographical distribution and exploration of the genus *Morus* L. (Moraceae), CSRTI, Mysore, India, pp. 4-7.
Scocchi A, Faloci M, Medina R, Olmos S, Mroginski L (2004). Plant recovery of cryopreserved apical meristems-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. Euphytica 135: 29-38.
Srivastava P, Vijayan K, Awasthi AK, Saratechandra B (2004). Genetic analysis of *Morus alba* through RAPD and ISSR markers. Ind. J. Biotech. 3: 527-532.
Tikader A, Dandin SB (2005). Biodiversity, geographical distribution, utilization and conservation of wild mulberry *Morus serrata* Roxb. Caspian J. Environ. Sci. 3:177-184.
Tikader A, Vijayan K (2010). Assessment of biodiversity and strategies for conservation of genetic resources in mulberry (*Morus* spp.). In: Nageswara-Rao, M., Soneji, J.R. (Eds), Tree and Forest Biodiversity. Bioremed. Biodiv. Bioavail. 4 (Special Issue 1): 15-27.
Vijayan K (2004). Genetic relationships of Japanese and Indian mulberry (*Morus* spp.) revealed by DNA fingerprinting. Plant Systemat. Evol. 243:221-232.
Vijayan K (2010). The emerging role of genomics tools in mulberry (*Morus*) genetic .improvement. Tree Genet. Genom. 6: 613-625.
Vijayan K, Chatterjee SN (2003). ISSR proofing of Indian cultivars of mulberry (*Morus* spp.) and its relevance to breeding programs. Euphytica 131:53-63.
Vijayan K, Kar PK, Tikader A, Srivastava PP, Awasthi AK, Thangavelu K, Saratchandra B (2004). Molecular evaluation of genetic variability in wild populations of mulberry (*Morus serrata* Roxb.). Plant Breed. 123: 568-572.
Vijayan K, Nair CV, Chatterjee SN (2005). Molecular characterization of mulberry genetic resources indigenous to India. Genet. Resour. Crop Evol. 52:77-86.
Vijayan K**,** Srivastava PP, Raghunath MK, Sarat-chandra B (2011). Enhancement of stress tolerance in mulberry. Sci. Hort. 129:511-519.
Vijayan K, Tikader A, Kar PK, Srivastava PP, Awasthi AK, Thangavelu K (2006). Assessment of genetic relationships between wild and cultivated mulberry (*Morus*) species using PCR based markers. Genet. Resour. Crop Evol. 53: 873-882.
Wang ZW, Yu MD (2001). AFLP analysis of genetic background of polyploid breeding materials of mulberry. Acta Sericol. Sinica 27:170-176.
Williams JGK, Kubelil AR, Livak KJ, Rafalski JA, Tingey VS (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic masters. Nucleic Acid Res. 18: 6531-6535.
Withers LA, Engelmann F (1997). *In vitro* conservation of plant genetic resources. In: Altman, A. (Ed.): Biotechnology in Agriculture. Marcel Dekker, New York. pp. 57-88.
Yakuwa H, Oka S (1988). Plant regeneration through meristem culture from vegetative buds of mulberry (*Morus bombycis* Koidz.) stored in liquid nitrogen. Ann. Bot. 62:79-82.
Yaltirik F (1982). *Morus.* In: Davis, P.H. (Ed.), Flora of Turkey. Edinburgh University Press, Edinburgh.
Zerega NJ, Clement WL, Datwyler SL, Weiblen GD (2005). Biogeography and divergence times in the mulberry family (Moraceae). Mol. Phylogene. Evol. 37: 402-416Y, Huang Y (2006). Genetic relatedness among cultivated and wild mulberry (Moraceae: *Morus*) as revealed by inter-simple sequence repeat (ISSR) analysis in China. Canadian J. Plant Sci. 86:251-257.
Zhai Z, Wu Y, Engelmann F, Chen R, Zhao Y (2003). Genetic stability assessments of plantlets regenerated from cryopreserved *in vitro* cultured grape and kiwi shoot tips using RAPD. CryoLett. 24:315-322.
Zhao W, Zhou Z, Miao X, Wang S, Zhang L, Pan Anonymous (2006). The Wealth of India- A Dictionary of Indian Raw Materials and Industrial products. Second supplement series. (Raw materials) Vol.1; A-F; NISCAIR, CSIR, Dr. K S Krishnan Marg N D--12, pp.188-201.

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Expression of an engineered tandem-repeat starch-binding domain in sweet potato plants

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In this study, the transgenic sweet potato Xu55-2 modified with an engineered tandem repeat of a family 20 starch binding domain (SBD2) was analyzed by Western dot blot to investigate whether SBD2 proteins are capable of granule-targeting during starch biosynthesis. Furthermore, the impact of SBD2 accumulation in granules on the physicochemical properties of the transgenic starches was also investigated. Our results demonstrate that the high levels of SBD2 protein could be accumulated in granules. The SBD2 expression affect granule morphology without altering the primary structure of the constituent starch molecules, suggesting that SBD2 could be used as an anchor for effector proteins to sweet potato starch granules during biosynthesis.

Key words: Sweet potato (*Ipomoea batatas*), tandem starch-binding domain, transgenic starch, granule morphology.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is one of the important starchy resources in Asia. It accounts for over 80% of world sweet potato starch production, most of which are in China. Every year, over 10% of the production of 100 million tons of sweet potato in China is processed into starch. Sweet potato starch is used in the manufacture of sweeteners, sizing of paper and textile, production of bio-ethanol (Chen et al., 2003; Tian et al., 2009; Yang et al., 2011) and it is particularly valued as a food starch used in starch noodles, bakery foods and snack foods production (Chen et al., 2003; Kitahara et al., 2007). The use of sweet potato starch is primarily determined by its physic-chemical properties. However, there are almost no natural starches with essential properties for a particular application. Thus, different modifications must be made before applying natural starch.

Improvement of natural starch properties for industrial purposes can be achieved by chemical or physical modification after isolation, but also through the *in planta* modification. Over the years, a number of starches with new or improved properties such as amylose-free starches (Kuipers et al., 1994; Noda et al., 2002; Kitahara et al., 2007), high-amylose starches (Schwall et al., 2000; Kitahara et al., 2007) and a heavily-branched amylo-

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Abbreviations: SBD2, Tandem starch-binding domain; **CGTase,** cyclodextrin glycosyl-transferase; **GBSS I,** granule bound starch synthase I; **PVDF,** polyvinylidene-fluoride; **AEC,** 3-amino-9-ethylcarbazole; **AM,** apparent amylose; **LM,** light microscopy; **SEM,** scanning electron microscopy; **DSC,** differential scanning calorimetry; **XRD,** x-ray diffractometer.

pectin starch (Kortstee et al., 1996) have been obtained by either inhibition of native genes or expression of foreign genes. The examples mentioned above show that genetic engineering may provide a promising approach to produce novel starches with the desired properties *in planta*. In previous study, we developed a starch-binding domain (SBD)-technology for modification starches with new or tailor-made properties, in which microbial SBD-encoding region of cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* is used as an anchor to target recombinant proteins into granules during the starch biosynthesis (Ji et al., 2003). Moreover, an engineered tandem repeat of SBD (SBD2) was expressed in potato, and the SBD2 shows much higher affinity for starch granules than single SBD, indicating that the SBD2 can be used as an alternative for making SBD fusion proteins (Ji et al., 2004). Sequentially, the SBD2 construct has been introduced into a sweet potato cultivar Xu55-2 to investigate whether the SBD2 can also be used as an anchor for its starch modification. The presence of the *SBD2* gene in the genomic DNA of transgenic sweet potatoes has been verified by polymerase chain reaction (PCR) amplification and confirmed by Southern blot analysis (Xing et al., 2008).

In this study, the transgenic sweet potatoes modified with the *SBD2* gene was investigated to determine whether SBD2 protein can accumulate in granules with a high level during starch biosynthesis, and whether accumulation of the SBD2 into granules has effects on the physicochemical properties of the transgenic starches.

MATERIALS AND METHODS

Preparation of transgenic plants

Agrobacterium tumefaciens strain EHA105 carrying the plasmid pBIN19/SBD2 was transformed into sweet potato cultivar Xu55-2 (Xing et al., 2008). The *SBD2* gene, in which two copies of SBD is linked through an artificial proline-threonine (PT)-rich linker, is expressed in sweet potato plants under the control of the tuber-specific potato granule bound starch synthase I (GBSS I) promoter. Amyloplast entry of SBD2 is mediated by the potato GBSS I transit peptide (Ji et al., 2004). Transgenic sweet potatoes Xu55-2 modified with the *SBD2* gene have been obtained in a previous study. The presence of the *SBD2* gene in the genomic DNA of 12 transgenic clones has been verified by PCR amplification and confirmed by Southern blot analysis (Xing et al., 2008). The resulting transgenic plants are referred to as 55-2-SSxx (where 55-2 represents sweet potato cultivar Xu55-2, SS represents the *SBD2* gene and xx represents the clone in a series of transformant). Untransformed control plants are referred to as 55-2-UT. In this study, the transgenic plants were clonally propagated and five plants of each transgenic clone were transferred to the greenhouse for tuberous-roots development. In addition, 10 untransformed controls were grown in the greenhouse.

Isolation of tuberous-root starch

All tuberous-roots from the five plants of each clone were combined, and the peeled roots were cut into small pieces and homogenized in a blender. The homogenate was filtered through a cheese-cloth to remove particulate material and allowed to settle for 20 min at 4°C. Subsequently, the root juice was collected and stored at -20°C for later use. The starch sediment was washed three times with distilled water, and air-dried at room temperature.

Western dot blot analysis

The amount of SBD2 proteins accumulated in transgenic starches was estimated with a Western dot blot procedure as described by Ji et al. (2003). 20 mg of (transgenic) starch was boiled for 5 min with 200 µL of a 2× sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970). After cooling to room temperature, the SBD2 proteins in the transgenic starch gels were electro-blotted onto the polyvinylidene-fluoride (PVDF) membrane (Roche, Germany) using a Fastblot B33 (Whatman Biometra, Germany; 150 mA, 4°C, 5 h). A 1:500 dilution of antiSBD antibody (Ji et al., 2003) was used as the primary antibody, and a 1:1000 dilution of Goat Anti-Rabbit IgG horseradish peroxidase (HRP) conjugate (Boster, China) was used as the secondary antibody for detection. The blot was stained with a 3-amino-9-ethylcarbazole (AEC) solution.

To investigate whether SBD2 protein was present in the solution fraction of tuberous-root, A 500 µL of juice sample was dried in a freeze-drier (LNG-T98, China). The resulting material was dissolved in 200 µL of a 2× SDS sample buffer. In order to make the sample suitable for the Western dot blot procedure, the mixture was boiled for 5 min in the presence of 20 mg starch from the untransformed control (Ji et al., 2004). The rest of the procedure was conducted in the same way as described above for root starch analysis.

Analysis of physicochemical properties of starch granules

Starch granule morphology was observed by light microscopy (LM; BX41-32H02, Japan) and scanning electron microscopy (SEM; HITACHI S-3000N, Japan). For light microscopy, starch granules were stained with a 20× diluted 1% I_2/KI solution. For scanning electron microscopy, starch samples were dried and coated with gold on the ion sputter (E-1010, Hitachi, Japan). Average granule size distribution of the transgenic starches was determined in triplicate with a particle size analyzer (CIS-50, Ankersmid, The Netherlands) according to the manufacturer's instructions. A 10 mg of starch sample was suspended in 100 µL of 20% iso-propanol. The apparent amylose content was determined in triplicate following the method as described by Hovenkamp-Hermelink et al. (1989).

Gelatinization properties of starch were determined by using a differential scanning calorimetry (DSC; DSC 27, PerkinElmer, USA). A 2.5 mg starch sample (dry basis) was weighed in aluminum sample pans and mixed with 7.5 µL of distilled water. The pan was sealed and the sample was equilibrated for 24 h at room temperature. The samples were heated from 30 to 110°C at a scanning rate of 10°C/min. An empty sample pan was used as a reference. For each endotherm, the onset temperature of gelatinization (T_0) and the difference in enthalpy (ΔH) were computed automatically. Crystallinity of starch granules was analyzed by an X-ray diffractometer (XRD; Scintag XDS 2000, USA). Prior to X-ray diffraction, the water content of the starch was equilibrated at 100% of relative humidity at room temperature in presence of iso-propanol. A 20 mg of starch sample was then sealed between two tape foils to prevent any significant change in water content during the measurement. X-ray diffraction patterns were obtained by the XRD working at 35 kV, 20 mA and producing Cu-k_α radiation at a wave-length of about 0.15405 nm. Starch

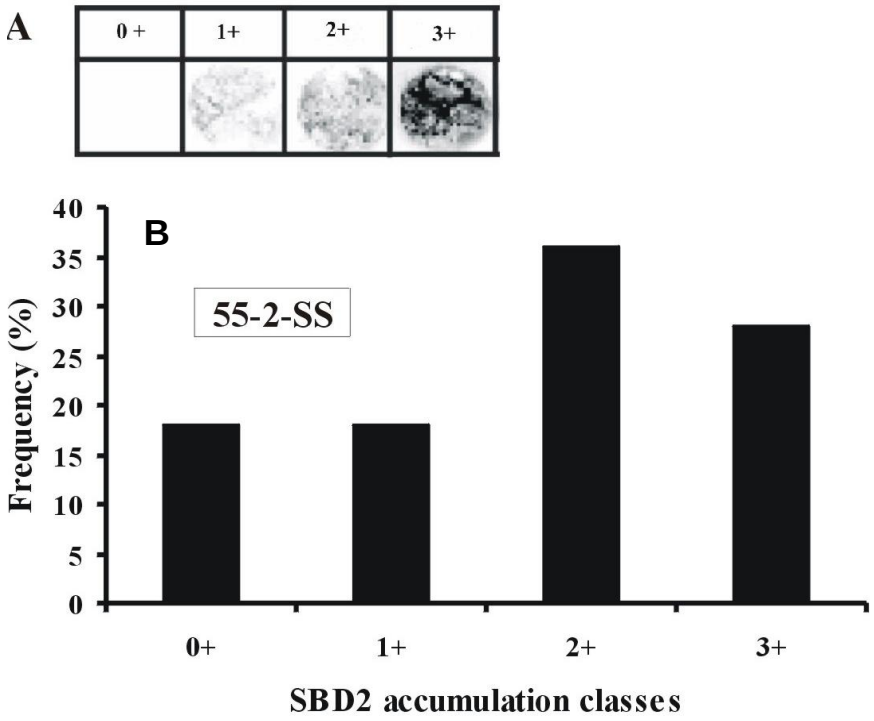

Figure 1. Accumulation levels of SBD2 in transgenic sweet potato starch granules. (A) The classes of SBD2 accumulation in starch granules, where the 0+,1+,2+ and 3+ classes represent no, low, intermediate and high levels of SBD2 accumulation, respectively. (B) The distribution of sweet potato transformants over the classes of SBD2 accumulation in the 55-2-SS series.

samples were scanned from 4 and 40º (2θ) at increments of 0.04º.

RESULTS

Preparation of transgenic plants

The 12 transgenic sweet potato clones obtained in a previous study have been analyzed to confirm the integration of the *SBD2* gene into the genomic DNA by PCR amplification and confirmed by Southern blot analysis (Xing et al., 2008). Five transgenic plants from each clone were multiplied and grown in the greenhouse to generate tuberous-roots. The morphology of plants and roots, as well as the root-yield, revealed no consistent differences between transformed and control plants (results not shown).

Western dot blot analysis

The levels of SBD2 accumulation in transgenic granules were analyzed by Western dot blot. The SBD2 accumulating clones were divided into four classes (ranging from 0+ to 3+) based on the amount of SBD2 protein associated with the starch granules, in which 0+, 1+, 2+ and 3+ represent no, low, intermediate and high accumulation levels, respectively (Figure 1A). SBD2 accumulation levels in the transgenic sweet potato starches are summarized in Figure 1B. The results clearly show that the highest level of SBD2 accumulation in sweet potato starch granules was the 3+ class. However, transformants belonging to the 2+ class were more abundant than in other classes. The results demonstrate that SBD2 protein could be accumulated in sweet potato starch granules during the biosynthesis process. The SBD2 protein concentration in the soluble fraction of roots from each class was also determined by Western dot blot. Table 1 shows the analysis results from one representative of each class. For comparison, the levels of SBD2 accumulation in relative transgenic granules were also indicated in the table. It appeared that SBD2 was only found in the root juices of transformants belonging to the 3+ class (the highest accumulator). The amount of unbound SBD2 in the root juice was 2+, corresponding to the dot with an intensity of 2+ in Figure 1A. This suggested that the amount of SBD2 in the 3+ class in starch granules was saturating.

Table 1. Accumulation levels of SBD2 protein in granules and juice of selected 55-2-SS transgenic roots, as estimated with Western dot blot analysis.

Clone	Amount of SBD2	
	In granules	In juice
55-2-SS8	0+	n.d[a]
55-2-SS7	1+	n.d
55-2-SS2	2+	n.d
55-2-SS4	3+	2+

[a]Not detected. The number representation (1+, etc) is according to the dot intensities in Figure 1A.

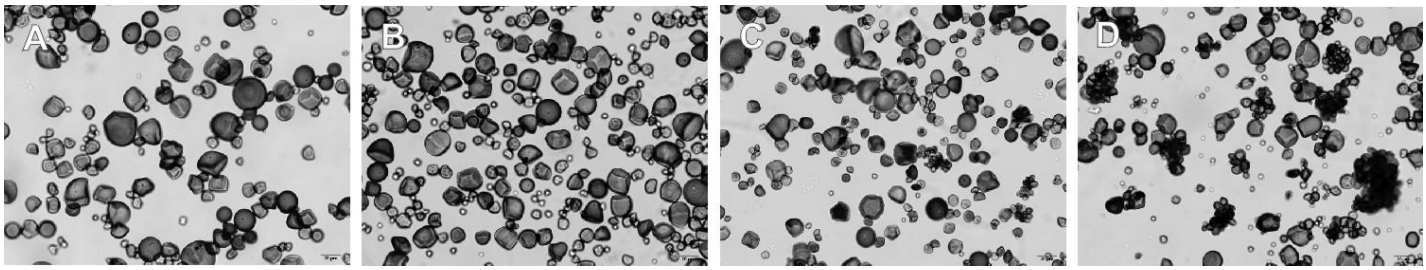

Figure 2. Light micrographs of 55-2-SS transgenic and untransformed starch granules: **(A)** 55-2-UT. **(B)** 55-2-SS7, 1+. **(C)** 55-2-SS2, 2+. **(D)** 55-2-SS4, 3+. Magnification of the starch granules: 200x.

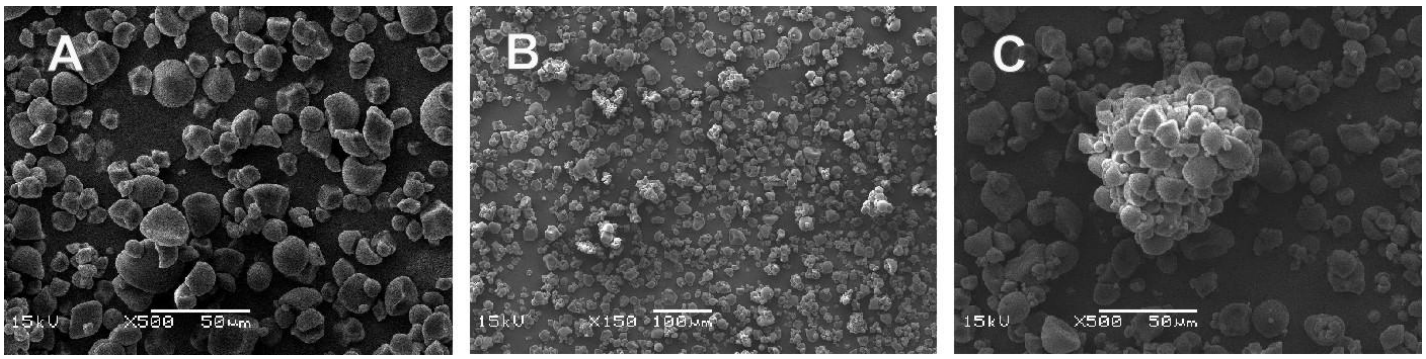

Figure 3. Scanning electron micrographs of transgenic and untransformed starch granules. **(A)** 55-2-UT. **(B)** and **(C)** 55-2-SS4, 3+.

Granule morphology

Starch granule morphology of each 55-2-SS transformed clone in the series was investigated by light microscopy. The micrographs of transgenic granules from one representative of each class and their control are shown in Figure 2. It can be seen that the morphology of 55-2-UT and 55-2-SS7 (1+) starch granules was more or less similar (Figures 2A and B), whereas higher SBD2 accumulation levels (2+ and 3+) in transgenic starch granules showed altered morphology in comparison with the control. Granules were sometimes organized in large clusters of many small ones (further referred to as “large clusters”) (Figures 2C and D). It seemed that this phenomenon was most pronounced in 55-2-SS4 (3+), although it could also be observed in 55-2-SS2 (2+). Based on the LM results, starches from 55-2-SS4 and the control were further investigated by scanning electron microscopy (Figure 3). The scanning electron micrographs of 55-2-SS4 granules (Figures 3B and C) revealed that large clusters were more loosely associated, suggesting that SBD2 accumulation could affect morpho-

Table 2. Gelatinization characteristics (T_0, ΔH), apparent amylose content AM (%), crystallinity (%) and starch granule size (d_{50}) measurements of starches from transgenic clones and the control.

Clone	AM (%)	T_0 (°C)[a]	ΔH (kJ/g)[b]	Crystallinity (%)	d_{50} (µm)[c]
55-2-UT	22.4 (±2.4)	68.1	9.9	31.2	15.3 (±0.4)
55-2-SS7(1+)	23.1 (±0.9)	66.9	9.6	30.5	14.1 (±1.1)
55-2-SS2(2+)	21.6 (±1.2)	68.5	10.0	31.9	13.7 (±1.4)
55-2-SS4(3+)	22.6 (±1.5)	67.4	9.9	30.9	14.8(±0.6)

[a]Temperature at onset of starch gelatinization, [b]enthalpy released, [c]median value of the granule size distribution. Data (±SD) are the average of three independent measurements.

logy of starch granule, at least when it was present in a high level.

Characterization of transgenic starches

The impact of SBD2 accumulation in granules on the physicochemical properties of starch was also investigated. Within the transgenic series, one transformant in each SBD2 accumulation class (0+, 1+, 2+ and 3+) was selected for further study. The untransformed starch was used as a control. Table 2 summarizes the results on starch granule size (d_{50}), granule-melting behaviour (T_0 and ΔH), crystallinity, and apparent amylose content (AM%).

From the table, it can be seen that no consistent difference was observed between transgenic starches and their control, sug-gesting that the altered granule morphology did not seem to lead to different physico-chemical properties of the granules. However, it should be noted although 55-2-SS4 and 55-2-UT starches gave similar results with respect to granule size (d_{50}), the granule size distribution of 55-2-SS4 (3+) was relatively wide compared to that of 55-2-UT (result not shown), which was in accordance with the SEM results (Figure 3B). Although it seemed that the large cluster granules of 55-2-SS4 dominated the SEM at first sight, their abundance was low compared with the small granules, and consequently they contributed relatively little to the granule size distribution.

DISCUSSION

In this study, the transgenic sweet potato modified with the engineered tandem-repeat starch-binding domain (SBD2) was investigated to determine if the SBD2 could be used as an anchor for the modification of starch. Our data shows that high levels of SBD2 protein could be accumulated in granules during starch biosynthesis, indicating that SBD2 could be used as tools to anchor effector proteins in sweet potato starch granules. In our previous study, the *SBD2* gene has been introduced into the amylose-containing potato genotype Kardal, and the highest levels of SBD2 accumulation in starch granules is 4+ (Firouzabadi et al., 2007). Sweet potato cultivar Xu55-2 used in this study is also an amylose-containing genotype. However, the highest level of SBD2 accumulation (3+) in transgenic sweet potato starch was lower than that in potato Kardal. This might be caused by using the potato GBSS I promoter and transit peptide sequence, which might not be suitable for expression of foreign genes in sweet potato starch granules.

The expression of SBD2 in sweet potato plants resulted in some clones containing starch with “large clusters” granules. Its occurrence seemed to be correlated with the amount of SBD2 accumulated in the granule, because they were most pronounced in 55-2-SS4 (3+ class). Interestingly, the “large clusters” granules is not observed when SBD2 is expressed in potato Kardal background, but large “amalgamated clusters” ones are encountered in transformants belonging to the 3+ class (Firouzabadi et al., 2007).

We know that both sweet potato Xu55-2 and potato Kardal are amylose-containing genotype and that there is presence of another granule-bound protein GBSS I (granule-bound starch synthase I) in the granules. When SBD2 is expressed in starch granules, SBD2 and GBSS I proteins are simultaneously present. We postulate that, in this situation, they might bind similar sites of the growing starch granule and the SBD2 has a higher affinity for starch granules than GBSS I.

As a consequence, GBSS I can be out-competed by the SBD2 protein in the granules. Additionally, it is known that there is a difference in size between potato and sweet potato starch granules. The mean granule size of potato Kardal is 27.9 µm (Ji et al., 2003), whereas that of sweet potato Xu55-2 was 15.7 µm. The former is approximately two times bigger than the latter. Thus, when SBD2 accumulation level in both starch granules is the same (3+ class), the surface of potato starch granules has more sites for both SBD2 and GBSS I binding. In this case, the surface of some granules is mostly covered by SBD2, in which due to the limitation of surface area, both SBD domains can not be accommodated on the same surface anymore.

Consequently, the SBD2 proteins may have only one SBD attached to a binding site, whereas the exposed SBD of SBD2 is available for interaction with amylose-like molecules produced by GBSS I, and/or with different growing granules (Firouzabadi et al., 2007). In this situation, the enzymes involved in starch biosynthesis are not hindered greatly in attaching material from the stroma to the growing granule. If the exposed SBD of SBD2 binds amylose-like molecules from a different nucleation sits, amylose may effectively fill the gap between loosely associated small granules, then the large “amalgamated clusters” granules may be formed in potato Kardal starch (Firouzabadi et al., 2007).

For sweet potato starch, the surface of granules is smaller and has fewer sites for both proteins binding. We speculate that, at the 3+ class of SBD2 accumulation, the surface of some granule might be fully covered by SBD2, in which also only one of SBD2 attached to a binding site. In this case, the SBD2 might hinder synthases from elongation amylopectin side-chains at the granule surface, and/or synthases from attaching side chains to the growing granule. Thus, small granule formation and additional nucleation sites for granule formation might be enforced by SBD2 (Ji et al., 2004), but it is possible to produce smaller starch granules without accompanying changes in contents of amylose and amylopectin. If the exposed SBD of SBD2 stuck to different growing granules, which might facilitate cross-linking of the small granules, the loosely associated “large clusters” might be formed (Figures 3B and C). This is also the reason why the analysis results of apparent amylose in the 55-2-SS4 starch did not reveal a reduction in the content in comparison with the control.

Except for the alteration of transgenic starch granule morphology, no consistent differences in the melting temperature of the granules, granule size distribution, crystallinity and apparent amylose content between transgenic SBD2 starches and the control were found, suggesting that SBD2 could be used as an anchor for targeting starch-modifying proteins to granules without having other side-effects. This study provides good perspectives for the applicability of SBD technology for sweet potato starch bioengineering. Our future research will focus on developing a number of applications in which SBD2 is used as an anchor to incorporate effector proteins in sweet potato starch granules.

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REFERENCES

Chen ZH, Schols HA, Voragen AGJ (2003). Physicochemical properties of starches obtained from three varieties of chinese sweet potato. J. Food Sci. 68:431-437.

Firouzabadi FN, vincken JP, Ji Q, Suurs LCJM, Buléon A, Visser RGF (2007). Accumulation of multiple-repeat starch-binding domains (SBD2-SBD5) does not reduce amylose content of potato starch granules. Planta 225:919-933.

Hovenkamp-Hermelink JHM, de Vries JN, Adamse P, Jacobsen E, Witholt B, Feenstra WJ (1989). Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue of potato. Potato Res. 31:241-246.

Ji Q, Oomen RJFJ, Vincken JP, Bolam DNB, Gilbert HJ, Suurs CJM, Visser RGF (2004). Reduction of starch granule size by expression of an engineered tandem starch-binding domain in potato plants. Plant Biotechnol. J. 2:251-260.

Ji Q, Vincken JP, Suurs CJM, Visser RGF (2003). Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis. Plant Mol. Biol. 51:789-801.

Kitahara K, Hamasuna K, Nozuma K, Otani M, Hamada T, Shimada T, Fujita K, Suganuma T (2007). Physicochemical properties of amylose-free and high-amylose starches from transgenic sweetpotatoes modified by RNA interference. Carbohyd. Polym. 69:233-240.

Kortstee AJ, Vermeesch AMG, de Vries BJ, Jacobsen E, Visser RGF (1996). Expression of *Escherichia coli* branching enzyme in tubers of amylose-free potato leads to an increased branching degree of the amylopectin. Plant J. 10:83-90.

Kuipers A, Jacobsen E, Visser R (1994). Formation and deposition of amylose in the potato tuber granule are affected by the reduction of granule-bound starch synthase gene expression. Plant Cell 6(1):43-52.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Noda T, Kimura T, Otani M, Ideta O, Shimada T, Saito A, Suda I (2002). Physicochemical properties of amylose-free starch from transgenic sweet potato. Carbohyd. Polym. 49:253-260.

Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, Shi Y-C, Gidley MJ, Jobling SA (2000). Production of very-high-amylose potato starch by inhibition of SBE A and B. Nat. Biotechnol. 18:551-554.

Tian Y, Zhao L, Meng H, Sun L, Yan J (2009). Estimation of un-used land potential for biofuels development in (the) People’s Republic of China. Appl. Energy S77-S85.

Xing YJ, Ji Q, Yang Q, Luo YM, Li Q, Wang X (2008). Studies on *Agrobacterium*-Mediated Genetic Transformation of Embryogenic Suspension Cultures of Sweet Potato. Afr. J. Biotechnol. 7(5):534-540.

Yang J, Bi HP, Fan WJ, Zhang M, Wang HX, Zhang P (2011). Efficient embryogenic suspension culturing and rapid transformation of a range of elite genotypes of sweet potato (*Ipomoea batatas* [L.] Lam.). Plant Sci. 181(6):701-711.

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Cytogenotoxic effects of cypermethrin, deltamethrin, lambdacyhalothrin and endosulfan pesticides on *Allium cepa* root cells

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Increased pesticides application in agriculture and public health has contributed to the pollution of the environment. This study evaluates the cytogenotoxic effects of emulsifiable concentrate of cypermethrin, deltamethrin, lambdacyhalothrin and endosulfan on *Allium cepa* root cells. Five concentrations (1.0, 5.0, 10.0, 20.0 and 40.0 ppm) of each pesticide were used for microscopic (48 h) and macroscopic (72 h) evaluations with distilled water as the control. Data were analyzed by Student's t-test. A dose dependent reduction in *A. cepa* root length was observed for the pesticides. Significant reduction in treated root length was observed at 10.0 ppm of deltamethrin, cypermethrin and lambdacyhalothrin, and at 20.0 and 40.0 ppm of all the pesticides compared to the control ($P<0.05$). The EC_{50} values showed growth inhibition in the order of lambdacyhalothrin > cypermethrin > deltamethrin > endosulfan, while that of total aberrant cells was cypermethrin > lambdacyhalothrin > deltamethrin > endosulfan. Microscopic aberrations observed in the pesticide-treated onions include sticky chromosomes, disturbed spindle and chromosome bridges. Dose dependent reduction was observed in the total mitotic dividing cells and mitotic index of the pesticide-treated *A. cepa,* except for 5.0 ppm of endosulfan. The pesticides induced growth inhibition and caused cytogenotoxic effects on the meristematic cells of *Allium cepa.* The data herein provide more information on the pesticides of which exposure to substantial concentration might constitute health risk to non-target organisms.

Key words: Pesticides, mitotic aberration, pyrethroid, organochlorine, growth.

INTRODUCTION

Pesticides are used to exterminate pests in order to increase yield and improve the shelf life of agricultural products. Besides, they are used in public health to reduce morbidity and mortality from pest related diseases. In recent years, there has been a tremendous increase in the use of these chemicals without paying much attention to the adverse effects they may have due to the toxic ingredients (Badr and Ibrahim, 1987; Anis et al., 1998). Reports have shown organochlorine pesticides like endosulfan to be toxic and have potential to be bioaccumulated in the environment and run off from field application of endosulfan leads to aquatic pollution. Animals that live in endosulfan-contaminated waters can bioaccumulate endosulfan in their bodies, the amount of which may be several times greater than in the surrounding water (ATSDR, 2008). Endosulfan has been reported to alter haematological profile in animals (Gimeno et al., 1994; Das et al., 2010; Modaresi and Seif, 2011; Yekeen and Fawole, 2011). Its accumulation in the environment led to its ban in most developed countries. However, it is still being used in most of the developing countries. Endosulfan is highly toxic and due to its persistence in the environment, its harmful effects are expected to manifest even in future generation of exposed population (Kumar and Chaudhary, 2012).

Bioaccumulative effects of organochlorine and high

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toxic effects of organophosphates especially on non-target organisms led to the increase use of pyrethroids as a potential alternative. Lambdacyhalothrin, deltamethrin and cypermethrin are type II pyrethroids extensively used in agriculture. Pyrethroids are also used in public health to reduce malaria morbidity and mortality (Zaim et al., 2000).

Although technical grades of pyrethroids were reported to have less to no toxic effects on non-target organisms, emulsifiable concentrate formulations of pyrethroids were two to nine times more toxic compared to the technical grades (Sanchez-Fortun and Barahona, 2005). Evaluations of some pyrethroids through different biological endpoints in animals show that they cause alteration in the haematological profile of exposed animals (Gimeno et al., 1994; Yekeen et al., 2007; Khan et al., 2012; Yekeen et al., 2013, Muthuviveganandave et al., 2013). Cypermethrin caused significant increase in chromosome aberration and in micronucleated erythrocytes frequency in farm workers (Carbonell et al., 1995; Lander et al., 2000). DNA damage was detected in tissue of workers involved in the production of cypermethrin (Grover et al., 2003).

Deltamethrin as a synthetic dibromo-pyrethroid insecticide and acaricide has been known to be three times more powerful than some other pyrethroids (Bradbury and Coats, 1989), which enhances its usage both indoor and outdoor. Cabral et al. (1990) reported that deltamethrin does not appear to be carcinogenic in mice or rats, while a very low dose of deltamethrin dis-plays harmful effects by disrupting hepatic and renal function and cause DNA damages in pubescent female rats (Chargui et al., 2012).

A non-significant induction of sperm cell aberra-ions in mice was reported for emulsifiable concentrate form of deltamethrin (Yekeen et al., 2007). Lambdacyhalothrin is used in public and animal health applications where it effectively controls a broad spectrum of insects and ectoparasites (Davies et al., 2000). The cytogenetic effects of lambdacyhalothrin were investigated in humans and various animal species using different endpoints such as micronucleus (MN) formation, induction of chromosomal aberrations and sister chromatid exchange (Fahmy and Abdalla, 2001; Celik et al., 2005), while studies on plant assay are limited.

The present study sought to evaluate the cytotoxic effects of cypermethrin, deltamethrin, lambdacyhalothrin and endosulfan in *Allium cepa*. This plant assay was selected because it is cost effective and as reliable as other methods for evaluation of chromosome aberrations (Rank and Nielsen, 1997) and can be easily used to assess toxicity via effective concentration determination (Yildiz and Arikan, 2008).

MATERIALS AND METHODS

Test chemicals

All pesticides were procured in the form (emulsifiable concentrate) commonly available in the market and widely used: Thionex® 35 EC (350 g/L) for endosulfan, Karate ® 2.5 EC for lambda-cyhalothrin, Deltaforce ® 2.5% EC for deltamethrin, and 10% EC for cypermethrin. Carmine salt was purchased from Zayo Sigma Chemicals Limited, Nigeria. All other chemicals used were of analytical grade.

Allium cepa assay

The onion bulbs (*Allium cepa* L.) used for experiment were sun-dried for three weeks, and the outer scales and brownish bottom plates were carefully removed, leaving the root ring primordial intact. Five concentrations (1.0, 5.0, 10.0, 20.0 and 40.0 ppm) of each pesticide were prepared with distilled water used as diluents as well as the control.

Twelve (12) onion bulbs were planted per concentration with each bulb placed on 50 ml capacity beaker filled separately with the prepared concentrations of the pesticides. Onion roots were grown at room temperature (25±1°C) in a dark cupboard. The contents of the beaker were replaced with freshly prepared pesticide solution at every 24 h.

The root tips used for microscopic evaluation were harvested from five onion bulbs per concentration at 48 h, and fixed in ethanol-ethanoic acid (3:1 v/v) before been transferred to 70% ethanol. The root tips were then hydrolyzed in 1 N HCl at 65°C for 3 min. Two root tips were squashed on slides, and then stained with acetocarmine for 15 min.

One thousand (1,000) cells per slide and a total of 5000 cells per concentration were scored for the frequency and occurrence of different types of chromosomal aberrations in the dividing cells at 1000x as previously described (Fiskesjo, 1985; Bakare et al. 2000; Lateef et al. 2007). The photomicrographs were taken with the Ocular VGA adapted Bresser Erudit DLX microscope (Germany). The mitotic index and mitotic inhibition were determined from the scores obtained for dividing cells based on these formulae:

$$\text{Mitotic Index (MI)} = \frac{\text{number of dividing cell in the treatment} \times 100}{\text{Total number of cell}}$$

$$\text{Mitotic Inhibition} = \frac{\text{Mitotic index of control} - \text{Mitotic index of treatment} \times 100}{\text{Mitotic index of control}}$$

The length of each root from the 5 onion bulbs per concentration and the control were measured at 72 h for macroscopic evaluation, and growth inhibition was evaluated. The EC_{50} was extrapolated from the graph of percentage root growth relative to control against pesticides concentrations.

Statistical analysis

The means with the standard errors for each of the concentrations per pesticide were calculated. The data obtained for the root length of the treated groups and the control was compared using t-test and considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

The mean root length of the treated *A. cepa* for the four pesticides in all concentrations was lower compared to the control (Table 1). A dose dependent reduction was observed in *A. cepa* root length for the pesticides except at 5.0 ppm of deltamethrin. Significant difference in root

Table 1. Macroscopic and microscopic evaluations of the pesticide treated *Allium cepa*.

Concentration (ppm)	Mean root length	% inhibition	Dividing cells	Mitotic index	Mitotic inhibition	Sticky chromosome	Disturbed spindle	Chr bridge	Chr fragment	Chr Laggard	C mitosis	Total aberration	% Frequency
Control													
0	2.88±0.08	-	162	3.24	-	-	-	-	-	-	-	-	-
Cypermethrin													
1.0	2.50±0.23	13.19	101	2.02	37.65	-	9	2	-	3	-	14	0.28
5.0	2.18±0.08	24.31	85	1.70	47.53	1	14	3	-	-	-	19	0.38
10.0	1.88±0.12*	34.72	65	1.30	59.88	3	10	5	-	2	-	20	0.40
20.0	1.54±1.12*	46.53	41	0.82	74.69	-	6	1	1	-	1	9	0.18
40.0	0.88±0.05*	69.44	26	0.52	83.95	-	6	3	-	-	-	9	0.18
Deltamethrin													
1.0	2.28±0.16	20.83	112	2.24	30.86	1	6	-	-	2	-	9	0.18
5.0	2.49±0.10	13.54	102	2.04	37.04	-	8	3	1	1	2	15	0.30
10.0	1.84±0.10*	36.11	78	1.56	38.27	-	-	2	-	-	-	2	0.04
20.0	1.58±0.12*	45.14	10	0.20	93.83	-	1	-	-	-	-	1	0.02
40.0	0.98±0.08*	65.97	0	0	100.0	-	-	-	-	-	-	-	-
Lambdacyhalothrin													
1.0	2.01±0.13	30.21	145	2.90	10.49	-	1	-	-	-		1	0.02
5.0	1.95±0.09	32.29	86	1.72	46.91	1	-	2	-	-		3	0.06
10.0	1.12±0.07*	57.99	73	1.46	54.94	-	15	-	-	1		16	0.48
20.0	1.04±0.08*	63.89	75	1.50	53.70	-	19	3	1	1		24	0.32
40.0	0.69±0.08*	76.04	32	0.64	80.25	-	3	1	-	1		5	0.10
Endosulfan													
1.0	2.84±0.10	1.39	102	2.04	37.04	-	-	-	-	-	-	-	-
5.0	2.53±0.08	12.15	136	2.72	16.06	-	5	1	-	1	-	7	0.14
10.0	2.12±0.06	26.39	79	1.58	51.23	-	2	-	-	1	-	3	0.06
20.0	1.78±0.05*	38.19	32	0.64	80.25	-	1	-	-	-	-	1	0.02
40.0	0.95±0.08*	67.01	17	0.34	89.51	-	2	-	-	-	-	2	0.04

5000 cells were scored per concentration; Chr: chromosome; *Student t-test show significant difference compared to control ($P \leq 0.05$).

length was observed at 10.0, 20.0 and 40.0 ppm of deltamethrin, cypermethrin and lambda-cyhalothrin, while endosulfan showed difference at 20.0 and 40.0 ppm ($P<0.05$). Highest percent-tage root inhibition was observed at 40 ppm of each of the pesticides. Figure 1 shows the percentage root length relative to control where the EC_{50} values of 9.0, 21.5, 23.5 and 29.00 ppm were obtained for lambdacyhalothrin, cyperme-thrin, deltamethrin and endosulfan, respectively, which indicate the decreasing order of their inhibitory effects on *A. cepa* root growth. The growth inhibitory effect of the pesticides is indicated by the significant reduction of root length compared to the control. Table 1 also shows the microscopic evaluation of the pesticides. A dose dependent reduction in the total mitotic dividing cells and mitotic index was observed in *A. cepa* treated with the pesticides, except for 5.0 ppm of endosulfan. However, complete cell arrest was observed only in deltamethrin at 40.0 ppm. The values of mitotic

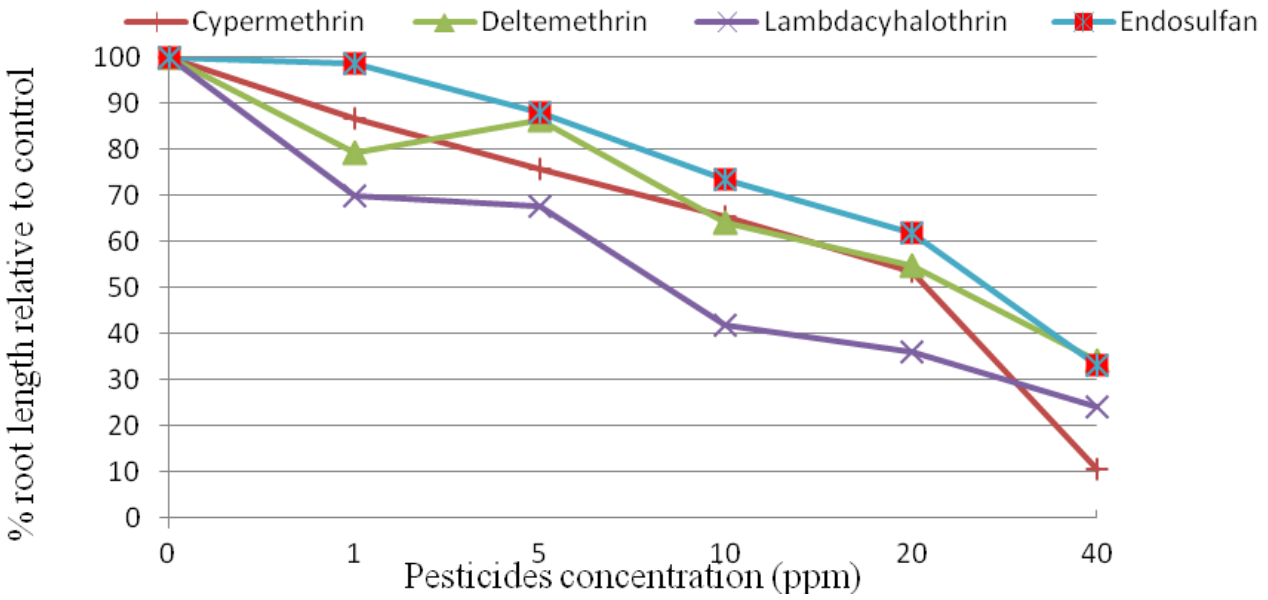

Figure 1. Growth inhibitions of pesticides treated *A. cepa* root.

index obtained for all pesticides at 10.0 (except in endosulfan), 20.0 and 40.0 ppm were lower than half of the negative control, which reflect their cytotoxicity. Similar observation was reported in *A. cepa* treated with different pesticides (Asita and Matebesi, 2010; Sibhghatulla et al., 2012). The total chromosomal aberrations induced were in the order: Cypermethrin > lambdacyhalothrin > deltamethrin > endosulfan.

The aberrations observed in the three pesticides included sticky chromosome, disturbed spindle, c-mitosis, chro-mosome-bridge and laggard chromosomes (Table 1 and Figure 2). Stickiness observed in the pyrethroid-treated onion roots may be due to physical adhesion of the proteins of the chromosome (Patil and Bhat, 1992). The occurrence of c-mitosis indicates that spindle formation was adversely affected (El-Ghamery et al., 2003). Disturbed spindle resulted in inability of chromosomes to move to the poles.

Chromosome bridge is formed by breakage and fusion of chromosomes and chromatids, the stickiness of chromosome and subse-quent failure of free anaphase separation, and unequal translocation or inversion of chromosome segments (Gomórgen, 2005). Permjit and Grover (1985) attributed laggard chromosomes to the delayed terminalization, stickiness of chromo-some ends or the failure of chromosomal movement.

Aberrations of mitotic cycle, change of mitotic index and chromosomal abnormalities observed after exposure to toxic metals, metalloids or organic pollutants were attributed to the disorganization and depolymerization of microtubules, which underlie these processes in higher plant cells (Liu et al., 2009; Xu et al., 2009; Dho et al., 2010; Eleftheriou et al., 2012, 2013; Adamakis et al., 2013). Cypermethrin among other pesticides tested in this study has the highest total chromosomal aberration. Seehy et al. (1983) reported that in mice, both technical and formulated products of alpha cypermethrin showed a dose dependent sister chromatid exchanges in dividing cells at all dose levels but the highest doses inhibited mitotic division.

Cypermethrin and alphamethrin were reported to elicit varying degrees of cytotoxic, turbagenic (toxicity to spindle) and clastogenic effects but generally more turbagenic and weak clastogenic (Rao et al., 2005). However, Asita and Makhalemele (2008) reported that alpha-thrin (active ingredient of alpha-cypermethrin) was only cytotoxic but not genotoxic at various concentrations in treated *A. cepa*. Cypermethrin has been classified as a possible human carcinogen (EPA, 2002).

The pesticides used induced significant growth inhibition at 10.0, 20.0 and 40.0 ppm. Also, at these concentrations, the mitotic index was lower than half of the values obtained for the control which indicate their cytotoxic effects. Induction of chromosomal aberrations at different concentrations shows the genotoxic effects on the meristematic cells of *A. cepa.* The aberrations observed were however not dose dependent, which may be due to fewer number of dividing cells at higher concentration of the pesticide and complete cell arrest observed at 40.0 ppm of deltamethrin.

Our results are in accord with the previous reports, where mitotic inhibition and genotoxicity of pesticides were demonstrated (Mosuro et al 1999; Chauhan et al., 1999; Kumar and Chaudhary, 2012). Reduction in mitotic activity could be due to the inhibition of DNA synthesis (Schneiderman et al., 1971; Sudhakar et al., 2001) or due to a block in the G2-phase of the cell cycle, thus preventing the cell from entering mitosis (Van't Hof, 1968).

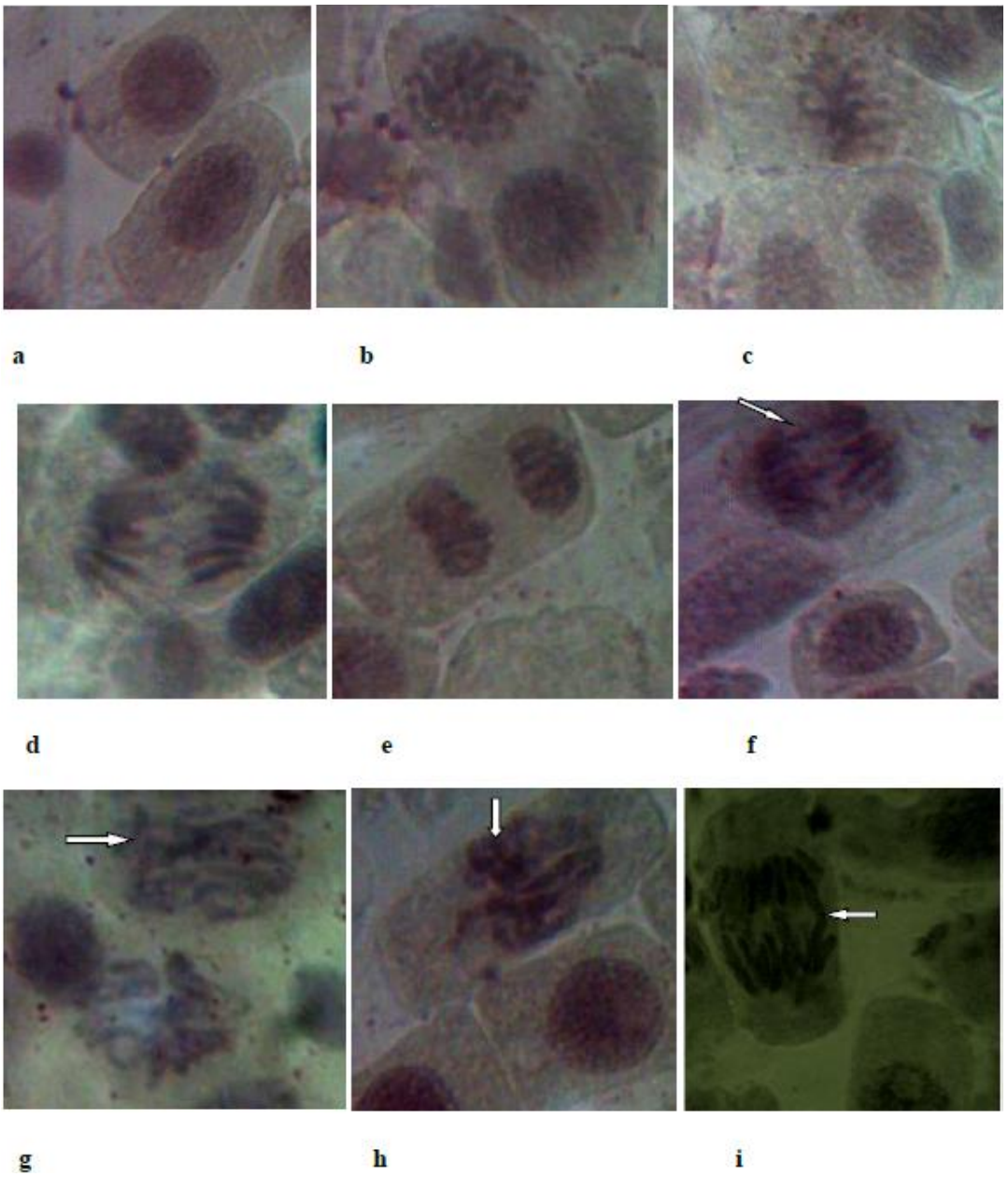

Figure 2. Normal and aberrant cells observed in *allium cepa* treated with pesticide. **a,** normal interphase; **b,** normal prophase; **c,** normal metaphase; **d,** normal anaphase; **e,** normal telophase; **f,** laggard chromosome; **g,** disturbed spindle and fragmentation; **h,** sticky chromosome; **I,** chromosome bridge.

Prior to occurrence of chromosome aberrations, there is always some growth restriction which is the cumulative response of all the damaging effects (Fiskejo, 1997).

Conclusion

The inhibition of growth and induction of chromosomal aberrations by the pesticides show their cytogenotoxic effects. This data provide more information on the cypermethrin, deltamethrin, lambdacyhalothrin and endo-sulfan of which exposure to substantial concentration may constitute health risk to non-target organisms and thus will assist in future ecotoxicological evaluations.

REFERENCES

Adamakis I-D, Panteris E, Cherianidou A, Eleftheriou EP, (2013). Effects of bisphenol A on the microtubule arrays of *Pisum sativum* L. root meristematic cells. Mutation Research - Genetic Toxicol. Environ. Mut. 750:111-120.

Anis M, Shiran B, Wani AA (1998). Genotoxic effect of aldrin and malathion on the root meristem of *Vicia faba*. J. Cytol. Genet. 33:35-42.

Asita AO, Matebesi LP (2010). Genotoxicity of hormoban and seven other pesticides to onion root tip meristematic cells. Afr. J. Biotechnol. 9(27):4225-4232.

Asita OA and Makhalemele R (2008) Genotoxicity of Chlorpyrifos, Alpha-thrin, Efekto virikop and Springbok to onion root tip cells. Afr. J. Biotechnol. 7(23):4244-4250.

ATSDR (2008) (Content Source);Sidney Draggan Ph.D. (Topic Editor) "Health effects of endosulfan". In: Encyclopedia of Earth. Eds. Cutler J. Cleveland (Washington, D.C.: Environmental Information Coalition, National Council for Science and the Environment). [First published in the Encyclopedia of Earth March 31, 2008; Last revised Date March 31, 2008; Retrieved May 1, 2013 <http://www.eoearth.org/article/Health_effects_of_endosulfan>

Badr A, Ibrahim AG (1987). Effects of the herbicide glean on mitosis, chromosomes and nucleic acids in *A. cepa* and *Vicia faba* root meristems. Cytologia*.* 52:293-302.

Bakare AA, Mosuro AA, Osibanjo O (2000). Effects of simulated Leachates on chromosomes and mitosis of *Allium cepa* (L). J. Environ. Biol. 21:263-271.

Bradbury SP, Coats JR (1989). Toxicokinetics **a**nd toxicodynamics of pyrethroid insecticides in fish. Environ. Toxicol. Chem. 8:373-380

Cabral JRP, Galendo D, Laval M, Lyandrat N (1990). Carcinogenicity studies with deltamethrin in mice and rats. Cancer LeU., 49:147-152

Carbonell E, Valbuena A, Xamena N, Creus A, Marcos R (1995). Temporary variations in chromosomal aberrations in a group of agricultural workers exposed to pesticides. Mutat. Res. 344:127-134.

Celik A, Mazmanci B, Camlica Y, Comelekoglu U, Askin A (2005). Evaluation of cytogenetic effects of lambdacyhalothrin on wisker rat bone marrow by gavage administration. Ecotoxicol. Environ. Safe. 61:128-133

Chargui I, Grissa I, Bensassi F, Hrira MY, Haouem S, Haouas Z, Bencheikh H (2012). Oxidative Stress, Biochemical and Histopathological Alterations in the Liver and Kidney of Female Rats Exposed to Low Doses of Deltamethrin (DM): A Molecular Assess Biomed. Environ. Sci. 25(6):672-683

Chauhan LKS, Saxena PN, Gupta SK (1999). Effects of deltamethrin on the ultrastructure of root meristem cells of *Allium cepa.* Pestic. Biochem. Physiol*.* 64:181-189.

Das B, Pervin K, Roy AK, Ferdousi Z, Saha AK (2010). Toxic effects of prolonged endosulfan exposure on some blood parameters in albino rat. J. Life Earth Sci. 5:29-32

Davies CR, Lanus - Cuentas EA, Campos P, Mange J, Leon E, Camales J (2000). Spraying houses in the Peruvian Andes with Lambda-cyhalothrin protects residents against cutaneous leshimaniasis. Trans. Roy. Soc. Med. Hyg. 94:631-636.

Dho S, Camusso W, Mucciarelli M, Fusconi A (2010). Arsenate toxicity on the apices of *Pisum sativum* L. seedling roots: effects on mitotic activity, chromatin integrity and microtubules. Environ. Exp. Bot. 69:17-23.

Eleftheriou EP, Adamakis I-D S, Fatsiou M, Panteris E. (2013). Hexavalent chromium disrupts mitosis by stabilizing microtubules in *Lens culinaris* Moench. root tip cells. Physiologia Plantarum 147:169-180.

Eleftheriou EP, Adamakis I-D S, Melissa P. (2012). Effects of hexavalent chromium on microtubule organization, ER distribution and callose deposition in root tip cells of *Allium cepa* L. Protoplasma 249:401-416.

El-Ghamery AA, El-Kholy MA, Abou El-Yousser MA, (2003). Evaluation of cytological effects of Zn2+ in relation to germination and root growth of *Nigella sativa* L. and *Triticum aestivum* L. Mutat. Res. 537(1):29-41.

EPA U.S. Environmental Protection Agency (2002). Chemicals Evaluated for Carcinogenic Potential, Science Information Management Branch, Health Effects Division, Office of Pesticide Program, May

Fahmy AM, Abdalla EF (2001). Cyogenetic effects by the natural pyrethrin and the synthetic lambda-cyhalothrin in mice *in vivo*. Cytologia. 66:139-149.

Fiskesjo G (1985) The Allium test as a standard in environmental monitoring. Hereditas 102:99-112.

Fiskesjo G (1997) 'Allium test for screening chemical evaluation of cytological parameters', in Wang, W., Gorsuch, J.W. and Hughes, J.S. (Eds), Plants for Environmental Studies New York, NY:CRC Lewis Publishers. pp.307-333.

Gimeno L, Ferrando MD, Sanchez S, Andreu E (1994). Endosulfan effects on liver and blood of the eel, *Anguilla Anguilla.* Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicol. Endocrinol. 108(3):343-348.

Gomórgen AN (2005). Cytological effect of the potassium metabisulphite and potassium nitrate food preservative on root tips of *Allium cepa* L. Cytologia 70:119-128.

Grover P, Danadevi K, Mahboob M, Rozati R, Banu BS, Rahman MF (2003). Evaluation of genetic damage in workers employed in esticide production utilizing the Comet assay. Mutagenesis 18:201-205.

Khan A, Ahmad L, Khan M.Z. (2012). Hemato-biochemical changes induced by pyrethroid insecticides in avian, fish and mammalian species. Int. J. Agric. Biol. 14:834-842.

Kumar G, Chaudhary N. (2012). Mitotoxic effect of 2, 4-D and endosulfan in root meristems of *Hordeum vulgare.* Chromosome Bot. 7:73-77.

Lander BF, Knudsen LE, Gamborg MO, Jarventaus H, Norppa H (2000) Chromosome aberrations in pesticide-exposed greenhouse workers. Scand. J. Work Environ. Health 26:436-442.

Lateef A, Yekeen TA, Ufoma P (2007). Bacteriology and Genotoxicity of some Pharmacaeutical wastewater in Nigeria. Int. J. Environ. Health. 1(4):551- 562

Liu D, Xue P, Meng Q, Zou J, Gu J, Jiang W (2009). Pb/Cu effects on the organization of microtubule cytoskeleton in interphase and mitotic cells of *Allium sativum* L. Plant Cell Rep. 28:695-702.

Modaresi M, Seif MR (2011). Effect of an organochlorine insecticide, endosulfan, on blood parameters in rat. Shahrekord University of Medical Sciences. 13(2); 26-32.

Mosuro AA, Bakare AA, Koofreh MA, Ngenwi AA (1999). Genetic safety evaluation of pesticides using *Allium cepa* assay J. Cytol. Genet. 34(2):173-182.

Muthuviveganandave V, Hwang I, Anita V, Malarani PS, Selvam C, Hemalatha M, Pandurangan M (2013). Synthetic Pyrethroid effect on blood plasma biomarker Enzymes and histological changes in *Catla catla*. Int. J. Exp. Path. 94:104-108.

Patil BC, Bhat GI (1992). A comparative study of MH and EMS in the induction of choromosomal aberrations on lateral root meristem in *Clitoria ternata* L. Cytologia*,* 57:259-264

Permjit K, Grover IS (1985). Cytological effects of some organophosphorus pesticides. II Meiotic effects. Cytologia*,* 50:199-211.

Rank J, Nielsen MH (1997). *Allium cepa* anaphasetelophase root tip chromosome aberration assay on NmethylNnitrosourea, maleic hydrazide, sodium azide, and ethyl methanesulfonate. Mut. Res. 390:121-127.

Rao BV, Narasimham TL, Subbarao MV (2005). Relative Genotoxic Effects of Cypermethrin, Alphamethrin and Fenvalerate on the Root Meristems of *Allium cepa*. Cytologia, 70(3):225-231.

Sanchez-Fortun S, Barahona MV (2005). Comparative study on the environmental risk induced by several pyrethroids in estuarine and freshwater invertebrate organisms. Chemosphere 59(4):553-559.

Schneiderman MH, Dewey WC, Highfield DP (1971). Inhibition of DNA synthesis in synchronized Chinese hamster cell treated in G1 with cycloheleximide. Exp. Cell Res. 67:147-155.

Seehy MA, Shalabi HG, Shaker N, Badr E (1983). In vivo induction of sister chromatid exchanges in mice by cypermethrin. Proceedings of the International Conference on Environmental Hazards of Agrochemicals. 1:659-673.

Sibhghatulla S, Nazia N, Mohammad IL, Waseem A (2012). Dichlorophen and Dichlorovos mediated genotoxic and cytotoxic assessment on root meristem cells of *Allium cepa* Science Diliman*.* 24(1):13-22.

Sudhakar R, Gowda KNN, Venu G (2001). Mitotic abnormalities induced by silk dyeing industry effluents in the cells of *Allium cepa.* Cytologia. 66:235-239.

Van't Hof J (1968). The action of IAA and kinetin on the mitotic cycle of proliferative and stationary phase excised root meristem. Exp. Cell Res. 51:167-176.

Xu P, Liu D, Jiang W (2009). Cadmium effects on the organization of microtubular cytoskeleton in interphase and mitotic cells of *Allium*

sativum. Biol. Plant 53:387-390.
Yekeen TA, Fawole OO (2011). Toxic effects of endosulfan on haematological and biochemical indices of *Clarias gariepinus* Afr. J. Biotechnol. 10(64),14090-14096
Yekeen TA, Fawole OO, Bakare AA (2013) Evaluation of toxic effects of lambdacyhalothrin on the haematology and selected biochemical parameters of African catfish *Clarias gariepinu.* Zool. Ecol. 23(1):45-52.
Yekeen TA, Okorie TG, Bakare AA (2007). Acute and Chronic Toxicity Effects of Deltamethrin in Mice. Asian Journal of Microbiology, Biotechnol. Environ. Sci. 9(1):11-17.
Yildiz M, Arikan ES (2008). Genotoxicity testing of quizalofop-P-ethyl herbicide using the *Allium cepa* anaphase-telophase chromosome aberration assay. Caryologia. 61(1):45-52, 2008).
Zaim M, Aitio A, Na-Kashima N (2000). Safety evaluation of pyrethroid treated mosquito nets. Med. Veter. Entomol. 14:1-5.

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Effects of sulphur preservative on phytochemical and antioxidant capacity of peels of mango cultivars (*Mangifera indica* L.) produced in South Africa

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Fruits have abundant phytochemicals that contribute as bioactive molecules with ability to lower incidence of diseases. Mangoes are rich in polyphenols and antioxidants. In this study, peels of six selected mango cultivars (Tommy Atkins, Peach, Saber, Sunshine, Keitt and Vhavenda) were treated with sulphur dioxide solutions (0, 10, 20, 50, 100, 150, 200, 250 and 300 ppm) as preservative of phytochemicals and antioxidants capacity. Regardless of cultivar, sulphur concentration had effect on composition of polyphenols and antioxidant capacity of mango peels, reaching a plateau at 50 ppm. Vhavenda cultivar has significantly highest polyphenols and antioxidant capacity than the other cultivars evaluated. This study reveals that mango peels are a prospective source of natural antioxidants as they constitute significantly higher total antioxidant capacity and phenolic content.

Key words: Antioxidants, mango cultivars, peels, phytochemicals, sulphur.

INTRODUCTION

Phytochemicals are compounds that act as free radical scavengers to help eliminate the highly charged oxygen molecules that are byproducts of metabolized oxygen (Khalid, 2007), and are believed to offer various health benefits (Van Duyn and Pivonka, 2000; Min et al., 2013). Antioxidants are substances that can prevent or delay the oxidative damage of lipids, proteins and nucleic acids by reactive oxygen species (ROS), which include free radicals such as hydroxyl, peroxyl and non-radicals such as hydrogen peroxides (Saikat et al., 2010). According to Pietta (2000), phenolic antioxidants are thought to neutralize ROS before they can cause damage.

The most of the abundant antioxidants in fruits are polyphenols, vitamin C, A and E and carotenoids to a lesser extent in some fruits (Lim et al., 2007). According to Fleuriet and Macheix (2003), most of these polyphenols are flavonoids and are present in the form of ester and glycoside in fruits. Flavonoids commonly found in fruits and vegetables have been linked to reduced risk of mortality from the coronary heart diseases (Wang et

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Abbreviations: ROS, Reactive oxygen species; **TPC,** total polyphenol content; **ABTS,** 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; **TAC,** total antioxidant activity.

al., 2011).

Phenolic compounds are plant secondary metabolites that are biosynthesized through the shikimic acid pathway (Tomas-Baraeran and Espin, 2001). Phenolic compounds are associated with the health benefits deriving from consuming high levels of fruits (Parr and Bolwell, 2000; Aberoumand, 2012). Mango is regarded as a valuable source of phytochemical compounds (Kim et al., 2007; Ashoush and Gadallah, 2011; Mudau et al., 2013); among these compounds, polyphenolics are widely distributed secondary metabolites and the predominant antioxidants present. Several studies have reported phenolic compounds in mango flesh and peels, including various flavonoids, xathones, phenolic acids and gallotannins (Schieber et al., 2000; Berardini et al., 2005) and variability of these compounds have been observed in different mango cultivars (Souza et al., 2011; Taing et al., 2012).

Mangoes are seasonal fruits with limited shelf-life with fruit quality dropping off rapidly once fully ripe. Mango fruits are processed for various products (Loelillet, 1994) and by-products such as peels and kernels are generated (Ashoush and Gadallah, 2011). These by-products are rich sources of natural bioactive compounds which play an important role in prevention of diseases (Ashoush and Gadallah, 2011).

Studies have been conducted on the utilization of mango kernels as a source of fat (Arogba, 2002), natural antioxidants (Kaur, 2004), starch (Moharram and Moustafa, 1982), flour (Puravankara et al., 2000) and feed (Ravindran and Sivakanesan, 1996) but studies on peels are scarce (Berardini et al., 2005). Most of the studies on the exploitation of mango peels dealt with their use as a source of pectin, which is considered a high quality dietary fiber (Beerh et al., 1976; Srirangarajan and Shrikhande, 1976; Tandon et al., 1991; Pedroza-Islas and Aguilar-Esperanza, 1994; Tandon and Garg, 1999).

The mango peels have been reported to a good source of dietary fiber containing high amount of extractable polyphenolics (Larrauri et al., 1996). According to Ojokoh (2007), mango peel fibre is a good source of dietary fiber and its chemical composition may be compared to citrus fibre. Mango peels also demonstrated higher values of anticancer properties because of polyphenolic extracts (Noratto, 2010) and glucose retardation (Reyers and Vega, 1988). Chemical in peels of certain mango cultivars have also been shown to prevent the formation of fat cells through disrupting adipogenesis (Taing et al., 2012) which is the key in development of obesity (Min et al., 2013).

The use of sulphur solution as a preservative in dried mango has become a commercial drying standard for the industry in South Africa with limited studies evaluating the phytochemical and antioxidant capacity of peels of mango cultivars. As a result, data that describe the use of preservatives in mango byproducts is lacking. Therefore, the objective of this study was to determine the effect of sulphur concentration on polyphenol content and total antioxidant capacity of mango peels for potential usefulness as a preservative.

MATERIALS AND METHODS

Location

The experiment was conducted at Agro-food Laboratory, University of Limpopo. The ripe fruits of mango cultivars *inter alia*; Tommy Atkins, Peach, Saber, Sunshine and Keitt were randomly collected from a commercial orchard in Hoedspruit. Vhavenda cultivar was collected from another commercial orchard in Vhembe District (23°N 50' E, 30°S 17'E); alt 610 m; subtropical-type climate (that is, summer rainfall and cold, dry winter) of Limpopo Province, South Africa.

Plant materials

Fresh, healthy and disease free fruits from six selected cultivars were washed and manually peeled. The peels were soaked in a sodium metabisulfite solution (BASF chemical company, Germany). Nine different concentrations of SO_2 (in 3 L of water) were used. Treatment concentrations were 0, 10, 20, 50, 100, 150, 200, 250 and 300 ppm. Mango peels from the six cultivars were soaked for 5 min in the solution, and then immediately dried in a hot air oven at 58°C for 28 h. Samples of dried peels were stored at -30°C until their analysis.

Sample preparations (extraction)

For the analysis, 10 g dried fruit peel samples were weighed and transferred to a waring commercial blender (Instrulab, Johannesburg, South Africa) containing 100 ml of methanol, and then blended at a high speed for 2 min (stopping occasionally to avoid accumulation of fumes). The mixture was removed and let stand in the beaker to achieve separation. After 6-8 min, the supernatant was collected, centrifuged at 12 000 x *g* for 10 min and stored. The residues were blended again with 50 ml methanol, supernatant decanted as above, combined with the first one, filtered with MN-615 (240 mm) filter papers (Bethlehem, USA) and stored at -4°C until analysis.

Determination of total phenolics content

Total polyphenol content (TPC) for fruit peel samples was determined using Waterman and Mole method (1994). In this method, 50 ml volumetric flasks were used, each containing 10 ml of water. Ten (10) ml of water, 0.5 ml of the sample extracts were added, as well as 2.5 ml of the Folin-Ciocalteau's reagent. Within 2-8 min, 7.5 ml of sodium carbonate was added and the flasks were filled with water to 50 ml of volumetric flask mark. The flasks were swirled and allowed to stand for 2 h in the dark. The absorbance was measured at 760 nm using Genesis 20 Spectrophotometer (Thermo Electron Corporation, Madison, USA). Data were calculated using a pre-prepared gallic acid calibration curve. A stock solution was prepared by dissolving 0.1 g gallic acid in 100 ml methanol. Results were expressed as milligrams of gallic acid per 100 ml of sample extracts.

Determination of total antioxidant activity

The 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS)

Table 1. Total antioxidant activity value and polyphenols content of dried mango peels treated with sulphur (different concentrations).

Sulphur concentration (ppm)	Saber				Peach				Keitt				Tommy Atkins				Vhavenda				Sunshine			
	AA	Std err	TP	Std err	AA	Std err	TP	Std err	AA	Std err	TP	Std err	AA	Std err	TP	Std err	AA	Std err	TP	Std err	AA	Std err	TP	Std err
0	393.9^{b}	±0.2	2.0^{b}	±0.1	392.7^{b}	±0.2	1.9^{b}	±0.1	382.7^{b}	±0.2	1.9^{b}	±0.1	372.8^{b}	±0.2	1.7^{b}	±0.1	393.7^{b}	±0.2	1.9^{b}	±0.1	382.7^{b}	±0.2	1.9^{b}	±0.1
10	409.9^{b}	±0.3	2.1^{b}	±0.1	409.6^{b}	±0.2	2.2^{b}	±0.1	409.6^{b}	±0.2	2.3^{b}	±0.1	419.3^{b}	±0.2	2.3^{b}	±0.1	429.9^{b}	±0.3	2.3^{b}	±0.1	411.6^{b}	±0.3	2.3^{b}	±0.1
20	436.6^{b}	±0.2	2.3^{b}	±0.1	435.6^{b}	±0.2	2.3^{b}	±0.1	435.6^{b}	±0.2	2.4^{b}	±0.1	435.5^{b}	±0.2	2.5^{b}	±0.1	446.6^{b}	±0.2	2.5^{b}	±0.1	425.6^{b}	±0.2	2.4^{b}	±0.1
50	465.9^{a}	±0.2	3.2^{a}	±0.1	467.3^{a}	±0.2	3.3^{a}	±0.1	457.3^{a}	±0.2	3.4^{a}	±0.1	467.1^{a}	±0.2	3.7^{a}	±0.1	475.9^{a}	±0.2	3.8^{a}	±0.1	487.3^{a}	±0.2	3.4^{a}	±0.1
100	416.5^{b}	±0.2	2.3^{b}	±0.1	416.3^{b}	±0.2	2.4^{b}	±0.1	426.3^{b}	±0.2	2.2^{b}	±0.1	446.3^{b}	±0.2	2.4^{b}	±0.1	426.5^{b}	±0.2	2.5^{b}	±0.1	426.3^{b}	±0.2	2.2^{b}	±0.1
150	408.9^{b}	±0.2	2.5^{b}	±0.1	410.5^{b}	±0.2	2.5^{b}	±0.1	420.5^{b}	±0.2	2.3^{b}	±0.1	430.6^{b}	±0.2	2.5	±0.1	418.9^{b}	±0.2	2.5^{b}	±0.1	420.5^{b}	±0.2	2.3^{b}	±0.1
200	409.4^{b}	±0.2	2.2^{b}	±0.1	410.2^{b}	±0.2	2.3^{b}	±0.1	420.2^{b}	±0.2	2.2^{b}	±0.1	420.1^{b}	±0.2	2.2^{b}	±0.1	419.4^{b}	±0.2	2.4^{b}	±0.1	410.2^{b}	±0.2	2.3^{b}	±0.1
250	398.2^{b}	±0.3	2.4^{b}	±0.1	405.3^{b}	±0.3	2.3^{b}	±0.1	425.3^{b}	±0.3	2.2^{b}	±0.1	415.3^{b}	±0.3	2.3^{b}	±0.1	400.2^{b}	±0.3	2.4^{b}	±0.1	425.3^{b}	±0.3	2.3^{b}	±0.1
300	384.9^{b}	±0.2	2.7^{b}	±0.1	405.1^{b}	±0.2	2.6^{b}	±0.1	395.1^{b}	±0.2	2.1^{b}	±0.1	395.3^{b}	±0.2	2.1^{b}	±0.1	394.9^{b}	±0.2	2.2^{b}	±0.1	415.1^{b}	±0.2	2.2^{b}	±0.1

AA, Total antioxidant activity (µmol/ g); TP, total polyphenol content (mg of gallic acid/100 mg). Means with different superscripts along the same column are significantly different ($P < 0.01$).

assay was used to measure the total antioxidant activity (TAC) of the mango peel extracts. 8 mM ABTS and 3 mM potassium persulfate ($K_2S_2O_8$) were dissolved in 25 ml distilled water each, and then the equal volumes of the two were mixed. The reaction mixture was left to stand at room temperature overnight (12-16 h) in the dark before usage. The resultant intensely coloured ABTS (mother solution) was diluted with phosphate buffered (pH 7.4) solution to make a working solution. The assay was first carried out on Trolox, the water-soluble α-tocopherol (vitamin E) analogue, which served as standard. Working solution (2900 µl) was added to 100 µl serial Trolox dilutions, swirled and left to react for 15 min.

For sample analysis, dilutions were made by adding 1 ml of the sample extract to 4 ml of the solvent (methanol), and then 2900 µl of the working solution was added to 100 µl sample extracts, swirled and left to react for 30 min. Absorbance was measured at 734 nm using Genesis 20 Spectrophotometer (Thermo Electron Corporation, Madison, USA). The assay was performed by triplicates and data were calculated using a Trolox calibration/standard curve. Fresh ABTS solution was prepared everyday due to self-degradation of the radical. The results were expressed as µmol Trolox equivalents (TEAC).

Statistical analysis

All data were reported as mean ± standard error of three replicates. Analyses of variance (ANOVA) were performed on data using the General linear model (GLM) procedure of SAS version 8.0 (SAS Institute Inc., 1999). Differences at $P<0.05$ were considered significant. Treatment means found were separated using Duncan's Multiple Range Test (DMRT).

RESULTS

Total phenolics composition of mango peels

The secondary metabolites composition of dried mango peels treated with sulphur at different concentrations showed that the total polyphenolic content at low concentrations (0, 10 and 20 ppm) was similar (Table 1). However, at 50 ppm of concentrations, the composition of polyphenols was significantly different to the low and high concentrations tested ($P<0.01$) (Table 1). The trend was shown in all six cultivars evaluated.

There were differences in the polyphenols on the different mango cultivars ranging from 2.3 mg/100 mg of Keitt mango peels to 2.5 mg of gallic acid/100 mg of Vhavenda mango peels (Table 2). The composition of polyphenols in Vhavenda mango peels was greater than that of the other five cultivars ($P<0.01$) (Table 2) whilst polyphenolic content of Peach, Saber, Tommy Atkins and Sunshine were significantly the same ($P<0.01$).

Total antioxidant capacity of mango peels

The total antioxidant capacity of dried mango peels treated with sulphur at different concentrations showed that the antioxidant activity at low concentrations (0, 10 and 20 ppm) was similar (Table 1), with the same trend shown in all mango cultivars. However, at 50 ppm of concentrations, the antioxidants activity was significantly different to the low and high concentrations tested ($P<0.01$) (Table 1). At high concentrations (100, 150, 200, 250 and 300 ppm), total antioxidants activity values were similar to the low concentrations (Table 1). There were no significant differences in the total antioxidant activity observed on the different mango cultivars ranging, with values ranging from 413.9 µmol/ g of Saber mango peels to 422.9 µmol/ g of Vhavenda mango peels (Table 2).

Table 2. Total antioxidant activity value and polyphenols content of dried mango peels (different cultivars).

Mango cultivar	Total antioxidants activity (µmol/ g)	Standard error	Total polyphenols content (mg of gallic acid/100 mg)	Standard error
Saber	413.9[a]	0.3	2.4[b]	0.2
Peach	417.0[a]	0.3	2.4[b]	0.2
Keitt	419.2[a]	0.3	2.3[c]	0.2
Tommy Atkins	422.5[a]	0.3	2.4[b]	0.3
Vhavenda	422.9[a]	0.3	2.5[a]	0.1
Sunshine	422.7[a]	0.3	2.4[bc]	0.2

Means with different superscripts along the same column are significantly different ($P < 0.01$).

DISCUSSION

Our results demonstrate that dipping mango peels in 50 ppm of SO_2 solution was effective in maintaining their bioactive compounds. In this way, previously it was demonstrated that the same concentration can effectively preserve dried mango flesh with maximum proximate composition attained at this level, and the pretreatment of most fruits prior to drying as it enhances inactivation of pathogenic bacteria during dehydration (Mudau et al., 2013; DiPersio et al., 2006). Besides reducing the pathogenic bacteria, treatment in preservatives also maintains the colour of dried fruit products (Davidek et al., 1990) increasing their marketability. Simple pretreatment methods prior to drying of mango slices also lead to significant retention of β-carotene of which it has high amount of vitamin A and antioxidative capacity (Muoki et al., 2009; Mercandate and Rodriguez-Amaya, 1998).

We found that mango peels possess high contents of the secondary metabolites than the flesh in this work. Similar results were reported by other authors, who found that mango peels containing high levels of polyphenols and dietary fiber (Larrauri et al., 1996; Kim et al., 2010). Also, it was reported that apple peels had higher antioxidant activities than the edible portion of the fruit (Wolfe et al., 2003).

In a recent study reported, it was suggested that mango peel extracts can inhibit adipogenesis likely due to higher concentrations and types of polyphenols in the peel extracts when they are compared with flesh extracts (Taing et al., 2012). For the same cultivars evaluated in this study, there are significantly more bioactive compounds in peels than the flesh (Mudau et al., 2013). Other studies have also shown the content of total polyphenol and antioxidant activities being higher in the peel than the pulp at any stage of mango fruit development (Lakshminarayana et al., 1979; Reyers and Vega, 1988; Ueda et al., 2000; Ajila et al., 2007) and several extraction techniques have been investigated for utilisation of these bioactive compounds (Ashoush and Gadallah, 2011; Palmeira et al., 2012, Meneses et al., 2013).

The variation in polyphenol content of peels of these cultivars as seen in this study may be associated to genetic factors (Mercandate and Rodriguez-Amaya, 1998) and also factors such as soil conditions (Rodríguez Pleguezuelo et al., 2012) and phytosanitary status (Tahir et al., 2002). This variability in polyphenol and antioxidant content has also been observed on mango flesh with causes extended to cultivar (Mercandate and Rodriguez-Amaya, 1998; Othman and Mbogo, 2009), cultivation practice (Hofman et al., 1995), climatic conditions (Léchaudel and Joas, 2006), ripeness at harvest (Jacobi et al., 1995; Lalel et al., 2003), and even postharvest storage conditions of the fruit (Hofman et al., 1997; Nunes et al., 2007).

Genetic variability in mango cultivars can ultimately lead to differences in carotenoids (Mitra, 1997) and anthocyanin (Lizada, 1991) content. Souza et al. (2011) reported that certain Brazilian mango cultivars have genomic similarities of as little as 7% showing a significant genetic diversity that could contribute to phytochemical differences between mango fruit cultivars. These phytochemical different cultivars offer potential in diverse uses, including in obese individuals.

In a study done by Taing et al. (2012), where mango peel extracts were reported to be more effective at inhibiting adipo-genesis in 3T3-L1 pre-adipocytes, it was further found that the degree of inhibition was cultivar-dependent. This difference in activity could be due to genetic variability between mango cultivars resulting in different types or relative amounts of phytochemicals (Taing et al., 2013).

Conclusion

From this work, we conclude that the natural bioactive compounds can effectively accumulate if mango peels are preserved at 50 ppm of SO_2 solutions in all cultivars evaluated. Also, from all cultivars studied in this work; Vhavenda has highest values of total polyphenols (2.5 mg/100 mg) and antioxidants (422.9 µmol/ g); and their peels could be used as a source of natural antioxidants. In general, mango peels with high total antioxidants activity showed the highest polyphenolic contents.

REFERENCES

Aberoumand A (2012). Screening of phytochemical compounds and toxic proteinaceous protease inhibitor in some lesser-known food based plants and their effects and potential applications in food. Inter J. Food Sci. Nutr. Eng. 3:16-20.

Ajila CM, Naidu KA, Bhat SG, Prasada Rao USJ (2007). Bioactive compounds and antioxidant potential of mango peel extracts. Food Chem. 105:982-988.

Arogba SS (2002). Mango seed kernel: Chromatographic analysis of the tannin, and stability study of the associated polyphenol oxidase activity. J. Food Compos. Anal. 13:149-156.

Ashoush IS, Gadallah MGE (2011). Utilization of mango peels and seed kernels powders as sources of phytochemicals in biscuit. World J. Dairy & Food Sci. 6:35-42.

Beerh OP, Raghuramaiah B, Krishnamurthy GV (1976). Utilization of mango wastes: peel as a source of pectin. J. Food Sci. Tech. 13:96-97.

Berardini N, Knodler M, Schieber A, Carle R (2005). Utilization of mango peels as a source of pectin and polyphenolics. Innov. Food Sci. Emerging Tech. 6:442-452.

Davidek J, Velisek J, Pokorny (1990). Chemical changes during fruit processing. Elsevier Science. Amsterdam. Netherlands. Pp. 230-310.

DiPersio PA, Kendall PA, Sofos JN (2006). Sensory evaluation of home dried fruit prepared using treatments that enhance destruction of pathogenic bacteria. J. Food Quality, 29:47-64.

Fleuriet A, Macheix JJ (2003). Phenolic acids in fruits and vegetables. In CA Rice-Evans & L Packer (Eds.), Flavonoids in health and disease. Marcel Dekker Inc.

Hofman PJ, Smith LG, Holmes R, Campbell T, Meiburg G (1995). Mango fruit quality at harvest is affected by production conditions. In: Proceedings of Mango 2000 marketing seminar and production workshop. Brisbane, Australia, pp.199-208.

Hofman PJ, Smith LG, Joyce DC, Johnson GI, Meiburg G (1997). Bagging of mango (*Mangifera indica* cv. 'Keitt') fruit influences fruit quality and mineral composition. Postharvest Biol. Technol. 12:83-91.

Jacobi KK, Wong LS, Giles JE (1995). Effect of fruit maturity on quality and physiology of high-humidity hot air-treated 'Kensington' mango (*Mangifera indica* Linn.). Postharvest Biol. Technol. 5:149-159.

Kaur M, Singh N, Sandhu KS, Guraya HS (2004). Physicochemical, morphological, thermal and rheological properties of starches separated from kernels of some Indian mango cultivars (*Mangifera indica* L.). Food Chem. 85:131-140.

Khalid R (2007). Studies on free radicals, antioxidants, and co-factors. Clin. Interv. Aging. 2:219-236.

Kim H, Moon JY, Kim H, Lee DS, Cho M, Choi HK, Kim YS, Mosaddik A, Cho SK (2010). Antioxidant and antiproliferative activities of mango (*Mangifera indica* L.) flesh and peel. Food Chem. 121:429-436.

Kim Y, Brecht JK, Talcot ST (2007). Antioxidant phytochemical and fruit quality changes in mango following hot water immersion and controlled atmosphere storage. Food Chem. 105:1327-1334.

Lakshminarayana S, Subhadra N, Subramanyam H (1979). Some aspects of developmental physiology of the mango fruit. J. Hort. Sci. 45:133-142.

Lalel HJD, Singh Z, Tan SC (2003). Maturity stage at harvest affects fruit ripening, quality and biosynthesis of aroma volatile compounds in 'Kensington Pride' mango. J. Hort. Sci. Biotechnol. 78:225-233.

Larrauri JA, Ruperez P, Borroto B, Saura-Calixto F (1996). Mango peel as a new tropical fiber: preparation and characterization. Trends in Food Sci. Technol. 329:729-733.

Léchaudel M, Joas J (2006). Quality and maturation of mango fruits of cv. Cogshall in relation to harvest date and carbon supply. Austr. J. Agric. Res. 57:419-426.

Lim YY, Lim TT, Tee JJ (2007). Antioxidant properties of several tropical fruits: A comparative study. Food Chem. 103:1003-1008.

Lizada MC (1991). Postharvest physiology of the mangos-A review. Acta Hortic. 291:437-453.

Loelillet D (1994). The European mango market: A promising tropical fruit. Fruit 49:332-334.

Meneses M, Guamán M, Adami R, Scognamiglio M, Caputo G, Reverchon E. (2013). Supercritical antisolvent fractionation of bio-active compounds from mango by-products. III Iberoamerican Conference on Supercritical Fluids Cartagena de Indias (Colombia), 1-7.

Mercandate AZ, Rodriguez-Amaya DB (1998). Effects of ripening, cultivar difference and processing on the carotenoids composition of mango. J. Agric. Food Chem. 48:128-130.

Min SY, Yang H, Seo SG, Shin SH, Chung MY, Kim J, Lee SJ, Lee HJ, Lee KW (2013). Cocoa polyphenols suppress adipogenesis *in vitro* and obesity *in vivo* by targeting insulin receptor. Inter. J. Obesity. 37:584-592.

Mitra SK (1997). Postharvest physiology and storage of tropical and subtropical fruits, CAB International, Wallingford, Oxon, OX10 8DE, UK.

Moharram YG, Moustafa AM (1982). Utilisation of mango seed kernel (*Mangifera indica*) as a source of oil. Food Chem. 8:269-276.

Mudau FN, Mabusela PJ, Ngezimana W (2013). Proximate, chemical compositions and sulphur concentrations on quality of selected dried mango (*Mangifera indica* L.). Afr. J. Biotechnol. 12:2678-2684.

Muoki PN, Makokha AO, Onyango CA, Ojijo NK (2009). Potential contribution of mangoes to reduction of vitamin A deficiency in Kenya. Ecol. Food Nutr. 48:482-498.

Noratto GD, Bertoldi MC, Krenek K, Talcott ST, Stringheta PC, Mertens-Talcott SU (2010). Anticarcinogenic effects of polyphenolics from mango (*Mangifera indica*) varieties. J. Agric. Food Chem. 58:4104-4112.

Nunes CN, Emond JP, Brecht JK, Dea S, Proulx E (2007). Quality curves for mango fruit (cv. Tommy Atkins and Palmer) stored at chilling and nonchilling temperatures. J. Food Qual. 30:104-120.

Ojokoh AO (2007). Effect of fermentation on the chemical composition of mango (*Mangifera indica* R) peels. Afr. J. Biotechnol. 6:1979-1981.

Othman OC, Mbogo GP (2009). Physico-chemical characteristics of storage-ripened mango (*Mangifera indica* L.) fruits varieties of eastern Tanzania. Tanz. J. Sci. 35:57-66.

Palmeira SMV, Gois LM, Souza LD (2012). Extraction of phenolic compounds from mango peels. Latin American App. Res.42:77-81.

Parr AJ, Bolwell JP (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. J. Food Sci. Agric. 80:985-1012.

Pedroza-Islas R, Aguilar-Esperanza E (1994). Obtaining pectins from solid wastes derived from mango processing. AIChE Symposium Series 300:36-41.

Pietta PG (2000). Flavonoids and antioxidants. J. Nat. Prod. 63:1035-1042.

Puravankara D, Bohgra V, Sharma RS (2000). Effect of antioxidant principles isolated from mango seed kernels on oxidative stability of buffalo ghee (buffer fat). J. Food Sci. Agric. 80:522-526.

Ravindran V, Sivakanesan R (1996). The nutritive value of mango seed kernels for starting chicks. J. Sci. Food Agric. 71:245-250.

Reyers TT, Vega WF (1988). Factors involved in storage of many fruits: A new evaluation proceedings International Society Citriculture. pp 227-231.

Rodríguez Pleguezuelo CR, Durán Zuazo VH, Muriel Fernández JL, Franco Tarifa D (2012). Physico-chemical quality parameters of mango (Mangifera indica L.) fruits grown in a Mediterranean subtropical climate (SE Spain). J. Agr. Sci. Tech. 14: 365-374.

Saikat S, Chakraborty CS, Reddy YSR, Biplab De (2010). Free radicals, antioxidants, diseases and phytomedicines: Current status and future prospect. Inter. J Pharmaceutical Sci. Rev. Res. 3:91-100.

Schieber A, Ullrich W, Carle R (2000). Characterization of polyphenols in mango puree concentrates by HPLC with diode array and mass spectrometric detection. Innov. Food Sci. Emerging Tech. 1:161-166.

Souza IG, Valente SE, Britto FB, de Souza VA, Lima PS (2011). RAPD analysis of the genetic diversity of mango (*Mangifera indica*) germplasm in Brazil. Genet. Mol. Res. 10:3080-3089.

Srirangarajan AN, Shrikhande AJ (1976). Mango peel waste as a source of pectin. Current Sci. 45:620-621.

Statistical analysis system institute Inc. (1999). User's guide, Version 8.0. 2nd ed. Vol.2, Cary NC, USA.

Tahir FM, Pervaz MA, Hameed C (2002). Losses of mango fruits after harvest and its control. Agri. Digest, 37: 62-64.

Taing MW, Pierson JT, Hoang VL, Shaw PN, Dietzgen RG, Gidley MJ, Roberts-Thomson SJ, Monteith GR (2012). Mango fruit peel and

flesh extracts affect adipogenesis in 3T3-L1 cells. Food Funct. 3:828-836.
Taing MW, Pierson JT, Shaw PN, Dietzgen RG, Roberts-Thomson SJ, Gidley MJ, Monteith GR (2013). Mango (*Mangifera indica* L.) peel extract fractions from different cultivars differentially affect lipid accumulation in 3T3-L1 adipocyte cells. Food Func. 3:481-491.
Tandon DK, Garg N (1999). Mango waste: a potential source of pectin fiber and starch. Indian J. Environ. Protection. 19:924-927.
Tandon DK, Kalrat SK, Singh BP, Garg N (1991). Characterization of pectin from mango fruit waste. Indian Food Packer 45:9-12.
Tomas-Barberan F, Espín JC (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J. Food Sci. Agric. 81:853-876.
Ueda M, Sasaki KS, Utsunimiya N, Inaba K, Bayashi YS (2000). Variation of total polyphenol oxidase activity during maturation of mango fruit (*Mangifera indica* L. Irwin) cultured in plastic greenhouse. Food Sci. Tech. Res. 6:299-305.
Van Duyn MA, Pivonka E (2000). Overview of the health benefits of fruit and vegetable consumption for the dietetics professional. Selected literature. JADA 12:1511.
Wang CZ, Sangeeta M, Tyler C, Yuan CS (2011). Botanical Flavonoids on Coronary Heart Disease. Am J Chin Med. 4:661-671
Waterman PG, Mole S (1994). Analysis of phenolic plants metabolites. Oxford Blackwell Scientific Publications. London.
Wolfe K, Wu X, Liu RH (2003). Antioxidant activity of apple peels. J. Agric. Food Chem.51:609-614.

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Full Length Research Paper

Physicochemical changes of raffia sap (*Raphia mambillensis*) contents during spontaneous fermentation

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The chemical content of a substance can influence its shelf life. Fermentation causes changes in freshly tapped sap and therefore makes its large scale use difficult. In order to study the effect of time on fermentation on the raffia sap property, its physico-chemical and microbiological characteristics were determined during five days. The results recorded for the harvest reveal an acidity, proteins and energy of about 67.7°D, 6.69 g/l, and 113.64 kcal/l respectively. Raffia sap acidity and protein content increase during fermentation when the energy value decreases. The microbiological analysis showed that this sap contains an average of 16.46 x 10^7 cfu/ml of total germs which increases during fermentation. This sap is therefore rich in useful and pathogenic micro-organisms. We noticed that this sap is very rich in lactic acid bacteria and yeasts which could be isolated and exploited for alcoholic and malolactic fermentations in order to obtain a very stable raffia sap.

Key words: Raffia sap, spontaneous fermentation, physicochemical property, lactic acid bacteria, yeast, nutrient content.

INTRODUCTION

The raffia sap commonly called raffia wine is a part of a series called "palm wine" which refers to all alcoholic beverages from the fermentation of sap of various palm trees. The raffia wine is an alcoholic traditional, sweet, effervescent beverage, usually consumed by the poor populations of Black Africa, Latin America and Asia (FAO, 1998).

In Cameroon, this sap is obtained from the palm tree *Raphia sp.* in the West region and particularly from the *Raphia vinifera* and *Raphia mambillensis* in the area of Dschang. It is largely consumed and presented in all traditional ceremonies. In response to humanitarian needs and environmental issues increasingly growing, the use of raffia sap increases more and more (Mbuagbaw et al., 2012). Indeed, it is used in almost all African tribes in particular during births, traditional rites, weddings, funerals. It is used in the treatment of venereal diseases, measles, and typhoid and is a significant contribution in the treatment of impotence. It also favors milk production in lactating women (Tachago, 2007; Mbuagbaw et al., 2012). This drink is rich in sugars, proteins, alcohol, minerals, and vitamins (Malaisse, 1997; Ogbonna et al., 2013) and contains a certain number of microorganisms (Okafor, 1972; Obire, 2005). With time, fermentation converts sugars to ethanol, then into lactic or acetic acid (Matthews et al., 2004), which is accompanied by a loss of sweet taste and palatability (Odunfa, 1985). Like other alcoholic traditional beverages, the fresh raffia sap has a very short life span; it cannot be preserved for more than one day; the sap becomes very sour which makes the product unacceptable by the consumers (Uzochuku, 2004; Mintah

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et al., 2011). The present work was undertaken to assess the physico-chemical and microbial evolutions of freshly harvested raffia sap during fermentation in order to understand the transformation that takes place in the sap and further set-up a process of conservation for this sap in Menoua division (West-Cameroon).

MATERIALS AND METHODS

Sample collection

The raffia sap used in this study was obtained from raffia palm plantations located at three different areas. Sixty-seven (67) samples of fresh raffia sap were collected (1500 ml each) from winegrower in Menoua division (West-Cameroon) during a three months period. Those samples were kept in clean sterile bottles and transported to the laboratory within 30 - 60 min of tapping. The samples were immediately distributed in three sterile bottles properly labelled and recorded; one for immediate analysis, another for the second day and the third bottle for the fifth day analysis. The last two bottles were preserved in the laboratory under sterile conditions at room temperature (approximately 25°C).

Analysis of samples for physico-chemical contents

Twenty milliliter (20 ml) portion of each sap sample were removed aseptically for each measurement. Moisture content and the density of the fresh wine were determined using the method described by AOAC (1995); acidity level was determined by titration of the sap with NaOH 1/9 N and pH using a pH-meter (SUNTEX TS-2) for the first, second, and fifth day. Each sample was repeated in triplicate.

Analysis of samples for microbial contents

The amount of micro-organisms was determined by serial dilutions and spread plate technique was used on appropriate selective media (Durgesh et al., 2008). Successive dilutions of the sap were prepared in screw test tubes and appropriate dilutions were poured into plates then enumerated for total plate count until scattered colonies were obtained. Ten milliliter (10 ml) of each sample was diluted with 90 ml of sterile buffered peptone water and well mixed. One milliliter (1 ml) of each dilution sample was inoculated in the following media: M17 Agar (Biolife) for the lactococci; the lactic acid bacilli on MRS Agar (Biokar Diagnostic); salmonellas and shigellosis on Salmonella-Shigellosis Agar (MERK) yeasts and moulds on Potato-Dextrose Agar; enteric bacteria on MacConkey Agar (SIGMA); staphylococci on Staphylococcus110 Agar (SIGMA) and total flora on plate count agar (SIGMA). All plates were incubated under aerobic conditions at room temperature (25 ± 1°C) and the colonies were counted using a colony counter (Stuart) after a period of 24 h to 72 h depending on the growth media. The mean number of colonies counted was expressed as log of colony forming units (cfu)/ ml. In order to check up the specificity of media, Gram stain test was done and colonies were observed under a light microscope with an appropriate dye to observed their shape and verify their membership in the considered group. Each experiment was performed on samples newly collected and those of the second and fifth days. Each sample was carried out in triplicate.

Analysis of samples for nutritional contents

Determination of total ash

Total ash was the residue obtained after incineration of the dried sample and represented as the mineral part of it. The method described by AOAC (1980) was adopted which consists of incinerating the sample in an oven (Hereaus) at 560°C in an oxidizing atmosphere until obtaining a residue of constant weight.

Determination of lipid content

Determination of fat quantity was done by Soxhlet extraction. The lipid was extracted in a Soxhlet extraction apparatus (Glassco) with hexane as solvent. The Soxhlet extraction procedure is a semi continuous process, which allows the buildup of the solvent for some time in the extraction chamber. A dried sample was crushed and introduced in an extraction thimble. The solvent surrounded the sample and was then siphoned back into the boiling flask. The lipid content was calculated at 0 % moisture by weight difference of the flask before and after complete extraction of the oil as described by IUPAC (1979).

Mass of lipid = (weight of the flask + extracted oil) − (weight of the flask)
Lipid content (%) = mass of lipid extracted (g)/sample weight (g) × 100).

Determination of total proteins

Determination of total protein was carried out by determining total nitrogen in each sample. Total nitrogen was determined by the Kjeldahl method as described by AOAC (1980). This method transformed organic nitrogen of the dried sap into mineral nitrogen by mineralization with concentrated sulfuric acid and a catalyst using ramp of mineralization all under hood. The nitrogen present in the sample was fixed in the form of $(NH4)_2SO_4$. After digestion, the solution was made alkaline by adding sodium hydroxide using an automatic distiller (Kjeltec System) to release ammonia. After distillation, the amount of ammonia was determined by acid-base titration with boric acid and the amount of protein was estimated using a conversion factor.

Determination of total carbohydrate

Total carbohydrates were determined using the current method of difference (AOAC, 1980). Total carbohydrate = [rate of dry matter - (ash + rate of lipid + rate of protein)] or
Total carbohydrate = [rate of organic matter - (rate of lipid + rate of protein)].

Statistical analysis

Data collected from physico-chemical, microbiological and nutritional content analyses of the sap at different stages of spontaneous fermentation were subjected to statistical analysis. All the results underwent the variance analysis (ANOVA) to the threshold of probability 0.01 and the test of Bonferroni was used to compare the averages by using software GRAPHPAD INSTAT.

RESULTS

The sap sources are shown in Table 1. After having transported the different sap samples collected in the laboratory, physical and chemical tests such as water content, density, Dormic acidity and pH were done on those samples.

During fermentation, the moisture contents of raffia sap

Table 1. Sources for raffia sap samples.

Month	Source			
	Bamendou	Foreke	Foto	Total
1st	7	4	14	25
2nd	5	3	8	16
3rd	8	3	15	26
Total	20	10	37	67

Table 2. Physico-chemical properties in the raffia sap during five days of spontaneous fermentation.

Number of days of fermentation	Parameters			
	Water content (%)	Density (g/ ml)	Dormic Acidity (°D)	pH
0	96.11 ± 1.21	1.00 ± 0.02	67.6 ± 06.38	4.08 ± 0.19
2	97.12 ± 0.72	0.97 ± 0.01	90.6 ± 20.00*	3.40 ± 0.07*
5	97.17 ± 0.05	1.00 ± 0.04	113 ± 13.68*	3.21 ± 0.16*

*, Significant difference; n, 67.

were: 96.11 ± 1.21%; 97.12 ± 0.72% and 97.17 ± 0.059% respectively at harvest, second and fifth days of spontaneous fermentation at room temperature (approximately 25°C). This content increases between harvest and the second day and then seems to be stabilized the fifth day (Table 2). However, there is not any significant difference at $p > 0.01$ from one sample to another and during five days of fermentation.

Values of the density were about 1.005 ± 0.02, 0.97 ± 0.01 and 1.002 ± 0.04 g/ml. We noticed that this content decreased slightly during the second day, then increased on the fifth day (Table 2). However this variation of density from one day to another was not significant. We obtained the following pH average values: 4.08 ± 0.19; 3.4 ± 0.07; 3.21 ± 0.16 respectively at harvest, second and fifth days (Table 2). This pH decreases during fermentation and was significantly different at $p > 0.01$ between harvest, second day and fifth day.

The values of Dormic acidity were: 67.6 ± 6.38°D; 90.6 ± 20°D and 113.4 ± 13.68°D. Dormic acidity of this sap increased during fermentation, what would also justify the reduction in the pH. In fact high Dormic acidity translates the presence of the lactic acid in the wine. This acidity as for the pH was significantly different at $p > 0.01$ between harvest and the fifth day, what translates the very acid character observed at this stage of fermentation.

In order to understand all these physico-chemical changes in the sap during spontaneous fermentation, we analyzed its wild microflora. We noticed that the microflora of the fresh raffia sap of *R. mambillensis* is very dense and it contains useful micro-organisms (Figure 1) and pathogenic micro-organisms (Figure 2); its total microflora increasesd during the five days. The most abundant micro organisms in the fresh sap were the lactic bacteria (lactobacilli and lactococci), then come yeasts (Figure 1). Then, we had the faecal coliforms, the staphylococci, few salmonellas and shigellosis (Figure 2), and other non-determined micro-organism but however present in the total flora. We noticed a positive correlation between lactic acid bacteria, yeasts, salmonellas and shigellosis and the density of the sap; also between enteric bacteria, staphylococci, acidity and the water content. Sap acidity seems to be unfavourable to useful micro-organisms and favourable to pathogenic micro-organisms.

During fermentation, there was no significant difference at $p > 0.01$ between the lactic bacilli, the lactococci, yeasts and moulds, total coliform bacteria; however these kinds are significantly different from staphylococci and salmonellas and shigellosis. Evolution of sap nutrient content during fermentation was studied and the results are presented per liter of sap in Table 3. From this, the average rate of protein increased during the fermentation while the lipid decreased and was significantly different at $p < 0.05$ between the first and fifth days.

Total carbohydrates average decreased during the fermentation with a significant difference at $p < 0.05$ between harvest, second and fifth days. Average ash decreased in the fermented raffia sap. There was no correlation between proteins, fat and total flora. Proteins significantly increased the fifth day. In general, there was a decrease in dry matter, lipids, carbohydrates, ash, and energy with the fermentation time; this decrease was significant at the fifth day.

DISCUSSION

Sap water content obtained at harvest (fresh sap) is similar to values reported by Malaisse (1997) with sap obtained from other palm tree species. Moisture content determines the shelf-life of sap. The lower the moisture content, the longer the expected shelf-life, thus moisture content is an important measure of sap quality as reported

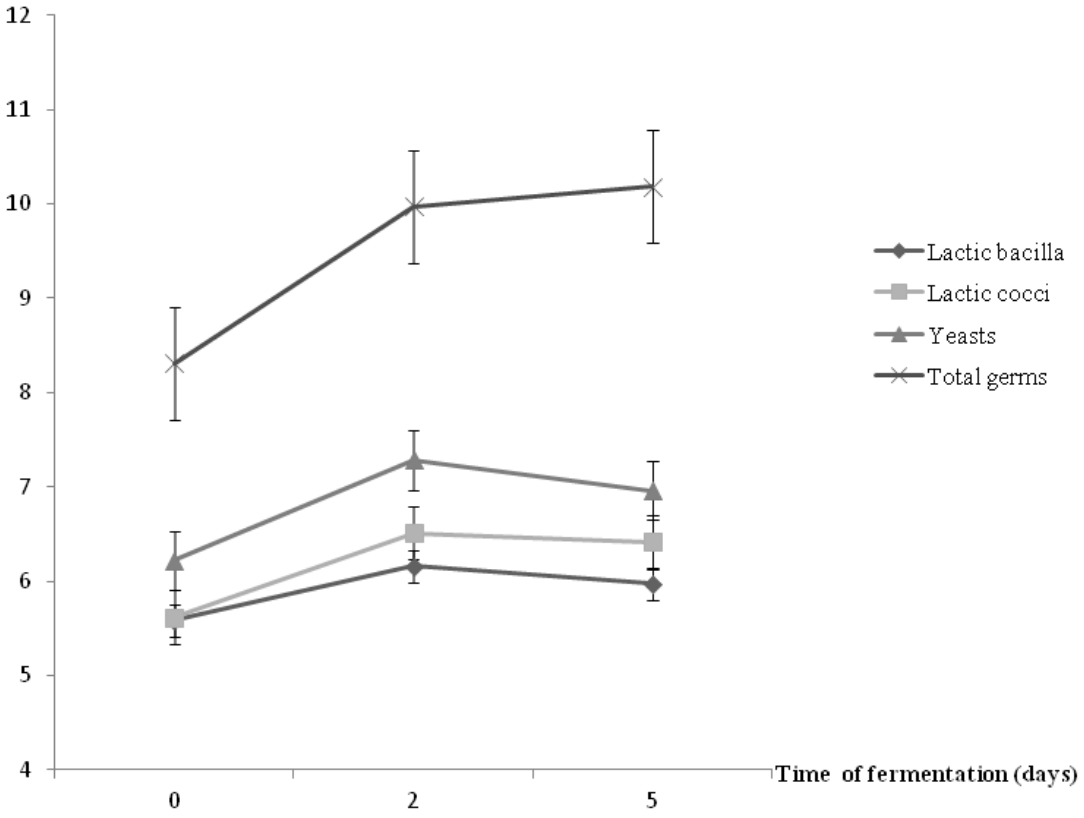

Figure 1. Evolution of useful microorganisms in the sap of *Raphia mambillensis.*

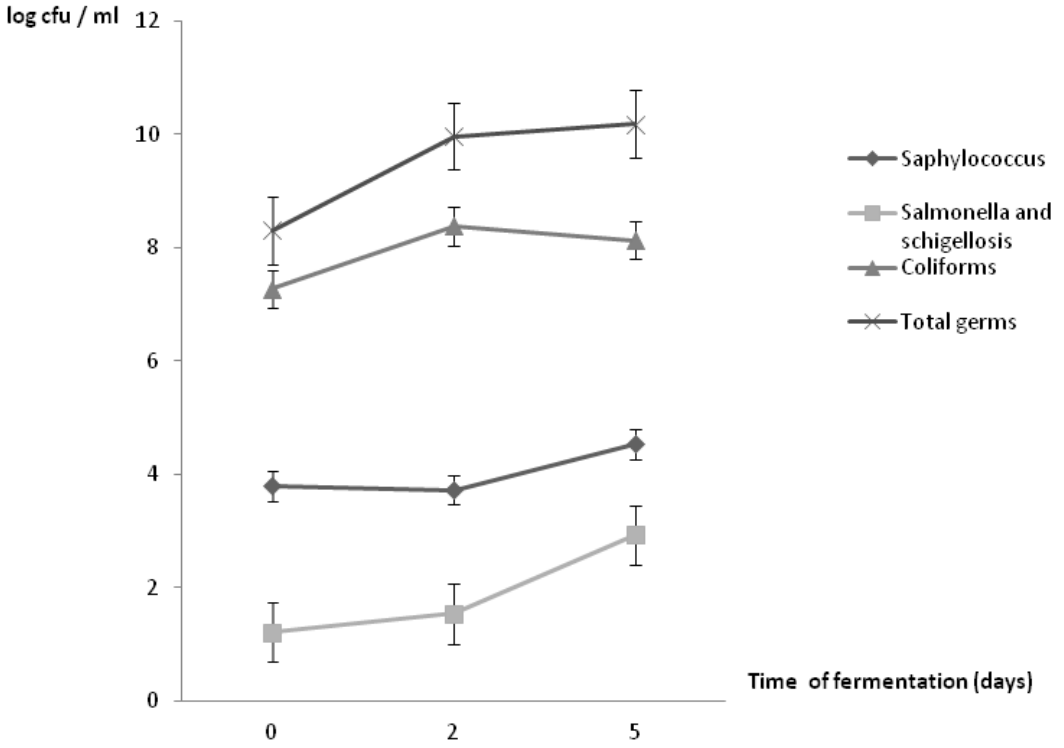

Figure 2. Evolution of pathogenic microorganisms in the sap of *Raphia mambillensis.*

Table 3. Nutritive composition of *Raphia mambillensis* sap during spontaneous fermentation.

Number of fermentation days	Parameter					
	Dry matter (g / l)	Protein (g / l)	Fat (g / l)	Carbohydrates (g / l)	Ash (g / l)	Energy (Kcal / l)
0	34.06 ± 0.51	6.69 ± 1.41	1.96 ± 0.47	17.31 ± 2.86	8.10 ± 1.23	113.64 ± 21.31
2	30.37 ± 0.74	7.01 ± 0.96	1.94 ± 0.39	13.51 ± 1.47	7.91 ± 0.70	99.54 ± 13.23
5	29.25 ± 0.16	8.85 ± 2.04 *	0.95 ± 0.51 *	11.84 ±2.02 *	7.71 ± 1.09 *	94.01 ± 20.83 *

* Difference is significant ($p < 0.05$) between the first and fifth days. n = 67

by Mintah et al. (2011). Increase in water content the second day corresponded to a reduction in the dry matter which could be due to substrate degradation such as ethanol to sugars with CO_2 production as reported by Matthews et al. (2004).

Between the second and the fifth day, there could be conversion of ethanol produced in lactic acid or acetic as described by Odunfa (1985) and Matthews et al. (2004). Density decrease observed the second day could be due to alcohol production which is lighter than water; between the second and the fifth day, there could be alcohol transformation into acetic acid and lactic acid. pH value obtained for the first day of spontaneous fermentation at room temperature is similar to that obtained by Malaisse (1997). According to this author, the chemical characteristics of the palm sap vary during the bleeding, making the comparisons sometimes delicate as indeed evidenced by several studies (Okafor, 1975; Mintah et al., 2011; Karamoko et al., 2012; Mbuagbow and Noorduyn, 2012; Ogbonna et al., 2013).

The microbiological analysis reveals that the most abundant micro-organisms in the fresh raffia wine are the lactic bacteria and the yeasts as underscored by Mattews et al. (2004). According to the same authors, the yeasts are responsible for the alcoholic fermentation of the sap and the lactic bacteria are responsible for malolactic fermentation (transformation of the L-malic acid into L-lactic). Raffia wine naturally contains micro-organisms (useful and pathogenic) as had already been described by certain authors such as Okafor (1972, 1975) and Obire (2005) for wine samples collected in Nigeria. At this stage of work, we recommend that raffia sap be consumed the first two days following the harvest because after this period it contains most pathogenic micro-organisms.

Nwachukwu et al. (2006) and Ogbulie et al. (2007) studied the effects of micro organisms on saps from other species and showed that these saps host a number of natural micro organisms that are responsible for their instability. Since these micro organisms catalyze the fermentation process, which leads to nutrient degradation or loss of sensory qualities of palm wine, loss due to the sour taste of fermented palm wine by the acids produced during the fermentation process as noticed by Malaisse (1997) and Ukhum et al. (2005).

The sap analysis showed that the average protein content increased during fermentation, which was due to cell death during fermentation. However, in our results, there is no correlation between changes in the rate of protein and that of the total flora during the fermentation. The protein average of fresh raffia sap was higher than the values obtained by Malaisse (1997) and Ghogomu (2004); this could be due to the influence of climatic and soil factors that influence the nutritive composition of sap samples.

Lipid decrease is due to degradation of raffia sap during fermentation by microorganisms. The energy value decreases, which is due to the reduction in the rate of reducing sugars which would be transformed into alcohol and acid. But if we had taken into consideration the increasing level of alcohol in the raffia sap from the work of Tachago in 2007 with the same species, the energy increased. The raffia sap naturally hosts microorganisms (pathogens and useful) as already described by Okafor in 1975 for sap samples collected in Nigeria. These pathogens originate from contact with the sap from the bark of the tree and plant debris (pulp rotten banana trunk) used to seal the entrance to the bud sectioned (Okafor, 1975; Uzochukwu, 2004; Ogbulie, 2007; Tachago, 2007).

The most abundant microorganisms in the raffia sap were lactic acid bacteria and yeasts as was underlined in Matthews et al. (2004) in red wine. Lactic acid bacteria, yeasts, salmonella and shigella decreased on the second day and subsequently increase as the density of the fifth day; we can say at this stage that these organisms have reached their stationary phase of growth. According to several authors such as Matthews et al. (2004), the yeasts are responsible for the alcoholic fermentation of a sap; lactic acid bacteria are responsible for malolactic fermentation (conversion of L-malic acid into L-lactic acid). Enteric bacteria and Staphylococcus increased until the fifth day as the acidity and water content. The sap acidity seems to be favourable to pathogenic micro-organisms and unfavourable to yeasts and lactic acid bacteria which are micro organisms with beneficial effects as reported by Tiepma et al. (2010). Sap acidity which seems to favour the growth of yeast and lactic acid bacteria led us to the perspective of isolating, characterizing for technological properties, identifying, and re-

seeding them in sterilized sap in order to control the fermentation.

Conclusion

A study of the impact of time of fermentation on raffia sap (*R. mambillensis*) property, its physico-chemical and microbiological characteristics during five days were carried out. The results of our study suggest that, the nutritional components as well as the microbiological content of the raffia sap from *R. mambillensis* change during spontaneous fermentation at room temperature. Wild microflora is very dense and includes both useful and harmful microorganisms with the majority being lactic acid bacteria and yeasts which could be isolated and exploited for alcoholic and malolactic fermentations, as well as for probiotic effects. In five days, the sap becomes very acidic and the most abundant acid was lactic acid. At the current state of our knowledge, these results constitute a database for studying the impact of fermentation period on the raffia sap and contribute to the creation of a stabilization process of this sap without completely changing its organoleptic qualities.

REFERENCES

AOAC (1980). Official methods of analysis. (13th ed.). Arlington V. A. Association of Official Analytical Chemists.

AOAC (1995). Official methods of analysis, (16th ed.). Arlington V. A. Association of Official Analytical Chemists.

Durgesh PM, Ranjana GK, Varsha KV (2008). Microbiological analysis of street vended fruit juices from Mumbai city, India. Internet J. Food Safe. 10:31-34.

FAO (1998). Fermented fruits and vegetables.With total prospect. Agricultural services. Agric. Report food Org. 134.

Ghogomu JN, Remigy JC, Aimar P, Aptel P (2004). Classification and stabilisation of palm juice and wine by membrane process, ICEF 9, International Congress on Engineering and Food. March, Montpellier, France:7-11.

IUPAC (1979). International Union of Pure and Applied Chemistry "Blue Book" Nomenclature of Organic Chemistry, Pergamon Press, Oxford, 1979.

Karamoko D, Djeni NT, N'guessan KF, Bouatenin KMJP, Dje KM (2012). The biochemical and microbiological quality of palm wine samples produced at different periods during tapping and changes which occurred during storage. Food control. 26:504-511.

Malaisse F (1997). To nourish itself in African clear forest: ecological and nutritional approach. *Agronomic presses of Gembloux* CTA.

Matthews A, Grimaldi A, Walker MR, Bartowsky E, Grbin P, Jiranek V (2004). Lactic acid bacteria have has potential source of enzymes for uses in vignification. Appl. Environ. Microbiol. 5715-5731.

Mbuagbaw L, Noorduyn SG (2012). The palm wine trade: Occupational and health hazards. The int. J. Occup. Env. Med. 3:157-164.

Mintah BK, Eliason AE, Barimah J, Oldham JH (2011). Development of syrup and “malt-like” drink from *Raphia hookeri* sap. Afr. J. Food Agric. Nutr. dev. 11:5203-5219.

Nwachukwu IN, Ibekwe VI, Nwabueze RN, Anyanwu BN (2006). Characterisation of palm wine yeast isolates for industrial utilization. Afr. J. Biotechnol. 5:1725-1728.

Obire O (2005). Activity of *Zymonas* species in palm-sap obtained from three areas in Edo State, Nigeria. Newspaper Appl. Sci. Environ. Manage. 9:25-30.

Odunfa SA (1985). African fermented foods. Microbiology of fermented foods. Elsevier applied Publishers Science, the UK.

Ogbonna AC, Abuajah CI, Akpan MF, Udofia US (2013). A comparative study of the nutritional values of palm wine and Kunu-zaki. Ann. Food Sci. Technol. 14:39-43.

Ogbulie TE, Ogbulie JN, Njoku HO (2007). Comparative study on the microbiology and shelf life stability of palm wine from Elaeis guineensis and Raphia hookeri obtained from Okigwe, Nigeria. Afr. J. Biotechnol. 6, 914-922.

Okafor N (1972). The source of the microorganisms in palm wine. Symposium Social Nigeria Microbioogly, 1.

Okafor N (1975). Microbiology of Nigerian Palm wine with particular reference to bacteria. Newspaper Appl. Bacteriol. 38:81-88.

Tachago DE (2007). Essai de stabilisation du vin de palme: études comparatives des effets anti-oxydants du benzoate de sodium et du meta bisulfite de sodium sur la fermentation du vin. *Thèse de Master*, Université de Dschang.

Tiepma NEF, Zambou NF, Tchouanguep MF (2010). Immune system stimulation in rats by *Lactobacillus sp.* isolates from raffia wine (*Raphia vinifera*). Cellular Immunol. 260:63-65.

Ukhum ME, Okolie NP, Oyerinde AO (2005). Some mineral profile of fresh and bottles palm wine-a comparative study. Afr. J. Biotechnol. 4:829-832.

Uzochukwu S (2004). Palm wine - Genetic engineering. Forum on Biotechnol. Food Agric.

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Full Length Research Paper

Tuberculosis treatment raises total cholesterol level and restores high density lipoprotein cholesterol (HDL-C) in patients with pulmonary tuberculosis

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The aim of this study was to determine whether tuberculosis (TB) treatment normalizes the lipid profile strongly affected by pulmonary TB. Serum levels of total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were determined in 83 patients with pulmonary TB before and after treatment, and compared to results obtained from 100 control subjects without TB. Before treatment, levels of TC ($p<0.005$), HDL-C ($p<0.005$) and LDL-C ($p<0.005$) were significantly lower in pulmonary TB patients than normal subjects. Unlike TC and LDL-C, HDL-C decrease was correlated ($r = 0.96$, $p<0.05$) with smear positivity extent (SPE). At the end of TB treatment, which lasted six months, TC ($p<0.01$) and HDL-C ($p<0.005$) levels were significantly increased than before treatment while LDL-C stayed relatively unchanged. The treatment significantly reduced the atherogenic indices TC/HDL-C ($p<0.001$), LDL-C/HDL-C ($p<0.001$) and log (TG/HDL-C) ($p<0.001$) levels. Our results show that tuberculosis treatment increases TC levels and normalizes HDL while reducing atherogenic indices to below levels of controls.

Key words: Pulmonary tuberculosis, lipid profile, treatment, atherogenic index.

INTRODUCTION

Cholesterol has received much attention in recent years mainly because of its involvement in cardiovascular disease (Smith et al., 1992). Current recommendations for treatment are all geared towards reducing the serum cholesterol levels (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). However, increasing evidence indicates a link between low blood cholesterol levels and a number of human diseases including tuberculosis (TB) (Perez-Guzman et al., 2005; Deniz et al., 2007). Specifically, it is reported that hypocholesterolemia promotes the development of TB whereas hypercholesterolemia confers some protection against infection with *Mycobacterium tuberculosis* (MTB) (Wilson et al., 2003; Perez-Guzman et al., 2005). Despite the existence of such links between cholesterol and TB, it is not known to which extent the treatment of the disease affects lipid indicators in patients with TB. We formulate the hypothesis that TB treatment is associated with restoration of normal levels of cholesterol in patients with pulmonary TB.

MATERIALS AND METHODS

Patients

This study was carried out as a pre-post test design study with a control group at *Centre National Hospitalier de Pneumo-Phtyisiologie Cotonou* (CNHPP) which is a reference center for TB diagnosis and treatment in West Africa. From October 2011 to June 2012, a total of 187 patients (72 females and 115 males) suffering from TB were admitted to CNHPP. Sputum and blood samples were systematically collected from all patients with clinical signs of pulmonary TB. Patients co-infected with MTB and human immunodeficiency virus, and those who underwent an incomplete TB treatment were excluded from the study. Furthermore, patients who were on antibiotic therapy or using any cholesterol lowering medication at the time of admission were also not included in the study. Eighty-three (83) out of 147 patients have met the criteria listed above and have been selected to participate in the study. All selected patients completed the treatment. One hundred (100) age- and sex-matched healthy subjects with no complaints and no known diseases were recruited at *Hôpital Saint-Luc* (Cotonou, Bénin) and enrolled as control group. This control group was tested negative upon TB diagnostic testing. Informed written consent was voluntarily obtained from each participant before entering the study and the local ethics board approved the study protocol.

Diagnostic criteria and bacteriological examination

On the basis of clinical signs, bacteriological diagnosis was requested by the physician for each patient. Two sputum samples were collected in two consecutive days in all patients. Sputum samples were examined by direct smear examination. Acid-fast mycobacteria (AFB) resist decolorization by acid-alcohol after primary staining owing to the high lipid (mycolic acid) content in their cell walls. The direct smear examination of sputum was carried out by auramine O stain. The technique consists of making a smear and drying it for 15-30 min, fixing it over the flame and staining for 15 min. The slides were decolorated with 0.5% acid-alcohol, rinsed, counterstained with acridine orange for 2 min and air dried. The smears were examined under oil immersion lens of microscope (objective 40X, total magnification, X400). To determine the smear positivity extent (SPE), the number of AFB were counted by an experienced technician. The results are expressed as the number of AFB per number of fields (F) observed as follows: +/-: 1-18/50F, +: 4-36/10F, ++: 4-36/F, +++: >36/F.

Measurement of biochemical parameters

Blood from fasting patients was collected in a dry tube (without anticoagulant) before and after treatment in experimental subjects and once in control subjects. In the event that the dosage was postponed, the serum was aliquoted into 1.5 ml Eppendorf tubes and stored at -20°C until needed. Total cholesterol (TC) (Biolabo, France), high density lipoprotein cholesterol (HDL-C) (Biolabo, France) and triglycerides (TG) (ELITech Group, France) were assayed by enzymatic methods. Low density lipoprotein cholesterol (LDL-C) was determined using the Friedewald formula.

Treatment

The treatment was based on a combination of isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E) and streptomycin (S) for a period of 6 months. It comprises an initial phase with a combination of ERHZ for two months, followed by a four month treatment of HR. In case of recurrence, the initial treatment comprised a two month treatment with ERHZS, followed by 1 month of ERHZ and five months of ERH for a total treatment time of 8 months.

Statistical analyses

Data were evaluated by Student's t-test using the SigmaPlot statistical analysis software (Systat Software, Inc. San Jose, CA, USA). Quantitative data are expressed as mean ± SEM. The null hypothesis was rejected at the level of 0.05.

RESULTS

This study involved 83 TB patients as the experimental group and 100 normal subjects who had previously not been diagnosed or received treatment for TB as the control group. TB patients were aged between 12 and 62 years with an average age of 32.37 ± 12.87 and included 57 men (68.67%) and 26 women (31.33%). The control group consisted of 35 women (35.00%) and 65 men (65.00%). The age of the control group was between 17 and 67 years with an average of 35.70 ± 12.48. There were no significant differences in age ($p=0.078$) between the two groups.

Lipid parameters were measured in 83 experimental subjects suffering from TB and 100 control subjects. In TB patients, the same individuals were tested before treatment and at the end of treatment. Before treatment, TC ($p<0.005$), HDL-C ($p<0.005$) and LDL-C ($p<0.005$) levels were significantly lower in TB patients as compared to normal subjects (Figure 1A). Triglycerides levels were unchanged between the two groups (Figure 1A). At the end of treatment, which lasted six months, the levels of TC remained significantly ($p<0.01$) lower in TB treated patients when compared to controls (Figure 1B). However, compared with the levels before treatment, TC levels increased significantly ($p<0.01$) (Figure 1C). TB treatment had not changed the levels of TG and LDL-C (Figures 1B and 1C). However, the levels of HDL-C were significantly increased after treatment compared to the control group ($p<0.02$) (Figure 1B) and with respect to non-treated TB patients ($p<0.005$) (Figure 1C).

In order to determine whether changes in the levels of lipid parameters correlated with the extent of bacterial load in TB patients, we compared the levels of TC, HDL-

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Abbreviations: TB, Tuberculosis; **MTB,** *Mycobacterium tuberculosis*; **CNHPP,** Centre National Hospitalier de Pneumo-Phtyisiologie Cotonou; **AFB,** acid-fast mycobacteria; **SPE,** smear positivity extent; **TC,** total cholesterol; **HDL-C,** high density lipoprotein cholesterol; **TG,** triglycerides; **LDL-C,** low density lipoprotein cholesterol; **H,** isoniazid, **R,** rifampicin; **Z,** pyrazinamide; **E,** ethambutol; **S,** streptomycin.

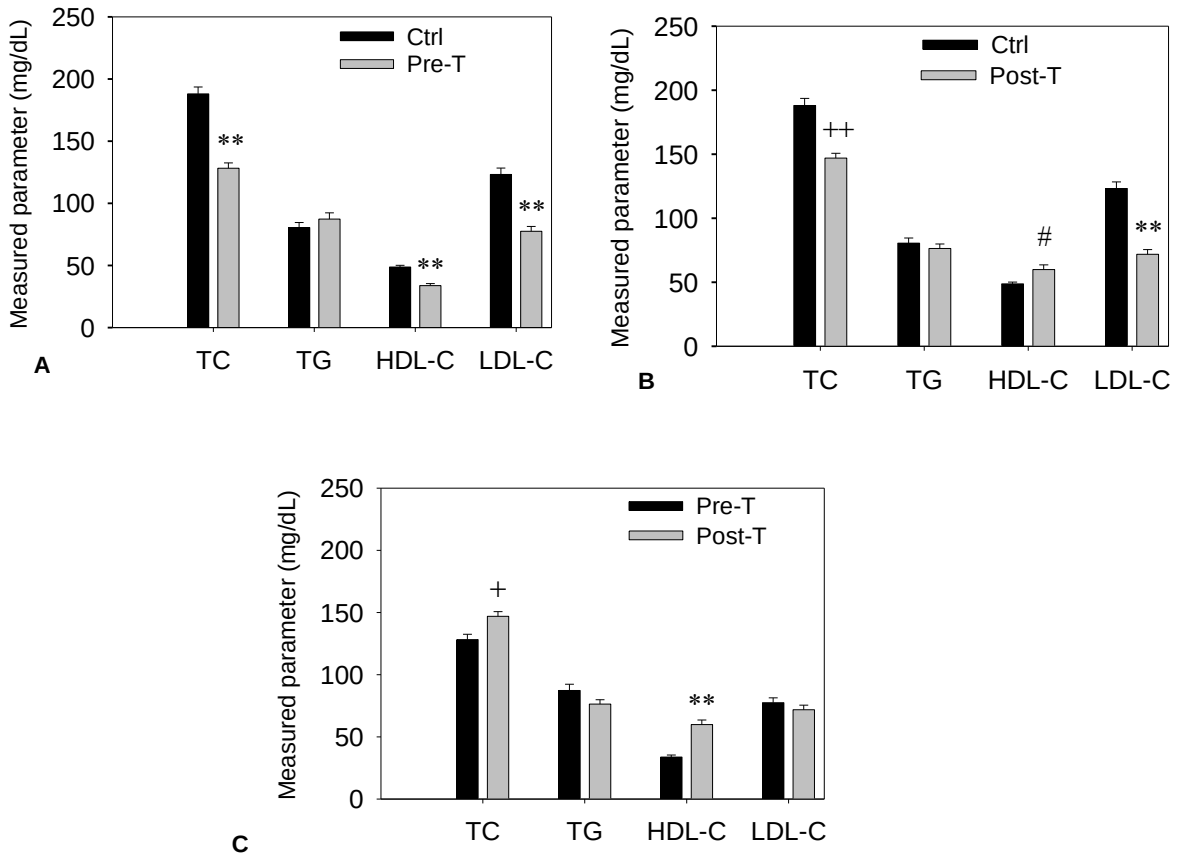

Figure 1. The mean levels of serum lipid parameters in treated and pre-treated tuberculosis patients. The mean levels of TC, TG, HDL-C and LDL-C of 83 tuberculosis patients prior to treatment (Pre-Treatment, Pre-T) (A) and at the end of treatment (Post-Treatment, Post-T) (B) were compared to that of 100 control patients. The values of lipid parameters in tuberculosis patients before (Pre-T) and after treatment (Post-T) were matched (C). The values shown are mean ± SEM. (**p<0.005; +p<0.01; ++p<0.001; #p<0.02).

C and LDL-C to corresponding SPE before treatment. We did not observe any correlations between the levels of TC (Figure 2A) and LDL-C (Figure 2B) and SPE. However, we found a strong negative correlation (r = - 0.96, p<0.05) between HDL-C and SPE (Figure 2C).

We calculated atherogenic indices in order to determine whether TB treatment affects these predictors of disease associated with dyslipidemia in TB patients. The results are summarized in Table 1. TC/HDL-C and LDL-C/HDL-C in TB patients were not significantly different from those in normal subjects. However, indices TC/HDL-C (p<0.001) and LDL-C/HDL-C (p<0.001) declined significantly after six months of treatment compared to controls and to patients prior to treatment. Log (TG/HDL-C) is another index referred to as the atherogenic index of plasma and reflects the size of circulating LDL (Dobiasova and Frohlich, 2001). Log (TG/HDL-C) was significantly increased (p<0.002) in TB patients before treatment compared to controls and then fell significantly (p<0.001) after 6 months of treatment to levels lower than those in control subjects. We compared atherogenic indices with SPE in patients before treatment. TC/HDL-C ratio increased significantly (p<0.01) in patients in whom SPE was the highest (Figure 3A). Log (TG/HDL-C) increased with the smear positivity to reach its maximum level (p<0.05) in patients with the highest SPE (Figure 3B). The index LDL-C/HDL-C did not vary significantly with increasing SPE (Figure 3C).

DISCUSSION

This study has shown that the level of TC was significantly lower in patients with TB. Similar results were reported elsewhere (Deniz et al., 2006 and 2007; Perez-Guzman et al., 2005). An adequate level of cholesterol is necessary for the proper functioning of the immune system against infection (Heiniger and Marshall, 1982). Our results support the observation that the level of circulating TC decreases in severe infections (Gonzales and Sande, 2000). Perez-Guzman et al. (2005) have shown that a cholesterol-rich diet accelerates bacteriologic sterilization in patients with TB. We showed here that treatment of TB during six months

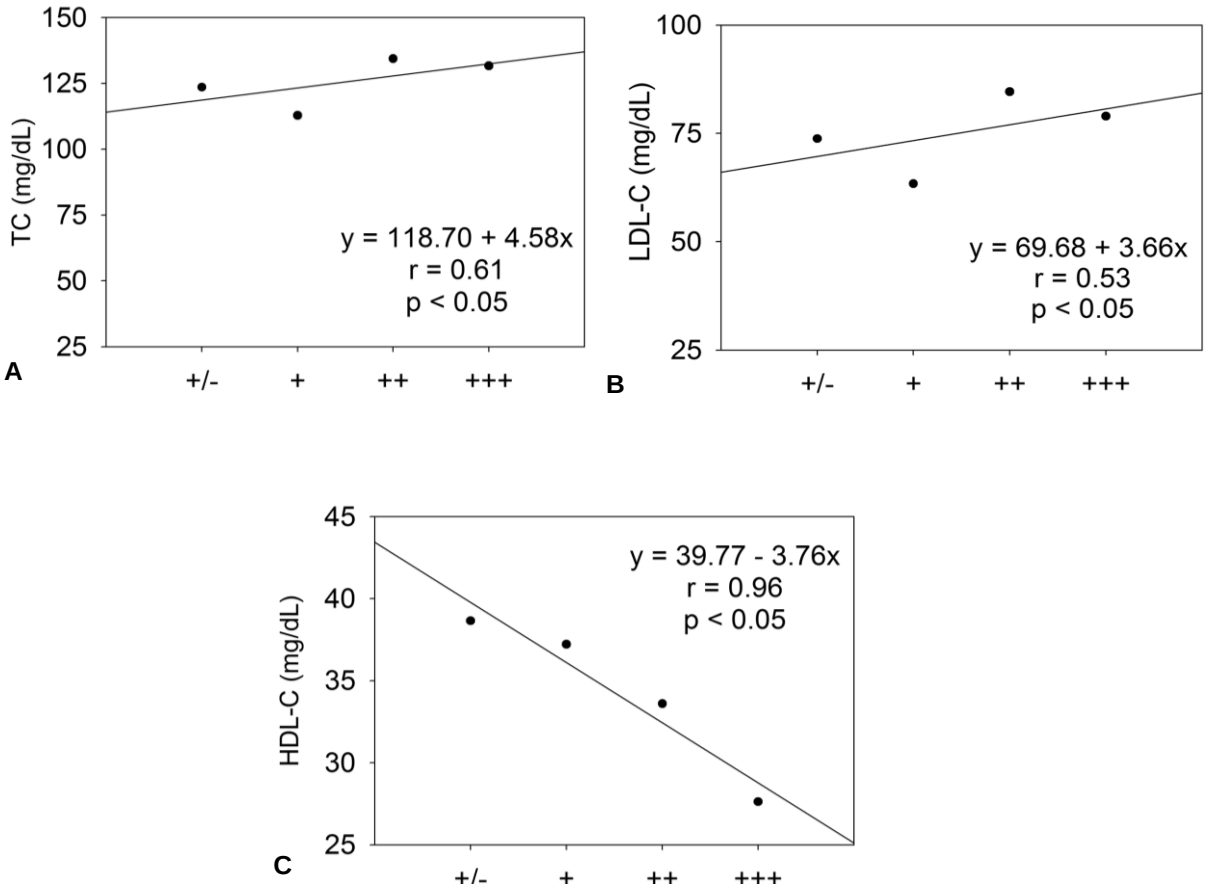

Figure 2. Correlations between smear positivity extent scores and lipid parameters. The values of SPE scores were correlated to the levels of TC (A), LDL-C (B) and HDL-C (C) in tuberculosis patients before the beginning of anti-tuberculosis treatment. (+/-, n = 15; +, n = 16; ++, n = 34; +++, n = 18).

Table 1. Atherogenic indices variation in TB patients.

Atherogenic index	Controls (n=100)	Pre-T (n=83)	Post-T (n=83)
TC/HDL-C	4.16 ± 0.18	4.55 ± 0.28	2.78 ± 0.11[a,a]
LDL-C/HDL-C	2.77 ± 0.16	2.89 ± 0.23	1.49 ± 0.10[a,a]
Log (TG/HDL-C)	0.19 ± 0.03	0.41 ± 0.04[d]	0.11 ± 0.03[b,a]

Atherogenic indices were determined in TB patients pre- and post-treatment and in controls. The values are the mean ± SEM of individual index in 100 control participants and 83 TB patients prior to treatment (Pre-T) and post-treatment (Post-T). ([a]p<0.001 Post-T vs Control or Pre-T; [b]p<0.05 Post-T vs Control; [d]p<0,002 Pre-T vs control).

under a normal diet significantly raises the TC level. This indicates that hypocholesterolemia can be seen as a consequence of TB disease although it cannot be excluded as also being a factor contributing to the development of pulmonary TB (Pérez-Guzmán et al., 2005). We have shown that there is no correlation between the bacillary load and TC variations. This suggests that the use of host cholesterol by MTB (Van der Geize et al., 2007) contributes little to the lower TC level in TB patients.

We showed that HDL-C level decreased significantly in non-treated TB patients compared to controls, and increased significantly after treatment above the control levels. Our results also showed that HDL-C decrease was strongly correlated with SPE. In general, HDL-C catabolism increases during inflammation. Response to inflammation during the acute phase of TB is characterized by an over expression of proteins such as phospholipase A2 and circulating amyloid A (Tietge et al., 2002) which stimulates HDL-C catabolism (Deniz et al., 2006). It is therefore expected that the levels of HDL-C increase after successful treatment in TB patients as

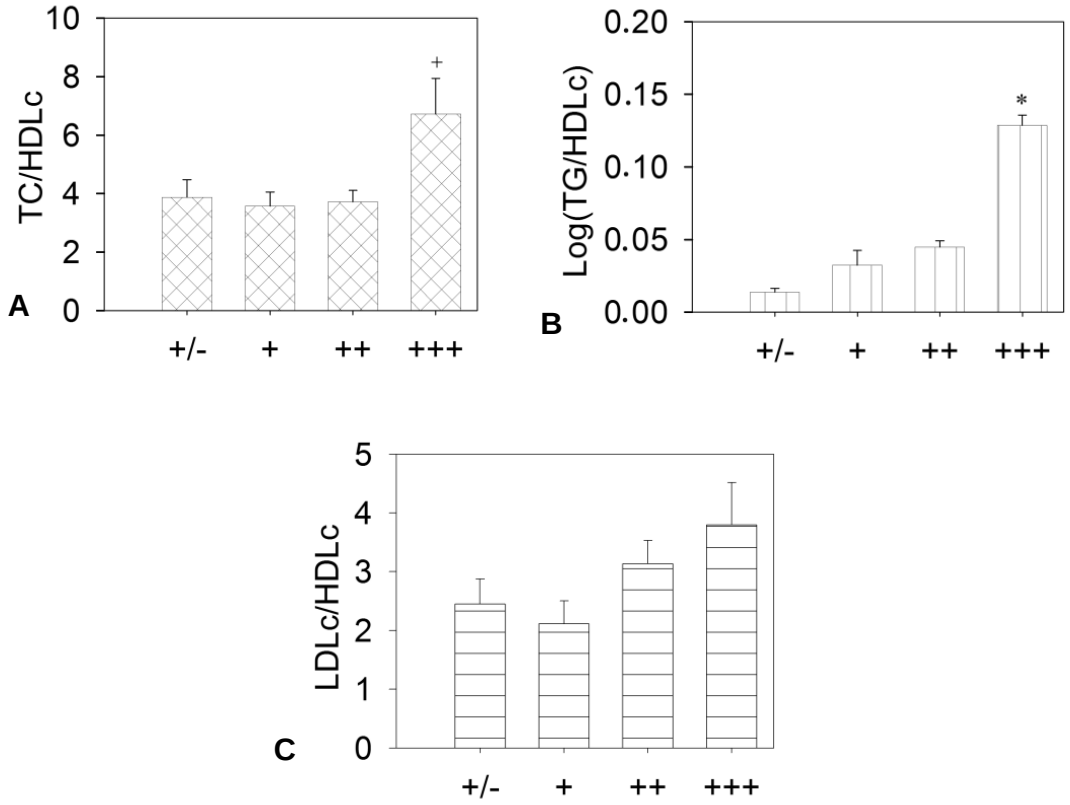

Figure 3. Smear positivity extent variation with atherogenic indices. Changes in atherogenic indices are represented according to the results of SPE scores in TB patients before treatment. The results are presented as mean ± SEM of atherogenic indices value within SPE scores groups. (+p<0.01, *p<0.05), (+/-, n = 15; +, n = 16; ++, n = 34; +++, n = 18).

shown by our results. LDL-C level decreased significantly in non-treated TB patients, but was not affected by the treatment. This suggests that the mechanism underlying LDL-C implication in MTB infection is different from HDL-C. Oxidation followed by removal of LDL-C is reported during infection in Hamsters (Memon et al., 2000) but it is not known if this is the case in humans.

Atherosclerosis and cardiovascular disease are common in clinical situations where dyslipidemia is present. We estimated the atherogenic indices TC/HDL-C, LDL-C/HDL-C and log (TG/HDL-C) in order to determine whether TB and/or its treatment represent cardiovascular diseases risks. Our results showed that the atherogenic index of plasma log (TG/HDL-C) was significantly increased in TB patients without reaching the critical level synonymous of atherogenic risk (Maron, 2000). We demonstrated that the atherogenic indices TC/HDL, LDL/HDL and log (TG/HDL) were significantly increased in patients with the highest SPE. We also showed that TB treatment induced significant reduction in all three atherogenic indices despite elevated total cholesterol. Improvement (decrease) in atherogenic indices reflects the increase in HDL-C after treatment as earlier reported by Deniz et al. (2006).

Conclusion

In this study, we showed that the recovery from TB is accompanied by normalization of lipid parameters such as cholesterol and HDL-C. Despite the rise of lipid parameter levels in TB treated patients, atherogenic indices were somewhat normal. Additional research is needed to more fully assess the link between TB treatment and levels of total cholesterol and its components in patients with pulmonary TB. It would be interesting to study the effectiveness of cholesterol supplement alone or in combination with antituberculosis therapy in TB patients care.

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REFERENCES

Deniz O, Gumus S, Yaman H, Ciftci F, Ors F, Cakir E, Tozkoparan E,

Bilgic H, Ekiz K (2007). Serum total cholesterol, HDL-C and LDL-C concentrations significantly correlate with the radiological extent of disease and the degree of smear positivity in patients with pulmonary tuberculosis. Clin. Biochem. 40:162-166.

Deniz O, Tozkoparan E, Yaman H, Cakir E, Gumus S, Ozcan O, Bozlar U, Bilgi C, Bilgic H, Ekiz K (2006). Serum HDL-C levels, log (TG/HDL-C) values and serum total cholesterol/HDL-C ratios significantly correlate with radiological extent of disease in patients with community-acquired pneumonia. Clin. Biochem. 39:287-292.

Dobiasova M, Frohlich J (2001). The plasma parameter log (TG/HDL-C) as an atherogenic index: correlation with lipoprotein particle size and esterification rate in apoB-lipoprotein-depleted plasma (FER_{HDL-C}). Clin. Biochem. 34:583-8.

Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (2001). Executive summary of the Third Report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 285:2486-2497.

Gonzales R, Sande MA (2000). Uncomplicated acute bronchitis. Ann. Intern. Med. 133:981-991.

Heiniger HJ, Marshall JD (1982). Cholesterol synthesis in polyclonally activated cytotoxic lymphocytes and its requirements for differentiation and proliferation. Proc. Natl Acad. Sci. 79:3823-3827.

Maron DJ (2000). The epidemiology of low levels of high-density lipoprotein cholesterol in patients with and without coronary artery disease. Am. J. Cardiol. 21:11-14.

Memon RA, Staprans I, Noor M (2000). Infection and inflammation induce LDL-C oxidation in vivo. Arterioscler. Thromb. Vasc. Biol. 20:1536-1542.

Perez-Guzman C, Vargas MH, Quinonez F, Bazavilvazo N, Aguilar A (2005). A cholesterol-rich diet accelerates bacteriologic sterilization in pulmonary tuberculosis. Chest 127:643-651.

Smith GD, Shipley MJ, Marmot MG, Rose G (1992). Plasma cholesterol and mortality: The Whitehall Study. JAMA 267:70-76.

Tietge UJ, Maugeais C, Lund-Katz S, Grass D, de Beer FC, Rader DJ (2002). Human secretory phospholipase A2 mediates decreased plasma concentrations of HDL-C cholesterol and Apoa-I in response to inflammation in human Apoa-I transgenic mice. Arterioscler. Thromb. Vasc. Biol. 22:1213-1218.

Van der Geize R, Yam K, Heuser T, Wilbrink MH, Hara H (2007). A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into Mycobacterium tuberculosis survival in macrophages. Proc. Natl Acad. Sci. 104:1947-1952.

Wilson RF, Barletta JF, Tyburski JG (2003). Hypocholesterolemia in sepsis and critically ill or injured patients. Crit. Care 7:413-414.

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Full Length Research Paper

Mechanism of ciprofloxacin resistance in *Shigella dysenteriae*

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Bacterial resistance to quinolones has emerged rapidly and such resistance has traditionally been attributed to the chromosomally mediated mechanisms that alter the quinolone targets and/or over-produce multidrug resistance efflux pumps. In the present investigation possible mechanism of ciprofloxacin (Cp) resistance in *Shigella dysenteriae* was studied. While the growth of sensitive *S. dysenteriae* was completely inhibited at 1 µg /ml concentration of ciprofloxacin, the resistant strain tolerated even 10 µg /ml concentration of ciprofloxacin. Mechanism of resistance was found to be the presence of plasmid.

Key words: Ciprofloxacin, *Shigella dysenteriae,* quinolone, plasmid, Resistance, SDS.

INTRODUCTION

Microbial resistance to antimicrobials is emerging as an important public health problem in both hospitals and the community. The 20th century has been considered the antimicrobial era whereas the 21st century may well present the post-antimicrobial era. The reason for this dramatic change, should it come to pass, is the development of bacterial resistance to antimicrobial agents. This emerging resistance is now challenging the clinical utility of many antimicrobial agents such that the chemotherapy of hospitalized patients with serious infections has been compromised. If the problem with resistance is to be successfully dealt with by clinicians, the mechanisms of such resistance must be known and understood. An understanding of these important microbial resistance mechanisms will help the clinician identify circumstances in which resistance may be a problem, as well as, evaluating the potential usefulness of an alternate antimicrobial agent against resistant microbes (Stratton, 2000).

Plasmid-mediated resistance to quinolones was first reported in 1998 in a *Klebsiella pneumonia* clinical strain solated in 1994 in Birmingham, Ala (Ghosh et al. 1997). Ciprofloxacin, a new fluoroquinolone, is a potent, broad spectrum antibacterial agent. It rapidly blocks spectrum of antibacterial agents. It rapidly blocks bacterial DNA replication by inhibiting DNA gyrase an essential prokaryotic enzyme that catalyzes chromosomal DNA supercoiling (Bhutta et al., 1999). Spence in Spence and Towner (2003) compared the *in vitro* activity of Moxifloxacin and Ciprofloxacin against 226 nosocomial isolates of *Acinetobacter baumanii* out of them 49% were resistant to Ciprofloxacin and 39.4% were Moxifloxacin resistant. A *Gyr A* mutation at *Ser*-83 was found in all ciprofloxacin resistant isolates.

Routine surveillance of antimicrobial susceptibility to all classes of clinically used agents is necessary to detect resistance trends in different parts of the world, detecting the emergence of new resistance mechanisms that guide infection control measures and public health guidelines such trends may help in identifying outbreaks of resistant organisms. Such a check seems to be the best way to find appropriate antibiotic regimens (Ashtiani et al.,

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2009).

Resistance has emerged even to newer, more potent antimicrobial agents. Therefore, to report resistance rates to antimicrobial agents, Ashtiani et al. (2009) isolated 2487 stool cultures from tertiary care hospital between 1996 and 2000, 2001 and 2005, out of the fecal cultures 53% of the cultures were *Shigella* spp. and 28% of the isolates were *Salmonella* spp. Resistance to antimicrobial agents increased among most of the pathogens between 2001 and 2005. An increase in the rate of resistance was observed in *Shigella* spp. for kanamycin and Ceftazidime and among *Salmonella* spp. for Nalidixic acid and Ceftazime (Ashtiani et al., 2009).

S. dysenteriae is a causative agent of bacillary dysenteries and the emergence of the drug resistance pattern was found to be these species. Infections caused by drug resistant *S. dysenteriae* are often serious sometimes even life threatening. The aim of the present investigations was to detect the exact mechanism of resistance to ciprofloxacin in *S. dysenteriae*.

Antibiotic resistance and associated genes are ubiquitous and ancient, with most genes that encode resistance in human pathogens having originated in bacteria from the natural environment (eg, ß-lactamases and fluoroquinolones resistance genes, such as qnr) (Finley et al., 2013). Consumption of raw vegetables represents a route of human exposure to antibiotic-resistant bacteria and resistance determinants naturally present in soil (Marti et al., 2013).

MATERIALS AND METHODS

Bacterial strain and its cultivation

The organism used in this study was one resistant *S. dysenteriae strain*, obtained from Agarkar research institute, Pune, India. One sensitive strain *S. dysenteriae* was obtained from Grant Medical College, Mumbai. *S. dysenteriae* cells were cultured on Nutrient agar (NA) (HiMedia, India) slants containing Beef extract 0.5; Peptone 2.5; Sodium chloride 2.5; Agar15 in a liter of distilled water pH was maintained at 7.4, slant were incubated at 37°C for 24 h.

Detection of antibiotic resistance and sensitivity in bacteria

A Standard agar diffusion method in the study of Reddish et al. (1929) was used here. Solution of ciprofloxacin in different concentrations was added to a well cut in the Muller Hinton Agar (MH) (HiMedia, India). MH agar plates were inoculated with McFarland 0.5 standard of each resistant and sensitive organism. Plates were incubated for 16 to 18 h at 37°C. Zone of inhibition was measured in millimeter scale and compared by following the NCCL standard. Tube dilution method in the study of Piddock (1990) was applied here. One hundred microgram (100 µg) of ciprofloxacin stock solution was prepared in distilled water and sterilized by filtration (Millipore filter size 0.45 nm was used). Nutrient broth was used as media. Different concentrations of ciprofloxacin were prepared in tubes. The innoculum was 10^8 cfu/ml (equivalent to Mc Farland 0.5 standard). Incubation was carried out at 37°C for 72 h on rotatory shaker. Turbidity was measured on LUMICHEM 20 at 540 nm. Results were compared with sensitive strain.

Isolation of plasmid

Plasmid DNA was isolated from *Shigella dysenteriae* resistant strain by alkaline lysis method (Lavery et al., 1997). Overnight cultures in Luria Bertani (LB) broth (1.5 ml) were harvested by centrifugation at 14,000 g for 3 min at room temperature. The cells were re-suspended in 100 µl of solution A (25 mM Tris HCL, 10 Mmedta, 50 mMGlucose + Lysozyme 20 mg/ml) and incubated at 37°C for 1 h. Freshly prepared 200 µl of solution B (0.2 M NaOH +1%SDS) was added for lysis of cells and mixed thoroughly by vortexing. It was then kept on ice for 5 min after which, 150 µl of solution C (5 M Potassium acetate) was added. It was then kept on crushed ice for further 15 min. Centrifugation of tubes was done at 14 000 g for 5 min at 4°C. The supernatant was transferred to new microfuge tubes. DNA was extracted with phenol: chloroform (25:24 ml) mixture and precipitation with the ice-cold absolute ethanol. After centrifugation, the supernatant was discarded and extracted DNA was air dried and dissolved in 50 µl of 50 mM Tris EDTA Buffer pH 8.0.

Spectrophotometric method

Qualitative determination of DNA was carried out by spectrophotometric method given by Maniatis et al. (1982).

Agarose gel electrophoresis

Agarose gel electrophoresis of plasmid was conducted for separation and analysis of nucleic acid using the method in the study of Meyers et al. (1976). Plasmid DNA (1 to 2 µg/ml) from *Shigella dysenteriae* resistant strain was electrophoresed in 2% agarose slab gels at 50V in Tris Acetate buffer(TAE) running buffer (0.15 m Tris base, 0.5M EDTA, 1 M Glacial acetic acid). Stained with ethidium bromide and photographed under U. V. illumination. The size of plasmid was estimated by comparing mobility with standard plasmid marker (pBR322/Hae III Digest, Banglore Genei, Banglore, INDIA).

Elimination of resistance due to plasmid

Elimination of resistance due to plasmid was carried out using the treatment of sodium dodecyl sulfate (SDS) and that of Tomoeda et al. (1968). An overnight culture of resistant (R) cells in penassay broth containing 10 µg/ml of ciprofloxacin was diluted to10^3 cells/ml in broth and add to the tubes containing 10% (w/v) SDS and shaken at 37°C. After appropriate dilution in saline cells were plated on nutrient agar. All colonies on the plate were tested for their ciprofloxacin resistance character on nutrient agar containing different concentrations of ciprofloxacin.

RESULTS

In agar diffusion test, when 0.25 and 0.5 µg/ml concentration of ciprofloxacin was tested, no zone of inhibition was observed in sensitive strain. When 1 to 10 µg/ml concentration of ciprofloxacin in sensitive *S. dysenteriae* strain was tested, zones of inhibition in each case were 21 to 29 mm (Figure 1; Table I). The resistant strain showed no zone of inhibition at 0.25 to 10 µg/ml concentration of ciprofloxacin (Figure 2). Effect of ciprofloxacin was studied on growth of *S. dysenteriae* sensitive strain and resistant strains. The growth in terms of turbidity was measured at 12 h interval at 540 nm

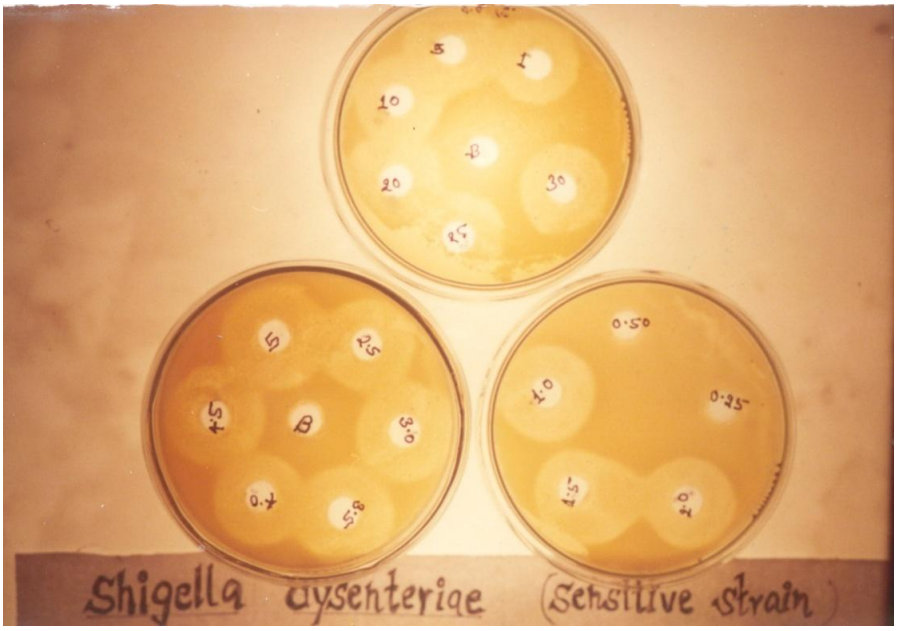

Figure 1. *S. dysenteriae* sensitive strain showing sensitivity to 1 to 10 µg/ml concentration of Ciprofloxacin.

Table 1. Diameter of zone of inhibition in *Shigella dysenteriae* to concentrations of Ciprofloxacin.

Concentrations of Ciprofloxacin (µg/ml)	Resistant strain (mm)	Sensitive strain (mm)
0.25	No zone of inhibition	No zone of inhibition
0.5	“	“
1	“	21
1.5	“	21.5
2.0	“	22
2.5	“	22.5
3.0	“	23
3.5	“	23.5
4.0	“	24
4.5	“	24.5
5	“	25
6	“	26
7	“	27
8	“	28
9	“	28.5
10	“	29

using colorimeter. Increased turbidity was observed in resistant strain and sensitive strain showed no turbidity. The inhibition in the growth of sensitive *S. dysenteriae* was 60 to 80% (that is, growth up to 20 to 40%) at 0.1 to 0.7 µg/ml concentration of ciprofloxacin, as compared to the control set. At 1 µg/ml concentration of ciprofloxacin, the growth of sensitive strain was completely inhibited (Figure 3) while the inhibition in the growth of resistant strain was 10 to 30% (that is, growth up to 70 to 90%) at 1 to 10 µg/ml concentration of ciprofloxacin as compared to the control set.

The resistant strain tolerated up to 10 µg/ml concentration of ciprofloxacin (Figure 4). Plasmid was isolated from resistant *S. dysenteriae* strain. Plasmid was not detected in sensitive strain. Agarose gel electrophoresis of plasmid DNA was carried out. The plasmid band was compared with standard plasmid marker. The single band was detected in Lane 2 (that is isolated plasmid DNA from resistant *S. dysenteriae*). The standard plasmid marker pBR322 /Hae III Digest showed

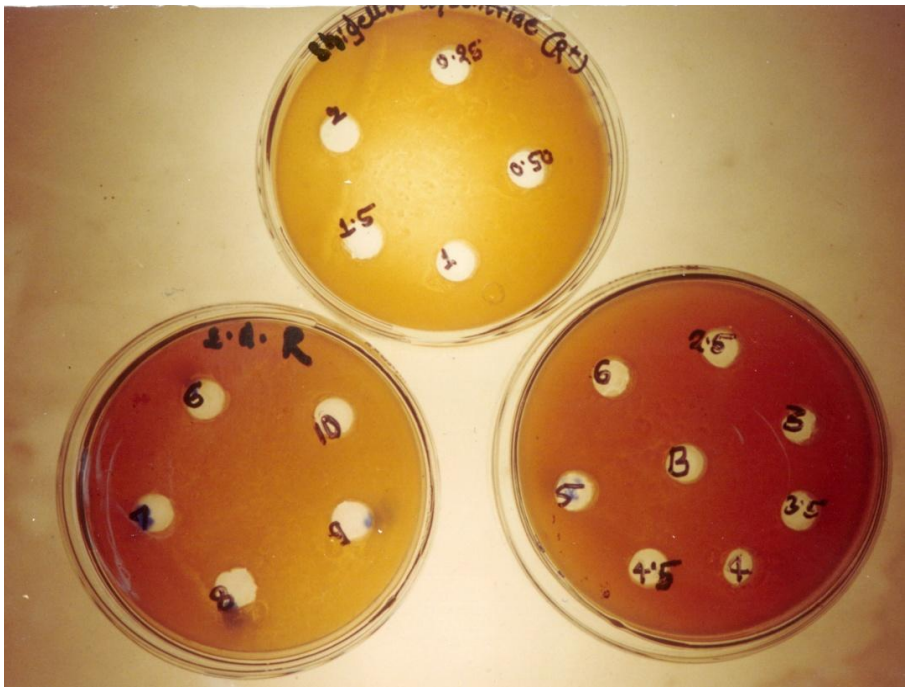

Figure 2. *S. dysenteriae* resistant strain showing resistance to 1 to 10 µg/ml concentration of Ciprofloxacin.

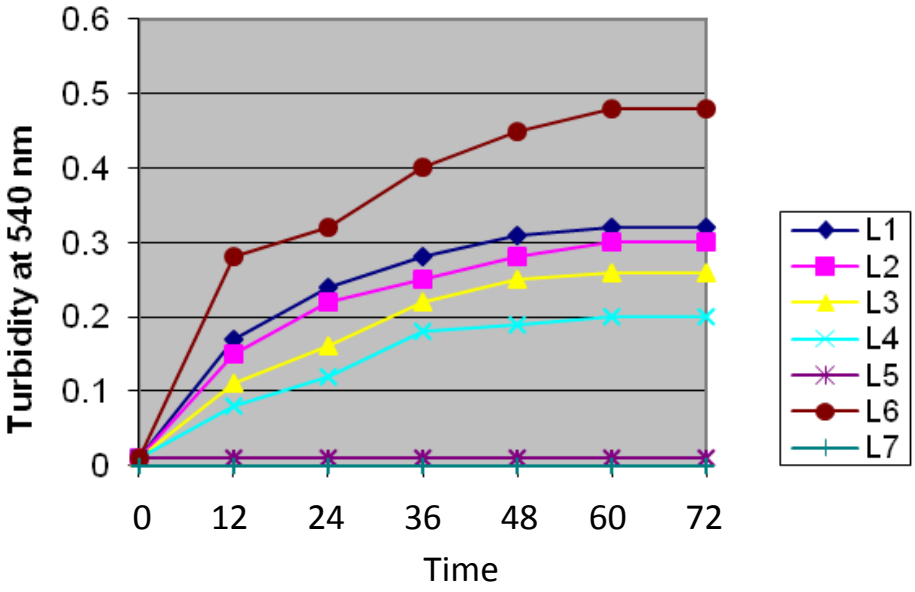

Figure 3. *S. dysenteriae* sensitive strain showing sensitivity to 1 µg/ml concentration of ciprofloxacilin. Different concentrations of ciprofloxacin were, L1- 0.1 µg/ml; L2-0.3 µg/ml; L3-0.5 µg/ml; L4-0.7 µg/ml; L5-1 µg/ml; L6-control; L7- blank.

bands (Figure 5). The size of isolated plasmid was 587(bp). It seems that mechanism of resistance was found to be the presence of plasmid.

Also the concentration of DNA in the S. *dysenteriae* was calculated. Concentration of DNA in sample solution was 1064 µg/ml. The ratio of protein to extracted DNA was 1.87 that means extraction of DNA was pure. *S. dysenteriae* resistant cell carrying plasmid was treated with sodium dodecyl sulphate at 2 to 10% concentrations. Sodium dodecyl sulphate was effective in producing

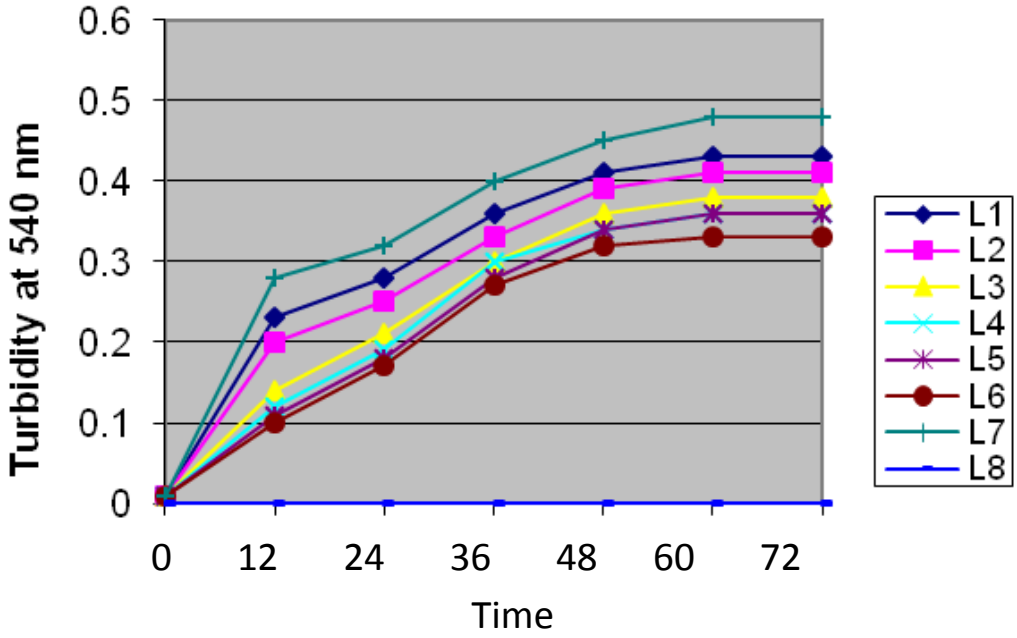

Figure 4. *S. dysenteriae* resistant strain showing resistance to 10µg/ml concentration of ciprofloxacin. Different concentrations of ciprofloxacin were, L1- 1 µg/ml; L2-2.5 µg/ml; L3-5 µg/ml; L4-7 µg/ml; L5-9 µg/ml; L6-10 µg/ml; L7-control; L8- blank.

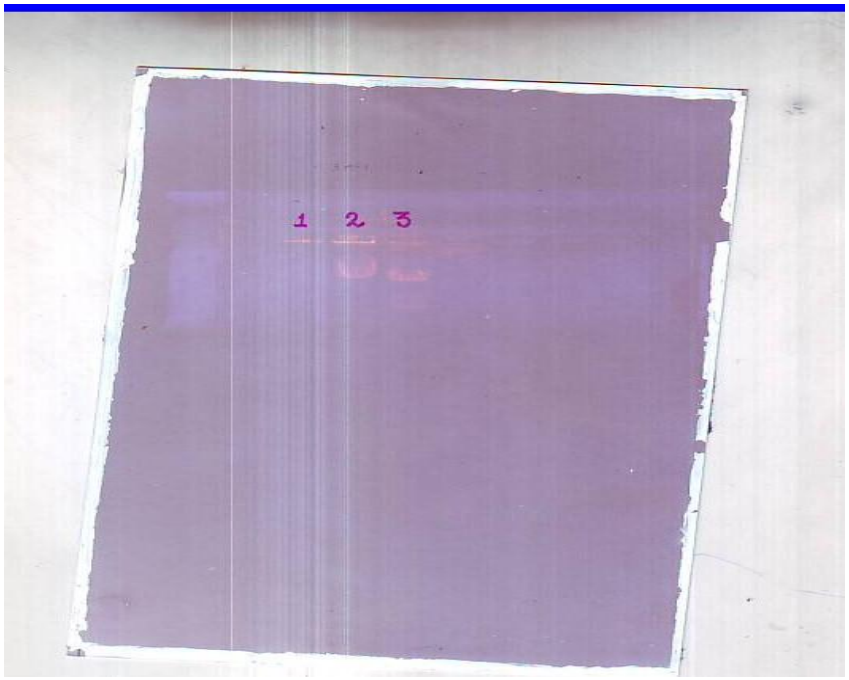

Figure 5. Agarose gel electrophoresis of plasmid DNA of *Shigella dysenteriae* resistant to ciprofloxacilin, Ampicillin and Zinc, showed single band (Lane 2) and Standard plasmid marker pBR322 DNA / Hae III Digest showed bands of different molecular sizes (Lane 3).

susceptible cells. The growth of resistant strain was 19 to 33% at 2 to10% SDS concentration as compared to control set. After SDS treatments, resistant strains were analyzed at interval for the presence or absence of drug resistance against different concentrations of ciproflo-xacin. The resistance was lost and the resistant strain

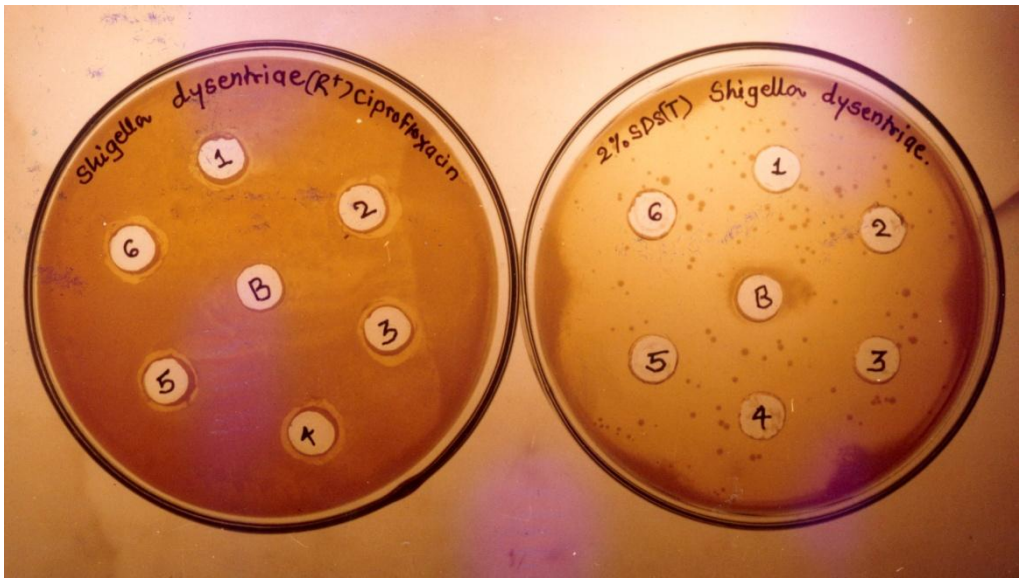

Figure 6. Elimination of resistance factor by treatment of Sodium dodecyl sulfate in *Shigella dysenteriae.*

strain was changed to sensitive strain (Figure 6).

DISCUSSION

The present investigation deals with the mechanism of ciprofloxacin resistance in *S. dysenteriae*. The *S. dysenteriae* resistant strain showed resistant to 10 µg/ml concentration of ciprofloxacin with no zone of inhibition, which was much superior result than the observation made by Cormican and Jones (1995), where they reported the MIC of ciprofloxacin as ≥4 µg/ml. Therefore it is indicated that the strain used in the present study seems to be more resistant. However, sensitive strain showed 21 mm zone of inhibition to1 µg/ml of ciprofloxacin. Hence, it indicates that the efficiency of ciprofloxacin varies according to resistant and non-resistant strains.

In the present investigation, plasmid was isolated from *S. dysenteriae* resistant strain while in sensitive strain, no plasmid was found. On elimination of plasmid from resistant strain sensitivity was observed in *S. dysenteriae*. This indicates that plasmid was responsible for resistance development in the microorganisms. The plasmid profile of the strain was studied. The single DNA band was detected on agarose gel electrophoresis. The plasmid molecular size of *S. dysenteriae* resistant strain was 587bp. This was in agreement with the studies of other workers, Ghosh et al. (1997) isolated plasmid from *A. aminolytica* and GS19h each possessed more than one plasmid. Kessie et al. (1998) reported that, *Staphylococcus lenticus* carried small plasmids of molecular size 2.0, 2.3, 2.7, 4.3 and 4.4 kb and *S. hylicus* isolated, harbored up to seven small plasmids ranging from 2.0 to 4.7 kb.

In the present study, elimination of resistance due to plasmid was carried out using sodium dodecyl sulphate (SDS) treatment. An anionic surface activating agent sodium dodecyl sulphate eliminated plasmid carrying resistance factor in *S. dysenteriae*. After SDS treatment, resistant cells lost their resistant character and changed to sensitive cells. SDS treated cells showed same zone of inhibition as that of sensitive strain. This is in agreement with the observations made by Tomoeda et al. (1968) that the action of SDS proved to be effective enough to eliminate R factor present in bacteria at frequencies up to 100%. Other workers in curing plasmid used different reagents. Rotimi and Duerden (1982) reported action of acridine dye on *E.coli* R^+ and *Bacteroides fragillis* cells leading to complete loss of R-factor. Pan et al. (1981), Attfield and Pinney (1985) used mytomicin C and bleomycin treatment for curing multi-copy plasmid. The relation of plasmid in development resistance was studied by Pan et al. (1981) in *Enterobacter aerogenes* against mercury.

Furthermore, he related mercury resistance due to the synthesis of outer member proteins coated by genes on plasmid. Denis and Moreau (1993) reported decrease in permeability in resistant strains compared with sensitive, due to modification of outer member proteins that was often evolved with resistance. Observation of Tran and Jacoby (2002) also supports the present finding, who reported a multi resistance plasmid that encodes transferable resistance to quinolones. Therefore it indicates that plasmid elimination which synthesizing membrane protein was responsible for drug resistance. It seems

that similar reason could be responsible for the ciprofloxacin resistance in the *Shigella dysenteriae* in the present study. The formation of the plasmid in the bacterial cell is mainly responsible for ciprofloxacin resistance in microorganisms.

REFERENCES

Ashtiani MTH, Monajemzadeh M (2009). Trends in antimicrobial resistance of fecal *Shigella* and *Salmonella* isolates in Tehran, Iran. Ind. J. Pathol. Microbiol. 52(1):52-55.

Attfield PV, Pinney RJ (1985). Elimination of multicopy plasmid R6K by Bleomycin. Antimicrob Agent Chemother. 27:985-988.

Bhutta Z, Black RE, Brown KH, Gardner JM, Gore S, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME, Rosado JL, Roy SK, Ruel M, Sazawal S, Shankar A (1999). Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries:pool analysis of randomizing controlled trails. J Pediatr. 135:689-97.

Cormican M, Jones RN (1995). Susceptibility to Levofloxacin predicted from *in vitro* susceptibility testing results obtained with ciprofloxacin and with Ofloxacin. J. Clinic Microbiol. 33:215-216.

Denis A, Moreau NJ (1993). Mechanisms of quinolone resistance in clinical isolates:accumulation of sparfloxacin and of fluoroquinolones of various hydrophobicity and analysis of membrane composition. J. Antimicrob Chemother. 32:379-92.

Finley RL, Collignon P, Joakim Larsson D. G, McEwen SA, LiX Z, Gaze WH, Reid-Smith R, Timinouni M ,Graham DW, Topp E (2013). The Scourge of Antibiotic Resistance:The Important Role of the Environment. Clin. Infect. Dis. 57(5):704-710.

Ghosh S, Mahaptra NR, Banerjee PC (1997). Metal resistance in Acidocella strain and plasmid mediated transfer of this character to *Acidophillium multivorum* and *Escherichia coli*. Appl. Environ. Microbiol. 63:4523-4527.

Kessie G, Ettabyebi M (1998). Plasmid profile and antibiotic resistance in coagulase negative *Staphylococcus* isolated from polluted water. J. Appl. Microbiol. 84:417-422.

Lavery A, Rossney S (1997) Incidence and detection of multidrug resistance in *Enterococci*. J. Bacteriol. 46:150-156.

Maniatis T, Frisch EF, Sambrook J (1982). Large isolation of Plasmid DNA. In Molecular Cloning:A laboratory manual ed. Maniatis T, Frisch EF, Sambrook J. Cold Spring Harbor Laboratory Press N.Y. 0879691360(pbk). 86-96.

Marti R, Scott A, Tien YC, Murray R, Sabourin L, Zhang Y, Topp E (2013) Impact of Manure Fertilization on the Abundance of Antibiotic-Resistant Bacteria and Frequency of Detection of Antibiotic Resistance Genes in Soil and on Vegetables at Harvest. Appl. Environ. Microbiol. 79(18):5701-5709.

Meyers JA, Sanchez B, Elwell LP, Falkow S (1976). Simple Agarose Gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

Pan-Hou HS, Nishimoto M (1981). Possible role of membrane protein in mercury resistance of *Enterobacter aerogenes*. Arch. Microbial. 130:93-5.

Piddock LJV (1990). Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. J. Appl. Bacteriol. 60:307-318.

Reddish GF (1929). Methods of testing antiseptics. J. Laborat. Clin. Med. 14:649-658.

Rotimi VO, Duerden BI (1982). Curing antibiotic resistance in Bacteroides species by aminoacridines and ethidium bromide. Afr. J. Med. Sci. 10:91-6

Spence RP, Towner KJ (2003). Frequencies and mechanism of resistance to Moxifloxacin in noscomial isolates of *Acinetobacter baumanii*. J. Antimicrob. Chemother. 52:687-690.

Stratton (2000). Mechanism of bacterial resistance to antimicrobial agents. J. Med. Liban. 48(4):186-198.

Tomoeda M, Inuzulka M, Kubo N, Nakamura S (1968). Effective elimination of drug resistance and sex factors in *Escherichia coli* by sodium dodecyl sulfate. J. Bacteriol. 95:1078-1089.

Tran JH, Jacoby GA (2002). Mechanism of plasmid mediated quinolone resistance. Proc. Natl. Acad. Sci. U. S. A. 99:5638-42.

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Full Length Research Paper

Morphology and anatomical structure of the larval salt gland of *Artemia tunisiana* under different salinities

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Brine shrimps of the genus *Artemia* is characterized by its high adaptability to adverse environmental conditions. To elucidate the effect of salinity on the neck organ (salt gland) of *Artemia tunisiana* nauplii, the morphology and fine structure of the ion transporting epithelium were examined following culturing under different salinities (25, 40, 70, 140 and 180 g/L). The expression of APH-1 mRNA, using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), was also determined. The morphology and anatomical structure of the salt gland varied according to the salinity degree. At low salinities, salt gland was small, thin and flat having many shallow canals, while at high salinities, it was more elongated with deeper canals and grooves. Ultrastructure examination showed low amplification of the plasma membrane at 25 g/L with no tubular tufts, while at 40 and 70 g/L salinities, the apical and central zones showed a large amplification of the surface area of the plasma membrane. At 140 g/L salinity, the epithelial cells were more elongated and the cuticle appeared to be composed of many layers. The general structure of the salt gland of nauplii cultured at 180 g/L disappeared. Semi-quantitative APH-1 mRNA analysis indicated that the gene was expressed in all tested salinities. The expression did not change remarkably between 25 and 40 g/L salinities. As salinity increased, the gene was up regulated at 70 g/L and reached the highest level at 140 g/L, while the expression level reduced significantly at 180 g/L. This coincides with the histological results and highlights the possible role of APH-1 in salinity protection in *Artemia.*

Key words: *Artemia*, nauplii, salt gland, salinity, APH-1 gene expression

INTRODUCTION

Salinity is one of the most important physical parameters affecting the life history of hypersaline organisms like *Artemia*. Genus *Artemia*, subdivided into six generally recognized bisexual species and a large number of parthenogenetic populations, is characterized by its adaptability to a wide range of salinity; 4 to 250 g/L (Varo et al., 2002). The ability of brine shrimp *Artemia* to exist in a broad salinity range results from an active excretion of sodium ions from the body into the external environment (Conte et al., 1972). The extent to which *Artemia* can tolerate extremes of salinity is evident in Wadi El-Natrun and Borg El-Arab, where both larvae and adults may be observed swimming amongst crystals of NaCl in saturated brine. Two structures seem to be actively involved in the osmoregulatory abilities of the adults, the epithelium of the gut (Croghan, 1958) and the metepipodite segments of the branchiae (Copeland, 1966). In *Artemia* nauplii, there is a lack of gut and metepipodites because at this stage of embryonic development, the analog of the legs segments have not formed their definitive structures. Nevertheless, nauplii possess a unique structure for coping with salinity differences via a larval salt gland or neck organ. This gland has been most thoroughly described for larval anostracans, because it is

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primarily a larval organ and because of the amount of attention lavished on the developmental biology of the genus *Artemia* (Criel, 1991). The salt gland in anostracans has been known since 1851 when Leydig described it in *Artemia salina*. Afterward, researchers described this structure in adults and in larvae (Sars, 1896 and Schrehardt, 1986), while others restricted the presence of salt gland in larvae of anostracans and its disappearance by the adult stage (Schrehardt, 1987). This structure is also known from notostracans, conchostracans and many species of cladocerans (Fryer, 1987; Martin and Laverack, 1992). The morphology and ontogeny of the gland were reviewed by Criel (1991); his description is based on the earlier work by Conte et al. (1972), Ewing et al. (1974) and Hootman and Conte (1975). Beside its role in ionic and osmotic regulation, other functions have been suggested for the salt gland including: (1) Adherence to external objects (Zenker, 1851), (2) respiration (Dejdar, 1930) and (3) support for the antennal and mandibular muscles (Weisz, 1947). Conte et al. (1972) found that larval brine shrimp like their adult counterparts are capable of living in saturated brines because they possessed this special organ that is capable of providing osmotic and ionic regulation (Hootman et al. 1972; Criel, 1991). Abel and Ellis (1966) investigated the need for such an organ. This function was supported by micropuncture studies (Russler and Mangos, 1978) and by the finding of Na^- K^- activated ATPase in the organ (Conte et al., 1977). All of them concluded that, brine shrimp use salt gland pump to maintain a relatively stable salt concentration within their bodies.

Artemia posses a drifter gene, designated *APH*-1 for *Artemia* POU-Homeoprotein, which is proved to be expressed in the salt gland (Chavez et al., 1999). This gene encodes a POU-III subclass homeobox-containing transcription factor highly related to the Drosophila drifter (Dfr) protein (Anderson et al., 1996). One prominent role of such gene is controlling tracheal development and transcription factor (Zelzer and Schilo, 2000) in addition to its possible role in salt regulation (Wang et al., 2012). Despite its early recognition, little definitive data concerning the morphology of the salt gland in *Artemia tunisiana* as well as the changes in gene expression due to culturing under different salinities. The herein work represents the results of light and electron microscopic study of the morphology and anatomy of the salt gland epithelium beside molecular expression of APH-1 in *Artemia* cultured under different salinities to understand the impact of this environmental stressor on the structure and function of *Artemia* salt gland.

MATERIALS AND METHODS

Collection of cysts

Local *Artemia* cysts were collected from inland saline lake Wadi El-Natrun situated in northern Egypt (30° 10′ N, 30° 27′ E). Cysts were stored and transported to the laboratory in plastic bags, then cleaned and dried using the bi-flotation technique described by Sorgeloos (1978). The dried cysts were stored at -20°C.

Culture techniques

The dried cysts were hydrated in a solution of instant Ocean Sea salt (40 g/L) at 28°C with continuous aeration. Approximately 20 to 24 h were needed for hatching at this temperature. Newly hatched nauplii (2000 to 2500 individuals) were transferred to 2 L culture flasks containing the different salinity experimental media (25, 40, 70, 140 and 180 g/L) made up with Ocean Sea salt and autoclaved deionized water. All cultures were kept at 28°C with continuous illumination and aeration. *Artemia* in each culture was fed with yeast and the culture medium was replaced every four days. When *Artemia* specimens reached the pre-adult stage (≈3 mm in length) their concentration was adapted to 100 individuals/flask, till reproductive time (when males are clasping females) as the number was adjusted to 10 couples/ flask. The first generation from the above cultures was collected. In the cases of 25, 40 and 70 g/L salinities, nauplii were immersed directly in the cultures, while at 140 and 180 g/L salinities, cysts were collected, dried and then hatched in the salinity experimental media as their parents. This gradual accommodation gives a depiction about what really happens in nature.

According to Weisz (1947) scheme, in which developmental stage is quantitatively assessed by the number of body segments present, stages 3-5 were used in this study (48 h post hydration). *Artemia* samples were either; collected and frozen (-80°C) in view of extracting RNA or processed for microscopy preparation.

Light and electron microscopy preparations

Nauplii were removed with disposable pipettes and placed on a rubber stopper under a dissecting microscope. The abdomen of each nauplius just posterior to the margin of the thoracic segments was removed using a fine metal needle to facilitate rapid fixation. The nauplii were transferred to a fixative solution [6% glutaraldehyde in 0.2M, S-collidine (pH 7.5) with 8.6% sucrose and a trace (0.002%) of $CaCl_2$] at 4°C as suggested by Bell et al. (1969). After 24 h, postfixation was carried out in 1% solution of OsO_4 in 0.2 M sodium phosphate (pH 7.4) with 20% sucrose at room temperature (26°C). The specimens were dehydrated through a graded series of ethanol (30-100%), transferred through propylene oxide and gradually embedded in Epon/Araldite resin mixture. Ultra-thin sections (0.5 µm) were cut on ultramicrotome and mounted. Some sections were mounted on glass slides and stained with methylene blue-azure II for light microscopic examination. The rest of sections were mounted on copper grids and stained with aqueous uranyl acetate for 30 min and lead citrate for 5 min. These sections were examined and photographed at a JEOL 100 Transmission Electron Microscope.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from three *Artemia* nauplii from each of the examined salinities using Trizol reagent (Invitrogen). All dissecting equipment and homogenizers were cleaned with RNAse-Zap (Ambion, Austin, TX, USA) and rinsed in RNAse-free water in order to work under RNAse-free conditions. Total RNA concentration, as well as the integrity and purity of each sample were determined to ensure the lack of genomic DNA contamination.

Of each sample, 1.5 µg RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 25 µl of reaction mixture

containing random hexanucleotides primer (0.2 µg/reaction), deoxyribonucleotide triphosphate (dNTP-0.5 mM of each), avian myeloblastosis virus reverse transcriptase (RT, AMV) (20 units/reaction), RNasin (20 units/reaction) and 2.5 µl of 10x PCR buffer. After an initial 5 min at 70°C in order to dissociate secondary RNA structures, samples were incubated at 42°C for 60 min. Reactions were terminated after 5 min at 95°C.

PCR was performed initially using primers for *Artemia* mitochondrial 16S rRNA (Palmero et al., 1988) (sense, 5′-AACAGACGCCTCTCTAGGCT-3′; antisense, 5′-CTTAAATGTACTTGGAGGGCG-3′), which served as a positive control for RT-PCR due to its constitutive expression. Products obtained using 20 cycles of amplification were within the linear range of signal amplification and allowed titration of the amount of template to be subsequently used in order to obtain consistent amounts of product between samples. The adjusted cDNA volumes were then used in the succeeding PCR reactions employing gene-specific primers for APH-1 (sense, 5′-AGTGCAGTCAGTTCTGAACCG-3′; antisense, 5′-GGGTACCATTCAAGGAGTCTC-3′) (Chavez et al., 1999). All specific primers were synthesized by Sigma-Aldrich (USA). Negative control reactions were performed either without cDNA template in order to ensure that no products arose due to contamination or primer-dimer effects, or with adults *Artemia* that are expected to lack the APH-1 transcription at this stage (Chavez et al., 1999).

The PCR reaction mixture was set up in a total volume of 50 µl, containing 2 µl of reverse transcriptase product, 5 µl of a 10x PCR standard buffer, 1.5 µl 50 mM MgCl2, 1 µl 10 mM dNTP mix, 1.5 units Taq DNA polymerase (Promega) and the selected primer pair (20 pmol/primer/reaction). For both APH-1 mRNA and mt 16S rRNA, amplifications were performed using 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 58°C), and extension (2 min at 72°C) followed by a final extension at 72°C for 10 min. Denaturation for 5 min at 95°C preceded cycling.

Semi-quantitative analysis of gene expression

Following PCR, a 10-µL aliquot of each PCR product was analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide and the bands were visualized in a UV- transilluminator for the presence of amplified DNA. Semi-quantitative assessments of mRNA levels were determined by quantifying the intensity of each band of PCR product through using Gel Analyzing Imager (Sharp-100). This relies on the quantification of the investigated genes expression on the basis of optical density of detected bands. The relative intensity of APH-1 to that of 16S rRNA was then determined. Corrected values were obtained by dividing the measured value for APH-1 by that of 16S rRNA. Mean values of three measurements of APH-1 expression relative to the corresponding 16S rRNA expression are presented.

RESULTS

General salt gland morphology

The salt gland of *Artemia* cultured at any investigated salinity was distinguished in early developmental stages; it was located on the mid line of the anterior head region, often just posterior to the eyes. The organ usually demarcated from the surrounding cuticle by thin cuticular festoon border and slightly elevated with respect to the surrounding cuticle. Irregular grooves extended throughout the gland surface and distinguished this area from the rest of the surface of the nauplius (Figure 1). With increasing salinity, the salt gland became enlarged and acquired different shape with several kinds of surface irregularities

At 25 g/L salinity

The salt gland was thin flat cap like structure; its diameter ranged from 117-160 µm and had many superficial canals (Figure 1A).

At 40 g/L salinity

The salt gland measured between 138-180 µm across the diameter of the circular base. The epithelium changed from a flat cap like structure into a hemispherical dome having more channels through the epithelium (Figure 1B).

At 70, 140 and 180 g/L salinities

At these high salinities, there were more development and growth of the gland (Figure 1C-F). The canals and grooves were deeper with the salinity increment. Also these grooves distinguished salt gland from the rest of the surface of the nauplius and transformed the gland from a smooth overlay into a covering that was mottled in appearance. The removal of the cuticular layer from the gland showed the secretory epithelium to have a striking surface pattern as illustrated in Figures 1E and F in nauplii cultured at 140 and 180 g/L salinities.

Histological and ultrastructure investigations

Histological investigation

Semi-thin sections of salt glands in nauplii cultured under different salinities showed a single layer of cuboidal-columnar epithelial cells. At 25 g/L salinity as illustrated in Figure 2A, the epithelial cells ranged from 23 to 45 cells with a diameter ranged from 20-28 µm from the apical (cuticular) to the basal surface (haemocoelic). The number of epithelial cells in those cultured at 40 and 70 g/L increased to become 35 to 52 cells with a diameter ranged from 26 to 32 µm (Figure 2B). With the elevation of salinity (140 and 180 g/L), the number of epithelial cells decreased (28 to 42 cells). The central portion of the salt gland was occupied with few scattered cells, characterized by many vacuoles especially in nauplii cultured at 180 g/L salinity (Figure 2C). In all cases, no boundaries between cells were distinct.

Ultrastructure investigations

Salt gland in nauplii cultured at 25, 40, 70, 140 g/L Salinity evidenced three zones; apical, central and basal

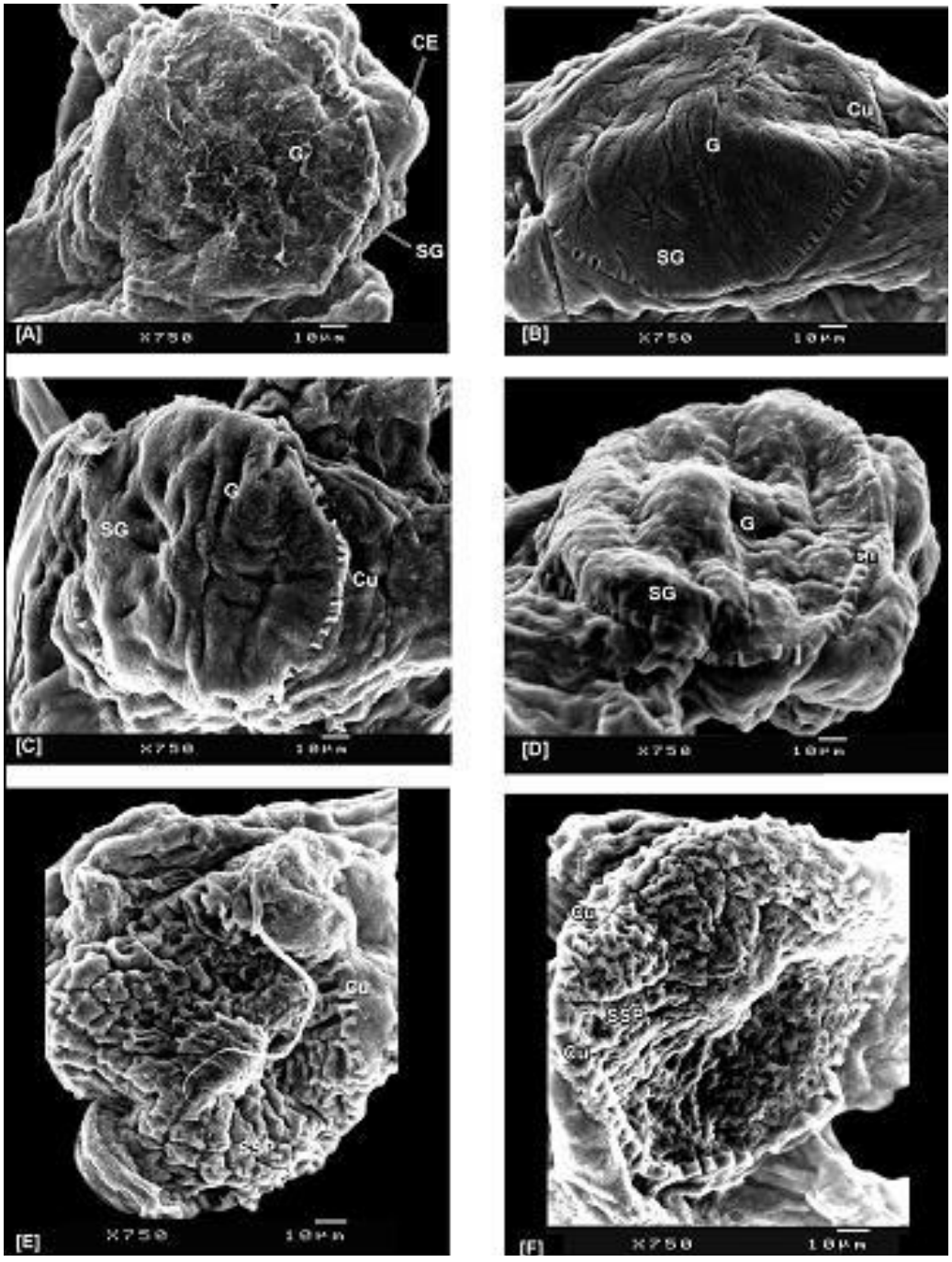

Figure 1. SEM photographs of salt gland of *Artemia tunisiana* (A-E stage 3 and F stage 5) at (A) 25 g/L, (B 40 g/L), (C) 70 g/L (D and E) 140 g/L and (F) at 180 g/L salinity, (E and F), the culticular layer has been removed during preparation. CE, compound eye; Cu, cutucus; G, groove; SG, salt striking surface pattern.

zone, while those cultured at salinity 180 g/L missed the previous zones pattern in both thickness and structure.

At 25 g/L salinity: Under the mentioned salinity the apical and central zones showed low amplification of the surface area of the plasma membrane (Figure 3A). The first pattern evolved from the plasma membrane was located at the apical surface and did not form tubular tufts beneath the cuticular surface as the other salinities. The second pattern formed smooth endoplasmic reticulum (labyrinth) which filled the central cytoplasmic zone. This

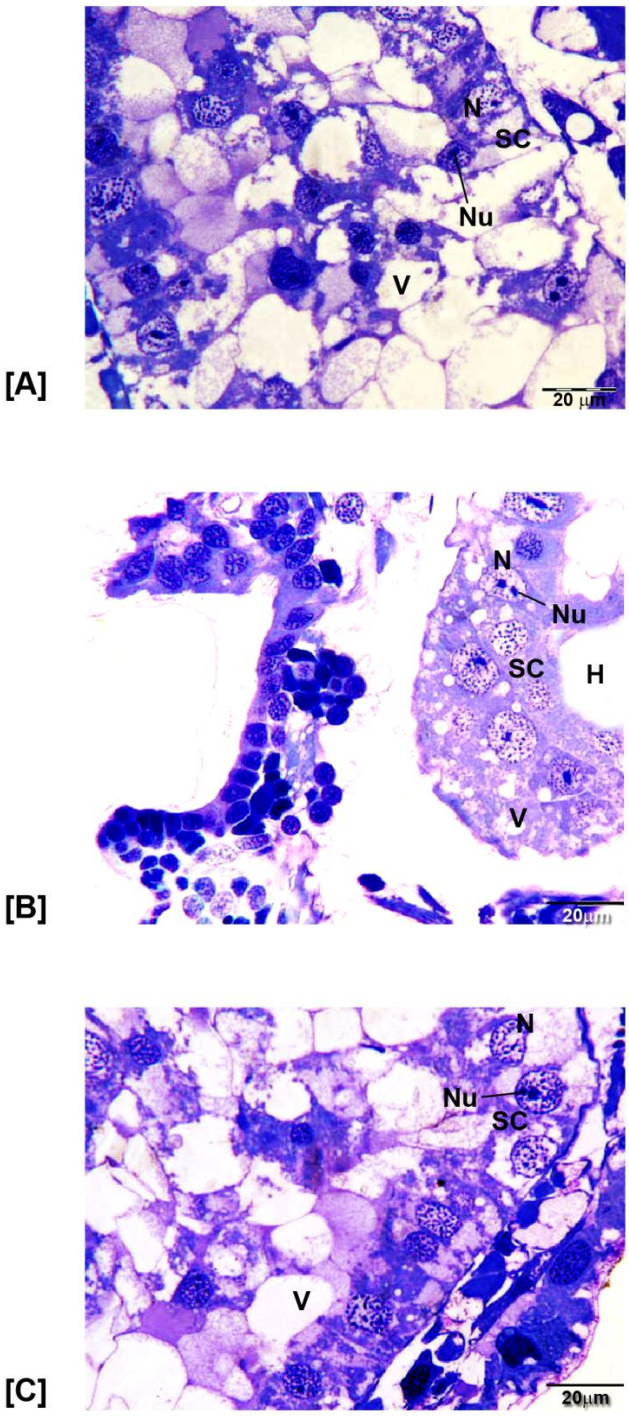

Figure 2. Light micrographs of semi-thin sections of salt gland epithelia (stage 3) cultured at A, 25 g/L; B, 40 g/L; C, 180 g/L sality. H, Haemocoeil; N, nucleus; Nu, nucleolus; SC, scattered cells; V, vacuoles.

network appeared to be composed of tubules of undetermined length and extended to the basal cell surfaces. Labyrinthine channels occupy the spaces between adjacent nuclei and form sinusoids open directly into the haemocoel. The central zone was characterized by an abundance of oval or round mitochondria. The basal zone contained most of the nuclei along with yolk platelets that vary greatly in size (2-6 µm in diameter) and other storage products. Nuclei were large, roughly oval, and contain intensely staining nucleoli and chromatin. The number of nucleoli visible may range from one to three. The entire epithelial complex of the gland did not rest upon a basal lamina. Other organelles as Golgi apparatus and membrane-bound vesicles were present.

At 40 g/L salinity: The apical and central zones showed a large amplification of the surface area of the plasma membrane. Two patterns have evolved from the surface boundary of the plasma membrane; the first (tubular tufts) located at the apical surface extended into large numbers of irregular shaped projection forming tubular tufts that lie beneath the cuticular surface. The second was the ramified network of smooth endoplasmic reticulum (labyrinth) which filled the central cytoplasmic zone. This network appeared to be composed of tubules of undetermined length and extended to the basal cell surfaces. Labyrinthine channels also occupied the spaces between adjacent nuclei and formed sinusoids that open directly into the haemocoel (Figure 3B). The tubular tufts did not associate with the mitochondria. The central zone was characterized by an abundance of mitochondria, while the basal zone contained most of the nuclei along with yolk platelets. Nuclei were large, roughly oval, and contain intensely staining nucleoli and chromatin.

At 70 g/L salinity: The salt glands structure of nauplii cultured under 70 g/L salinity resembled those cultured at 40 g/L salinity with more amplification of the apical and central zones (Figure 3C). Mitochondria were the most abundant organelles, they were oval or round and distributed throughout the cells, but were numerous in the labyrinth. Mitochondria (single or aggregated) were found to be closely associated with the tubules of the labyrinth. Single mitochondrion appeared to be enclosed in capsule-like extensions of cytoplasm, while aggregated ones were separated from each other by a narrow canaliculus (Figure 3D). Cristae were numerous and iregular. Other organelles as Golgi apparatus and membrane-bound vesicles were present.

At 140 g/L salinity: The epithelial cells were more elongated (Figure 4A) than those cultured under lower salinities. The cuticle appeared to be composed of many layers and not two as in the case of salt glands cultured under other investigated salinities (25, 40 and 70 g/L). The thickness of the cuticle layer increased to 30-42 nm. The apical plasmalemma showed minor infolding and some areas exhibited blocks of tubules (Figure 4B). Nuclei were elongated and contained intensely staining hetero-chromatin. The central zone was characterized by

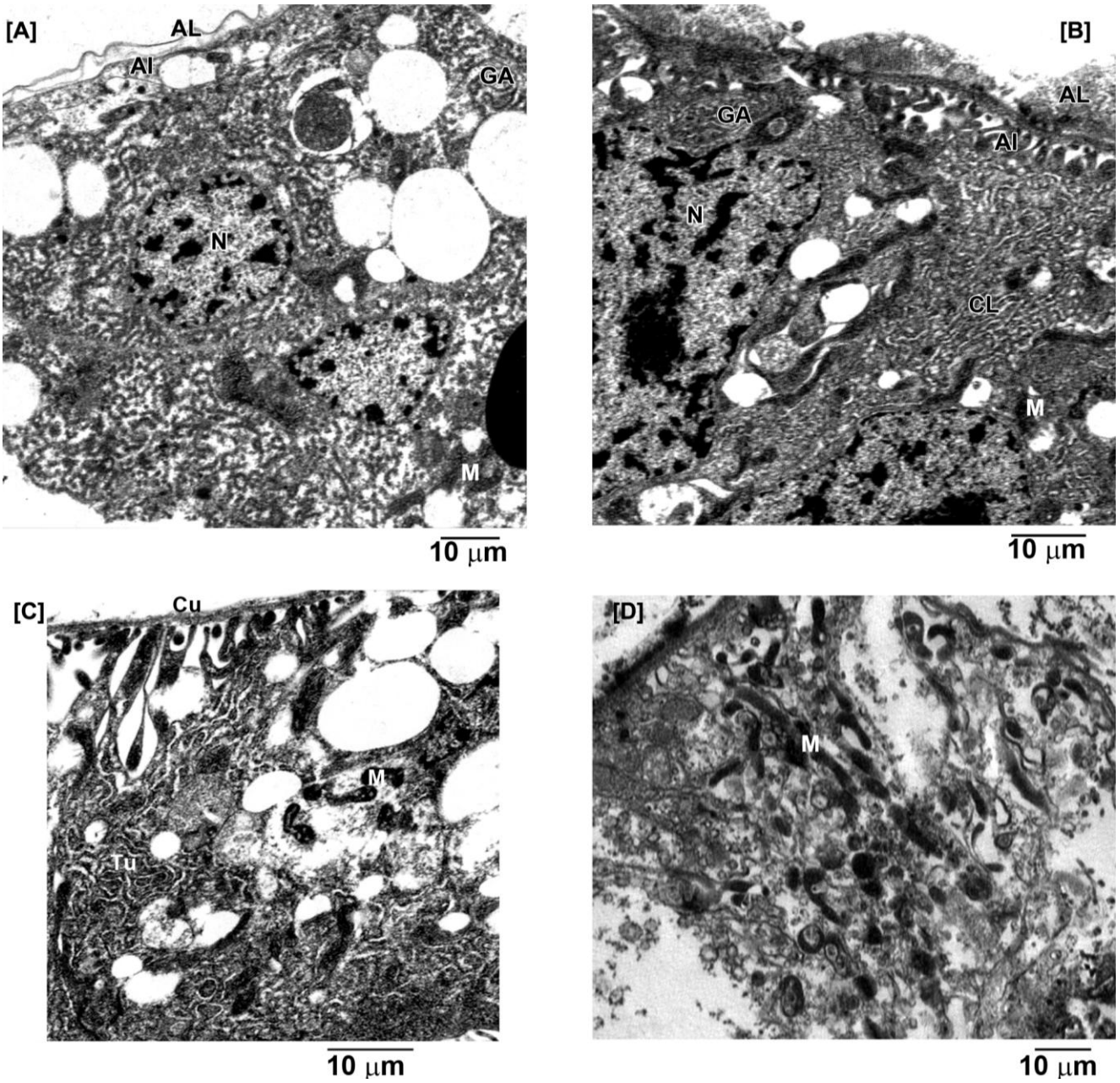

Figure 3. Photomicrograph of TEM of transverse section through the salt gland of nauplii cultured at A, 25 g/L; B, 40 g/L; C, 70 g/L salinity and D, the labryrinth from the previous sector showing a typical mitochondrial pump. AI,Apical infolds; AL, apical layer; CL, cytoplasmic labyrinth; GA, golgi apparatus; M, mitochondria; N, nucleus; R, tubular reticulum.

an abundance of mitochondria with numerous and irregularly arranged cristae. Yolk platelets were completely consumed.

At 180 g/L salinity: The general structure of the salt gland disappeared in *Artemia* cultured at 180 g/L. The cuticle layer became thicker and composed of many layers (Figure 4C). The salt pumps were destroyed and only scattered mitochondria were present and appeared to be separated from each other with less number of cristae (Figure 4D). The apical and central zones showed no amplification of the surface area of the plasma membrane. The labyrinth was absent. Clusters of electron lucent vesicles and membrane bound vesicles of various types were present and occupied the majority of cell. Nuclei were smaller than in other salinities, roughly oval and contain intensely staining nucleoli and chromatin.

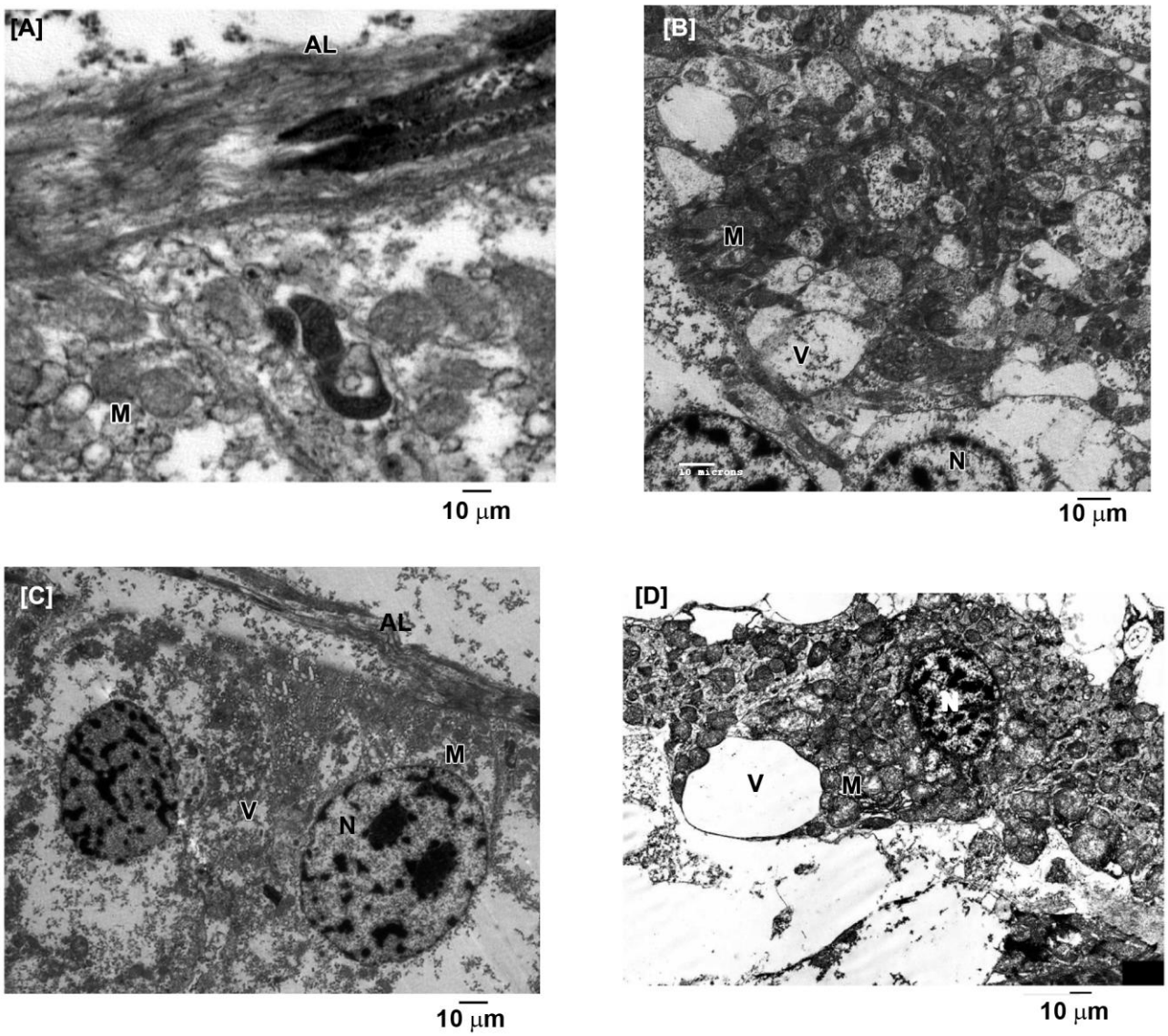

Figure 4. Photomicrograph of TEM of transverse section through the salt glands cultured at A, 140 g/L salinity; B, the basal zone from the previous section showing mitochondria; C, cultured at 180 g/L salinity and D, enlarged portion of the previous section showing the scattered mitochondria. AL, apical layer; M, mitochondria; N, nucleus; V, vacuole.

RT-PCR and analysis of APH-1 gene expression

RT-PCR of mitochondrial 16S rRNA resulted in the amplification of the expected 302 bp fragment in every sample, which indicated the integrity of the total RNA used for RT-PCR as well as the successful first-strand cDNA preparation. Meanwhile, amplification of the APH-1 gave the anticipated 422 bp fragment. In addition, no signal was observed in adult *Artemia* (data not shown) consistent with the lack of APH-1 transcription at this stage.

APH-1 mRNA was expressed in all tested salinities. Low level of expression was noticed in both 25 and 40 g/L salinity concentrations. However, as salinity increased up to 70 g/L the gene was up regulated and higher levels of expression was detected. The APH-1 expression level reached its maximum at 140 g/L salinity. On the other hand, the expression level reduced significantly at salinity 180 g/L (Figures 5 and 6).

DISCUSSION

In the present work, *Artemia* nauplii tolerance to the salinity extremes indicated their proficiency to cope with different NaCl concentrations despite the lack of thoracic appendages and epithelial gut. Under the first four salinities (25, 70, 120 and 140 g/L), *Artemia* was an excellent hypoosmotic regulator; it maintained its low osmotic concentration, not by being very impermeable to water, but by active pumping of NaCl as demonstrated

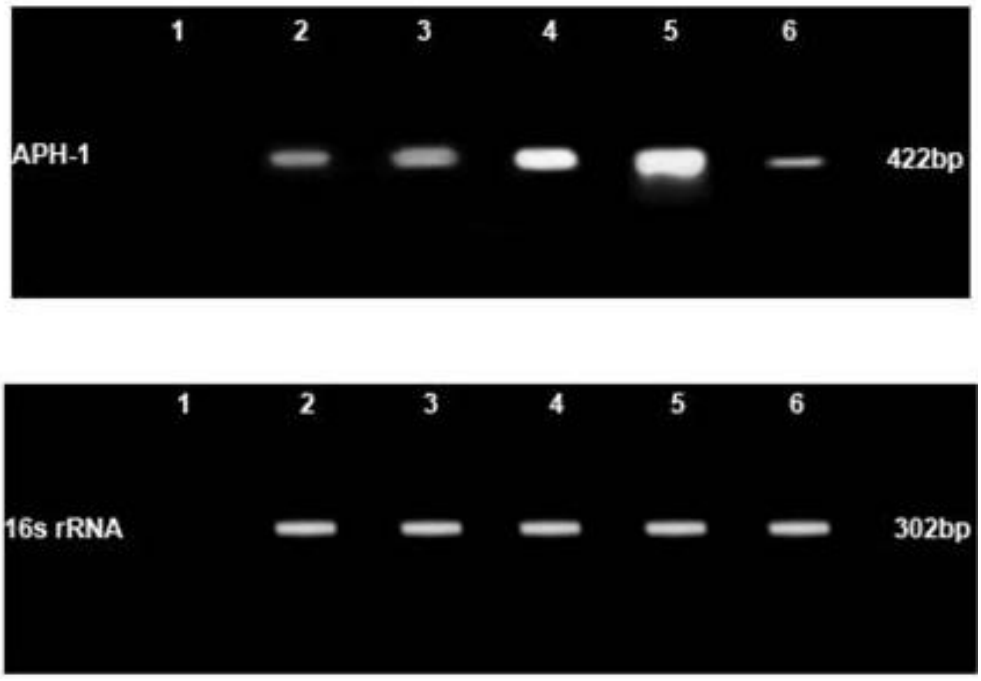

Figure 5. Expression of APH-1 mRNA in *Artemia tunisiana.* Electrophoresis of RT-PCR products of APH-1 (422 bp and 16S rRNA (302 bp) mRNA was performed in ethidium bromide-stained agarose gel (1.5%).Shown are amplicons; lane 1, negative control; lanes 2-6, 25, 40, 70, 140, 180 g/L salinities respectively.

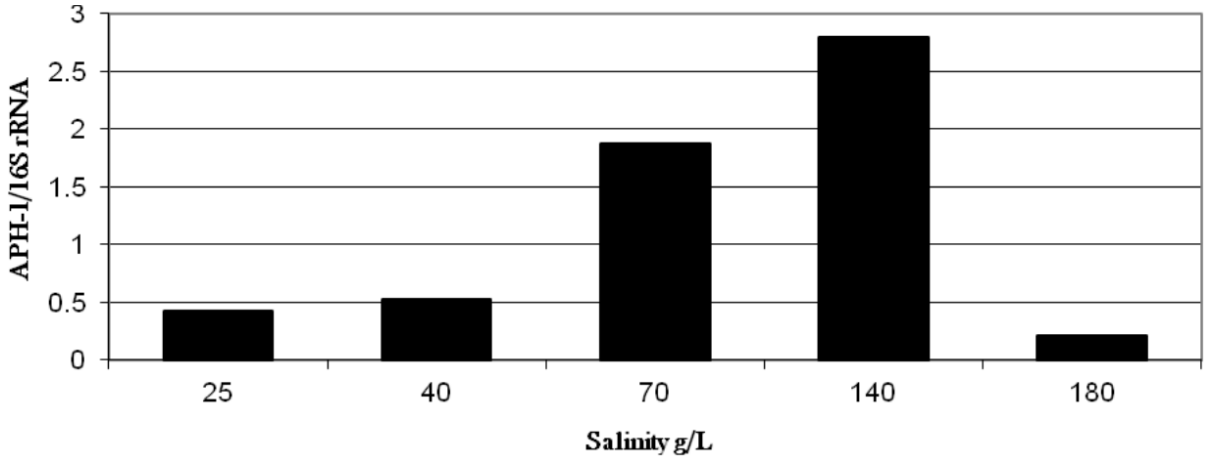

Figure 6. *Artemia tunisiana* APH-1 expression.

from the huge number and distribution of mitochondria and a large amplification of the surface area of the plasma membrane. At 180 g/L salinity *Artemia* survived this treatment but they lost their ability to osmoregulate and became strict osmo conformers.

The present study using scanning electron microscope gave morphological evidence to support the function and adaptation of salt gland due to the different salinities. The shape, size, number of channels and ridges, all of them were influenced by salinity increment. The epithelium with salinity became enlarged may be to engage the new acclimatations as the more amplification of the apical and central zones and the profusion of mitochondria. Also, the hemispherical dome with surface irregularities of epithelium at the high salinities (70, 120, 140 and 180 g/L) incite more surface area for salt excretion. The ultrastructure of the salt gland especially at 40, 70 and 140 g/L was resembled to the branchial gills of the adult.

Hootman and Conte (1975) studied the salt gland ultrastructure and showed its similarity to that of the branchial gills of the adult described by Copeland (1966).

The fine structure of the salt gland in the nauplius confirmed its role in both osmotic and ionic regulation. Ewing et al. (1972) studied the effects of several kinds of inhibitors on survival of nauplii at various salinities and concluded that unusual macromolecular events may be occurring which permit nauplii to live in high concentrations of salt. The modification of the epithelium, the amplification of plasma membrane, and the large populations of mitochondria in the herein species are described in *A. salina* (Hootman and Conte, 1975) and in ion transporting epithelia of other crustaceans as in isopod hindgut (Holdich and Ratcliffe, 1970); the midgut and branchial chamber of the brown shrimp (Talbot et al., 1972); the antennal gland of the fiddler crab (Schmidt-Nielsen et al., 1968); the gills of crabs (Copeland, 1968); and crayfish (Fisher, 1972). The folding of the plasma membrane into an extensive network of tubular reticulum is similar to that observed in the teleost chloride cell (Kessel and Beams, 1962; Karnaky, 1972) and the cells of the avian salt gland (Martin and Philpott, 1973). The amplification of the apical plasmalemma under high salinities (70, 120 and 140 g/L) may serve to increase the surface area available for active solute transport. Also the ramified network of smooth endoplasmic reticulum, which filled the central cytoplasmic zone indicated a salt secretory role of the neck organ under high salinities. Kikuchi (1972) has shown that the extent of smooth tubular network which fills the central cytoplasmic zone (labyrinth) is related to the salinity of the culture medium. The most abundant organelles were mitochondria that also related in their shape, number and size to the degree of salinity. They formed aggregations in intimate contact with the tubular membranes reminiscent of mitochondrial pumps. These mitochondrial pumps were described by Copeland (1967) in the neck organ of *Artemia salina*. Ewing et al. (1972) concluded that the coincident transcription and translation of both nuclear and mitochondrial genes are involved in the development of the naupliar salt gland. *Artemia* at 180 g/L with burnt salt pumps were restricted to narrow range of osmotic regulation. The previous indication is confirmed by the thickness of cuticlular layer in this high salinity concentration in comparison to other tested salinities, which indicated more impermeability to the salt. The survival of these nauplii may be due to the earlier formation of gut (stage 2 instead of 4) than those cultured at lower salinities. The consumption of yolk platelets which contain a unique storage compound in the salt glands of nauplii cultured at 70, 120, 140 and 180 g/L than those cultured at low salinities indicates the high metabolic rate required for nauplii to overcome the high salinities. Also, large quantities of glycogen granules in salt gland at high salinities may provide the cell with metabolic advantages for ATP production.

The most frequent source of evidence for a given protein to be considered relevant in osmoregulation is when its expression or activity is altered after the organism has gone from high to low salt, or vice versa (Saez et al., 2009). Changes in gene expression have been suggested as an important component for adaptation to different environmental conditions and stress management (Schulte 2001; Xu, and Liu, 2011). Several studies examining osmotic pressure stress in animal identify salinity stress through the expression of osmoregulation-related genes (Towle and Weihrauch, 2001; Wu et al., 2011; Barman et al., 2012). Chavez et al. (1999) cloned and sequenced a partial cDNA of *Artemia franciscana* APH-1 (named Af-APH-1). This gene encodes a POU domain family of transcription factors that observed expression in the larvae salt gland but not in the adult *Artemia*. More recently, and by the time of writing up this manuscript, Wang et al. (2012) isolated a full-length cDNAs of *A. sinica* POU-homeoprotein enco-ding gene, designated As-APH-1. The gene encoded a protein of 388 amino acid polypeptide with a calculated molecular mass of 42.85 kDa and an isoelectric point of 6.90 and the protein belongs to the POU III family. The authors determined, by semi-quantitative RT-PCR and whole-mount embryonic immunohistochemistry, an early and persistent expression of As-APH-1 in the naupliar stages, which suggests that As-APH-1 functions very early in the salt gland and may be required continuously in this organ, while later in development, the expression of the gene begins to dramatically decrease and disappear in salt gland and appears in the thoracic epipods in sub-adult. In addition, they tested the expression under different salinities (50, 75, 100, 125 and 150 g/L) and realized that As-APH-1 increased obviously as the salinity increased, with almost no expression in 50 g/L, initial increase in expression in 75 g/L, constantly increase in 100 g/L and a highest expression level was reached in 150 g/L salinity. In the present study, APH-1 mRNA was expressed in all tested salinities. Contrary to Wang et al. (2012), detectable level of expression was noticed in both 25 and 40 g/L salinity. As salinity increased up to 70 g/L the gene was up regulated and higher level of expression was detected. The APH-1 expression level reached its maximum at 140 g/L salinity. On the other hand, the expression was reduced significantly at salinity 180 g/L, which demonstrates that the nauplii became less capable of coping with greatly increased salt load. This reduction in gene expression also concurs with the recorded structure diminishing of the salt gland in this salinity concentration.

In conclusion, *Artemia* represents a valuable model for a salinity tolerated organism. Our results demonstrate differences in the morphology and structure of *A. tunisiana* salt gland as well as in the expression of APH-1 reflecting salinity changes. Although it can be used as a good indicator of salinity stress, the observation that this expression is lowered in the highest tested salinity, with *Artemia* ability to withstand very high salinity concentra-

tions, highlights the possible contribution of other gene(s) to compensate this salinity augment. As the mechanism of adaptability to hypersaline water is expected to be a complex physiological and molecular process, further investigation of this issue is auspicious.

REFERENCES

Abel JH, Ellis RA (1966). Histochemical and electron microscopic observations on the salt secreting lachrymal glands of marine turtles. Amer. J. Anat.118:337-358.

Anderson MG, Certel SJ, Certel K, Lee T, Montell DJ, Johnson WA (1996). Function of the Drosophila POU domain transcription factor drifter as an upstream regulator of breathless receptor tyrosine kinase expression in developing trachea. Development 122:4169-4178.

Barman HK, Patra SK, Das V, Mohapatra SD, Jayasankar P, Mohapatra C, Mohanta R, Panda RP, Rath SN (2012). Identification and characterization of differentially expressed transcripts in the gills of freshwater prawn (*Macrobrachium rosenbergii)* under salt stress. The Sci. W. J.

Bell AL, Barnes SN, Andrson KL (1969). A fixation technique for electron microscopy which provides uniformly good preservation of the tissues of a variety of marine invertebrates. Biol. Bull. 137:393.

Chavez M, Landry C, Loret S, Muller M, Figueroa J, Peers B, Rentier-Delrue F, Rousseau GG, Krauskopf M, Martial JA (1999). APH-1, a POU homeobox gene expressed in the salt gland of the crustacean *Artemia franciscana*. Mech. Dev. 87:207-212.

Conte FP, Droukas PC, Ewinf RD (1977). Development of sodium regulation and *de novo* synthesis of Na+K-activated ATPase in the larval brine shrimp *Artemia salina*. J. Exp. Zool. 202:339-361.

Conte FP, Hootman SR, Harris PJ (1972). Neck organ of *Artemia salina* nauplii: a larval salt gland. J. Comp. physiol. 80:239-246.

Copeland DE (1966). Salt transport organelle in *Artemia salenis* (brine shrimp). Science. 151:470-471.

Copeland DE (1967). A study of salt-secreting cells in the brine shrimp (*Artemia salina*). Protoplasma, 63:363-384.

Copeland DE (1968). Fine structure of salt and water uptake in the land crab, *Gecarcinus lateralise*. Amer. Zool. 8:417-432.

Criel RJ (1991). Morphology of *Artemia*. In, *Artemia* biology. (Brown RA, Sorgeloos, P , CNA Trotman, eds) CRC Press, Boca Raton, FL, Pages 155-185.

Croghan PC (1958). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut. J. Exp. Biol. 35:243-249

Dejdar E (1930). Die Korrelationen zwischen Kiiemensackchen und Nackenschild bei phyllopoden. (Versuch einer Analyse mit Hilfe elective vitalfarbung.) Z. wiss. Zool. 136:422-452.

Ewing RD, Peterson GL, Conte FP (1972). Larval salt gland of *Artemia salina* nauplii. Effects of inhibitors on survival at various salinities. J. Comp. Physiol. 80:247-254.

Ewing RD, Peterson GL, Conte FP (1974). Larval salt gland of *Artemia salina* nauplii: localization and characterization of sodium and potassium-activated adenosine triphosphatase. J. Comp. Physiol. 88:217-234.

Fisher JM (1972). Fine-structural observations on the gill filaments of the freshwater cryfish *Astacus pallipes* Lereboullet. Tissue and cell, 4:287-299.

Fryer G (1987). A new classification of the branchiopod Crustacea. Zool. J. Linn. Soc. 91:357-383.

Holdich DM, Ratcliffe NA (1970). A light and electron microscopic study of the hindgut of the herbivorous isopod, *Dynamene bidentata* (crustacean: pericarida). Z. Zellforsch., 111:209-227.

Hootman SR, Conte FP (1975). Functional morphology of the neck organ in *Artemia salina* nauplii. J. Morph. 145(3):371-386.

Hootman SR, Harris PJ, Conte FP (1972). Surface specialization in the larval salt gland in *Artemia salina* nauplii. J. Comp. Physiol.79:97-104.

Karnaky KJ (1972). Osmoregulation in euryhaline teleosts: cytological and biochemical studies on the branchial epithelium of *Cyprinodon variegateus*. PhD Thesis. Rice University. Houston, Texas.

Kessel RG, Beams HW (1962). Electron microscopic studies on the gill filaments of *fundulus heteroclitus* from sea water and fresh water with special reference to the ultrastructural organization of the “chloride cell”. J. Ultr. Res. 6:77-87.

Kikuchi S (1972). Three-dimensional network of a tubular system in the salt-transporting cells of the gill and the neck organ of *Artemia salina* (brine shrimp). Annual Report of the Iwate Medical University school of Liberal Arts and Science 7:15-22.

Martin BJ, Philpott CW (1973). The adaptive response of the salt glands of adult mallard ducks to a salt water regime: an ultrastructural and tracer study. J. Exp. Zool. 186:111-122.

Martin JW, Laverack MS (1992). On the distribution of the crustacean dorsal organ. Acta Zoologica 73:357-368.

Palmero I, Renart J, Sastre L (1988). Isolation of cDNA clones for mitochondrial 16S ribosomal RNA from the crustacean *Artemia*. Gene 68:239-248.

Russler D, Mangos J (1978). Micropuncture studies on the osmo-regulation in the nauplius of Artemia salina. American J. Physiol. 234:R216-R222.

Sáez AG, Lozano E, Zaldívar-Riverón A (2009). Evolutionary history of Na, K-ATPases and their osmoregulatory role. Genetica. 136 (3):479-490.

Sars GO (1896). Fauna Norvegiae, Vol. I. Descriptions of the Norwegian species at present known belonging to the suborders Phyllocarida and Phyllopoda. Joint-stock, Christiania.

Schmidt-Nielsen B, Gertz HK, Davis LE (1968). Excretion and ultrastructure of the antennal gland of the fiddler crab, Uca modax. J. Morph. 125:473-496.

Schrehardt A (1986). Der Salinenkrebs Anemia 2. Die postembryonale Entwicklung- Mikrokomos 75:334-340.

Schrehardt A (1987). A scanning electron-microscopic study of the post-embryonic development of *Artemia*. In *Artemia*: Research and Applications, Volume 1, (P. Sorgeloos et al., eds), Universa Press, Wetteren, Belgium, pp.5-32.

Schulte PM (2001). Environmental adaptations as windows on molecular evolution. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128:597-611.

Sorgeloos P (1978). The culture and use of brine shrimp *Artemia salina* as food for hatchery raised larval prawns, shrimp and fish in South East Asia. FAO Report THA/75/008/78/WP3, 50pp.

Talbot PW, Clark HJ, Lawrence AL (1972). Fine structure of the midgut epithelium in the developing brown shrimp, *Penaeus aztecus*. J. Morph. 138:467-486.

Towle DW, Weihrauch D (2001). Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. Amer. Zool. 41:770-780.

Varo I, Navarro JC, Amat F, Guilhermino L (2002). Characterization of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. Chemosphere 48:563-569

Wang JQ, Hou L, Yi N, Zhang RF, Zou XY (2012). Molecular analysis and its expression of a pou homeobox protein gene during development and in response to salinity stress from brine shrimp, *Artemia sinica*. Comp. Biochem. Physiol. 161(A):36-43.

Weisz PB (1947). The histological pattern of metameric development in *Artemia salina*. J. Morph. 81:45-95.

Wu G, Zhang H, Sun J, Liu F, Ge X, Chen WH, Yu, J, Wang W (2011). Diverse LEA (late embryogenesis abundant) and LEA-like genes and their responses to hypersaline stress in post-diapause embryonic development of *Artemia franciscana*. Comp. Biochem. Physiol. 160(B):32-39.

Xu Q, Liu Y (2011). Gene expression profiles of the swimming crab *Portunus trituberculatus* exposed to salinity stress. Mar. Biol. DOI 10.1007/s00227-011-1721-8.

Zelzer E, Shilo BZ (2000). Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein drifter specifies tracheal cell fates. Mech. Dev. 91:163-173.

Zenker W (1851). Physiologische bemerkungen uber die Daphnoiden. Arch. Anat. Physiol. Med. 1851:112-121.

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Full Length Research Paper

Study of seasonal sexual activity variations in Algerian rams: Sexual behaviour, testosterone concentration control and environmental factors

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This study focuses on the determination of seasonal effect on two main andrological sexual activity parameters within young and adults rams of Rembi breed from Algeria for a period of one year. The experiment involved a weekly evaluation of males' sexual behaviour and a monthly measurement of serum testosterone concentration in order to know the main testicular endocrine activity. The purpose of this experiment was to assess better the characteristics of the reproductive activity between two categories of rams age-wise and to define the variations and interactions between the two parameters during each season. Data shows no statistically significant effect of age on the testosterone concentration but showed a highly significant difference between age groups in terms of sexual behaviour. Seasonal variations of the studied parameters were statistically significant in both ram ages with higher values during spring and autumn and lower values during summer and winter.

Key words: Rembi, ram, testosterone, sexual activity, season, age.

INTRODUCTION

In Algeria, sheep represent the main animal resource; about 23 million heads cover 40 million hectares of grazing arid regions, where 12 million hectares are steppe. Seventy five percent of sheep are thus concentrated in the steppe and are reared in extensive system (Nedjraoui, 2006). This system is characterized by a heavy dependence on the natural vegetation composed mainly of Alfa and therefore remains heavily influenced by climatic conditions (Nedjraoui, 2002).

The economic importance of sheep farming constitutes an important source of animal protein (meat and milk) and has a significant contribution in livestock products (skins and wool). For this purpose, it is essential to find ways to improve sheep productivity in Algeria. This improvement is associated with the control of reproduction which is the center piece of the economic efficiency of any breeding program (Nedjraoui, 2006). For successful productivity of sheep, herds must pass through the control of ram's reproduction (Ólafur and Jón Viðar, 2011). The Rembi race represents 12% of the national sheep flock and constitutes one of the most interesting Algerian races based on its physical, productive and reproductive skills by two lambings per year with a rate of twinning quite acceptable (Nedjraoui, 2006). It is the biggest sheep in Algeria; the ram weighs 90 kg, while the weight of the sheep is 60 kg. The proportional body shape of this race ensures excellent criterion for the success of sexual activity for both male and female.

The sexual behaviour and testosterone level point out

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the effectiveness of male's reproduction because they are influenced by race and testicular size partly (Zamiri and Khodaei, 2005) and by the geographic location and season of the year (Karagiannidis et al., 2000). The control of sexual behaviour constitutes a key for improving the performance of and/or managing of producing animals. In most species, the expression of this behaviour depends on the rate of steroid hormones, nutritional statue, physical and social environment and structure of the social group (Fabre, 2000).

Androgens or male hormones are steroid hormones that are able to develop and maintain male sexual characters involved in spermatogenesis and therefore command males' sexual behaviour. They are found principally in the internal testes secretions, adrenal and ovarian. Testosterone is the hormone that controls the operation of testis, epididymis and accessory glands as well as the expression of sexual behaviour; its dosage could provide explanation for the high and low levels of dead and abnormal sperm observed respectively from April to October (Issa et al., 2001).

In several rams' breeds programs, it was established that the release of the hormone testosterone into the blood was characterized by high peaks in European, American and Australian breeds. The frequency of testosterone peaks increases when the rams pass to the breeding season. Seasonal variations of testosterone have also been reported in adult rams breed "Chios and Daglic" in the province of Afyonkarahisar in Turkey when the highest concentrations of the testosterone in the plasma were recorded during autumn (Gundogan, 2007).

Sexual behaviour and hormone levels of testosterone vary also between breeds of rams age-wise (Langford et al., 1999; Kafi et al., 2004; Zamiri and Khodaei, 2005); this is because for there to be good improvement in the productivity of flocks of sheep, an attention must be focused on the proper earliest selection of the future genitors. It is to be noted that the seasonal variations of testosterone in sheep, even for Rembi rams, in our country including arid and steppe areas are still not yet considered. The unique use of adult dominant rams that may have infertility problems can induce significant losses in reproduction that may go undetected for some time especially if a good level of libido is maintained in these rams.

For a proper management of sheep farms, knowledge of the optimal season for breeding rams is of great importance, especially in Algeria, where majority of farmers prefer spring and autumn seasons for breeding periods. In these periods, births coincide with the time of food availability and the most favourable climatic conditions as well as the choice of the best time for marketing. Performance and management of sheep farms can be improved, in particular by reducing the variability of fertility and controlling the optimal time of reproduction. Sexual behaviour is of obvious interest from this point of view. The performance of a farm depends on reproduction which is the function of the willingness and ability of animals to engage in sexual behaviour and fertilize at the right time. In order to control the expression of sexual behaviour, it is necessary to know the various influencing factors such as age, season, environment, food, climate, testosterone level etc. Our work describes the state of knowledge about sexual behaviour and evolution of the plasma testosterone levels.

The aim of our study was to determine the factors and parameters involved in rams' sexual activity by studying the evolution of the sexual activity of Rembi race and the intervening factors in this activity during the four seasons of the year.

MATERIALS AND METHODS

Experimental design

The study was conducted in an experimental farm located in a steppe pastoral region (geographic coordinates: longitude 2°19'E, latitude 35°10'N, altitude 839 m.s.l). The climate is arid with cold and wet winter and hot and dry summer; the temperature varies from -1.1 to 16.4°C in winter and from 21.9 to 39.5°C in summer. The daily photoperiod varies from 9.34 h during the winter solstice to 14.23 h during the summer solstice. Rembi is from the leading Algerian sheep breeds, located in the Northwest of Algeria; it has a low size, tawny head, very strong members and carcass of red fawn colour. It is an interesting race based on its physical, productive and reproductive abilities.

Ten (10) Rembi rams aged between 02 and 06 years in addition to three live wire sheep aged between 02 and 04 years were selected for our experiment in 2010. Males were separated into two groups (young: n = 03 aged between 02 and 03 years and adults: n = 07 aged between 04 and 06 years). Males were in contact with the sheep during the time of sexual activity test. Prophy lactic treatments were recommended, and only healthy subjects were selected. Each ram was identified and medically examined with emphasis on the integrity of the genital area. These animals belonged to a semi ranching, in addition to grazing on natural woody plants (Alpha, sagebrush, Atriplex). They received a nutritional complement of barley, corn, soybeans and hay (600 g/day) while water was provided ad libitum.

The sexual behaviour of males is under the control of testosterone or its metabolites. In castrated males, testosterone treatment restores males' sexual behaviour, whereas before treatment, it tends to persist for several months after castration in sexually experienced animals. In seasonal breeds, these steroid secretions vary with the season. However, hormonal changes are gradual and it takes several weeks after a change in plasma level to observe an effect on sexual behaviour.

Evaluation of sexual behaviour (Libido)

The evaluation of the sexual activity of rams is a very important aspect in reproduction and sheep industry. The tests assess libido sexual motivation (number of mounts/time) and sexual efficiency (number of ejaculations/ram/time). Rams used in this study were separated from females, and were brought into them during tests of sexual activity. All tests were performed once a week in the afternoon between 13 and 16 h (Ahmed and Noakes, 1995).

Tests of sexual behaviour were conducted by exposing rams individually to ewes (1 to 3) in oestrus for 10 to 15 min. These sheep were treated by Estradiol Benzoate (0.5-1 ml by intramuscular 24 h before test) in order to stimulate their sexual activity (Price et al., 1988). Thereafter, these tests were carried out

Table 1. Seasonal variations of testosterone concentrations and sexual behaviour of rams.

Season	Testosterone concentration (ng/ml)			Sexual behaviour		
	All	Young	Adult	All	Young	Adult
Spring	2.80 ± 0.84	3.28 ± 0.54	2.60 ± 1.05	8.87 ± 1.14	6.94 ± 2.71	9.70 ± 0.51
Summer	0.60 ± 0.07	0.48 ± 0.09	0.65 ± 0.07	6.58 ± 1.82	4.02 ± 2.09	7.67 ± 1.72
Autumn	2.68 ± 0.54	2.64 ± 0.34	2.69 ± 0.64	8.66 ± 0.90	6.66 ± 1.81	9.52 ± 0.51
Winter	0.93 ± 0.39	1.22 ± 0.65	0.81 ± 0.30	5.66 ± 1.45	2.63 ± 1.33	6.96 ± 1.61

by exposing the whole set of rams of the two age categories to the sheep in oestrus to determine their behaviour. During the trial period, behaviours were recorded by an observer located outside the site of action at a distance of approximately two meters in order to calculate the overall level of physical activity. During each test, we recorded the number of anogenital flairages and the various components of Flehmen and consummatory phase of ram's sexual behaviour (advancement of the foreleg, mounts attempted, mounted with or without ejaculations) (Price et al., 1988). This allowed us to record the total number of mounts of one or more rams for one or more sheep during a defined test period. From these observations, a score of 10 points was calculated (Ahmed and Noakes, 1995). The ram that shows no interest in sheep (not more than one goes without ejaculation) will receive a score of 0 (marked as low and insignificant libido); the ram that rides twice with or without projection (at least 01 or 01 up ejaculation) will get a score of 05 (marked as acceptable, average libido); the ram that engages in two or more times ejaculations (at least 02 ejaculations or 05) and still shows an interest in the female will get a score of 10 (marked as high strong libido).

This test will mainly present results on levels of sexual behaviour and reproductive performance of rams during mating with the flock throughout the active sexual period. The purpose of this experiment is to study and monitor the characteristics and components of the sexual activity of Rembi race during each season.

Hormone dosage

Monthly samplings of blood tests (at 09 h am) for each ram were performed during the whole year of our study to estimate their blood testosterone. The operation took place in the quiet to avoid males' stress. The blood was collected from the jugular vein of each ram in individual tubes heparinised and immediately placed in a cooler. Blood samples were then transported to the laboratory, centrifuged at 3000 rpm/min for 20 min. The collected plasma was stored at -20°C until radioimmunoassay of testosterone was done (Kafi et al., 2004). Testosterone was measured by radioimmunoassay method testosterone (RIA), direct REF IM 1119 ISO 9001/13485 certified (IMMUNOTECH FRENCH). Assayed sera were incubated in antibody coated tubes with a testosterone tracer labelled with Iodine 125. After incubation, the tube contents were emptied by inspiration and the bound radioactivity was measured. A calibration curve was established and values were determined.

Statistical analysis

Data were arranged into a single matrix. The variance of homogeneity of the data was assessed and conformed to the model which would permit analysis of variance (ANOVA) on the data set in order to determine seasonal or monthly significant differences between sexual behaviour and testosterone concentration of rams for each age group and for both groups together. The term, significance indicates differences in which $P<0.05$ under the confidence level of $\alpha=95\%$. Collected data were used also for calculation of correlations based on the coefficient of Pearson among examined traits. Data were analyzed using the General Linear Model (GLM) procedure implemented in the statistical software SPSS v16 (Chicago, 1986).

RESULTS

The statistical analysis did not show significant effect of ram's age on testosterone concentration ($P>0.05$); however, a very high significant difference was found between the two age groups with regard to sexual behaviour ($P <0.001$). Our results show that rams' sexual behaviour varied significantly between different examined seasons of the year ($P <0.001$). Overall, the sexual behaviour was at its peak during spring from March to May and during autumn from September to November. Plasma testosterone concentration varied significantly between months as well as between seasons of the year ($P<0.001$), but did not vary significantly between the two groups in terms of age ($P>0.05$). Monthly variations of testosterone concentrations were similar to the previous changes regarding seasons. Maximum values were recorded during months of spring and autumn while lowest values were observed during the months of summer and winter. This observation was similar for young as well as adult rams with higher testosterone concentration synthesis during spring (3.28±0.54 ng/ml against 2.60±1.05 ng/ml, respectively). Lower testosterone concentrations were mainly reported during summer from June to August for both age groups (0.48±0.09 ng/ml for young and 0.65±0.07 ng/ml for adult) and then during winter (December to February) (Table 1).

Ejaculate latency was lesser during spring and autumn when the sexual behaviour of males was very clear. A slight decrease in the intensity of the sexual activity with an acceptable copulatory activity was observed during the warm periods of summer mainly in August as well as when temperatures decreased in January for both groups. Higher values were observed for adult rams compared to young rams at any season.

Significant correlations were found between the plasma testosterone concentration and the sexual behaviour in rams and were even more pronounced in adult than in young group. Thus, testosterone concentration and libido

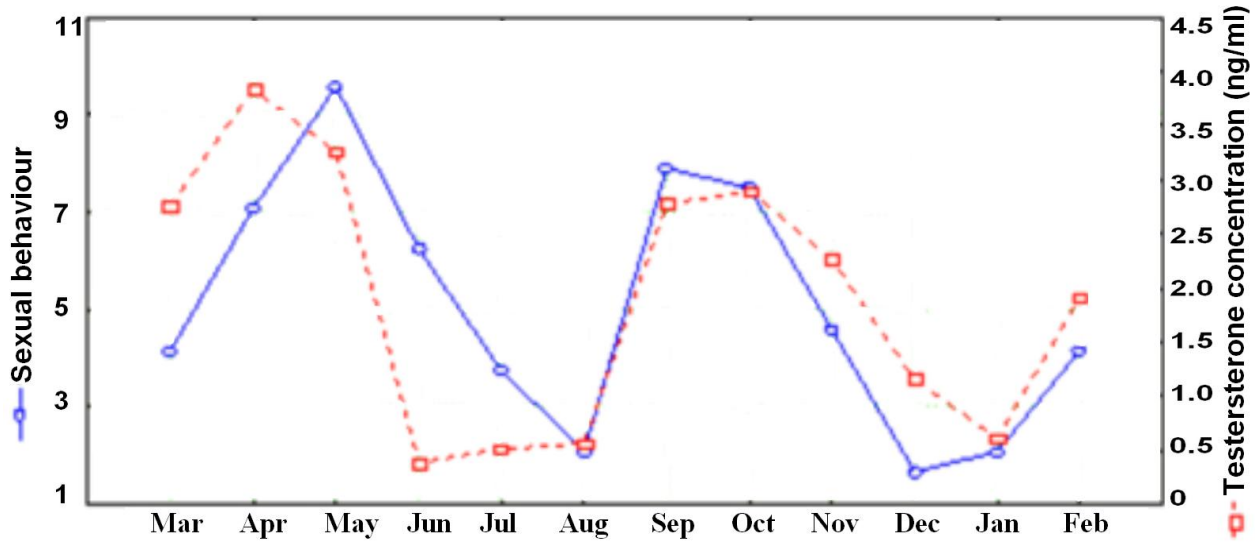

Figure 1. Monthly changes of the sexual behaviour and testosterone concentration in young rams.

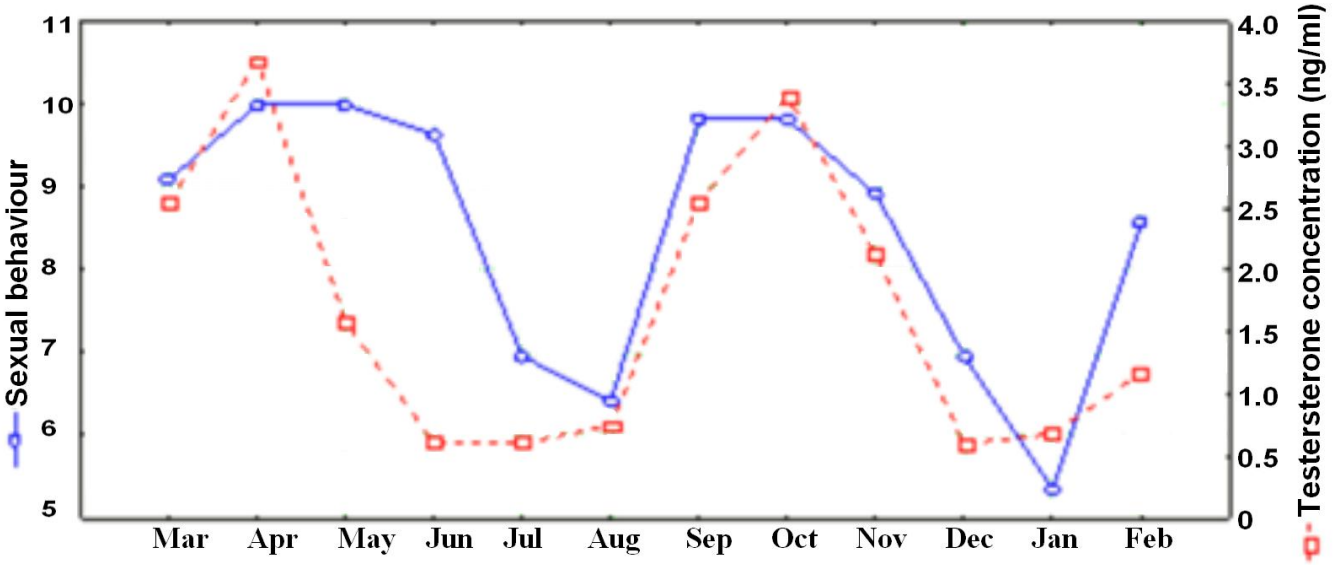

Figure 2. Monthly changes of the sexual behaviour and testosterone concentration in adult rams.

scores were negatively correlated with season (r=-0.23* and r=-0.27** respectively). It appears that the sexual activity of Rembi breed adult rams did not vanish throughout the year. This activity was higher in spring and autumn but lower in summer and winter (Figures 1 and 2).

Our study shows that the sudden introduction of stimulated ewes into the group of rams induced some changes in male's behavioural state. The presence of females generally improved the sexual activity level. Interestingly, these effects appeared to be less pronounced during anoestrus periods. At the beginning of each experiment, the sexual activity remained fairly limited with rams faltering parades. In contrast, males engaged in fighting, usually between males of the same age. Females gradually became more tolerant towards males' approaches and the sexual activity level increased. Later, males' sexual parades became shorter, giving way to a more expeditious and brutal behaviour. Thus, the physical contact and dominance among the strongest rams for mating were significantly marked in the presence of all the other rams around stimulated

females.

The rams showed high levels of sexual performance and we found that mature rams were highly efficient and had a significantly greater effect on the ewes in oestrus compared to young rams which had less sexual activity. However, analysis of behavioural data showed that young rams spent more time near the ewes than adults. The adult rams were more efficient and experienced and demonstrated their libido with ease compared to young rams. Males of 04 and 05 years were the most involved in reproduction and young rams showed a significant participation. It should be noted that the presence of a viewer dominant ram can inhibit sexual activity of a young subordinate.

During oestrus, each female may mate with several males (young and adult) and each male with several females (from 2 to 4). Females in natural or artificial oestrus play an important role in facilitating the full expression of sexual behaviour of males. Rams are particularly sensitive to the effects of the environment on sexual responsiveness, which sometimes can be inhibited or stimulated. Increasing the time between successive couplings with a single sheep could be interpreted as resulting from the physiological evolution of an internal mechanism. However, the presentation of a new receptive sheep induced intense recovery of copulatory activity.

DISCUSSION

Our results show that monthly changes in sexual behaviour were not marked. The ram's sexual behaviour began to grow in early spring and reached its highest during this period; and then it was followed by a decrease during the summer season and increased again during autumn (Table 1). It is to be noted that there was no stopping of the ram's sexual activity throughout the year; the lowest scores recorded were during winter. Through observed variations and despite a constant supply throughout the duration of the experiment, this result indicates that the season had a great influence on males' reproductive physiology regardless of changes in food resources.

There is some association between testosterone concentrations and the different scores of sexual behaviour throughout the year with some lag. Indeed, the increase in testosterone levels preceded the four weeks of elevation of sexual behaviour scores during spring season. In rams, the level of sexual activity fluctuates during the year in conjunction with the rate of testosterone.

Ahmad and Noakes (1995) reported that sexual function in sheep appears in adulthood; the information acquired during ontogeny appears to be involved in its organization. Price et al. (1988) reported that inexperienced younger rams showed a gradual increase in sexual behaviour from the age of puberty. A brief exposure (white cross) to ewes in oestrus may increase their level of sexual behaviour at a level very similar to that of experienced adult rams. Other studies have shown that young ruminants also manifested an increase of sexual behaviour level over time (Godfrey et al., 1993).

According to our results, the performance of adult rams' sexual behaviour (from 4 years) did not improve with age; no change in latency to protrude between the first and the fourth ejaculates. In contrast, repeated stimulation by females was effective. By contrast, these performances were higher within the older rams (06 years old). We noticed that the sexual activity of all rams correlated with food context. In times of flushing, the degree of sexual behaviour and libido increased significantly. The same result was observed in spring season (April-June) while the rams were grazing. Knowing that during the most difficult periods of the year, a decrease of sexual activity is directly related to the exclusion of food resources in both sexes.

Baril et al. (1993) reported that in rams, libido decreases severely from five to ten weeks after the beginning of undernourishment (observed in a long-term deficiency of vitamin A). Rams of tropical and subtropical breeds (if well fed) do not show seasonal variations in their behavioural and spermatogenic activity. In some cases, however, the situation may be complicated by the fact that in tropical and subtropical countries, high temperatures during hot seasons cause the appearance of dead and abnormal sperm (Baril et al., 1993). These authors reported that while spermatogenesis within sheep living in middle and high latitudes does not stop the number of sperm produced by the testis, it decreases at certain seasons of the year. Outside the breeding season, the total number of sperm per ejaculate decreases more rapidly with the order numbers than successive ejaculates during the breeding season. This goes in line with the findings of our research. The intensification of sexual behaviour during the sexual season is due to an increase in testosterone that occurs earlier. This hormone is responsible for the proliferation of Leydig cells, Sertoli cells and germ cells, resulting in increased testicular size and sexual activity.

Male sexual behaviour has no short-term variations. In temperate zones, sheep sexual activity is seasonal; the male's sexual responsiveness varies slowly and gradually over the years in a parallel manner, but offsets with respect to the development of androgen production. In the short term, testosterone is secreted within males in the form of discrete episodes. These rapid changes in circulating levels do not show a direct relationship with sexual behaviour (Signoret and Balthazart, 1983). The importance of seasonal effect in sheep depends more on latitude as we get close to the equator, and less is important (the main factor responsible for this seasonality is photoperiod). Temperature can also play a role by artificial manipulation of illumination and can possibly

lead to changes in the breeding behaviour (Fabre-Nys et al., 1993).

Sexual behaviour and plasma testosterone levels were reported to be highest during spring and autumn. This trend coincided with a decrease in the length of day and ambient temperature. The pronounced increase in the level of testosterone during autumn was also reported in the studies done by Kaya et al. (1999) and Keskin and Keçeci (2001). These researchers suggested that stimulation of the pituitary gland in the ram is more likely to begin in autumn during low and ambient temperatures and reduced length of day. A similar trend of our results was observed in several varieties of sheep in Northern Turkey, showing a maximum testicular androgen activity in autumn and a minimum in summer (Aral and Tekin, 1996; Ataman et al., 1996; Gündõgan and Demirci, 1999; Gündõgan et al., 2003).

A slight modification of the environment (change of mating or feeding location), can inhibit or activate the sexual behaviour of rams. Sexual activity and reproductive health are reduced or inhibited in subordinate rams having suffered social stress. Motivation and sexual performance of rams may be modified by competition and existing hierarchy in a group. In males, the dominant can block or reduce the activity and gonadal androgen secretion within the dominated party (Signoret and Balthazart, 1983).

In dominated male, androgen secretion is often inhibited by the presence of dominant or is not in conjunction with high levels of corticosteroids and sexual activity is itself reduced. Social stress and adrenocortical activation are also involved in rams and found when the population density increases (Fabre-Nys et al., 1993). Where there are stable relations of dominance-subordination between males, the dominant has preferential access to receptive females. Competition can lead to fierce battles, especially when foreign males are involved.

Subordinates may be excluded and the group becomes a permanent or temporary harem. However, the existence of dominance does not necessarily lead to a complete exclusion of subordinate males. Changes in levels of aggression/tolerance of the dominant, the degree of synchronization of receptivity in females and their dispersion in space are all factors that may allow access of subordinate males to reproduction (Fabre-Nys et al., 1983).

Conclusion

Rembi breed rams living in the area of Ksar Chellala are constantly nurtured whether young or adults and are sexually active throughout the year. This reflects the conservation of sexual activity at all stages (sexual behaviour and plasma testosterone concentration) with some seasonal variations. Regarding reproductive parameters, sexual behaviour was positively correlated with testosterone that is much more pronounced within adults. Young rams of the Rembi breed were quite sensitive than adults to the adverse effects of heat, stress and food. We noted a decrease in sexual activity of the youth category during times of very high temperatures "ram sterility of summer", during winter, periods of food restriction and poor pasture. However, the reproductive activity of all rams continued during the year with spring and autumn at its peak (decrease of ejaculation latency; increase of servings by test) with a decrease during summer and winter (increased latency of ejaculation with a decreased number of overlaps per test). The presence of a dominant ram can cause a kind of hierarchy within a group of males, resulting in a slight decrease in reproductive performance of subordinate subjects.

In conclusion, we can say that the importance of these seasonal effects was not marked to prevent the rams of the Rembi breed to present an acceptable sexual activity (quantity and quality) throughout the year. This requires a fight against severe weather while food intake must be adequate with a supplement in seasons of struggle and during bad times; however, a good clinical follow-up of breeding rams could be useful.

REFERENCES

Ahmad N, Noakes DE (1995). Seasonal variations in testis size, libido and plasma testosterone concentrations in British goats. Anim. Sci. (61):553-559.

Aral F, Tekin N (1996). Influence of season on the semen quality of rams. J. Cent. Anim. Res. Int. (6):15-20.

Ataman MB, Kaya A, Karaca F, Yıldız C, Çoyan K, Ergin A, Aksoy M (1996). Relationships between morphometric measurements of testes and semen characteristics in breeding and nonbreeding season to select breeding yearling rams. J. Cent. Anim. Res. Int. (6):1-7.

Baril G, Chemineau P, Cognie Y, Guerin Y, Leboeuf B, Orgeur P, Vallet JC (1993). Manuel de formation pour l'insémination artificielle chez les ovins et les caprins. Rome: Organisation des Nations Unies pour l'Alimentation et l'Agriculture (FAO), (Etude FAO. Production et Santé Animales; 83). 230 p.

Fabre-Nys C (2000). Le comportement sexuel des caprins : contrôle hormonal et facteurs sociaux. INRA Prod. Anim., (13) :11-23.

Fabre-Nys C, Martin G.B, Venier G (1993). Analysis of the hormonal control of female sexual behavior and the preovulatory LH surge in the ewe:Roles of quantity of estradiol and duration of its presence. Hormones and Behaviour. (27):108-121.

Godfrey SI, Walkden-Brown SW, Martin GB, Gherardi SG, Lindsey MJ, Porter BL (1993). Immuno-castration of adult Cashmere bucks with Vaxstrate. Proceedings of the Australian Society for Reproductive Biology.

Gündoğan M (2007). Seasonal variation in serum testosterone, T3 and andrological parameters of two Turkish sheep breeds.

Gündõgan M, Baki D, Yeni D (2003). Reproductive seasonality in sheep (review). Acta Agric. Scand. Sec. A: Anim. Sci. (53):175-179.

Gündõgan M, Demirci E (1999). The effects of scrotal heating on spermatogenesis and other semen characteristics in the rams. Fırat Univ. J. Health Sci. (13):193-200.

Issa M, Yenikoye A, Marichatou H, Banoin M, (2001). Spermogramme de béliers Peuls bicolores et Touaregs : influence du type génétique et de la saison. Revue Elev. Méd. vét. Pays trop., (54):269-275.

Kafi M, Safdarian M, Hashemi M (2004). Seasonal variation in semen characteristics, scrotal circumference and libido of Persian Karakul rams: technical note. Small Rumin. Res. (53):133-139.

Karagiannidis A, Varsakeli S, Alexopoulos C, Amarantidid I (2000). Seasonal variation in semen characteristics of Chios and Friesian

rams in Greece. Small Rumin. Res. (37):125-130.
Kaya A, Yıldız C, Lehimcioğlu NC, Ergin A, Aksoy M (1999). Seasonal variation in sperm quality, testicular size and plasma testosterone concentrations in Konya Merino rams. J. Cent. Anim. Res. Int. (9):1-5.
Keskin E, Keçeci T (2001). Blood serum thyroid hormone levels and rumen protozoa in Merino rams at different environmental temperatures. Tr. J. Vet. Sci. (17):115-118.
Langford GA, Sanford LM, Maqrcus GJ, Shrestha JNB (1999). Seasonal cyclic pituitary and testicular activities in rams. Small Rumin. Res. (33):43-53.
Nedjraoui D (2002). Sources Statistiques Agricoles 1990-1999 and FAO database 2002. Rapport FAO. Country Pasture/Forage Resource Profiles. Algeria p56.
Nedjraoui D (2006). Livestock grazing systems & the environment. Rapport FAO. Country Pasture/Forage Resource Profiles. Algeria. p. 28.
Ólafur RD, Jón Viðar J (2011). The management of replacement ewe and ram lambs for breeding in Iceland. EAAP Sheep and Goat Commission. 29 Theatre, 10435. Session 32. p 17.
Price EO, Katz LS, Wallach SRJ, Zenchak JJ (1988). The relationship of male-male mounting to the sexual preferences of young rams. Appl. Anim. Behav. Sci., (21):347-355.
Signoret JP, Balthazart J (1983).Le comportement sexuel, In "La Reproduction chez les mammifères et l'homme", C Thibault & MC Levasseur coordonnateurs, INRA, Ellipses, pp. 515-536.
Zamiri MJ, Khodaei HR (2005). Seasonal thyroidal activity and reproductive characteristics of Iranian fat-tailed rams. Anim. Reprod. Sci. (88):245-255.

UPCOMING CONFERENCES

2nd Conference of Cereal Biotechnology and Breeding (CBB2), Budapest, Hungary, 5 Nov 2013

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014

ICBEE 2014 : International Conference on Biotechnology and Environment Engineering

GB, London
January 20-21, 2014

Conferences and Advert

November 2013
2nd Conference of Cereal Biotechnology and Breeding (CBB2), Budapest, Hungary, 5 Nov 2013

December 2013
International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013

International Conference on Agriculture and Biotechnology, Kuala Lumpur, Malaysia, 29 Dec 2013

January 2014
International Conference on Biotechnology, Chemical and Environmental Engineering, London, UK, 19 Jan 2014

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014

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