African Journal of Biotechnology

Volume 15 Number 24, 15 June 2016

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

African Journal of Biotechnology

Table of Content: Volume 15 Number 24 15 June, 2016

ARTICLES

Application of biotechnology for the domestication of *Dacryodes edulis* (G. Don) H. J. Lam in Cameroon: A review 1177
TCHATCHOUA Dorothy Tchapda, TCHOUNDJEU Zac and CASPA Roseline Gusua

Growth performance and nutrient utilization of *Clarias gariepinus* fed with different dietary levels of processed cassava leaves 1184
Odo Gregory E., Agwu Juliana E., Nweze Nkechi O., Clement Sunday, Alfreda Nwadinigwe O., Onyishi Grace, Uche Nzekwe, Ajuziogu Godswill, Ikegbunam Clara, Nelson Ossai I., Ezea Osita C., Osayi Emmanuel E., Onyeke Christopher C. and Eneje Victor

Influence of aluminum on root growth and of anatomy *Stenocalyx dysentericus* (DC.) O. Berg 1193
Rodrigues, A. A., Vasconcelos Filho, S. C., Rodrigues, D. A., Rodrigues, C. L., Sales, J. F. and Vital, R. G.

Physicochemical analysis of cellulose from microalgae *Nannochloropsis gaditana* 1201
BABA HAMED Samia, BABA HAMED Mohamed Bey, KASSOUAR Schéhérazade and ABI AYAD Sidi– Mohammed El - Amine

Effect of different microencapsulation materials on stability of *Lactobacillus plantarum* DSM 20174 1207
Lulwah Y. Al-Furaih, Ibtisam M. Ababutain, Azzat B. Abd-El-Khalek and Ahmed M. Abdel-Salam

Potential biosurfactant producing endophytic and epiphytic fungi, isolated from macrophytes in the Negro River in Manaus, Amazonas, Brazil 1217
João Marcelo Silva Lima, José Odair Pereira, Ieda Hortêncio Batista, Pedro de Queiroz Costa Neto, Jucileuza Conceição dos Santos, Solange Pires de Araújo, Mozanil Correia Pantoja, Adolfo José da Mota and João Lúcio de Azevedo

Foot-and-mouth disease (FMD) prevalence and exposure factors associated with seropositivity of cattle in north-central, Nigeria 1224
Wungak, Y. S., Olugasa, B. O., Ishola, O. O., Lazarus, D. D. and Ularamu, G. H.

African Journal of Biotechnology

Table of Content: Volume 15 Number 24 15 June, 2016

ARTICLES

***GhNAC18*, a novel cotton (*Gossypium hirsutum* L.) NAC gene, is involved in leaf senescence and diverse stress responses** 1233
Ondati Evans, Lingling Dou, Yaning Guo, Chaoyou Pang, Hengling Wei, Meizhen Song, Shuli Fan and Shuxun Yu

Fatty acids and amino acids contents in Scomber *scombrus* fillets from the South East of Tunisia 1246
EL Oudiani Salma, Darej Cyrine and Moujahed Nizar

Biochemical and hematological profile of different breeds of goat maintained under intensive production system 1253
Samira Ahmed Mohammed, Mohammed Abdur Razzaque, Anaam E. Omar, Sheeba Albert, and Waleed M. Al-Gallaf

Chitosan from shrimp shell (*Crangon crangon*) and fish scales (*Labeorohita*): Extraction and characterization 1258
Suneeta Kumari, Rath P. and Sri Hari Kumar A.

***In vitro* regeneration of a common medicinal plant, *Ocimum sanctum* L. for mass propagation** 1269
Mohammad Abu Hena Mostofa Jamal, Imdadul Hoque Sharif, Md. Mostofa Shakil, A. N. M. Rubaiyath-Bin Rahman, Nilufa Akhter Banu, Md. Rezuanul Islam and Md. Nazmuzzaman

academicJournals

Vol. 15(24), pp. 1177-1183, 15 June, 2016
DOI: 10.5897/AJB2016.15245
Article Number: D1039FE58856
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Application of biotechnology for the domestication of *Dacryodes edulis* (G. Don) H. J. Lam in Cameroon: A review

TCHATCHOUA Dorothy Tchapda[1,2]*, TCHOUNDJEU Zac[1] and CASPA Roseline Gusua[3]

[1]World Agroforestry Center (ICRAF), West and Central Africa Regional Office, B.P 16713, Yaounde, Cameroon.
[2]Department of Agriculture, Animal Husbandry and Derived Products, The Higher Institute of the Sahel University of Maroua, BP 46 Maroua, Cameroon.
[3]Forest and Wood Program, Institute of Agricultural Research for Development (IRAD), P.O. Box 2123 Yaounde, Cameroon.

Received 29 January 2016, Accepted 23 May, 2016.

Cultivation of *Dacryodes edulis* (G. Don) H. J. Lam commonly known as safou to improve the livelihood of the local population has been growing rapidly in the range of occurrence of safou and recent emergence of the market chain introduced by the World Agroforestry Center (ICRAF) experts has stimulated this further. Domestication through conventional genetic improvement (breeding) of safou has relied on phenotype selection and quantitative genetics through field trials breeding. Technologies to increase productivity, increase sustainable resource use, respond to climate change and enhance efficiency are required to meet demand. Biotechnology applications give a scope for rapid improvement and also facilitate the breeding program. Advantages of biotechnology application using molecular markers in breeding programs includes: study of genetic diversity, DNA fingerprinting of individuals, easy identification of specific traits or genes of interest, rapid propagation of improved genotypes and integration of gene(s) of interest into the species. It also provides genetic basis for selecting individuals and particular regions of the genome in a breeding program, reduce breeding population, can lead to early selection of traits and the development of a new variety with combination of characteristics. The complementary role of these techniques will be necessary for a successful genetic improvement program in the species. This review examines the achievements obtained using classical techniques, emphasizes missing gaps for the application of molecular techniques and discusses the complementary role of biotechnology techniques for a sustainable genetic improvement program in the species.

Keywords: *Dacryodes edulis,* domestication, population, genetic diversity, multiplication, biotechnology and genetic improvement

INTRODUCTION

Dacryodes edulis (G. Don) H. J. Lam belongs to the family *Burseraceae* and is known in Cameroon as African plum, bush butter or safou. It is a dioecious, small to medium-sized tree up to 20–25 m tall. It is an important fruit tree at the national and international levels, has high nutritional values and plays an important role in the

economy of the rural communities (Tchiegang et al., 1998). The species originates from Central Africa and the Gulf of Guinea and is presently cultivated from Sierra Leone to Angola along the Atlantic and further inland as far as Ugandaand northern Zimbabwe. Safou is an important fruit tree nationally and internationally, whose production has improved the living standard of many rural communities through the domestication program by the World Agroforestry Center (ICRAF).

Cultivation of safou to improve local livelihoods has been growing rapidly in the range of occurrence of the species and recent emergence of the market chain introduced by ICRAF experts has further stimulated this (Tabuna, 2002). Technologies to increase productivity and increase sustainable resource use, respond to climate change and enhance efficiency are required to meet demand. Domestication through genetic improvement is the alteration of the genetic composition of species through the selection of desirable traits from the original (wild) population to the planted population. The achievements obtained using classical technique includes: selection of plus trees from natural stands, farmlands and homestead gardens and phenotypic diversity studies. This was followed by testing their progenies with quantitative genetic studies in field trials (forward selection) for control crosses or interspecies hybridization. The best parents were selected (Backward selection) for the establishment of clonal trials. The main purpose of the tree improvement programs is geared at enhancing the genetic value of the population while maintaining genetic diversity (Namkoong et al., 1998). Each generation of selection changes the genetics structure of the population by changing allele frequencies and the association of alleles on chromosomes. Early generation of selection maintain high levels of genetic diversity within the large population size (Williams et al., 1995).

However, after a few generations of selection there will be a trade-off between increase genetic gain and maintaining genetic diversity. In addition, domestication by phenotypic selection methods could take many decades to increase the productivity of forest species (Bradshaw and Strauss, 2001). The rapid developments in plant biotechnology in the last decade have witnessed an explosion of efforts to utilize molecular tools in identifying genes and understanding their functions and the application in safou cannot be an exception. The field of biotechnology tools in the study of the entire genome has significantly impacted agricultural production and many reviews have been cited for forest trees (Riemenschneider et al., 1988; Jain and Priyadarshan, 2009; Schnell and Priyadarshan, 2012). Biotechnology techniques (genetic markers and recombinant DNA technology/genetic engineering) can be used to facilitate the breeding program. The advantages of genetic markers in breeding programs includes: the study of genetic diversity, DNA fingerprinting, easy identification of specific traits or genes of interest, rapid propagation of improved genotypes and integration of gene(s) of interest into species. It provides the genetic basis for selecting individuals and particular regions of the genome in a breeding program, reduces the breeding population, can lead to early selection of traits and the development of a new variety with combination of characteristics. The complementary role of these techniques will be necessary for a successful genetic improvement program in the species. This review examines the achievements obtained using classical techniques, emphasizes the missing gaps for the application of molecular techniques and discusses the complementary role of these techniques for a sustainable genetic improvement program in the species.

DOMESTICATION INITIATION PROGRAM

Prior to domestication program, studies on safou focused on the reproductive biology (Kengue, 1990), economic importance, uses and management (Ayuk et al., 1999) which are all factors which affect breeding operation. Management activities in Safou can be ascertained since many farmers have kept on working on what can be done for their trees to produce more fruits with the available manure, fertilizers and land with little emphasis on the genetic potential of the planting stock being used. Domestication through genetic improvement is the alteration of the genetic composition of species due to selection of trees with desirable traits from the original population to the planted population. International organizations such as the World Agroforestry Centre (ICRAF), Center for International Forestry Research (CIFOR) and some national agricultural research centers like the Institute of Agricultural Research for Development (IRAD), have been involved in the domestication of high value indigenous forest trees of West and Central Africa for the last ten years. Five species (*Irvingia gabonnensis*, *Dacryodes edulis*, *Ricinodendron heudelotii*, *Garcinia kola* and *Pausinystalia johimbe*) were selected during the initial process of domestication based on farmer's preference and the market potentialities of the species. *D. edulis* (G. Don) H. J. Lam was ranked second amongst trees that farmers selected for the domestication program (Franzel et al., 1996).

*Corresponding author. E-mail: tchatchouad@yahoo.com. Tel: (237) 677598313.

Germplasm collection and conservation

Several literatures have indicated that safou is cultivated and marketed in the Humid Lowland Zone (HLZ) of Cameroon notably in the South West, West, Littoral, Center, South and East regions (Tabuna, 2002). The collection of plus-trees was concentrated on populations located in these regions. Collected germplasms (plus-trees) were from homestead gardens, cultivated farmlands and secondary forests. The distance between populations was 200 km and between plus-trees was 100m. Phenotypic mass selection and progeny test have been the most common practice for the quantitative evaluation and selection of superior trees (Tchoundjeu et al., 2002; Tchatchoua et al., 2012). The efficiency of selections when trees are growing in natural forests or in an unimproved plantation depends on the traits, species and the experience of the investigator. Only by careful study of the open-pollinated progeny, controlled-pollinated progeny and replicated clonal trials, can we determine if a given tree is really genetically superior (Zobel and Talbert, 1984). The selected plus-trees supplied seeds for the provenance-progeny trial and seedlings for cuttings while marcots were obtained for the vegetative propagation stands in Mbalmayo and Minkoameyos field trials. Unfortunately, many of the identified and labeled plus-trees on farm lands were subsequently destroyed or the farmers may not be available, so future genetic improvement program will need to be centered on the available genebank stocks.

Population variation

ICRAF started with the collection of germplasm in 1998 with desired characteristics in the range of the distribution of this species (Tchoundjeu et al., 2002). Safou has a great deal of variability in fruit traits. Selected traits for on farm establishment included: fruit characteristics such as size, shape, taste, colour and nutritional qualities. Many farmers and through market survey records indicated that fruits with particular traits are preferred by the population. In such cases, selecting superior individuals with potential traits for planting can increase production but such an approach needs the testing programs, that is, testing of the progenies or clones.

A genetic improvement strategy in its broadest sense includes the whole process of selection, breeding and testing from species level, through the population and family, down to the clonal level, and conservation of variation within them. Understanding the genetic structure among populations for the desired traits is essential for developing tree improvement programs by selecting population for conservation and use. Research straggling in safou and suggest the presence of population variability in fruit traits. Leakey and Ladipo (1996) reported continuous variation in fruit characteristics from different regions in Cameroon. Youmbi et al. (1989) distinguished two morphological types based on their chemical compositions. Waruhiu (1999) identified tree to tree variation in fruit traits from three regions in Humid Forest Zone of Cameroon. Additional work by Kengue and Singa (1998) indicated existing population variations on growth traits from safou collections grown in Barombi-kang, Cameroon. Population selection indicates a method designed to improve the phenotypic performance of an inter-mating population by increasing the frequency of favorable alleles controlling traits of interest in which case the improved population can be directly used as a cultivar.

Genetic variation

The genetic characterization of plant is usually the initial stage after species selection whereby the germplasms are collected in the distribution range of the species to find and create enough genetic diversity in the population. Selecting trees from natural or planted tree populations must have a certain degree of genetic diversity. Genetic diversity is of fundamental importance to the long term survival and evolution of the species and it gives an opportunity to tree breeders to carry out selection from their breeding populations and thus conservation and management of the germplasm (Poltri et al., 2003). Improving all the traits of interest simultaneously due to pleiotropy effect of genes was identified by Leakey and Ladipo (1996) and Tchatchoua et al. (2012). It was shown that selection for yield favoured seed traits and unfavourable for nut production. This is scientifically attractive because one could promote significant gain for yield, even with a small breeding program. However, it is important to keep in mind that unfavorable genetic correlations can offset the advantage brought by the traits making the ideotype. Several methods for the study of genetic diversity includes: Phenotypic and molecular techniques. Phenotypic characterisations are influenced by environmental conditions and can lead to overestimation of the diversity of important traits. Also, major traits for which improvement is desired are the results from complex interaction of genes at multiple loci as such improvement of these traits can be possible using biotechnology tools. Work has been initiated on the study of genetic diversity using microsatellite markers in some Cameroonian populations (Benoit et al., 2011; Donfagsiteli et al., 2016).

MULTIPLICATION OF GERMPLASM

Propagation by seeds

Propagation through seed has been the common method used by farmers for decades until the introduction of

grafting by ICRAF. Propagation through seed provides opportunity for variation and genetic improvement in the species. Propagation by seeds has been a very successful method for safou production in Cameroon. Many seedling management experiments have been conducted in the nursery of ICRAF leading to a provenance-progeny trial established in Minkoameyos from four populations representing two agro-ecological zones in Cameroon. A seedling stand from four populations and a comparison stand of seedling and marcots from five families were established in Mbalmayo. The establishment of the trial consisted of field visits to identify candidate trees in terms of desired fruit traits which was conducted by ICRAF technical staff in 2001 with the help of farmers (Tchoundjeu et al., 2002). Fifty seeds were collected from each tree and sown and nursery bred in polythene bags at ICRAF's research nursery in Yaounde-Cameroon. After six months of growth, 30 seedlings per population were planted in manure-filled holes of 40 x 40 x 60 cm in the experimental field trial at Minkoameyos near Yaounde. Distance between trees was 5 by 5 m. The field trial was intercropped with maize for weed control and manual weeding was done three times a year. The experimental design consists of four populations planted in separate plots of 15 replicates with 5 families per population and two-tree plot per family per replication. Border trees from unknown provenances were planted around the separate population plots.

Vegetative propagation

Vegetative propagation method is an effective means of mass propagating and deploying genetically improved materials. The use of clones in tree improvement programs cannot be overemphasized as clones not only fruit earlier but they accumulate high genetic gain through clonal trials. Clonal trees whether grafted or cuttings can be used to measure and understand environmental effects and the importance of genotype by environment interaction can be determined by genetically comparable studies on multiple sites. Clonal trials established though with a few clones and many unknown populations indicated that using clones in plantations and on farms can be feasible in safou.

Multiple approaches for vegetative propagation of safou including grafting, cuttings and marcotting have been reported. There has been a transition in vegetative propagation by cutting, from a very difficult to root species in the 50ths (Philippe, 1957) to about 100% success in rooting of young seedlings with the development of new techniques of propagation. The published report by Mialoundama et al. (2002) recorded 80% rooted cuttings from juvenile leafy stem cuttings in sawdust or sand/sawdust medium. Clonal propagation to capture all the genetic effects (dominance, additive and epistatic) of selected individuals or plus trees have been employed in clonal trials established in Minkoameyos in June 2001 and some selected farmer's fields in West and Centre regions (ICRAF personal comm.). Use of mycorrhiza to improve rooting ability was investigated by Mbeuyo et al. (2013) and many investigations still need to be done on the effect of clones on rooting, use of mycorrhiza to improve rooting ability, source of rooting materials from the tree, etc. In addition, nut grafting process involving the removal of the hypocotyls and root from a germinated nut followed by inserting of the scion into a slit cut into the nut as reported in some forest trees (Jaynes and Messner, 1967) is yet to be investigated. Vegetative propagation by marcotting has yielded very excellent results from experiments using different branch types, branch diameter, substrate and hormone concentrations (Mialoundama et al., 2002). Many marcots from ICRAF plus-trees have been planted on farm and in comparison, a stand of seedlings and marcots from five families in Mbalmayo. A clonal orchard was established in Mbalmayo with five families. Experimental design is complete randomized block with single tree-plot of five replicates. More research needs to be done on using silvicultural methods to improve marcots and cutting growth. Clonal variation is an important issue to be investigated in the genebanks to help in future breeding opportunities. Knowledge of the methods by which each tree can be propagated will be useful in our future research endeavours. Limitations in propagation through seed range from biological factors unusable variation that may occur in the production population while vegetative propagation may be limited by genotype specificity which can be overcome by *in-vitro* or micropropagation techniques.

Hybrids through control-cross pollination

Cross-pollination programs have started very timidly. Controlled cross pollination are necessary to efficiently combine traits into single genotype and to study the genetic influence of both simple and complex traits. Controlled crosses that produce full-sibling families are expected to be almost twice efficient as open-pollinated half-sib families for meeting improvement goals (Kung et al., 1974). Controlled crossing of elite individual trees is an important method to understand both the specific genetic combining ability of trees and to develop superior individuals for seed orchards or clonal propagation. The problem in using controlled crosses is that the cost of producing a large number of progeny is high, and if only a relatively small number of progeny are produced, the rate at which genes are fixed is much greater. In addition, the large size of trees of reproductive age makes controlled breeding difficult and the length of time from seed

germination to flowering can slow down breeding programs. Interspecific crossing based on DNA fingerprints will provide variation means for improving genetic variation in population while selection for important traits may be further increased by the use of DNA markers.

BIOTECHNOLOGY APPLICATION

The continuous application of traditional breeding methods in a given species could lead to the narrowing of the gene pool from which cultivars are drawn, rendering crops vulnerable to biotic and abiotic stresses and hampering future progress. This technology deals with the complementarity between parents characteristics without identifying the gene responsible for the control of the trait or traits of interest. The use of biotechnology tools in the study of the entire genome has significantly impacted agriculture. Several different markers have been developed for trees (Neale et al., 1992; Haines, 1994) and specifically for tropical forest trees (Muchugi et al., 2008) and new marker types are developed every year. These markers range from biochemical markers (monoterpenes and allozymes) to the most complex molecular markers: single allelic dominant markers (AFLPs, RAPD) and the multiallelic codominant markers (RFLPs, SSRs, CAPs, EST and SNPs). Molecular markers are classified into two groups based on DNA–DNA hybridization or on polymerized chain reaction (PCR) with properties of a good molecular marker as follows:

1. Highly robust and repeatable across different tissue types and different laboratories, that is, it can be reproducible in any laboratory experiment or between different laboratories performing identical experiments.
2. Unaffected by environmental and developmental variation
3. Inexpensive to develop and apply
4. Polymorphic, this is the variability among individuals and reveals high levels of allelic variability.
5. Reveal markers that are codominant. Depending on the type of application, the selected technology must be able to detect the marker's different forms, distinguishing between homozygotes and heterozygotes (codominant inheritance). A heterozygous individual shows simultaneously the combined genotype of the two homozygous parents.
6. Evenly distributed throughout the genome. Polymorphism is better assessed when the marker is more distributed and densely covers the genome.
7. Discriminating, that is, can be able to detect differences between closely related individuals.
8. Neutral. The allele present at the marker locus is independent of, and has no effect on, the selection pressure exerted on the individual.

The advantages these genetic markers offer in breeding programs include:

a) Rapid and easy method to study genetic diversity. Genetic diversity is the diversity that occurs in genes of individuals and species. Genetic diversity can be found among species, populations, within populations and within individuals. If we consider the long-term genetic improvement of yield for the major crops, plant breeding has worked whenever there is genetic variation within the germplasm pools accessible to plant breeders and selection has focussed on the right traits measured in the right environments. Several markers have been identified in the study of genetic diversity which include biochemical markers used to determine the differences in chemical composition to molecular markers (restricted fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), single nucleotide polymorphism (SNPs), etc) which can detect variation ranging from mutation at single nucleotide to variation due to changes in an entire chromosome.
b) Genetic fingerprinting. DNA fingerprinting is the method by which an organism is genotyped across a range of genetic loci (markers) to develop their individual genetics fingerprint. It was originally developed in the 1980s (Jeffrey et al., 1985a). Microsatellite loci are used most frequently in DNA fingerprinting, however single nucleotide Polymorphisms (SNPs) are becoming more frequently used. Plant genotyping is required for a variety of end uses including marker assistance selection MAS, associating phenotype with polymorphism, DNA barcoding, genetic diversity studies, conservation genetics and improving genome assemblies. Methods of genotyping depend on the facilities available and questions to be answered.
c) Easy identification of specific traits or genes of interest for marker assistance selection (MAS). A marker can either be located within the gene of interest or be linked to a gene determining a trait of interest. As such MAS can be executed as a selection for a trait based on the genotype using associated markers rather than the phenotype of the trait. MAS program for a given trait involves: 1) Characterizing germplasm for useful traits; 2) Selection of diversified parents; 3) Developing mapping population; 4) Selection of suitable combination of molecular markers and genotyping of parents and mapping population; 5) Construction of genetic or linkage map; 6) Phenotyping of mapping population for the selected traits; 7) QTL, analysis by combining the data of linkage and phenotyping; 8) Identifying mapping and validation of QTLs and 9) Executing MAS for the target traits with benefits as follows: early selection, decreasing breeding cycle, increase selection intensity and relative efficiency of selection on low heritability traits (Meuwissen

et al., 2001). The development of whole genome sequencing using next generation sequencing technology has made SSPs and SNP the markers of choice for genetics and plant breeding. Its application in this species can speed up or revolutionise the present research. This new DNA marker technology can be used to facilitate the breeding program.

d) GMO for rapid introduction of trait of interest. Biotechnology involves *in vitro* propagation approaches including micropropagation (axillary shoot multiplication), organogenesis (advertitious shoot production) and somatic embryogenesis are techniques that can be used in the production of plus trees and elite individuals for establishment on farms and plantation. These techniques can also be useful in gene transfer for the genetic engineering process (GMO). Transgenic technology offers the possibility of transferring single traits, without the problems encountered in traditional breeding that arise from the introduction of often additional undesirable genetic material.

CONCLUSION

Globally, *Dacryodes edulis* (safou) is an important fruit tree whose production has improved the living standard of the rural communities through the domestication program by the World Agroforestry Center (ICRAF). During the past decades, ICRAF has undertaken efforts for the conservation of safou genetic resources and major collections have been established. Collection of data in different sites and location permit selection of germplasm on a geographical scale. There is the need for future germplasm characterisation that combines both the classical and genetic characterisation using biotechnology tools, to ensure a more complete and informative characterisation that reveals the true genetic diversity of the population.

The reliability of selection based on field trials may be further increased by the use of DNA markers whereas interspecific crossing based on parent genotypes will provide means for improving the introduction of genetic variation. Genetic engineering will provide the means for introducing traits of interest from other species that are not available in the target plant gene pool or its wild relative. Phenotypic selection and quantitative genetics are very important for germplasm conservation, management and improvement. However, knowledge of genetic diversity and relationship among elite germplasm using biotechnology tools will improve breeding efficiency in the domestication efforts.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ayuk ET, Duguma B, Franzel S, Kengue J, Mollet M, Tiki-Manga T, Zekeng P (1999). Uses, management and economic potential of *Dacryodes edulis* (Burseraceae) in the humid lowlands of Cameroon. Ecol. Bot. 53(3):292-301.

Bradshaw HD, Strauss SH (2001). Breeding strategies for the 21st century: domestication of *poplar*. pp383-394. In: Dickman DI, Isebrands JG, Eckenwalder JE, Richardson J (eds.). *Poplar* Culture in North America, Part B. NRC Research Press, Ottawa. pp. 383-394.

Benoit L, Born C, Vignes H, Chevallier MH, Todou G, Debain C, Joly H (2011). Polymorphic microsatellite loci from *Dacryodes edulis* (Burseraceae), A Central African rainforest and fruit-tree species. Am. J. Bot. pp. 274-275.

Donfagsiteli TN, Waswa BW, Muchugi A, Fotso, Nzweundji G, Omokolo ND, Skilton R (2016). Genetic diversity and gene flow revealed by microsatellite DNA markers in some accessions of African Plum (*Dacryodes edulis*) in Cameroon. Afr. J. Biotechnol. 15(13):511-517.

Franzel S, Jaenicke H, Janssen W, Ayuk E, Boland D (1996). Choosing the right trees setting priorities for multipurpose tree improvement. Int. Serv. Nation Agric. Res. Rep. 8:87.

Haines R (1994). Biotechnology in forest trees improvement. Food and Agriculture Organisation of the United Nations Rome FAO Forestry paper No. 118.

Jain SM, Priyadarshan PM (2009). Breeding Plantation tree Crops. Vol. I and II. Springer.

Jaynes RA, GA Messner (1967). Four years of nut grafting chestnut. In: Proc. Int. Plant Propagators Soc. 17:305-310.

Jeffreys AJ, Wilson V, Thein SL (1985a). Hypervariable'minisatellite' regions in human DNA. Nature. 314:67-73.

Kengue J (1990). Le Safoutier (*Dacryodes edulis*) Don G, Lam HJ. Premières Données sur la Morphologie et la Biologie. Doctorat de 3eme Cycle ThesisUniversity of Yaounde. p154.

Kengue J, Singa EM (1998). Preliminary characterization of some African Plum accessions in Barombi-Kang germplasm collection. Pp113-122 in Kapseu C, Kayem GJ (eds.). Proceedings of the 2nd International Workshop on African pear improvement and other new sources of vegetable oils. ENSAI. Presse Universitaires de Yaounde.

Kung FH, Bey CF, Larson JL (1974). Nursery Performance of Black Walnut Twins. In: Garrett PW (ed). Proceedings, 22nd Northeastern Forest Tree Improvement Conference; 1974 August 7-9. Syracuse, NY. Upper Darby PA, US. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station: pp. 184-190.

Leakey RRB, Ladipo D (1996). Trading on genetic variation in fruits of *Dacryodes edulis*. Agroforestry Today 8:16-17.

Mbeuyo M, Donfagsiteli NT, Youmbi E, Mbita Messi HJC, Amougou A (2013). Performance of *Dacryodes edulis* mycorrhized layers under different cropping conditions in Makenene, Cameroon. Turk. J. Agric For. 37:335-343

Meuwissen THE, Hayes BJ, Goddard ME (2001). Prediction of total genetic value using genome wide dense marker maps. Genetic 157: 1819-1829.

Mialoundama F, Avana ML, Youmbi E, Mampouya PC, Tchoundjeu Z, Mbeuyo M, Galamo GR, Bell JM, Kopguep F, Tsobeng AC, Abega J (2002). Vegetative propagation of *Dacryodes edulis* (G. DON) HJ. Lam by marcots, cuttings and micropropagation. Forests trees Livelihoods 12:85-96.

Muchugi A, Kadu K, Kindt R, Kipruto H, Lemurt S, Olale K, Nyadoi P, Dawson I, Jamnadass R (2008). Molecular Markers for Tropical Trees. A Practical Guide to Principles and Procedures. ICRAF Technical Manual no. pp. 9-91. Dawson I, Jamnadass R (eds). Nairobi: World Agroforestry Center.

Namkoong G, Kang HC, Brouard JS (1988). Tree breeding; Principles and strategies (Monographs on Theoritical and Applied Genetics, Edited by Frankel R, Grossman M, Linskens H, Maliga FP. Springer Verlag, New York.

Neale DB, Devey ME, Jernstad KD, Ahuya MR, Alosi MC, Marshall KA (1992). Use of DNA markers in forest tree improvement research. New Forests. 6:391-407.

Philippe J (1957). Essai de reproduction végétative du safoutier. Bulletin

d'information INEAC 6(5):319-327
Poltri SNM, Zelener N, Traverso JR, Gelid P, Hopp HE (2003). Selection of a seed orchard of Eucalyptus dunnii based on diversity criteria calculated using molecular markers. Tree Physiol. 23:625-632.
Riemenschneider DE, Haissig BE, Bingham ET (1988). Integrating biotechnology into woody plant breeding programs. pp433-449. In: Hanover JW, Keathley DE (eds.). Genetic Manipulation of Woody Plants. New York, Plenum Press pp. 433-449.
Schnell RJ, Priyadarshan PM (2012). Genomics of Tree Crops. Springer.
Tchatchoua DT, Tchoundjeu Z, Ebenezar A, Tsobeng A, Weber JC, Kalinganire A (2012). Methodological approaches for the selection of genotypes in a progeny trial of *Dacryodes edulis* (G. Don) H. J. Lam in Cameroon. Int. J. Biol. Chem. Sci. 6(4):1480-1491
Tchiegang C, Kapseu C, Parmentier M (1998). Chemical composition of oil from *Coula edulis* (Bail.)nuts. J. Food Lipids 5(2):103-111.
Tchoundjeu Z, Kengni J, Leakey RRB (2002). Domestication of *Dacryodes edulis;* State of – the – art. Forests, trees Livelihoods. 12:3-13.
Waruhiu QN (1999). Characterisation of fruit traits towards domestication of an indigenous fruit tree of West abd Central Africa. A case study of *Dacryodes edulis* in Cameroon. MSc. Thesis. University of Edinburgh, Scotland. 56 p.
Williams CG, Hamrick JL, Lewis PO (1995). Multiple-population versus hierarchical breeding populations: a comparison of genetic diversity levels. Theor. Appl. Genet. 90:584-594.
Youmbi E, Clair-Macrwulajtys Bory G (1989). Variations de la composition chimique des fruits de *Dacryodes edulis* (Don) Lam. Fruits. 44:149-153.
Zobel BJ, Talbert JT (1984). Applied forest tree improvement. Wiley and Sons, Inc. New York 505 p.

academicJournals

Vol. 15(24), pp. 1184-1192, 15 June, 2016
DOI: 10.5897/AJB2015.14958
Article Number: 6F6165B58858
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Growth performance and nutrient utilization of *Clarias gariepinus* fed with different dietary levels of processed cassava leaves

Odo Gregory E.[1]*, Agwu Juliana E.[1], Nweze Nkechi O.[3], Clement Sunday[1], Alfreda Nwadinigwe O.[3], Onyishi Grace[1], Uche Nzekwe[3], Ajuziogu Godswill[3], Ikegbunam Clara[3], Nelson Ossai I.[2], Ezea Osita C.[1], Osayi Emmanuel E.[3], Onyeke Christopher C.[3] and Eneje Victor[2]

[1]Department of Zoology University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Enugu State University Science and Technology, Nigeria.
[3]Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 1 September, 2015; Accepted 29 March, 2016

The growth performance of *Clarias gariepinus* fed with different dietary levels of processed cassava leaves and their nutrient utilized were studied for a period of 24 weeks. One hundred and forty four experimental Catfish (*Clarias gariepinus*) with 0.75±0.20 g mean weight and 3.9±0.31 cm mean total length were collected from Aqua-fish Consult, Awka, Anambra State, Nigeria. Eighteen rectangular plastic tanks were used for this study. Green cassava leaves (*Manihot esculenta*) were collected from a farm in Uda, Igbo-Eze North of Enugu State. The leaves were soaked for 36 h and sun-dried for one week to reduce the presence of cyanogenic glycosides. The dried leaves were ground into fine powder and analyzed for proximate composition. The experimental design used was Complete Randomized Block Design (CRBD) consisting of 18 treatments. Triplicate culture tanks in treated (L_{25}-L_{100}), and control groups (L_0 and $A_{c)}$ were stocked with eight fingerlings per square meter, respectively. The cost benefit of *C. gariepinus* fingerlings was estimated using weight gain and specific growth rate against management and construction cost. Weight gain, specific growth rate (SGR), feed efficiency (FE) and total length (TL) of catfish in different treatments were plotted against water chemistry parameters. This trail was conducted to access the possibility of replacing maize (*Zea mays*) with varying levels of whole cassava leaf meal in the diet of *C. gariepinus* catfish. Five isocaloric and isonitrogenous diets were formulated to contain 0, 25, 50, 75 and 100% cassava leaves to replace equal weight of maize meal. Internationally made fish feed (coppens) was also used as a control feed. All the diets were fed to catfish (*C. gariepinus*) fingerlings in replicate for 24 weeks. The results revealed that optimum requirement of cassava leaves level in the formulation of practical diets for improved growth of *C. gariepinus* was 25%.

Key words: Growth performance, nutrient utilization, *Clarias gariepinu*, cassava leaves.

INTRODUCTION

The overall effect of food insecurity is not only inadequate food production but also imbalances in the nutritional status of the populace at large (Aderemi et al.,2012).The dearth of animal products in the diet of an average

Nigeria increases yearly, mainly due to poverty, high cost of animal feeds, political and economic instability coupled with decreased interest in animal production with greater efforts directed towards petroleum exploitation. Leaf meals of most tropical plants for example cassava leaf meal (Anyanwu, 2009) are available and cheap. Cassava is a multipurpose plant that thrives well in the tropics. It is a very good energy source widely grown in Nigeria. It has a wide range of adaptability, resistance to drought and tolerance to poor soils.

African Catfish (*C.garepinus*) are known to be omnivorous in their food habits (Anyarnwu et al., 2012). Besides, they are hardy and tolerant to a wide range of environmental conditions (Nwani et al., 2015). These attributes have indicated the fish as highly and veraciously disposed to accepting unconventional dietary feeds, such as leaf meals. The quest to intensify the culture of the fish so as to meet its ever increasing demand has made it vital to develop suitable diets either in supplementary forms in ponds or as whole feed in tanks (Olukunle, 2006).Feed is one of the major inputs in aquaculture production and fish feed technology has become one of the least development sectors of aquaculture particularly in Africa and other developing countries of the world (Gabriel et al., 2007). High cost of fish feed ingredients (maize and fish meal) is observed as one of the problems militating against aquaculture development in Nigeria (Gabriel et al., 2007). This leads to malnutrition of fish which subsequently results in decline in reproduction of individual fish. This eventually causes scarcity of fish species in the market which invariably results in high cost of fish. Leaf meals of most tropical plants, for example, cassava leaf meal (Anyanwu, 2009) are available and cheap.

Cassava roots and leaves are readily available and have also been found to support the growth of different fish species such as C. *gariepinus* (Catfish), *Oreochromis niloticus* (Tilapia), *Mystus cavasinus* (Catfish); but this can be limited by the presence of anti-nutrients such as linamarin. Linamarin is cyanogenic glycosides (2-B-D-glucopyranosy 1oxy-isobutryo nitrite) found in leaves and tuberous roots of cassava, which release high toxic cyanide (HCN) during hydrolysis at the time of digestion (Presston, 2004; Aderemi et al., 2012; Akapo et al., 2014). Previous researchers have attempted to increase non-conventional plant and animal materials to replace conventional feed ingredients like maize and fish meal in fish feed ration (Falaye, 1988; Fagbenro, 1992; Olatunde, 1996; Baruah et al*.,* 2003; Eyo and Ezechie, 2004; Azaza et al., 2015). According to Olurin et al. (2006), maize is the major source of metabolizable energy in most compounded diets for *Hetorebranchus x clarias* hybrid species because it is readily available and digestible. However, the increasing prohibitive cost of this commodity has necessitated the need to search for an alternative source of energy.

Few works are available on the replacement of maize with cassava root and cassava leaf in fish diet. These include those on mirror carp, *Cyprinus Carpio* (Ufodike and Matty, 1983), Rainbow trout, Salmo trutta (Ufodike and Matty, 1984), Tilapia, *Oreochromis niloticus* (Faturoti and Akinbote, 1986), *Oreochromis mossambicus* (Wee and Ng, 1986); catfish, *C. gariepinus* fingerlings (Olurin et al., 2006); *C. gariepinus* advance fry (Olukunle, 2006), *C. gariepinus* (Anyanwu et al., 2009, 2012) and *Tinca tinca* (Garcia et al., 2015); hence the quest to determine the growth performance and nutrient utilization of *C. gariepinus* species fed with varying dietary levels of cassava leaf meal as substitute for maize.

MATERIALS AND METHODS

Procurement of *C.gariepinus*

One hundred and forty four experimental Catfish with 0.75±0.20 g mean weight and 3.9±0.31 cm mean total length were collected from Aqua-fish Consult, Awka, Anambra State, Nigeria. After collection, they were transported to the wet laboratory of Zoology Department, University of Nigeria, Nsukka and allowed to acclimatize for 2 weeks with rectangular plastic tanks.

Preparation of culture tanks

Eighteen rectangular plastic tanks were used for this study for a period of twenty four weeks. Each tank has a water-holding capacity of 95 L, but water was maintained at 60 L level within the period of the research (Eyo, 1994). However, they were covered with mosquito net to prevent escape of fish, as well as entry of predators, and leaves from falling in. All tanks were continuously aerated using air pump.

Collection and processing of cassava leaves

Green cassava leaves (*Manihot esculenta*) were collected from a farm in Uda, Igbo-Eze North of Enugu State. The leaves were soaked for 36 h and sun-dried for 1 week to reduce the presence of cyanogenic glycosides. The dried leaves were grounded into fine powder and analyzed for proximate composition according to the procedure of APHA (2006).The proximate composition of the nutrient contents is shown in Table 1.

Formulation of fish feed with cassava leaves

Proximate analysis of dietary ingredients was carried out. Then, five isocaloric and nitrogenous diets were prepared: they contain 0, 25, 50, 75 and 100% processed cassava leaves, which were labeled L_0, L_{25}, L_{50}, L_{75}, and L_{100} respectively, to replace equal weight of maize. Prior to formulation of the five inorganic diets, experimental feeds were ground to a fine powder by using hammer mill machine. One kilogram of the feed was weighed out using a triple beam

*Corresponding author. E-mail: gregory.odo@unn.edu.ng.

Table 1. Proximate composition of nutrient content of cassava leaf.

Parameter	Percentage composition
Moisture	9.911
Ash	2.787
Fats	10.365
Fibre	4.386
Protein	21.628
Carbohydrate	50.923

Table 2. Percentage composition of experimental diets in different treatment (cassava leaves inclusion) .

Ingredients	Treatment				
	L_o (Control 0% cassava leaves inclusion)	L_{25} (25% cassava leaves inclusion)	L_{50} (50% cassava leaves inclusion)	L_{75} (75% cassava leaves inclusion)	L_{100} (100% cassava leaves inclusion)
Maize meal	40	30	20	10	0
Cassava leaves	0	10	20	30	40
Fish meal	15	15	15	15	15
Soya beans	31	31	31	31	31
Wheat middling	8	8	8	8	8
Vitamin premix	1	1	1	1	1
Mineral premix	1	1	1	1	1
Vegetable oil	2	2	2	2	2
Bone meal	1.5	1.5	1.5	1.5	1.5
Salts	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100

balance model 700. The diets were homogenously mixed with 200ml of water and placed in sealed heat resistant polythene bags. The polythene bags were steamed for an hour. This was done to facilitate the gelatinization of starch and binding of diets. The diets were run through meat mincer fitted with 3mm dye and the resulting nodule shaped strands were cut into pellets and oven dried at 50°C for 3 h. The proximate analysis of the diet was carried out (AOAC, 1975) and shown in Table 2.

Experimental design

The experimental design used was Complete Randomized Block Design (CRBD) involving 18 treatments. Triplicate culture tanks in treated (L_{25}-L_{100}), and control groups ($L_0$and $A_{c)}$ were stocked with eight (8) fingerlings M^{-2} respectively (Anibeze et al., 2003).

Control group A_c was fed with internationally made feed (coppens) while Control group L_0 was fed with 0% dietary level cassava leaves. Prior to feeding, the fish in each tank was left for 2 days during which no artificial diet was administered to them, but was starved to allow utter digestion of any food in their stomach. At the end of acclimatization, fish in each tank were weighed (with mettler top loading balance) to determine their initial mean weight. Also mean total length and mean standard length measurements were taken using fish measuring board. The fish were fed daily in two rations (at 9:00am and 4:00pm) at a rate equivalent to 5% of the total body weight of the fish in each tank. The quantity of the diet administered was adjusted fortnightly using weight gain data.

Acceptability of diet

The acceptability of diet was accessed using the "time to strike index" (Eyo, 1994). The fish in every treatment was starved overnight to induce hunger. Pellet of diets for each treatment was dropped into the aquarium; the time which elapsed from the time the pellet penetrates the water and the moment the last fish struck the pellet with its mouth was recorded in seconds. The acceptability index was calculated as the reciprocal of the "time to strike".

Production parameters

All the fingerlings in each treatment were harvested and weighed collectively using mettler electronic balance (PC 2000) to the nearest 0.01g.

Weight gain

Weight gain was calculated using W_2-$W_{1,}$ where W_2 = final weight and W_1= initial weight over a period.

Specific growth rate (SGR)

Specific growth rate (SGR) was calculated using,

$$SGR = \frac{\log W_2 - \log W_1}{T_2 - T_1} \times \frac{100}{1}$$

Where W_2= weight at time T_2 (days); W_1=weight at time T_1 (days) (Brown, 1975).

Feed efficiency (FE)

Feed efficiency for catfish in different treatments was calculated using the formula;

$$FE = \frac{\text{Weight gain (b)}}{\text{Feed intake (a)}}$$

Where, feed intake (a) = feed eaten by the fish on a matter basis; weight gain (b) = a weight increase on wet matter basis (Boonyaratpalin, 1989).

Condition factor (K)

Condition factor of catfish in difference treatments was calculated using the formula:

$$K = \frac{100W}{L^3}$$

Where *W* = Weight of fish and L = total length of fish (Adikwu, 1992).

Total length (TL)

Total length of juvenile catfish in different treatments was recorded using fish measuring board to the nearest 0.01 cm.

Also, standard length of Juvenile catfish in different treatments was measured and recorded using fish measuring board to the nearest 0.01 cm.

Length-weight relationship (LWRs)

The length-weight relationship (LWRs) of the catfish in different treatments was plotted using the pooled data on length and weight of catfish in different treatments.

Water chemistry

Water quality parameters were measured during each sampling fortnightly. Temperature of the water was measured using mercury-in-glass thermometer, water pH was measured using Jenway P^H metre, dissolved oxygen was determined using dissolved oxygen metre, total hardness mg/L, total ammonia mg/L and nitrite were measured using spectrometer.

Growth parameters-water chemistry relationship

Weight gain, SGR, FE and TL of catfish in different treatments were plotted against water chemistry parameters. The TW was used to calculate the Normalized Biomass Index (NBI)(Beck, 1979):

$$NBI = (W_F \times N_F)(W_1 \times N_1) \times \frac{1}{100}$$

Where, W_F = Final weight of catfish in milligram, N_F = Final number of catfish, W_1 = Initial weight of catfish in milligram and N_1 = Initial number of catfish.

Cost benefit analysis

The cost benefit of *C. gariepinus* fingerlings was estimated using weight gain and specific growth rate against management and construction cost.

Feed cost

The cost of feeding the fish was computed using (Lipton and Harmel, 2004):

$C_{feed} = P \times W_A \times FCR/1-[0.5(1-S)]$

Where, C_{feed} = cost contribution of feed to produce a pound of fish, P=per pound price of fish, W_A = Weight added from purchase seed to harvest size (Harvest size - seed weight), FCR = Feed Conversion Ratio, and S=Percentage of fish surviving from seed to market size.

Seed cost

Cost of stocking at different densities was computed using:

$C_{seed} = P_{feed}/W \times S$ (Liption and Harmel, 2004).

Where, C_{seed} = Cost of contribution for producing a pound of fish, P_{feed}= Purchase price of seed (Cassava leaves), W= Average weight of harvestable fish, S = Percentage of fish surviving from seed to market size.

Management cost

Management Cost was computed using (Lipton and Harmel, 2004):

$C_{variable} = C_{seed} \times C_{feed}$

Where, C_{seed} = Cost of producing pound of fish; C_{feed} = Cost contribution of feed to produce a pound of fish.

All costs will be reduced to Naira (Nigeria National Currency) using Nsukka Urban Market Price.

Statistical analysis

Data resulting from the experiment were subjected to a two way analysis of variance using SPSS (Statistical package for social sciences) version 12. Turkey HSD, Dunkan, Fisher LSD and Tanhane were used to compare differences among individual means at P = 0.05.

RESULTS

Palatability of diets with different levels of cassava leaf meal inclusion fed to *Clarias gariepinus* juveniles

It was observed that it took the catfish 4.43 ± 0.08 s to strike diet A_c (coppens fish feed) as against 12.98 ± 0.12 s spent in striking diet L_{100} (100% cassava leaf meal substitution) (Figure 2). There was inconsistency in the

Table 3. Acceptibility index of *C. gariepinus* fed different dietary levels of CLM.

Diet	Week												
	0	2	4	6	8	10	12	14	16	18	20	22	24
L_o (0%CLM)	0.16	0.17	0.16	0.17	0.16	0.16	0.17	0.18	0.16	0.17	0.17	0.16	0.16
L_{25}(25%CLM)	0.19	0.17	0.17	0.19	0.18	0.18	0.18	0.20	0.17	0.20	0.20	0.19	0.19
L_{50}(50%CLM)	0.14	0.14	0.14	0.15	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.15
L_{75}(75%CLM)	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
L_{100}(100%CLM)	0.08	0.07	0.08	0.07	0.08	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08
A_c (IMCF)	0.24	0.24	0.25	0.24	0.20	0.23	0.21	0.24	0.23	0.22	0.22	0.20	0.24

*CLM = cassava leaf meal; *IMCF= Internationally made Coppens fish feed.

Table 4. Total Length (cm) of C. *gariepinus* juveniles fed graded levels of cassava leaf meal (CLM) substitution for 24 weeks.

Diet	Week												
	0	2	4	6	8	10	12	14	16	18	20	22	24
L_o (0%CLM)	4.0	4.4	5.1	6.2	7.4	8.5	10.5	13.1	15.1	17.3	20.6	23.2	26.7
L_{25}(25%CLM)	4.2	4.6	5.2	6.1	7.3	8.3	9.8	12.3	14.7	17.0	20.2	22.9	25.8
L_{50}(50%CLM)	4.7	5.1	5.9	6.8	7.5	8.3	9.4	10.7	13.4	16.5	19.7	22.2	25.7
L_{75}(75%CLM)	4.8	5.3	6.0	7.1	7.7	8.4	9.5	11.9	13.7	15.1	19.0	21.7	26.0
L_{100}(100%CLM)	4.5	4.9	5.8	6.4	7.4	8.6	9.7	10.8	12.5	15.1	16.0	17.2	19.6
A_c (IMCF)	3.8	4.0	4.9	6.3	7.5	8.5	10.7	14.1	16.0	18.7	20.9	24.0	27.6

*CLM = cassava leaf meal; *IMCF = Internationally made Coppens fish feed.

time spent to strike pellets from diets L_{25}; 5.42 ± 0.09 , L_{50}; 7.00 ± 0.04 , L_{75}; 9.07 ± 0.04 and L_0; 6.04 ± 0.05 s respectively. Similarly, the acceptability indices indicated that diets L_{25}, L_{50}, L_0 andA_c (0.19 ± 0.00, 0.14 ± 0.00, 0.17 ± 0.00 and 0.23 ± 0.00 respectively) were easily accepted compared to L_{75} and L_{100} (0.11 ± 0.00 and 0.08 ± 0.00 respectively). All diets' acceptability indices were significantly different from control diet and from one another (P< 0.05) (Table 3).

Growth response

The total length of group Ac was the highest (12.85 ± 2.22a) while L100 had the lowest total length (10.65 ± 39^a). The mean total length decreases in the following order: Ac > Lo > L_{25}> L_{75}> L_{50}> L_{100} (Table 4).

The mean weight of catfish fed diet L_{100} (14.98 ± 4.61) was very low compared to those fed other diets. Also, diet L_{25} had the highest mean weight (43.69 ± 17.51) among the varied levels of substitution of cassava leaf meal (Table 4 and Figure 1).

Furthermore, the mean condition factor (1.10± 0.01^a) of fish in diet was the highest followed by diet Ac. L0 > (50 > L_{75} > L_{100} giving the trend L_{25}> A_c> L_0> (50 > L_{75}> L_{100} (Table 4)

Weight gain

The mean weight of catfish fed diet L_{100} (100% Cassava leaf meal) - 14.98 ± 4.61 was very low compared to the mean weight of catfish fed diets L_0,L_{25}, L_{50}, L_{75} and A_c (41.17 ± 16.03, 43.69 ± 17.51, 32.45 ± 13.03, 26.85 ± 10.35 and 49.98 ± 19.43 respectively). Apart from group A_c (Internationally made coppens fish feed) which mean weight is relatively high, that is, 49.98 ± 19.43, group L_{25} had the highest mean weight gain (43.69 ± 17.51) among the varied levels of substitution of cassava leaf meal (Table 5).

Mean values with alphabets were compared with time to strike. Mean values with different alphabets differ significantly (P<0.05). Mean values with figures were compared with acceptability index. The time to strike differed significant (P<0.05) when diet groups were compared, with mean time to strike decreasing in the following order: L_{100}<L_{75}<L_{50}<L_0<L_{25}<A_C.The acceptability index of A_C group was highest and it differed significantly (P<0.05) from those of the other groups. L_{25} had the next high acceptability index followed by that of L_0, L_{50}, L_{75} and L_{100}.

Water chemistry

The highest mean temperature occurred in diet L_{50} 27.5 ± 0.21) followed by diet L_{75} (75% - 27.4 ± 0.67), diet A_C- 27.4± 0.55), while the least mean temperature was observed in diet L_{25} (25% - 27.3± 0.88). Also, the D.O in diet A_c- 4.49 ± 1.23) was highest and this was followed by

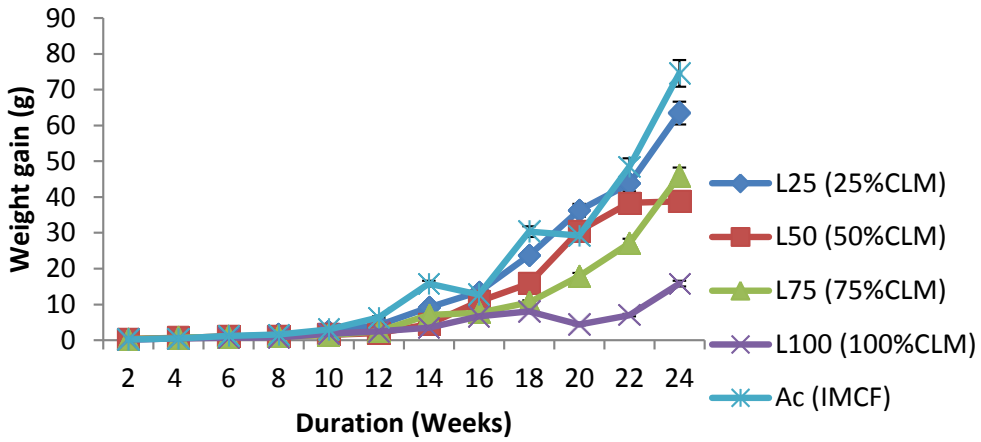


Figure 1. Weight gain of *Clarias gariepinus* juveniles fed different dietary levels of cassava leaf meal substitution for 24 weeks.

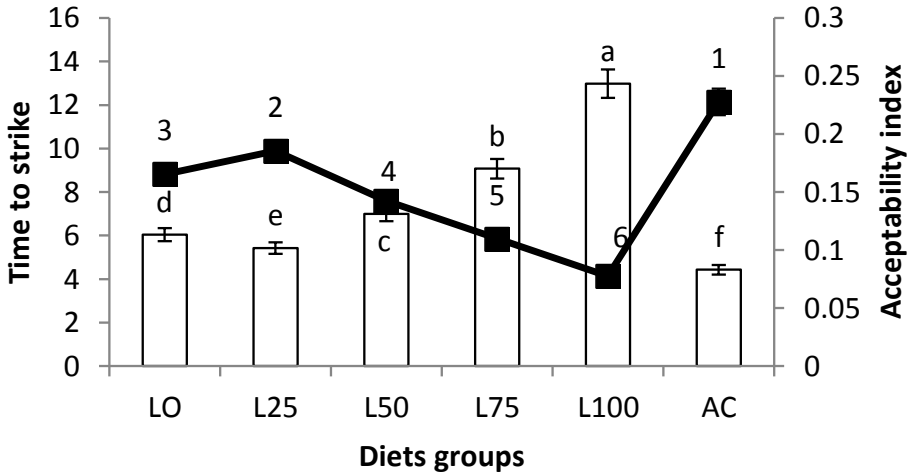


Figure 2. Time to strike and acceptability.

mean D.O. occurred in diet L_{100} (100% CLM substation - 4.22 ± 1.06) (Table 6). Similarly, the pH during the culture period ranged from 6.40 to 7.90 and the highest mean pH occurred in diet L_0 (0% CLM, first control diet - 7.10± 0.45), followed by diet L_{75} (75% CLM - 7.10± 0.2). The least mean P^H was observed in diet L_{50} (50% CLM substation - 6.9 ± 0.32). Also the highest mean NH_3-N occurred in diet L_{75} (75% CLM inclusion - 0.00029 ± 0.00001), followed by diet L_{100} (100% CLM - 0.00026 ± 0.00001). The least mean NH_3-N reading was observed in diet L_{25} (25% CLM - 0.000018± 0.00001) (Table 6).The highest mean nitrate was noticed in diet L_{25} (25% CLM - 0.00004 ± 0.00001) while the least mean nitrate was found in diet L_{100} (100% CLM inclusion - 0.00002 ± 0.00001) (Table 6).The highest mean nitrite occurred in diet L_{25} (25% CLM - 0.0003 ± 0.00004) while the least mean nitrite was observed in diet L_{50} (50% CLM - 0.00023 ± 0.00001) (Table 6).

DISCUSSION

The mean values for the water condition of the experimental aquaria (27.4± 0.55, 4.49± 1.23, and 7.1± 0.20) for temperature, pH and dissolved oxygen respectively fall within the optimal requirements for fish production (Ochang et al., 2007; Anyanwu et al., 2012). The highest mean feed efficiency was observed in diet Ac (coppens fish feed), followed by diet L_0 and then L_{25}; the lowest mean feed efficiency occurred in diet $L_{100.}$ The relatively low feed efficiency recorded in diet L100 (100% CLM - 9.82± 0.82) may be attributed to the presence of anti-nutrients in cassava leaf such as Linamarin (Presston, 2004; Azaza et al., 2015; Garcia et al., 2015).The chemical composition of cassava leaf meal showed very high level of crude fibre and low energy level, a future that is very common with leaf meals. The metabolizable energy value of the diets decreased with

Table 5. Growth performance and nutrient utilization of *Clarias gariepinus* fed different dietary levels of cassava leaf meal substitution for 24 weeks.

Group	Experimental											
	Total length (cm)	Standard Length (cm)	Weight (g)	Condition factor (k)	Time to strike (s)	Acceptability Index (cm^{-1})	5% biddy weight of feed (g)	Feed intake (g)	Weight gain (g)	Feed efficiency ratio	Specific growth rate	Feed conversion ratio
Lo	12.47 ±2.10^{a}	11.50±11.91^{a}	41.12±16.03^{a}	1.00±0.01^{b}	6.04± 0.05^{d}	0.17±0.00^{c}	2.06±0.80^{a}	1.92±0.77^{a}	15.64±5.75^{a}	14.66±1.16^{a}	1.48±0.10^{b}	0.12±0.01^{a}
L25	12.18±2.03^{a}	11.25±1.8^{a}	43.69±17.51^{a}	1.10±0.01^{a}	5.42±0.09^{e}	0.19±0.00^{b}	2.19±0.88^{a}	1.85±0.79^{a}	16.64±6.02^{a}	14.66±1.04^{a}	1.48±0.10^{b}	0.12 ± 0.01^{a}
L50	11.99±1.93^{a}	11.05±1.74^{a}	32.45±13.03^{a}	0.92±0.02^{c}	7.00±0.04^{c}	0.14±0.00^{d}	1.62±0.65^{a}	1.43±0.58^{a}	12.14±4.39^{a}	13.06±1.03^{a}	1.34 ± 0.01^{bb}	0.13 ± 0.01^{a}
L75	12.12±1.87^{a}	11.12±1.70^{a}	26.85±10.35^{a}	0.86±0.02^{d}	9.07±0.04^{b}	0.11±0.00^{e}	1.34±0.52^{a}	1.20±0.49^{a}	10.26±4.02^{a}	11.99±0.81^{a}	1.18 ± 0.05^{b}	0.13 ± 0.01^{a}
L100	10.65±1.39^{a}	9.78±1.24^{a}	14.98±4.61^{a}	0.81±0.02^{e}	12.98±0.12^{a}	0.08±0.00^{f}	0.75±0.23^{a}	0.67±0.21^{a}	4.33±1.31^{a}	9.82±0.82^{a}	1.08 ± 0.09^{b}	0.16 ± 0.02^{a}
Ac	12.85±2.22^{a}	11.87±2.05^{a}	49.98±19.43^{a}	1.07±0.01^{a}	4.43 ± 0.08^{f}	0.23 ± 0.00^{a}	2.50 ± 0.97^{a}	2.21 ± 0.89^{a}	18.67± 6.72^{a}	14.80± 1.31^{a}	1.56 ± 0.13^{b}	0.12 ± 0.02^{a}

Table 6. Water quality fluctuations during the 24 week feeding trial of *C. gariepinus* juveniles fed graded levels of CLM substitution.

Treatments	Water chemistry parameters											
	Temp(°C)	Mean±SD	DO(mg/l)	Mean±SD	PH	Mean±SD	NH_3–N (mg/l)	Mean± SD	NO_3- N	Mean±SD	NO_2- N	Mean±SD
L_o (0%CLM)	26.1-29.1	27.3±1.02	3.43-5.60	4.43± 0.60	6.78-7.80	7.1± 0.45	0.00018-0.00022	0.0002± 0.00001	0.00002-0.00004	0.00003±0.00001	0.00021-0.00034	0.00029±0.00001
L_{25} (25%CLM)	26.1-29.1	27.3±0.88	3.45-5.40	4.41± 1.20	6.52-7.69	6.9± 0.48	0.00014-0.00021	0.00018± 0.00001	0.00003-0.00004	0.00004±0.00001	0.00028-0.00032	0.00030±0.00001
L_{50} (50%CLM)	26.1-29.0	27.5±0.21	3.30-5.35	4.36± 0.70	6.44-7.60	6.9± 0.32	0.00017-0.00022	0.00021± 0.00001	0.00002-0.00003	0.00003±0.00001	0.00021-0.00024	0.00023±0.00001
L_{75} (75%CLM)	26.1-29.1	27.4±0.67	3.24-5.30	4.32± 1.33	6.40-7.90	7.1± 0.20	0.00026-0.00031	0.00029± 0.00001	0.00002-.0.00003	0.00003±0.00001	0.00025-0.00034	0.00029±0.00001
L_{100} (100%CLM)	26.1-29.0	27.3±1.28	3.11-5.20	4.22± 1.06	6.40-7.60	7.0± 0.50	0.00024-0.00029	0.00026± 0.00001	0.00001-0.00003	0.00002±0.00001	0.00024-0.00028	0.00026±0.00001
A_c (IMCF)	26.1-29.0	27.4±0.55	3.55-5.50	4.49± 1.23	6.66-7.88	7.0± 0.40	0.00021-0.00028	0.00025± 0.00002	0.00002-0.00004	0.00003±0.00001	0.00022-0.00030	0.00027±0.00001

increased levels of the leaf, indicating low energy status.

According to Lagler et al. (1977), the interaction of the factors affecting internal motivation or drive for feeding on specific diets range from intrinsic factor relating to physiology, genetics and morphology to extrinsic factors involving the living conditions especially food and feeding habits of the fish. Moreso, specific features such as size, age, sex, season and site of collection as well as species of fish, are of primary importance in determining the nutritional status and diet acceptability in catfish fed specific dietary types (Eyo, 1994). These factors however were taken care of since the catfishes were hatchery-raised, reared in the same pond and fed the same diet until they were collected for laboratory studies.

The incorporation of cassava leaf meal in the diet fed to *C.gariepinus* increased the palatability of the diets. The least mean time to strike (4.43± 0.08) was observed in diet Ac, followed by diet L_{25} (5.42± 0.09). There was observed inconsistency in the mean-time to strike the graded levels of substitution of cassava leaf meal. Statistically, each diet was significantly different ($p<0.05$) from another. Furthermore, despite the high acceptability observed in diet Ac. (Coppens fish feed), the acceptability index recorded in diet L_{25} was significantly higher than those of other test diets.

The growth pattern revealed that C*. gariepinus* performed better in diet L_{25} than all other diets. It has been documented that 50% replacement of maize with cassava root meal in broiler diet showed no depression in growth or unfavourable feed conversion ratio (Essers et al., 1995) and that the best growth performance was recorded in layers fed 10% cassava root meal. Olurin et al. (2006) reported a replacement level of 50% cassava meal for maize without a depression growth in C*. gariepinus*. In this present study, the best growth performance and nutrient utilization were recorded in fish fed 25% level of whole cassava leaf meal. This implies that the inclusion of 25% level of whole cassava leaf meal in the

diet of *C. gariepinus* catfish enhanced growth rate. This is in line with the work of Ernesto et al. (2000; Amisah et al., 2009), where broiler had the best growth performance at 25% cassava root meal inclusion level.

However, the poor growth performance recorded in diet L_{100} (100% CLM inclusion) may be attributed to the amount of anti-nutritional factors such as hydrogen cyanide (HCN) present in cassava leaf. This was indicated by the condition factor (k) of catfish in diet L_{100}. In the present study, it is shown that the inclusion of cassava leaf meal at 75% and above led to poor growth performance of the catfish. Feed stuffs which have anti-nutritional factors recorded poor growth performance in fish when supplemented at high levels (Ugwu and Mgbenka, 2006; Adewolu, 2008).Replacement of corn meal with cassava leaf meal recorded the highest mean weight gain and specific growth rate among catfishes fed diet L_{25} (25% CLM inclusion). This represents the highest level of CLM incorporation. On the contrary, the least mean weight gain and specific growth rate were recorded in diet L_{100} (100% CLM inclusion).

Furthermore, the ability of an organism to convert nutrients especially protein positively influences its growth performance. This was justified by the growth performance in 25% whole cassava leaf meal inclusion diet. Lower feed conversion ratio indicates better utilization of the feed by the fish. According to De Silva and Anderson (2001), feed conversion ratio is between 1.2 to 1.8 for fish fed carefully prepared diets, and the results from the present study fall within this range when multiplied by 10. The least mean feed conversion ratio was observed in diet L_{25}, indicating that fish in this diet had the best utilization of the feed. However, the mean feed conversion ratio was highest in diet L_{100} (100% CLM inclusion), indicating that fish in this diet had the worst utilization of the feed. Diet L_{100} was significantly different ($p<0.05$) from all other diets.

In addition, condition factor (k) of fish in diet Ac (internationally made coppens fish feed) and diet L_{25} (25% CLM inclusion) were not significantly different ($p>0.05$). Both had the best condition factor, implying that 25% CLM inclusion is the best rate of CLM substitution for maize in the growth of C*. gariepinus* catfish (Azaza et al., 2015). The optimum survival rates recorded in this study indicate that feeding C*. gariepinus* catfish with processed cassava leaf meal does not lead to mortality of the fish. This may probably be due to the substantial reduction in the cyanide content (by boiling and drying) of the whole cassava leaf meal (Garcia et al., 2015). Cardoso et al. (2005) observed that good processing of cassava enhanced survival and healthy state of fish at all stages of their lives.

Conclusion

Based on the results obtained in this study, it is recommended that under standard culture conditions, up to 25% cassava leaf meal can be used to substitute maize. Cassava leaf is abundant in Nigeria and extremely less expensive compared to maize. This will reduce over-dependence on imported fish feed and maize feed used in aquaculture.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Adewolu MA (2008).Potentials of potato leaf as dietary ingredient for *Tilapia zillii* fingerlings. Pak. J. Nutr. 7:444-449.

Adikwu P (1992). Dietary Carbohydrate utilization in Tilapia (*Oreochromis niloticus*). J. Agric. Sci. Technol. 1:33-37.

Akapo AO, Oso AO, Bamgbose AM, Sanwo KA, Jegede AV, Sobayo RA, Idowu OM, Fan J, Li L, Olorunsola RA (2014) .Effect of feeding cassava (Manihot esculenta Crantz) root meal on growth performance, hydrocyanide intake and haematological parameters of broiler chicks. Trop Anim Health Prod. 46(7):1167-1172.

Amisah S, Oteng MA, Ofori JK (2009).Growth performance of the Afirican cat fish Clarias gariepinus, fed varying inclusion levels of Leucaena leuccocephala leaf. J. Appl. Sci.Environ. Manage. 13:21-26.

Anibeze CIP, Amulu UC, Anibeze CI (2003). Evaluation of the Biomass and Profitability of *Heterobranchus Longifilis* under different Environmental Stocking Densities Enugu State Agricultural Development (ENADEP) Annual Report Zoo. 63 p.

Anyanwu DC, Udedibie ABI, Esonu BO, Osuigwe DI (2009).Growth and nutrient utilization of heteroclarias (Heterobranchui bidorsalis xClarias gariepinus fed dietary levels of Microdesmis puberurla leaf meal. Int. J. Agric. Rural Dev. 11:10-13.

Anyanwu DC, Umeh OI,Maduka BC,Mbchu MU (2012).Nutrient utilization and growth of catfish *(Clarias gariepinus* fed dietary levels of cassava leaf meal. Pak. J. Nutr.11(11):1037-1040.

AOAC (1975). Official Methods of Analysis. Kethed Association of Analytical Chemists, Washington, DC., USA.

APHA-American Public Health Association (2006). Standard methods for the examination of water and waste water. APHA Washington DC. 14th ed. P. 107.

Azaza MS, Khiari N,Dhraief MN,Alonui N Kratem MM,Elfeki A (2015). Growth performance oxidative stress indices and hepatic carbohydrate metabolic enzymes activities of juvenile Nile tilapia ,Oreochromis niloticus L.in response to dietary starch to mprotein ratios. Aquac. Res. 46:14-27.

Baruah K, Sahu NP, Denath D (2003). Dietary Phytase: An ideal approach for a cost effective and low polluting aquafeed. NAGA, WorldFish Center Quarterly 27(3-4):15-19.

Beck A (1979). Parel discussion of live feed versus artificial feed in feed fry. In: E & Styezriska – Junewises, T. Backnel, E. Jaspers and G. Personne (eds). Cultivation of fish fry and IBS line feed. European Mariculture Society, Special Publication No. 4. Bredere Belgum. pp. 515-527.

Boonyaratpalin M (1989). Methodologies for Vitamin requirements studies. In: De Silver S.S. (ed) Fish Nutrition Research in Asia. Proceedings of the third Asia fish Nutrition Network Meeting. Asia fish society, special publication Asian Fisheries Society, Maula, Philippines. pp. 58-67.

Brown ME (1975). Experimental Studies on Growth. The physiology of fishes. In: Brown M.E. (Ed) Academic Press, London, 1:401.

Cardoso AP, Mirione E, Ernesto M, Massazer F, CLIFF J, Haque RM , Bradbun HH (2005). Processing of Cassava Roots to remove cyanides. J. Food Compost. Anal. 18:451-460.

De Silva SS, Anderson TA (1995). Fish Nutrition in Aquaculture. London, UK: Chapman and Hall Aquaculture series 1.

Ernesto M, Cardoso AP, Nicala D, Mineone E, Massasa F, Cupp J,

Haque RM, Brasubry JH (2000). Persistent Konzo and cyanogens toxicity from cassava in Northern Mozambique. Acta Trop. 82:357-360.

Eyo AA (1994). The requirement for formulating standard artificial fish feed. Paper presented at the 11th Annual Conference of The Fisheries Society of Nigeria (FISON) held at the Lagos State Auditorium Secretariat Alausa Ikeja, Lagos State, 22nd -24th February, 1994, P 15.

Eyo JE, Ezechie CV (2004). The effects of rubber (*Havea brasilienis*) seed meal based diets on diets acceptability and growth performance of *Heterbranchus bidorsalis* (male) x *clarias gariepinus* (female) hybrid. J. Sustain. Trop. Agric. Res. 10:20-25.

Fagbenro OA (1992) dried fermented fish silage in diets for *Oreochromia niloticus.* Isr. J. Aquac. 46(3):140-147.

Falaye AE (1988). Utilization of Agro-Industrial Wastes as Fish Feed stuffs in Nigeria. Proc. of the 10th Annual Conf. FISON, Abeokuta, 16 – 20 November, pp. 47-56.

Faturoti EO, Akinbote RE (1986). Growth response and nutrient utilization in *Oreochronis niloticus* fed varing levels of dietary cassava peel. Niger. J. Appl. Fish. Hydrobiol. 1:47-50.

Gabriel UO, Akinrotimi OA, Ananwu PE, Bekibele DO , Onunkwo DN (2007). Locally produced fish feed potential for aquaculture development in sub-Saharan Africa. J. Agric. Res. 2:287-295.

Garcia V, Celada JD, González R, Carral JM, Sáez-Royuela M, González Á (2015). Response of juvenile tench (*Tinca tinca* L.) fed practical diets with different protein contents and substitution levels of fish meal by soybean meal. Aquat. Res. 46(1):28-38.

Lipton D, Hanel R (2004). Figuring Production Cost in Finfish aquaculture. Publication number: UM – SG – MAP – 90:02. 10 p. http://www.mdsg.umd.edu/ Assessed August 12, 2011.

Nwani CD, Ifo CT, Nwamba HO, Ejere VC, Onyishi G, Ikwuagwu EO, Odo GE (2015). Oxidative stress and biochemical responses in the tissues of African catfish *Clarias gariepinus* juvenile following exposure to primextra herbicide. Drug Chem. Toxicol. 38(3):278-285.

Ochang SN, Fagbenro OA, Adebayo OT (2007). Growth performance, body composition haematology and product quality of the African catfish (*Clarias gariepinus*) fed diets with palm oil. Pak. J. Nutr. 6:452-459.

Olatunde AA (1996). Effect of supplementary of soyabean diet with L. and D.L. methionine on the growth of mud fish *Claria anguillaris.* Niger. J. Biotechnol. 9(1):9-16.

Olukunle O (2006). Nutritive potential sweet potato peel meal and root replacement value for maize in diets of African catfish (*Clarias gariepinus*) advanced Fry. J. Food Technol. 4:289-293.

Olurin KBE, Olujo AA , Olukoya OA (2006). Growth of African Catfish *clarias gariepitus fingerlings,* fed different levels of cassava. World J. Zool. 1:54-56.

Presston TR (2004). Potential of cassava in integrated farming systems. Livest. Resour. Rural Dev. 10(8):20-28.

academicJournals

Vol. 15(24), pp. 1193-1200, 15 June, 2016
DOI: 10.5897/AJB2016.15383
Article Number: AC39E8258860
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Influence of aluminum on root growth and of anatomy *Stenocalyx dysentericus* (DC.) O. Berg

Rodrigues, A. A.*, Vasconcelos Filho, S. C., Rodrigues, D. A., Rodrigues, C. L., Sales, J. F. and Vital, R. G.

Federal Institute of Education and Technology Goiano, Campus Rio Verde, South Highway Goiana Km 01, Zona Rural, Rio Verde, GO, Brasil.

Received 4 April, 2016; Accepted 18 May, 2016

This study aimed to evaluate the effect of aluminum (Al) on root growth and root anatomical structure of *Stenocalyx dysentericus* seedlings. Newly emerged plants were grown in simple solution composed of 0.1 µM of $CaCl_2.2H_2O$ and five Al concentrations of 0, 150, 300, 600, and 1200 µM for 37 days in a hydroponic system. Subsequently, the seedlings were evaluated for root growth, relative root elongation, and anatomical studies using bright-field and fluorescence microscopy techniques. The results showed tolerance by *S. dysentericus*, with more root relative elongation in treatments with 150, 300, and 600 µM of Al. The anatomical studies revealed the presence of Al in root tissue, through the morin reagent, mainly in the 1200 µM treatment, characterizing some internal detoxification mechanism. *S. dysentericus* demonstrated tolerance in the tests with Al, principally at lower doses. These results may be entirely linked to its wide distribution in the cerrado domain, demonstrating to be a species adapted to soils with higher Al concentration. *S. dysentericus*, when subjected to treatment with Al, showed a stimulating effect on root growth; for this species, low concentrations of Al may be essential for better root growth.

Key words: Cerrado, acidic soils, plant toxicity, tolerance.

INTRODUCTION

Aluminum (Al) is the third most abundant chemical element in the earth's crust, with 8%; however, a small amount of this element occurs in a soluble, toxic form to plants. Its toxic form is observed when the pH is below 5, the Al^{3+} ion predominating, which gives way to the Al ions $(OH)^{2+}$, Al $(OH)_2^{+}$, and Al $(OH)_3$ as the soil pH value increases (Mossor-Pietraszewska, 2001; Frankowski et al., 2013). In acid soils, high Al levels and calcium deficiency are often considered the main limiting factors of plant growth. Under these conditions, the roots may have thickening and yellowing at the tips, degenerated and tortuous (Codognotto et al., 2002; Peixoto et al., 2007). On the other hand, some native cerrado species with distribution in acid soils show tolerance to the toxic

*Corresponding author. E-mail: arthuralmeidaeng@gmail.com. Tel: 66. 55 (64) 9237-4049.

effects of Al, being able to develop characteristics that make them resistant to higher concentrations of the metal and thus allowing their establishment in more acidified soils (Furley and Ratter, 1988; Andrade et al., 2011). As an example, there are *Qualea grandiflora, Vochysia thyrsoidea* and *Salvertia convallariaeodora* (Haridasan, 1982; Haridasan, 2008).

The study of plants tolerant to various concentrations of Al is considered the best alternative for increasing agricultural production in acid soils with high concentrations of this cation (Sanchez-Chacón et al., 2002; Echart and Cavalli-Molina, 2001). In addition, such plants provide important information on Al tolerance mechanisms that are necessary in breeding programs that aim to select the most productive plants with greater adaptability under stress conditions and can alleviate productivity problems in acid soils, caused by high Al levels (Freitas et al., 2006).

Among the diverse native cerrado species, cagaita (*Stenocalyx dysentericus* DC. O. BERG.), a representative of the Myrtaceae family, demonstrates high Al tolerance potential, has characteristics adaptive to sandy, acid and nutrient-poor soils. This species can be found in the extensive vegetation of the Cerrado area, mainly in the North, Southeast and Midwest (Vieira Neto et al., 2009). Therefore, the objective herein is to evaluate the effect of Al on root growth and root anatomical structure of *S. dysentericus* at the seedlings stage grown hydroponically in a simple nutrient solution.

MATERIALS AND METHODS

The fruits of *S. dysentericus* were collected on the Gameleira farm, located in the city of Montes Claros, Goiás, whose geographical coordinates are 16° 06'20 " S - 51° 17'11"W, at 592 m of altitude. The fruits were later pulped to obtain the seeds which were treated with fungicide, 30% Vitavax Tiran®, according to the manufacturer recommendations. Initially, the seeds were sown in beds containing washed sand as substrate. After 18 days emergence occurred and the standardization of size was at 40 days of cultivation, when the seedlings had an average of 14 cm in height. After the selection, the seedlings were transferred to hydroponic cultivation. Before immersing the roots solution, length of the principal root was measured, and the presence and visual stage of leaflets verified (cotyledonary and issued, up to this period).

The seedlings were then fixed in plastic caps with cotton support and placed in plastic pots containing 3 L of simple solution, consisting 0.1 µM de Ca L^{-1} in the form of $CaCl_2.2H_2O$, prepared according to the methodology proposed by Jacob Neto (1993). The pH of the solution was adjusted to 4.0 ± 0.2 with 1 M of HCl solution and the use of 0.1 M NaOH. The solution was changed every three days and constantly aerated using a compressor. To evaluate the effect of Al, concentrations of 0, 150, 300, 600, and 1200 µM of Al were adopted in the form of Al sulphate ($Al_2(SO_4)_3 \cdot 18H_2O$) in simple solution. From the start of the experiment, the seedling roots were maintained in solution containing the Al treatments and measured every 2 days for a period of 37 days, evaluating relative root elongation (RRE%), calculated according to the equation proposed by Vasconcelos et al. (2002), and shoot and root dry mass.

$$RRE = (LeAl_x - LiAl_x)/(Le\ Al_0 - LiAl_0) \times 100$$

where RRE is relative root elongation; iAlx is length initial root measured before exposure to the solution with “x” in Al; LeAlx is length end root measured before exposure of the solution with “x” in Al; $LiAl_0$ is length initial root before exposure to solution no Al; $LeAl_0$ is length end root measured after 37 days of exposure to the solution with Al.

The experimental design was completely randomized with 5 treatments with 4 repetitions each, with each replicate consisting of 4 seedlings per pot, totaling 20 experimental units. Data were subjected to analysis of variance by the F test and regression analysis.

Anatomy of root tips

After 37 days of hydroponic cultivation, samples with approximately 0.5 cm of root tips were collected with the help of disposable razor from one seedling per pot and fixed in Karnovsky solution (Karnovsky, 1965) for 24 h. After fixation, the samples were dehydrated in an ascending ethanol series, pre-infiltrated and infiltrated using historesin (Historesin, Leica) according to the manufacturer recommendations. The root tips were longitudinally sectioned to 5 mm with a rotary microtome (Model 1508R) and subsequently stained with toluidine blue-polychromatic staining, 0.05% in 0.1 M phosphate buffer, pH 6.8 (O’Brien et al., 1964), for structural analysis.

To evaluate Al location in the *S. dysentericus* seedling root tips, Morin fluorochrome was used (Eticha et al., 2005). 4',6 - Diamidino-2-phenylindole (DAPI), 1 µg ml^{-1} for 20 min, was also employed in order to evaluate the effect of Al on the DNA of meristematic cells. DAPI is a fluorochrome which binds strongly to DNA-rich regions. The anatomical images were obtained in an Olympus, BX61 bright-field and fluorescence microscope with a DP-72 camera. Fluorescence analysis was performed using a UV excitation cube (DAPI) 330-385.

RESULTS

Seedling growth under hydroponic cultivation with simple solution

The relative root elongation rate increased in treatments with 150, 300, and 600 µM of Al, providing evidence of Al tolerance for this species, since such concentrations stimulated root growth (Figure 1). Beyond the 600 µM dose, Al seedlings presented a root growth rate decrease, and phytotoxicity effects were observed from this dose onward. In the dry mass of root analysis, increase of 2.83 and 6.53% can be verified at the 300 and 600 µM Al dose and 26.98% reduction in the 1200 µM Al dose when compared with the control (Figure 2). For dry weight of shoot, the 300 µM Al dose promoted 6.02% increase as compared to the treatment without Al, while others had average lower than the control (Figure 2).

Visual analysis of seedlings at the end of the period of treatment showed greater root growth with Al treatments of 150, 300, and 600 µM (Figure 3). The apical region growth was stimulated up to the 600 µM Al dose. As for the Al dose of 1200 µM, a reduction was observed in root growth and the emergence of slight yellowing of the leaf edges, followed by premature leaf drop.

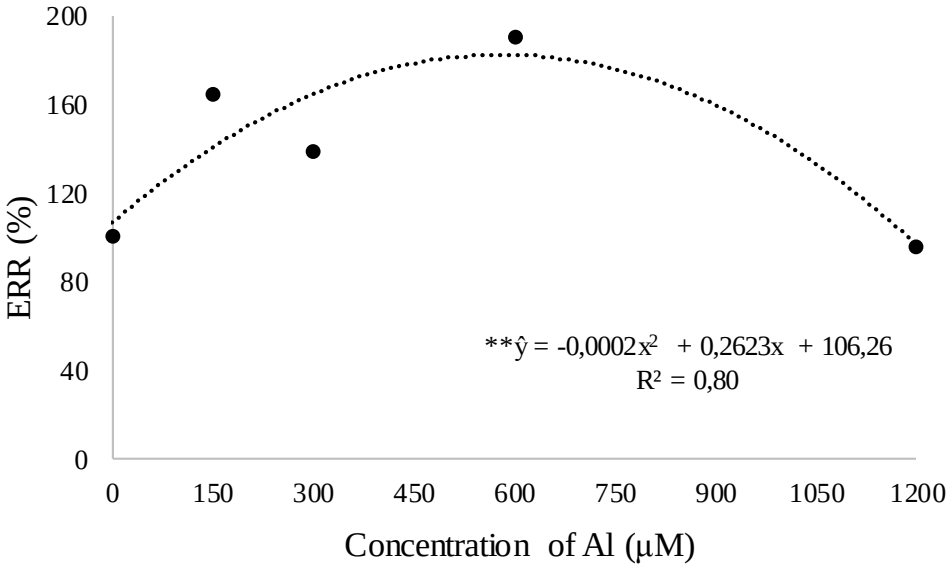


Figure 1. Relative root elongation (ERR%). ** Significant at the 5% level of probability. CV (%).

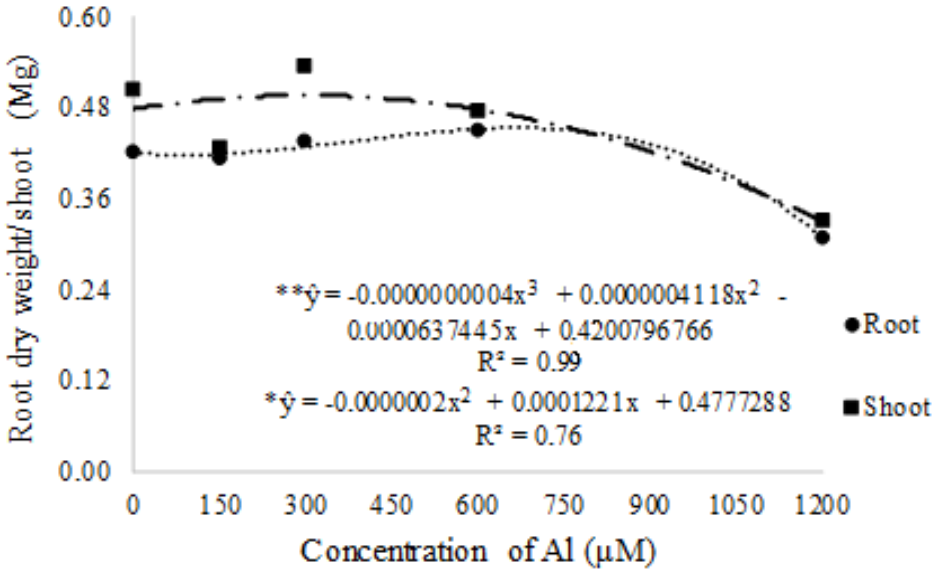


Figure 2. Root dry weight/shoot (Mg). ** Significant at the 1% level of probability and *Significant at 5% probability CV (%) = 13.55 (root) and 16.31 (shoot).

Root tip anatomy

Figure 4 shows *S. dysentericus* root tip sections exposed to different Al treatments, stained with toluidine blue. In analyzing Figure 4A and B, it was observed that the root apical meristems of the seedlings cultivated without Al consist of small juxtaposed cells with dense cytoplasm and an evident nucleus, tiny vacuoles also occur, the hood has uniform formation with apex cell integrity. In treatment with Al (Figure 4C, D, E, F, G and H), thicker roots were observed, consisting of increasingly larger cells and vacuoles in accordance to the Al dose increase. However, with 1200 µM of Al, the roots were thinner noting meristemic cells with walls of irregular outline, very large vacuoles with accumulation of content stained by toluidine blue, the presence of intercellular spaces and deformities in the epidermis; the promeristem is absent in this treatment, characterizing disorganization of the apical

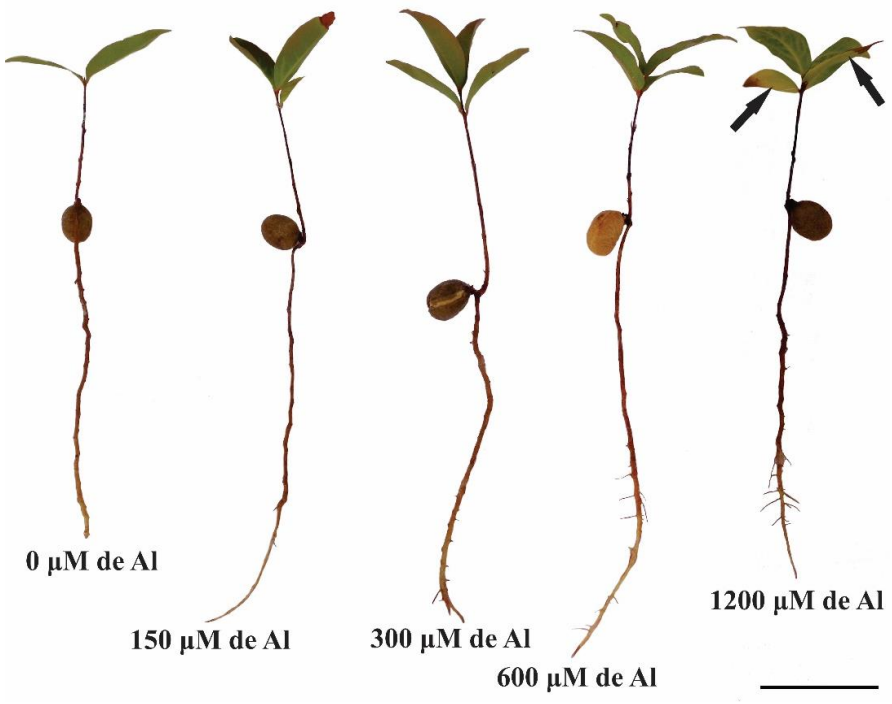


Figure 3. Visual appearance of seedlings at the end of the treatment period (*S. dysentericus*). Scale bar 5 cm.

meristem, which consequently leads to lower root growth (Figure 4I and J).

Tests with Morin show that for increasing Al doses promoted the highest intensity of green fluorescence (Figure 5A, C, E, G and I), especially from Figure 5I with a strong fluorescence signal in the cell wall, cytoplasm and nucleus demonstrating higher concentration of Al. The blue fluorescence with DAPI was with higher intensity to the treatments with 150, 300 and 600 µM of Al (Figure 5D, F and H), when compared with control (Figure 5B). For the treatment of 1200 µM Al, the blue fluorescence was lower, proving that for this does, Al adversely affects cell division inhibiting root growth (Figure 5J).

DISCUSSION

The central hypothesis of this study was that *S. dysentericus* presents Al tolerance, as do many native, usually woody, perennial species of the Cerrado that develop Al tolerance characteristics, being able to accumulate high concentrations in leaves, with levels above 1,000 mg Al kg^{-1} as a root Al detoxification method. These species are also called Al hyperaccumulators, frequent in families Euphorbiaceae, Myrtaceae, Rubiaceae, Melastomataceae, and Vochysiaceae (Cuenca et al., 1991; Jansen et al., 2002a, b).

S. dysentericus demonstrated tolerance and stimulated growth at Al doses up to 600 µM. As has been demonstrated by research works, Al can be beneficial when used in low concentration. Root growth inhibition tendency via Al application did not occur in some treatments, as reported for tea (Morita et al., 2008), corn (Comin et al., 1999) and apple (Stolf et al., 2008). Foy (1983) reported that in some species of plants, low doses of Al can be beneficial to growth. Silva (1992) found that the growth of rice plants was stimulated by the addition of up to 5 mg of Al^{3+} L^{-1} nutrient solution. For Silva (2007), in maize, sugar beet, and some species of tropical legumes, Al concentrations that result in stimulation of growth varied from 71.4 to 185 µM. However, the nature of the beneficial effects of Al is still unknown, but Huang and Bachelard (1993) postulated that this growth stimulation occurs under H^+ stress conditions, concluding that Al^{3+} minimizes the toxicity of H^+.

When a species shows sensitivity to Al, the first visible symptom is the inhibition of root elongation, although this root response has presented different behavior among plant species and even among cultivars (Matsumoto and Motoda, 2012). As an example, Matsumoto (2002) reported that the root growth elongation of Al-sensitive wheat was inhibited by a 3 h treatment with 5 µM of Al, while in the tolerant cultivar elongation was inhibited by a 10 fold higher concentration. The relative root elongation refers to a percentage assessment of the treatments effects on the root growth of seedlings in order to

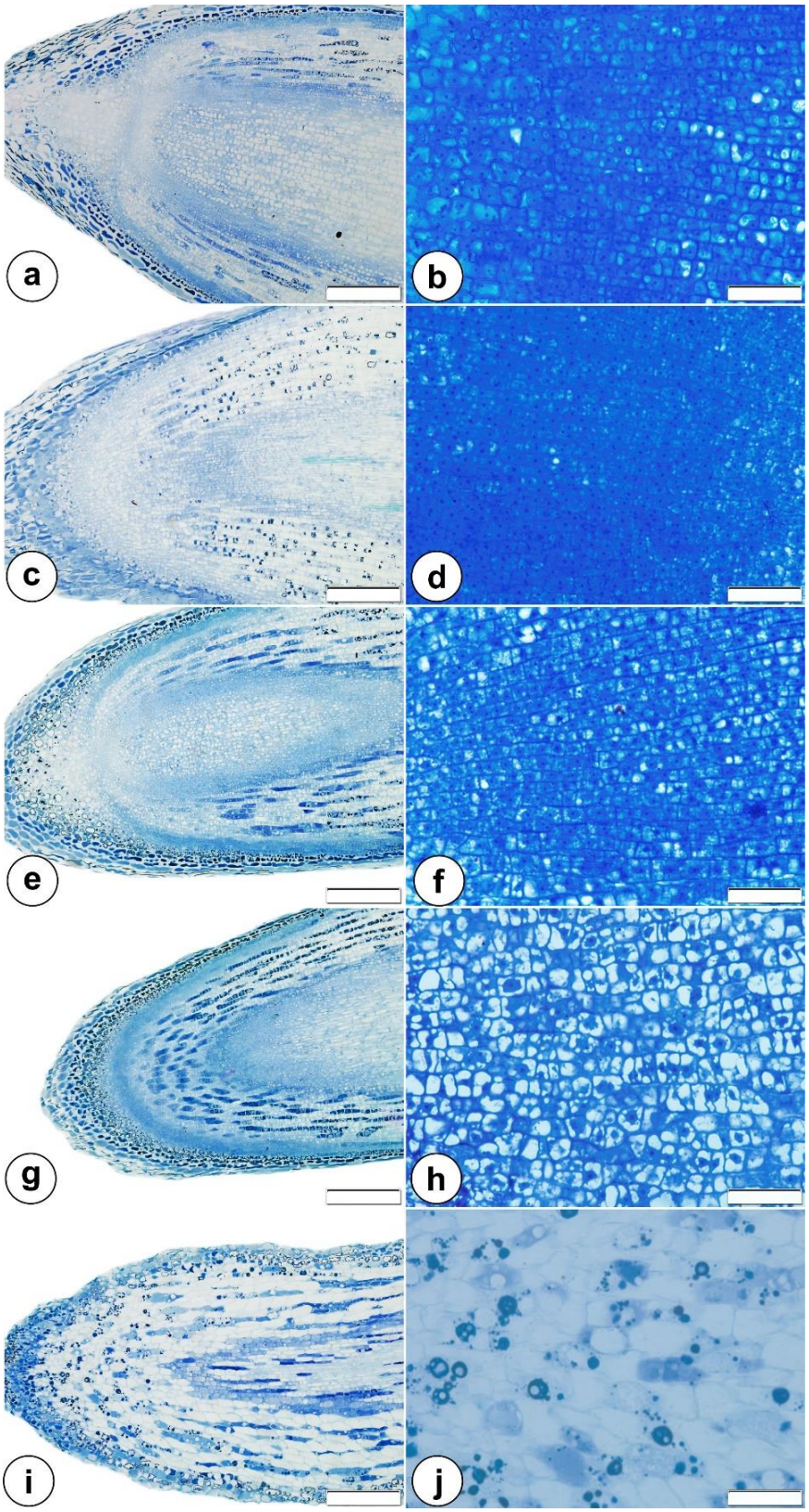


Figure 4. Optical micrographs of longitudinal sections of seedling roots *S. dysentericus*. Stained with toluidine blue. Where A - without Al; B - 150 µM of Al; C - 300 µM of Al; D - 600 µM of Al; and E - 1200 µM of Al A, C, E, G and I Bars = 200 µm; B, D, F, H and J Bar = 50 µm.

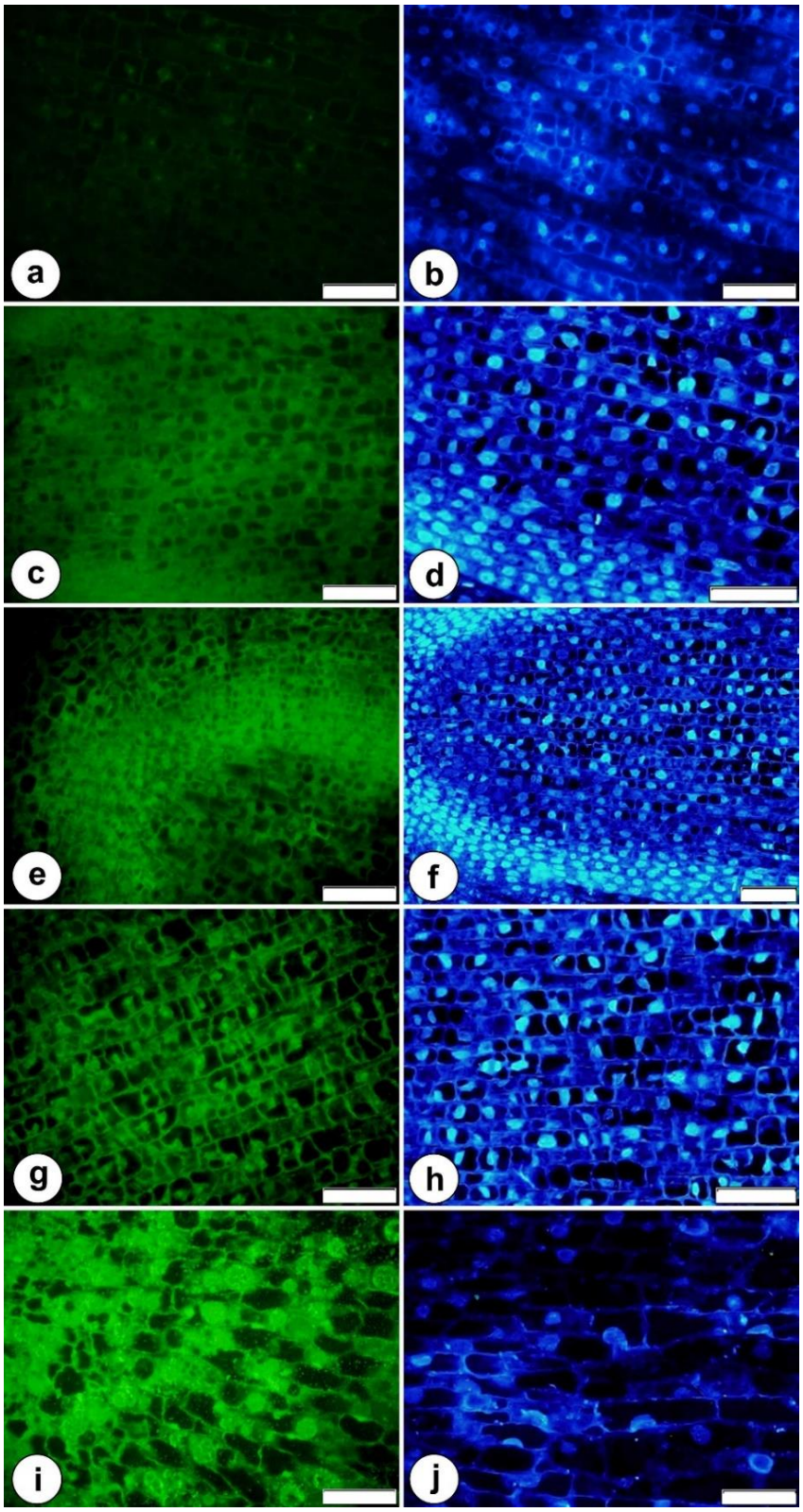


Figure 5. Fluorescence micrographs of cross sections of seedling roots *S. dysentericus*. Treated with morin fluorochrome (picture left) and fluorochrome DAPI (photo right). where: A and B - No Al; C and D - 150 µM dose of Al; E and F - 300 µM dose of Al; G and H - 600 µM dose of Al; I and J -. 1200 µM dose Al The green fluorescence indicates the presence of aluminum and blue presence of DNA. Bar 50 µm.

demonstrate the sensitivity or tolerance of species to Al. Vasconcelos et al. (2002), in a study using RRE% for evaluating the toxicity of Al in rice cultivars, concluded that this parameter was sufficient to identify differences in tolerance among cultivars, even at low concentrations.

The shoot dry mass at an Al dose of 300 µM presented the highest averages. Similar results were observed in castor beans by Lima et al. (2007), regarding the shoot dry matter, in which the increase was 6.3 times in treatments without Al and 15.8 times with a high degree of Al. Probably, the 300 µM Al concentration stimulated the best shoot development, demonstrating that, for this species, the solution with Al promoted greater growth of roots and shoots.

Root tip anatomy

The negative influence of Al in sensitive species alters the growth and cell expansion rate (Barceló et al., 1996). Thus, the cell volume increase at doses of 600 and 1200 µM of Al can be explained by the fact that the roots have a decreased pressure potential, which reduces the apparent hydraulic conductivity, thereby indicating that Al severely affects the proportion of water in the root (Echart and Cavalli-Molina, 2001).

Lima and Copeland (1994) indicated that the effects on the meristematic cells, that is, root growth reduction, become evident only after prolonged exposure to Al^{+3}. Thus, simultaneous alterations in cell elongation and thickness suggest that the effect of Al^{+3}, directly or indirectly, affect many cell expansion-related processes (Nichol et al., 1993). This information corroborates that of this present study, in which the effect of Al on increased cell size was observed at doses of 600 and 1200 µM (Figure 4H and I). In addition, other evidence indicates that cell elongation inhibition may be due to the result, at least in part, of changes in the cap cells, which act as environmental stress sensors (Marschner et al., 1991).

The toxicity of Al in the treatment with 1200 µM can be associated with the gross changes in the root morphology. Briefly, Al results in toxicity in root elongation, inhibiting root development, producing dark colored, thick apices and little secondary root formation due to the high saturation in such treatment for the seedling. Root damage results in a reduced root system, damaged, limiting water and mineral nutrient absorption (Delhaize et al., 1993; Maron et al., 2010). In characterizing the presence of Al in root tips, Garzon et al. (2011) reported that control plant root tips showed low fluorescence when stained with Morin and Al accumulation in the cell wall for treatments with higher doses of this element. Al internalization in the root and root growth stimulation may be associated with mechanisms of complexation by organic acids or internal detoxification mechanisms, impeding the genotoxic action of Al at doses of 150, 300, and 600 µM.

Achary and Panda (2010), working with *Allium cepa*, showed that at high concentrations, Al induces DNA damage, however, when in small concentrations, it can provide adaptive responses conferring genomic protection against genotoxic risk posed by the ion and promoting greater root system growth. When comparing the areas marked by Morin and DAPI, it was observed that increasing Morin fluorescence is related to the decrease of the DAPI fluorescence, that is, Al accumulation in cells causes cell death in the root apex, since such characteristics were demonstrated at higher Al doses at which root growth loss was observed.

Conclusion

S. dysentericus, when subjected to treatment with Al, showed that the Al may be essential for root growth, with smaller root tip diameter and nuclei division stimulus in the treatments with 150, 300 and 600 µM; however, the 1200 µM dose promoted a root growth decrease with cell expansion, showing that for this species, low concentrations of Al may be essential for better root growth.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors acknowledged the Federal Institute Goiano, Brasil.

REFERENCES

Achary VMM, Panda BB (2010). Aluminium-induced DNA damage and response to genotoxic stress in plant cells are mediated through reative oxygen intermediates. Mutagenesis 25(2):201-209.

Andrade LVM, Barros LMG, Echevarria GF, Lourdes I, Amaral V, Cotta MG, Rossato DR, Haridasan M, Franco AC (2011). Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. Environ. Exp. Bot. 70(1):37-42.

Barceló J, Poschenrieder C, Vázquez MD, Gunsé b (1996). Aluminum phytotoxicity. A challenge for plant scientists. Fertil. Res. 43:217-223.

Codognotto LM, Santos DMM, Leite IC, Marin A, Madaleno LL, Kobori nn, Banzatto DA (2002). Aluminum effect on chlorophyll contents of bean seedlings -mungo e labe-labe. Mag Ecosyst, Espírito Santo do Pinhal, 27(2):27-39.

Comin JJ, Barloy J, Bourrie G, Trolard F (1999). Differential effects of monomeric and polymeric aluminium on the root growth and on the biomass production of root and shoot of corn in solution culture. Eur. J. Agron. 11:115-122.

Cuenca G, Herrera R, Mérida E (1991). Distribution of aluminum in accumulator plants by X-ray microanalysis in Richeria grandis Vahl leaves from a cloud forest in Venezuela. Plant Cell Environ. 14:437-441.

Delhaize E, Ryan PR (1993). Randall, Aluminum tolerance in wheat (*Triticum aestivum* L.): II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol. 103:695-702.

Echart CL, Cavalli-Molina S (2001). Aluminum phytotoxicity: effects of tolerance mechanisms and their genetic control. Rev. Ciênc. Rural 31(3):531-541.

Eticha D, Stass A, Horst WJ (2005). Localization of aluminium in the maize root apex: can morin detect cell wall-bound aluminium? J. Exp. Bot. 56:1351-1357.
Foy CD (1983). The physiology of plant adaptation to mineral stress. Iowa State J. Res. 57:355-391.
Frankowski M, Zioła-Frankowska A, Siepak J (2013). From soil to leaves – aluminum fractionation by single step extraction procedures in polluted and protected areas. J. Environ. Manage. 127(30):1-9.
Freitas FA, Kopp MM, Sousa RO, Zimmer PD, Carvalho FIF, Oliveira AC (2006). Absorção de P, Mg, Ca e K e tolerância de genótipos de arroz submetidos a estresse por alumínio em sistemas hidropônicos. Rev. Ciênc. Rural 36(1):72-79.
Furley PA, Ratter JA (1988). Soil resources and plant communities of central Brazilian cerrado and their development. J. Biogeogr. 15:97-108.
Garzon T, Gunse B, Moreno AR, Tomos AD, Barcelos J, Poschenrieder C (2011). Aluminum induced alteration of ion homeostasis in root tipo vacuoles of two. Plant Sci. 180:709-715.
Haridasan M (1982). Aluminium accumulation by some cerrado native species of central Brazil. Plant Soil 65:265-273.
Haridasan M (2008). Nutritional adaptations of native plants of the cerrado biome in acid soils, Brazil. J. Plant Physiol. 20:183-195.
Huang JW, Barchelard EP (1993). Effects of aluminum on growth and cation uptake in seedlings of *Eucalyptus mannifera* and *Pinus radiata*. Plant Soil 1:121-127.
Jacob Neto J (1993). The interactions of H+/OH- exchanges between roots and rhizosphere with plant nutrition and aluminium effects. Dundee: University of Dundee. (PhD Thesis). 281p.
Jansen S, Broadley MR, Robbrecht E, Smets E (2002a). Aluminum hyperaccumulation in Angiosperms: a review of its phylogenetic significance. Bot. Rev. 68:235-269.
Jansen S, Watanabe T, Smets E (2002b). Aluminium accumulation in leaves of 127 species in Melastomataceae, with comments on the Order Myrtales. Ann. Bot. 90:53-64.
Karnovsky MJA (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell. Biol. 27:137-138.
Lima MLD, Copeland L (1994). Changes in the ultrastructure of the root tip of wheat following exposure to aluminum. Funct. Plant Biol. 21(1):85-94.
Lima RLS De, Severino LS, Ferreira GB, Silva Mil Da, Albuquerque RC, Beltrão NEM (2007). Growth of castor bean in soil with high aluminum content in the presence and absence of organic matter. Rev. Bras. Ol Fibros. 11(1):15-21.
Maron lg, Pineros MA, Guimaraes CT, Magalhaes JV, Pleiman JK, Mao CZ, Shaff J, Belicuas SNJ, Kochian LV (2010). Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. Plant J. 61:728-740.
Marschner H, Mechanisms of adaptation of plants to acid soils. In: Wright RJ, Baligar VC, Moorman RP (1991). (eds), Plant-soil interactions at low pH. Proceedings of the Second International Symposium on Plant Soil Interactions at Low pH, Beckley, West Virginia, USA. Kluwer Academic Publisher pp. 683-702.
Matsumoto H (2002). Plant roots under aluminum stress: toxicity and tolerante. In. Weisel T., Eshel A., Kafkafi U., Plant Roots: The hidden half, 3. Ed; Marcel Dekker. New York. pp. 821-838.
Matsumoto H, Motoda h (2012). Aluminum toxicity recovery processes in root apices. Possible association with oxidative stress. Plant Sci. 185:1-8.
Morita A, YanagisaWA O, Takatsu S, Maeda S, Hidrate S (2008). Mechanism for the detoxification of aluminum in roots of tea plant (*Camellia sinensis* L. Kuntze). Phytochemistry 69:147-153.
Mossor-Pietraszewska T (2001). Effect of aluminium on plant growth and metabolism. Acta Biochim. Pol. 48:673-686.
Nichol BE, Oliveira LA, Glass ADM, Siddiqi MY (1993). The effects of aluminum on the influx of calcium, potassium, ammonium, nitrate, and phosphate in an aluminum-sensitive cultivar of barley (*Hordeum vulgare* L.). Plant Physiol. 101(4):1263-1266.
O'Brien TP, Feder N, Mccully ME (1964). Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368-373.
Peixoto PHP, Pimenta DS, Cambraia J (2007). Changes in morfology and phenolics accumulation in sorghum (*Sorghum bicolor* (L.) Moench) plants under aluminum stress. Bragantia 66(1):17-25.
Sanchez-Chacón CD, Federizzi LC, Milach SCK, Pacheco MT (2002). Genetic variability and inheritance of tolerance to aluminum toxicity in oats. Pesq. Agropec. Bras. 35(9):1797-1808.
Silva AAP (1992). Biometric parameters and physiological rice plant growth (*Oriza sativa* L.) cv. Rio Paraíba subjected to increasing levels of aluminum in hydroponics. Botucatu. Dissertation (Masters in Botany), Basico Institute of Biology and Agricola, Universidade Estadual Paulista. 116p.
Silva LM (2007). Root growth and nutrient absorption of rice cultivars submitted to aluminum in nutrient solution. Thesis College of Agricultural Sciences UNESP.
Stolf EC, Dantas ACM, Boneti JL, Comin JJ, Nodari RO (2008). Establishment of criteria to select apple rootstocks tolerant to aluminum in nutrient solution. Rev. Bras. Frutic. 30:476-481.
Vasconcelos SS, Rossielo ROP, Jacob-Neto J (2002). Morphological parameters to establish differential tolerance to aluminum toxicity in rice cultivars. Pesq. Agropec. Bras. 37(3):357-363.
Vieira Neto RD, Silva Junior JF Da, Ledo AS Da. (2009). Mangaba. In: Santos-Serejo JÁ Dos, Dantas JL, Coelho CVS, Coelho YS Da (Org.). Fruti trop: Regional and exotic species. Brasilia: Embrapa Inform. Technol. pp. 323-338.

academicJournals

Vol. 15(24), pp. 1201-1207, 15 June, 2016
DOI: 10.5897/AJB2016.15321
Article Number: B051B1658862
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Physicochemical analysis of cellulose from microalgae *Nannochloropsis gaditana*

BABA HAMED Samia*, BABA HAMED Mohamed Bey, KASSOUAR Schéhérazade and ABI AYAD Sidi– Mohammed El - Amine

Laboratory of aquaculture and bioremediation (*AQUABIOR*), Department of Biotechnology, University of Oran 1 - Ahmed Ben Bella, Algeria.

Received 4 March, 2016; Accepted 18 May, 2016

Nannochloropsis gaditana **is a microalgae belonging to the class of Eustigmatophyceae. This particular microalga is the most studied species. For its richness in lipids, it is used for the biodiesel production. The aim of this work is to advance another important metabolite which is cellulose. This represents 25% of the dry weight of the *N. gaditana.* A low concentration of NaOH (2%) and sodium hypochlorite (6%) allowed the obtainment of relatively pure cellulose analyzed by Fourier transform infrared (FT-IR) and X-Ray Diffraction (XRD). The progress of the microalgae mass production could help in the substitution of the cellulose of microalgae for the vegetal cellulose, as seen in the simple technical extraction, the yield and the procurement of uncontaminated molecule with lignin. This substitution will contribute in protecting the environment.**

Keywords: Cellulose, *Nannochloropsis gaditana*, procedure extraction, structural characterization.

INTRODUCTION

Nannochloropsis gaditana is a microalgae that belongs to the class of Eustigmatophyceae (Andersen, 1998). It has five species and they are: *N. gaditana, N. salina, N. oculata, N. oceanica and N. limnetica* (Hibberd, 1981; Lubian,1982; Karlson et al.,1996; Krienitz et al.,2000 Suda et al.,2002) . The *gaditana* and *salina* species are recently reported as two strains belonging to the same species (Shawn et al., 2014). *N. gaditana* is rich in lipids and produces mass biodiesel (AttilioConverti et al., 2009; Rodolfi et al., 2009; Pal et al., 2011; Bondioli et al., 2012). The cell wall of *N. gaditana* is comprised a bilayer structure consisting of a cellulosic inner wall (~75% of the mass balance), protected by an outer hydrophobic algaenan layer (Scholz et al., 2014). To meet with the global needs of cellulose (the textile, paper, plastic, paint, chemistry, pharmaceutical and cosmetic field) and vegetable, a wide deforestation and environmental damage is involved. The substitution of the parietal compounds of the plant source by those microalgae is possible and can present several advantages. Reasons being that: (i) the growth of microalgae is quick and harvesting is done continually throughout the year (Gouveia, 2011); (ii) they can grow in saline aqueous areas, waste water and brackish water (Gouveia, 2011)

*Corresponding author. E-mail: samiababa13@gmail.com. Tel: ++213(0)540622220.

or even in wasteland (Demirbas, 2011); (iii) their production contributes to a better CO_2 sequestration than plants (Gouveia, 2011).

The mechanical properties of the cellulose determine its use. These properties depend on the spatial structure obtained after extraction by chemical treatment (Morán et al., 2008). The aim of this study is to extract the main parietal compound of *N. gaditana* (cellulose) at different concentrations of extraction reagents. The quality of cellulose is analyzed by Fourier transform infrared (FT-IR) and by X-Ray Diffraction (XRD). Cellulose is a linear homopolymer consisting of anhydroglucopyranose units (AGU), connected by glycosidic linkages in β - (1-4). The chains are linked by hydrogen bonds fashioned by covalent bonds formed between C1 of one monomer in one strand and a C4 in an adjacent strand.

MATERIALS AND METHODS

Biological material

N. gaditana is mass produced by the company: PARTISANO BIOTECH Algeria in Oran and the samples are provided in dried powder form.

Cellulose extraction

The extraction method of algae cellulose was a modification of Jayme-Wise method (Leavitt and Danzer, 1993; Gaudinski et al., 2005; Rosli et al., 2013), initiated with the de-waxing of algae by treating micralgae with toluene: ethanol. Five gram (5 g) of micro algae dry powder were treated with a mixture of toluene/ ethanol (68:32) (v/v) for 24 h using magnetic stirring. After filtration, the parietal residue was separately treated with 2 or 4% NaOH for 2 h at 80°C, to eliminate Hemicelluloses. Bleaching is performed with sodium hypochlorite (6 or 10%) at pH 4.8, stirred for 2 h at 70°C. The cellulose pellet recovered by centrifugation was dried using lyophilization and weighed. The extraction was carried out in triplicate.

Characterization of cellulose

Analysis of cellulose by FTIR spectroscopy

FTIR spectra of the cellulosic samples were measured with FTIR alpha Brucker .The resolution of 26 scan were taken with the frequency range of 4000 to 400 cm^{-1} in the transmission mode.

Analysis of cellulose by DRX, (DRX D8 Advance Brucker)

The cellulose samples were analyzed from 4 to 70° (2θ) with a pitch of 0.02° and measuring 1s of acquisition time by an R-X diffractometer.

RESULTS AND DISCUSSION

Quantification of cellulose

Cellulose obtained after extraction and dried is 25% of the dry weight of the microalgae, *N. gaditana.*

Characterization of cellulose

Analysis of the cellulose by FT-IR

The infrared spectra of cellulose were studied in the literature (Nelson and O'Connor, 1964; Oh et al., 2005; Alvarez and Vazquez, 2006; Yang et al., 2007). The analysis by FTIR of the extracted cellulose from 2% NaOH and 6% $NaClO_2$ (Figure 1) is carried out to identify polysaccharide and to determine its contaminants such as: hemicelluloses, lipids and proteins.The spectrum obtained (Figure 1) revealed a broad band in the 3600 to 3100 cm^{-1}which correspond to the (- OH). The peak occurred at 3283 and 3272 cm^{-1}, which indicated the presence of hydroxyl groups in algae cellulose and carboxymethyl cellulose respectively. The peaks 2929/2857 and 2880 cm^{-1} corresponded to the (-CH) groups stretching vibration in cellulose algae and caboxymethyl cellulose (Oh et al., 2005; Nelson and O'Connor, 1964).

The peak present at 1069 cm^{-1} corresponding to 950 to 1200 cm^{-1} range is attributed to C-O vibration of carbon C_2, and also the 1632 cm^{-1} band corresponded to the carbonyl groups. In addition, the FTIR absorption bands at 1458/1378 and 1412/1320 cm^{-1} was assigned to O-H bending vibration.The occurrence of the peak at 1156 cm^{-1}, located in 1170 to 1082 cm^{-1} range corresponds to asymmetric C-O-C stretching vibration. The appearance of the 1524 cm^{-1} peak at interval (1600 to 1500cm^{-1}) corresponds to the aromatic rings. This group is a contaminant due to the existence of peptides or proteins. Proteins represent about 1% of the parietal content of *N. gaditana* (Scholz et al., 2014).

The peak presented at 1735 cm^{-1} in the spectrum corresponding to the cellulose obtained from 2% NaOH and 6% $NaClO_2$ could be, due to the presence of small amounts of hemicelluloses, which contain higher C=Olinkage at 1765 to 1715 cm^{-1}. Another possibility is that carboxyl or aldehyde absorption (1736cm^{-1}) could be arising from the opened terminal glycopyranose rings (Morán et al., 2008). The carboxyl may be due to the presence of lipids. The Cellulosic wall *N. gaditana* is protected by algaenan which is a lipid structure (Scholz et al., 2014). The current analysis of the cellulose extracted in our study by NMR reveals the presence of lipids (Stuart Jones, personal comm.).However, the extraction of cellulose in higher concentrations NaOH2 to 4% to get a better solubilization of hemicelluloses followed by bleaching with hypochlorite6% (Figure 2) does not reduce the peak intensity1735cm^{-1}.The hemicelluloses are principally extracted with 2% NaOH. This peak also corresponds with the functional groups aldehyde and carboxyl (Morán et al., 2008).

The aldehydes and carboxyl groups are more accessible at 4% NaOH. There is an increase in the peak intensity 1736cm^{-1}. The increase in the concentration of NaOH 4% did not only affect the peak 1736 cm^{-1}, but also the other peaks representing other functional groups. The

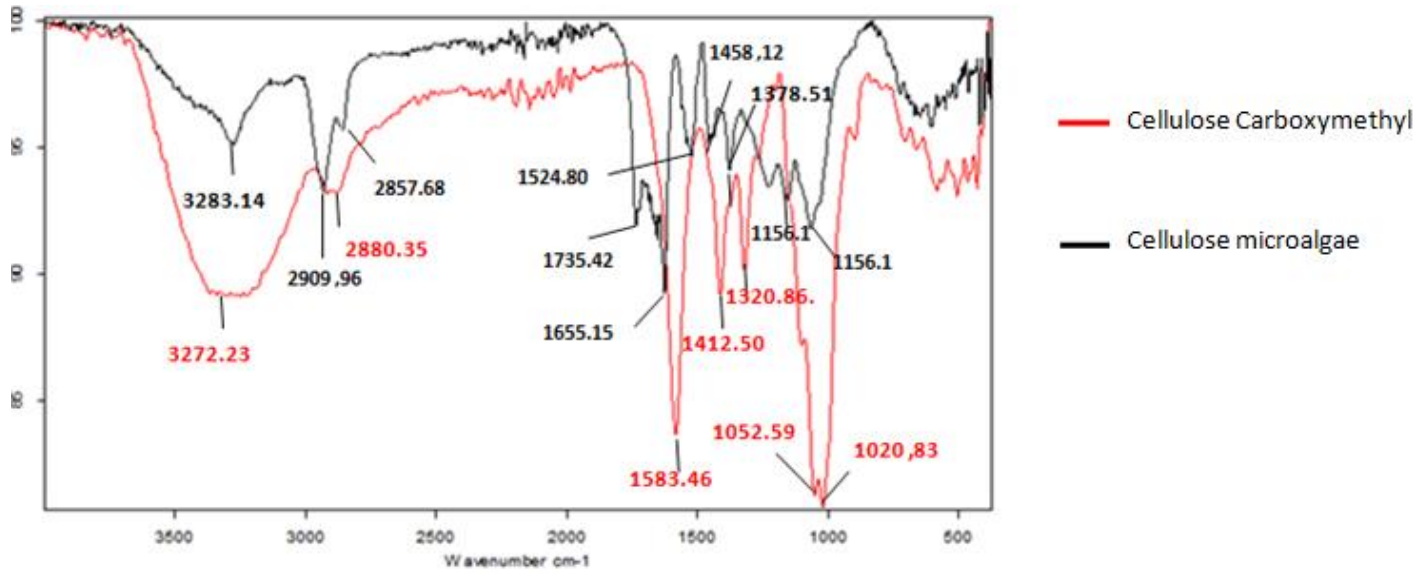


Figure 1. FT-IR spectra of carboxymethyl cellulose and cellulose extracted from *N. gaditama* by 2% NaOH and 6% $NaClO_2$.

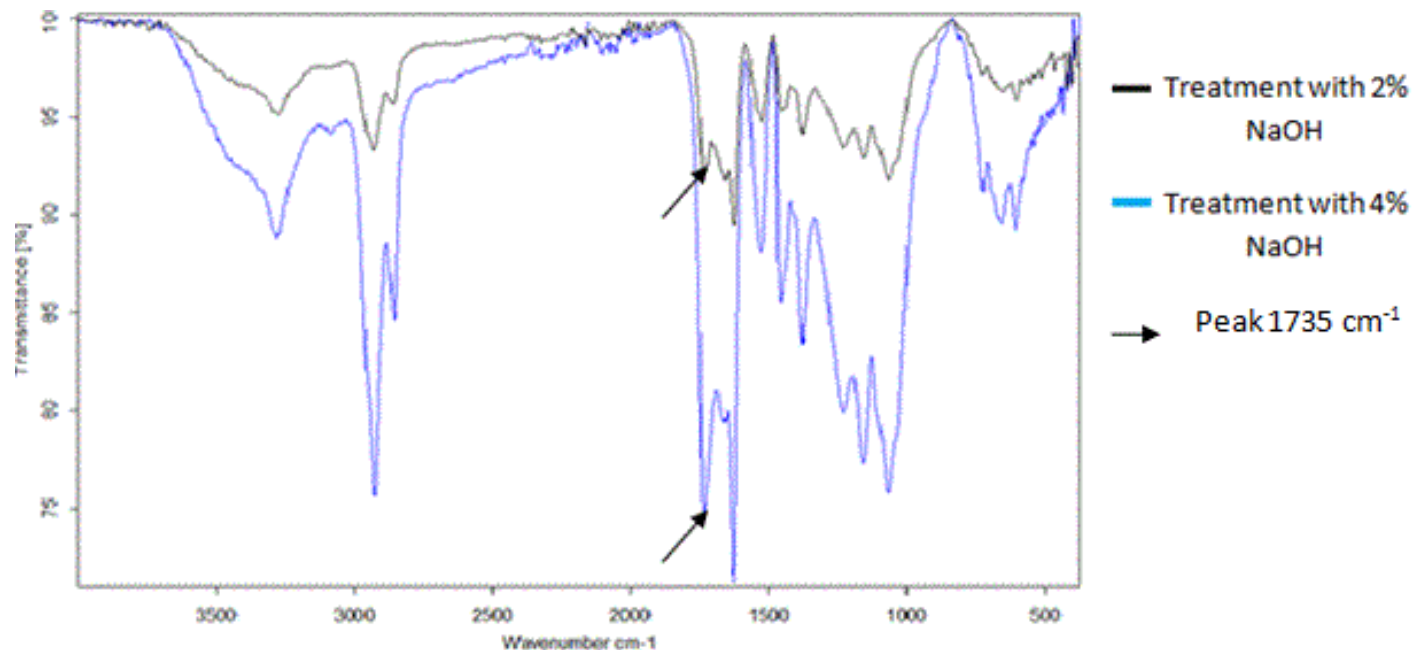


Figure 2. Cellulose FT-IR spectrum extracted from *N. gaditama* by 2 and 4% NaOH followed by 10% of $NaClO_2$.

concentration of 4% NaOH transforms the semi crystalline structure of cellulose to amorphous structure. The increase in the concentration of $NaClO_2$ from 6 to 10% (Figure 3) highlights the functional groups of the cellulose expressed by the increase of peaks intensity. The concentrations of 10% $NaClO_2$ would act on the cellulose structure while making its functional groups more accessible to the infrared rays. The semi crystalline structure of the cellulose became more amorphous.

Analysis of cellulose by XRD

In order to identify the crystalline and amorphous areas of celluloses obtained in this work, a diffractometry X-Ray was carried out. Cellulose samples obtained at different concentrations of NaOH (2 and 4%), followed by bleaching with 6% sodium hypochlorite, and another obtained after treatment with 2 and 4% NaOH, followed by 10% hypochlorite bleach were analyzed (Figure 4).

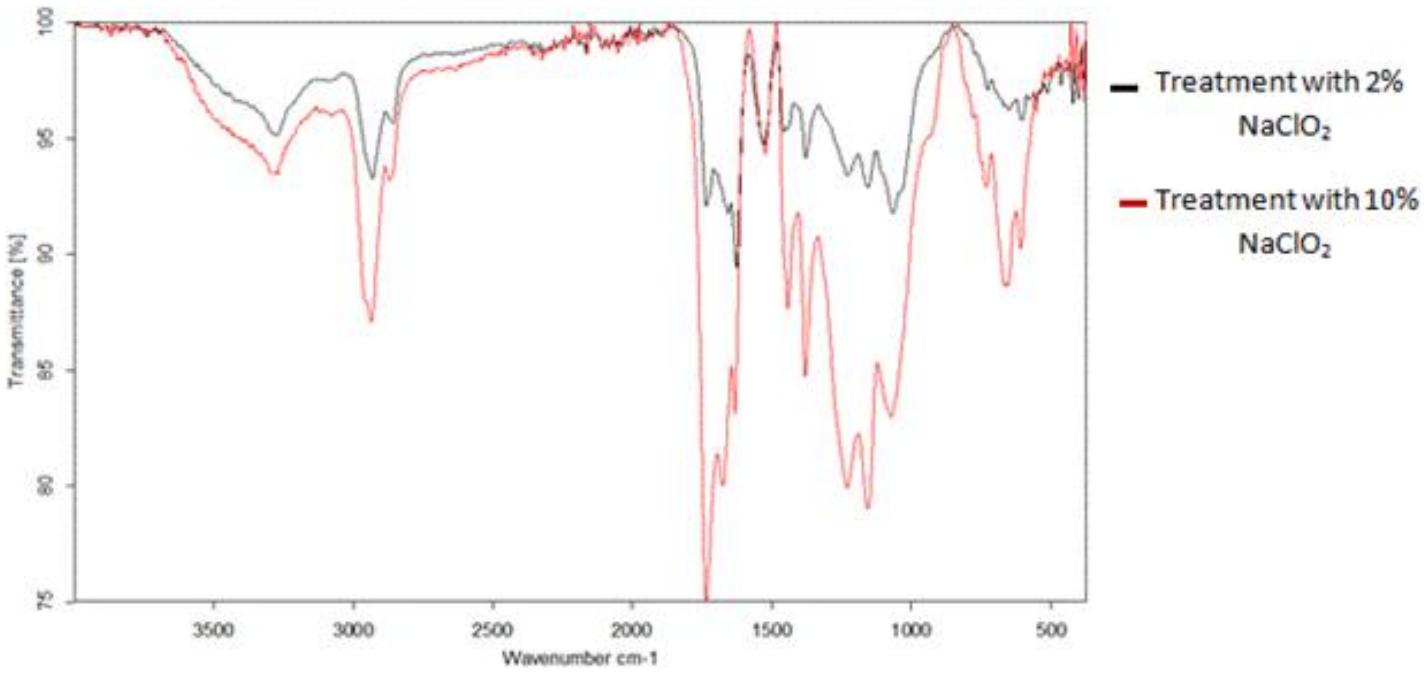


Figure 3. Cellulose FT-IR spectrum extracted from *N. gaditama* by 2% NaOH followed by 6 and 10% of $NaClO_2$.

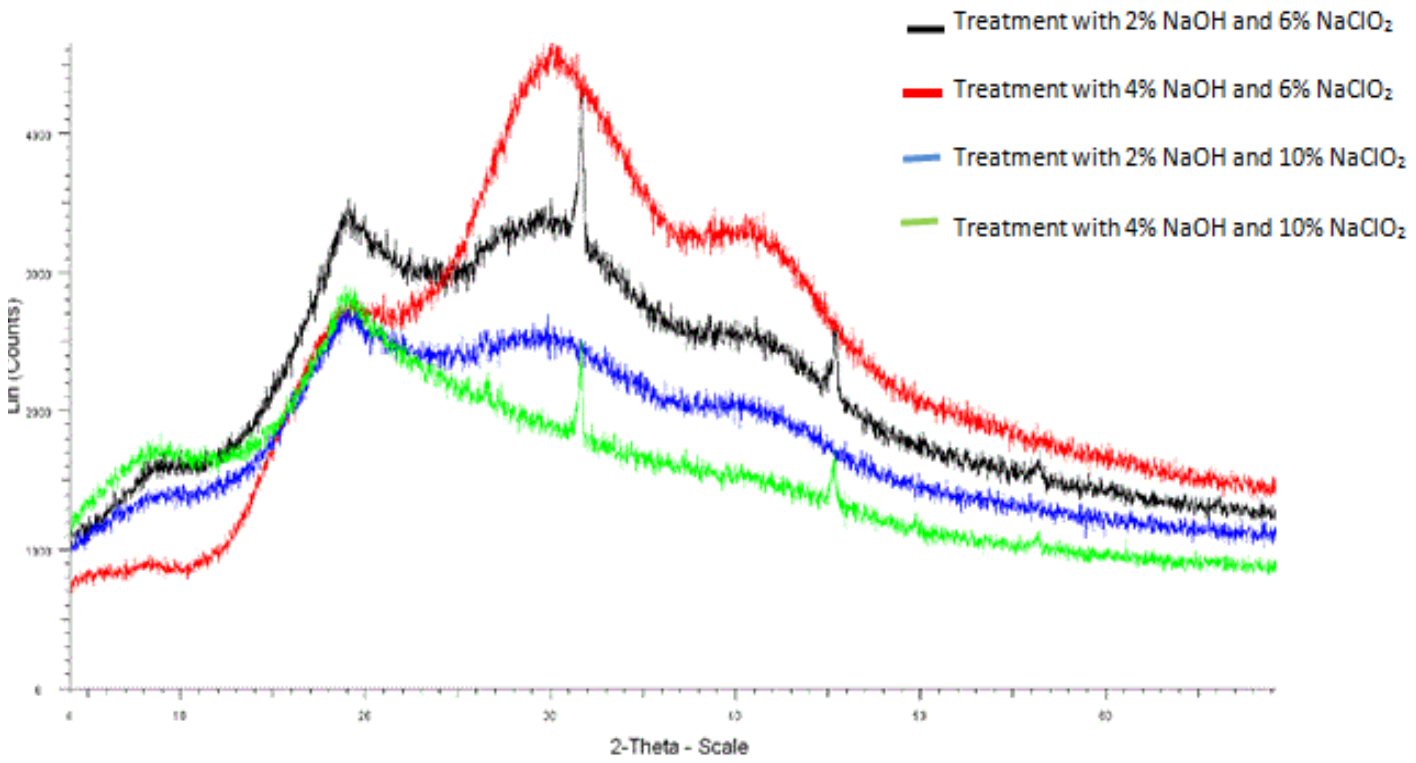


Figure 4. X-ray diffractogram of cellulose extracted from 2 and 4% of NaOH followed by bleaching 6 and 10% of $NaClO_2$

The XRD confirmed the hypothesis that the variable of NaOH concentration of 2 to 4% shows an amorphous structure, which becomes increasingly abundant to a maximum concentration of 10% sodium hypochlorite.

Conclusion

N. gaditana is rich in cellulose. This compound would represent 25% of the dry weight of the microalgae. The FTIR analysis of the cellulose extract identified the functional groups of the cellulose and its contaminants. The XRD analysis tracked the structural change (crystalline, amorphous) of the cellulose taken out based on the extraction mode. During extraction, the NaOH is used to remove hemicellulose and the $NaClO_2$ bleaching removes other contaminants. According to the results, 2% NaOH seemed sufficient to extract most of the hemicellulose. An increase to 4% NaOH did not diminish the intensity of the peak 1735 indicative of the hemicellulose carbonyl group. However, the intensity of the functional groups of the cellulose peaks is important. The concentration of 4% NaOH appears to have altered the semi-crystalline structure in amorphous structure making them a more accessible functional group. XRD analysis confirmed the hypothesis that Cellulosic extract obtained in 2% NaOH is semi-crystalline, and at 4% NaOH, the cellulosic extract is amorphous. Similarly, bleaching seemed to affect the cellulosic extract structure. Whereas, 2% NaOH followed by a bleach of 6% $NaClO_2$ cellulosic extract is semi-crystalline. An increase in the concentration to 10% $NaClO_2$ yielded the amorphous structure. These observations were concluded from the FTIR spectra and RXD diffraction patterns. This "amorphous" property has an industrial interest. The functional groups are more accessible and thus esterifiable. However, the cellulosic extract obtained remained contaminated with lipids and proteins. These two compounds are incorporated into the cell wall. Aggressive physicochemical treatment given to remove these contaminants, may damage the fragile spatial structure of the cellulose extracted. The extraction method must be gentle not to alter the structure of cellulose and should be strong enough to remove contaminants. *N. gaditana* is a unicellular organism. Its cell wall is less complex than that of higher plants known for their high lignin. Lignin is the major contaminant in the extraction of cellulose. Cellulose microalgae would be an alternative to replace the cellulose of higher plants. However, the choice of the extraction process is important. Improper process can damage the cellulose structure (Moon et al., 2011).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

The author extends their thanks to Partisano Biotech Company Algeria, for providing the microalgae *N. gaditana* to laboratory AQUABIOR. They are also thankful to Professor BELBACHIR Mohamed for the help, as well as Chemistry Laboratory, Polymers University of Oran 1 - Ahmed Ben Bella, Algeria.

REFERENCES

Alvarez VA, Vazquez A (2006). Influence of fiber chemical modification procedure on the mechanical modification procedure on the mechanical properties and water absorption of MaterBi/sisal fiber composites. Compos. Part A Appl. Sci. Manuf. 37(10):1672-1680

Andersen RA, Brett RW, Potter D, Sexton JP (1998). Phylogeny of the Eustigmatophyceae based upon 18SrRNA gene, with emphasis on Nannochloropsis. Protist 149:61-74.

Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (209). Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsisoculata and Chlorella vulgaris for biodiesel production. J. Chem. Eng. Process. 48:1146-1151

Bondioli P, Della Bella L, Rivolta G, ChiniZittelli G, Bassi N, Rodolfi L, Casini D, Prussi M, Chiaramonti D, Tredici MR (2012). Oil production by the marine microalgae Nannochloropsis sp. F&M-M24 and Tetraselmissuecica F&M-M33. J. Bioresour. Technol. 114:567-672.

Demirbas MF (2011). Biofuels from algae for sustainable development. J.Applied Energy, 88:3473-3480.

Gaudinski JB, Dawson TE, Quideau S, Schuur EA, Roden JS, Trumbore SE, Sandquist DR, Oh SW, Wasylishen RE (2005). Comparative analysis of cellulose preparation techniques for use with 13C, 14C, AND 18O isotopic measurements. J. Anal. Chem. 77:7212-7224.

Gouveia L (2011). Microalgae as a Feedstock for Biofuels. Springer, London. pp.1-69.

Hibberd DJ (1981). Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae (synonym Xanthophyceae). Bot. J. Linn. Soc. 82:93-119.

Morán JI, Alvarez VA, Cyras VP, Vázquez A (2008). Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose 15:149-159.

Karlson B, Potter D, Kuylenstierna M, Andersen RA (1996). Ultrastructure, pigment composition, and 18S rRNA gene sequence for *Nannochloropsisgranulata* sp. nov. (Monodopsideaceae, Eustigmatophyceae), a marine ultraplankter. Phycologia 35:253-260.

Krienitz L, Hepperle D, Stich HB, Weiler W (2000). *Nannochloropsislimnetica* (Eustigmatophyceae), a new species of picoplankton from freshwater. Phycologia 35:219-227.

Leavitt SW, Danzer SR (1993). Method of batch processing small wood samples to holocellulose of stable-carbon isotope analysis. Anal. Chem. 65:87-89.

Lubian LM (1982). Nannochloropsis gaditana spec. nov., una nueva Eustigmatophyceae marina. Lazaroa 4:287-293.

Moon RJ, Martini A, Nairn J, Simonsen J, Youngblood J (2011). Cellulose nanomaterials review: structure, properties and nanocomposites. Chem. Soc. Rev. 40(7):3941-3994.

Nelson ML, O'Connor RT (1964). Relation of certain infrared bands to cellulose crystallinity and cristal lattice type. Part II: a new infrared ratio for estimation of crystallinity in cellulose I and II. J. Appl. Polym. Sci. 8(3):1328-1341

Oh SY, Yoo DI, Shin Y, Seo G (2005). FTIR analysis of celluloses treated with sodium hydroxide and carbon dioxide. J. Carbohydr. Res. 340:417-428.

Pal D, Khozin-Goldberg I, Cohen Z, Boussiba S (2011). The effect of light, salinity, and nitrogen availability on lipid production by Nannochloropsis sp. Appl. Microbiol. Biotechnol. 90:1429-1441.

Rodolfi L, Chini-Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, Tredici M (2009). Microalgae for oil: strain selection,induction of lipid synthesis and outdoor mass cultivation in a low cost photobioreactor. J. Biotechnol. Bioeng 102:100-112.

Scholz MJ, Weiss TL, Jinkerson RE, Jing J, Roth R, Goodenough U, Posewitz MC, Gerken HG (2014). Ultrastructure and composition of the *Nannochloropsisgaditana* cell wall. Eukaryot. Cell 13(11):1450-

1464.
Starkenburg SR, Kwon KJ, Jha RK, McKay C, Jacobs M, Chertkov O, Twary S, Rocap G, Cattolico RA (2014). A pangenomic analysis of the *Nannochloropsis*organellar genomes reveals novel genetic variations in key metabolic genes. BMC Genomics 15:212.
Suda S, Atsumi M, Miyashita H (2002). Taxonomic characterization of a marine *Nannochloropsis*species, *N. oceanica*sp. nov. (Eustigmatophyceae). Phycologia 41:273-279.
Yang H, Yan R, Chen H, Dong HL, Zheng C (2007). Characteristics of hemicelluloses, cellulose and lignin pyrolysis. Fuel 86:1781-1788.

academicJournals

Vol. 15(24), pp. 1207-1216, 15 June, 2016
DOI: 10.5897/AJB2016.15215
Article Number: 3105BFF58864
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of different microencapsulation materials on stability of *Lactobacillus plantarum* DSM 20174

Lulwah Y. Al-Furaih[1], Ibtisam M. Ababutain[1]*, Azzat B. Abd-El-Khalek[2] and Ahmed M. Abdel-Salam[3]

[1]Department of Biology, College of Science, University of Dammam, P.O. Box 1982, Dammam 31441, Saudi Arabia.
[2]Dairy Science Department, National Research Centre, Dokki, Cairo, Egypt.
[3]Food Science and Human Nutrition Department, College of Agriculture and Veterinary Medicine, Qassim University, P.O. Box 6622 Buraidah 51452 Al-Qassim, Saudi Arabia.

Received 8 January, 2016; Accepted 23 May, 2016

The aim of this work was to investigate the effect of different microencapsulation materials on the stability of probiotic bacterium (*Lactobacillus plantarum* DSM 20174). Microencapsulation methods with alginates were carried out using sodium chloride, canola oil, olive oil, and chitosan. The recorded data showed that the encapsulated probiotic bacterium was more stable compared with free cells. Olive oil capsules recorded the highest stability at pH 2 after incubation period of 24 h with stability up to 0.00004%. Olive oil and chitosan capsules showed stability with high concentration of bile salts (0.5%) with stability percent of 82 and 65% respectively, after 2 h of incubation. Sodium chloride and chitosan capsules gave the best stability percent of 0.026 and 0.00005%, respectively, at heat treatment up to 65°C for 30 min. Storage treatment at 4°C for 17 days reduced the stability of all capsule types, whereas sodium chloride and chitosan capsule showed stability percent up to 59 and 56%, respectively.

Key words: Microencapsulation, *Lactobacillus plantarum,* olive oil and alginate.

INTRODUCTION

Probiotic bacteria are described by the World Health Organization (WHO) as “live organism, which when administered in adequate amounts confer health benefits to the host” (FAO/WHO, 2002). Probiotic can provide beneficial effects on the human body by keeping the healthy gut microflora, inhibiting the growth of pathogenic bacteria, relieving constipation, stimulating the immune system, synthesizing vitamins working as antimicrobial agents, and improving the absorption of calcium, when there are enough probiotic in colon (Rokka and Rantamäki, 2010). To produce such beneficial effects, probiotics have to be able to survive and multiply in the host. In this regard, probiotics should be metabolically stable and active in the product, survive passage through the stomach and reach the intestine in large amounts (Laparra and Sanz, 2010). In fact, there are still, a number of problems related to the low survival of probiotic bacteria under gastrointestinal conditions, pH, hydrogen peroxide, oxygen and storage (Martín et al., 2015).

Recent research indicates that the microencapsulation

*Corresponding author. E-mail: Iababutain@uod.edu.sa or Dr.king2007@hotmail.com.

of probiotic cells presents one of the most promising and efficient techniques for the enhancement of probiotic survival (Chávarri et al., 2008). Microencapsulation can be defined as a process in which cells are retained within an encapsulated membrane to reduce cell injury or cell lose, in a way that results in appropriate microorganism release in the gut (Sultana et al., 2000).

To enhance the efficacy of probiotic, micro-encapsulation has been introduced by entrapping cells into a polymer matrix (Dianawati et al., 2013; Dong et al., 2013). Alginate has been widely used as a micro-encapsulation material as it is cheap, non- toxic and compatible with most other materials. It is also able to absorb water quickly (Rowley et al., 1999), which assists in gel formation. Sodium alginate is a generally regarded as safe (GRAS) material certified by FDA (George and Abraham, 2006). Coating alginate capsules containing probiotic with chitosan has been shown to promote cell protection and enhance the effectiveness of encapsulation either in food and beverages (Nualkaekul et al., 2012; Brinques and Ayub, 2011; Krasaekoopt et al., 2006).

Whereas probiotic are living cells, the conditions for implementation of this technology are designed to maintain cell viability. In fact, selecting the encapsulation technology is very important. Whereas probiotic are living cells, the conditions for implementation of this technology are designed to maintain cell viability. Therefore, the main aim of this work was to study the effect of different microencapsulation materials on stability and survival of *Lactobacillus plantarum* DSM 20174 during cold storage, low pH, bile salt and temperature.

MATERIALS AND METHODS

Preparation of bacterial culture

L. plantarum DSM 20174 obtained from Deutsche Sammlung von Mikroorganismen DSM und Zellkultturen-GmbH, Germany, was reactivated on De Man, Rogosa, Sharpe broth (MRS) for 2 times at 30°C for 24 h before use. The cells were harvested by centrifugation (Optima L-100XP, Beckman Preparative Ultra-centrifuge, Shanghai, China) at 5,000 rpm for 20 min, at 4°C. Then bacterial cells were washed twice using 1.0% peptone solution and re-suspended in 5 ml of 0.1% peptone solution.

Microencapsulation technique

The microencapsulation technique of *L. plantarum* DSM 2017 with alginates was carried out using sodium chloride, canola oil, olive oil and chitosan.

Extrusion microencapsulation (EM)

Microencapsulation using alginate with sodium chloride

Following the procedure of Klinkenberg et al. (2001), suspension of bacterial cells was mixed with an equal volume of sodium alginate. The mixture was passed through a syringe into 60 ml sterile solution consisting of a mixture of 20 ml sodium chloride (0.5%) and 40 ml calcium chloride (0.05%) and homogenized until alginate beads were formed. The beads were left for 30 min to harden at room temperature, then washed twice with 1.0% peptone water solution and stored in a refrigerator (4°C) until further use.

Microencapsulation using alginate and vegetable oil

To form beads, an amount of bacterial cell suspension was mixed with sodium alginate solution, vegetable oil (Canola or olive oil) and Tween 80 solution at ratio 3:3:1:0.5, respectively (Klinkenberg et al., 2001). Then the mixture was dropped through a syringe into 60 ml calcium chloride solution (0.5%). The beads were left to harden at room temperature as mentioned earlier and stored for further use.

Microencapsulation using alginate and chitosan

Alginate beads previously prepared as mentioned earlier were transferred to 100 ml of Chitosan solution and mixed gently using magnetic stirrer at a speed of 100 rounds for 50 min (Krasekoopt et al., 2006). The resulting alginate coated-chitosan beads were washed twice with peptone water solution (1.0%) and stored at 4°C until further use.

Capsules morphology

Morphology of capsules was studied using photographic camera (Nikon D7000), light microscopy (Model SZ61, Olympus) following Chan et al. (2011) and scanning electron microscopy as described by Alaş et al. (2010).

Stability of the encapsulated bacterium under different conditions

Dismantling the capsules

To determine the viable count of trapped *L. plantarum* DSM 20174, cells were released from the microcapsules using the method of Kim et al. (2008). The released bacterial cells were plated on MRS agar plates using ten-fold dilutions and incubating at 30°C for 24 to 48 h. The viable population in terms of colony forming units (CFU) per gram of the sample was counted according to Stukus (1997), the experiment was conducted in triplicate.

Survival of free and encapsulated bacterium in low pH

The pH of MRS growth medium was adjusted to pH 2 and 3 according to Lo et al. (2004). The media were inoculated separately with 1% of the encapsulated and free bacterial cells. Plates were incubated at 30°C for 0 to 24 h. The encapsulated cells were periodically released from the capsules and the total CFU count per g was performed. Comparison between the released bacterial cell count and free cells were recorded. The experiment was conducted in triplicate.

Survival of free and encapsulated bacterium at high concentrations of bile salts

Resistance to bile salts was determined according Lo et al. (2004)

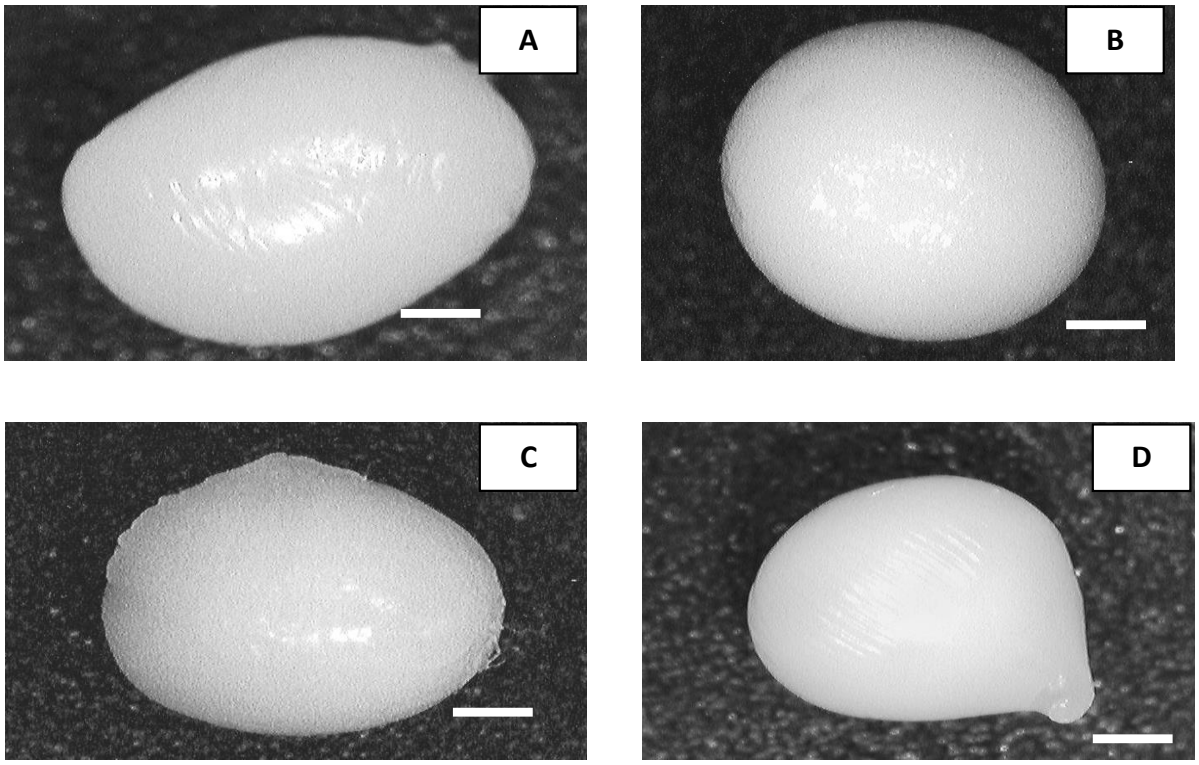


Figure 1. Photograph using Nikon D7000 Camera: (A) Alginate with sodium chloride capsule showed oval shape, (B) Alginate capsule coated with canola oil showed spherical shape with smooth surface, (C) Alginate capsule coated with olive oil showed semi spherical shape with rough surface, (D) Alginate capsule coated with Chitosan showed drop shape with Shell-like surface. Bar marker represents 2 mm.

by inoculating 1% free and microencapsulated cells separately to MRS broth containing 0.3 and 0.5% of Oxgall bile salts. Then samples were withdrawn after incubation at 30°C for 0, 2 and 4 h to determine cell count of *L. plantarum* DSM 20174. Comparison between the released bacterial cell count and free cells were recorded. The experiment was conducted in triplicate.

Survival of free and encapsulated cells after heat treatments

Encapsulation was assessed in terms of viable cell protection efficiency during thermal processes according to Kim et al. (2008). In brief, 1 g of broth free and microencapsulated *L. plantarum* DSM 20174 were assayed for heat resistance at 65°C for different periods: 0, 15, and 30 min with MRS broth as a suspending medium. After the end of each heat treatment, samples were cooled down to room temperature (25°C) using water bath. The survived of free and encapsulated *L. plantarum* DSM 20174 were enumerated in triplicate in MRS agar plates as previously described.

Viability of free and encapsulated cells under refrigerated conditions

The viability of both encapsulated and free *L. plantarum* DSM 20174 cells were monitored by counting the CFU/ml after 17 days of storage at 4°C according to Hou et al. (2003). Survivals of free and encapsulated *L. plantarum* DSM 20174 were enumerated after 0, 4, 7, 11, 14 and 17 days. The experiments were also performed in triplicates.

Statistical analysis

The results were reported throughout the experiments as mean ± standard deviation. Statistical analysis of the data was conducted using analysis of variance (ANOVA) and t-test, Version 17 of SPSS. Values $P \geq 0.05$ were considered statistically significant.

RESULTS

Microencapsulation using alginate with sodium chloride

Capsules of alginate with sodium chloride photograph appear as oval shape (Figure 1A). Scanning electron microscopy photographs of the capsules showed smooth and uniform round declines surface (Figure 2A). Dimensions of the capsules ranged between 2120 and 2646 µm by using Optical microscope (Figure 3A) and the sphericity factor (SF) was 0.169±0.227 (Table 1).

Microencapsulation using alginate and vegetable oil

Alginate capsule coated with canola oil

Capsules of alginate coated with canola oil photograph

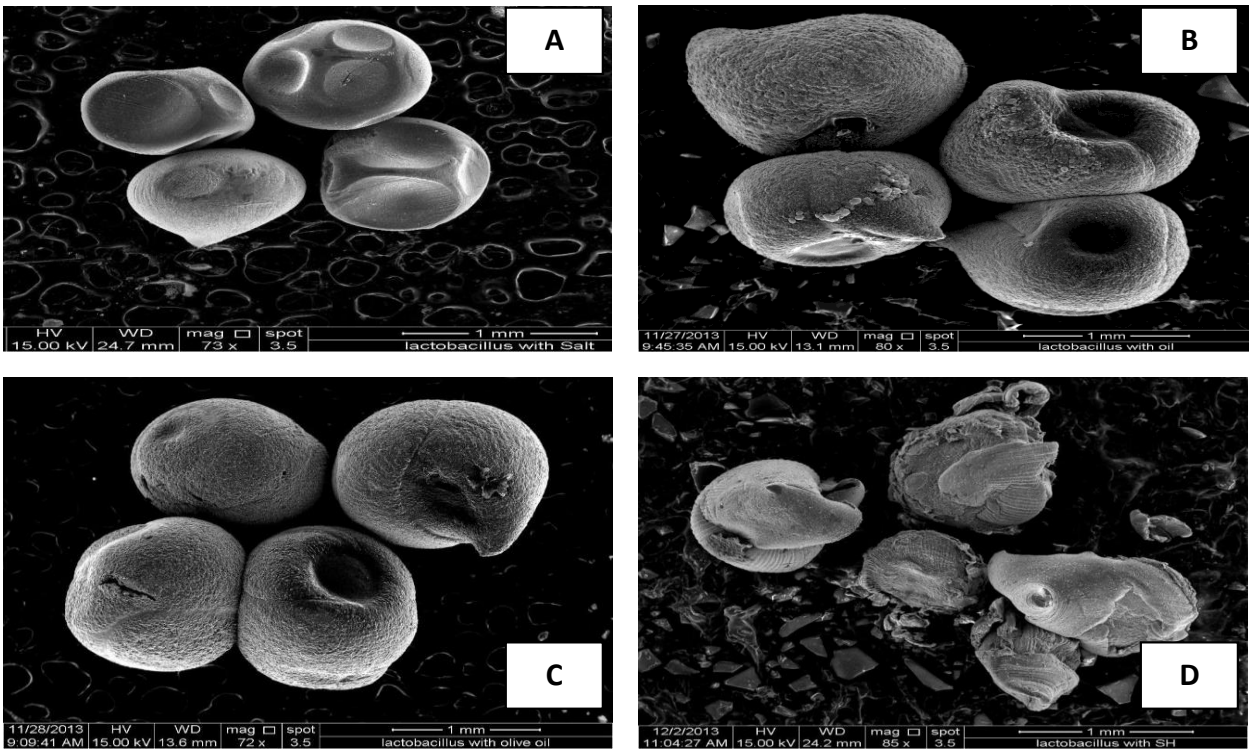


Figure 2. Scanning electron microscopy photographs showed: (A) Alginate with sodium chloride capsules size 73x, (B) Alginate capsules coated with canola oil size 80x, (C) Alginate capsule coated with olive oil size 72x, (D) alginate capsules coated with chitosan size 85x.

appear as spherical shape (Figure 1B). Scanning electron microscopy photographs of the capsules showed coarsely appears as some bacterium cells appear on the surface with a round declines from the middle (Figure 2B). Dimensions of the capsules ranged between 1991 and 2398 µm by using Optical microscope (Figure 3B) and the SF was 0.024±0.023 (Table 1).

Alginate capsules coated with olive oil

Capsules of alginate coated with olive oil photograph appear as spherical shape with curvy surface (Figure 1C). Scanning electron microscopy photographs of the capsules showed rough and round declines in the center (Figure 2C). Dimensions of the capsules ranged between 1988 and 2447 µm by using Optical microscope (Figure 3C) and the SF was 0.061±0332 (Table 1).

Microencapsulation using alginate and chitosan

Capsules of alginate coated with chitosan photograph appear as drop shape (Figure 1D). Scanning electron microscopy photographs of the capsules showed rough and shell-like broken-rough surface also shows grooves resembling shells notes lack of resistance to the capsule settings imaging electron microscope and appeared in a distorted manner (Figure 2D). Dimensions of the capsules ranged between 2214 and 1970 µm by using Optical microscope (Figure 3D) and the SF was 0.050±0.069 (Table 1).

Stability of the encapsulated *L. plantarum* DSM 20174 under different conditions

Stability at low pH

The stability of free and encapsulated *L. plantarum* DSM 20174 under low pH conditions (pH 2 and 3) is as shown in Figure 4. At pH 2 free cells of *L. plantarum* DSM 20174 and all capsules were not stable except alginate capsules coated with olive oil (Fig. 4A). While at pH 3 showed slightly effect on the stability of all samples, however, there were differences in the percentage of stability and it was in descending order: alginate with sodium chloride, alginate coated with chitosan, alginate coated with olive oil, and alginate capsules coated with canola oil (84, 63, 58, 56, and 49%), respectively.

Stability at high concentrations of bile salts

The effect of bile salt on the stability of free and encapsulated *L. plantarum* DSM 20174 is as shown in

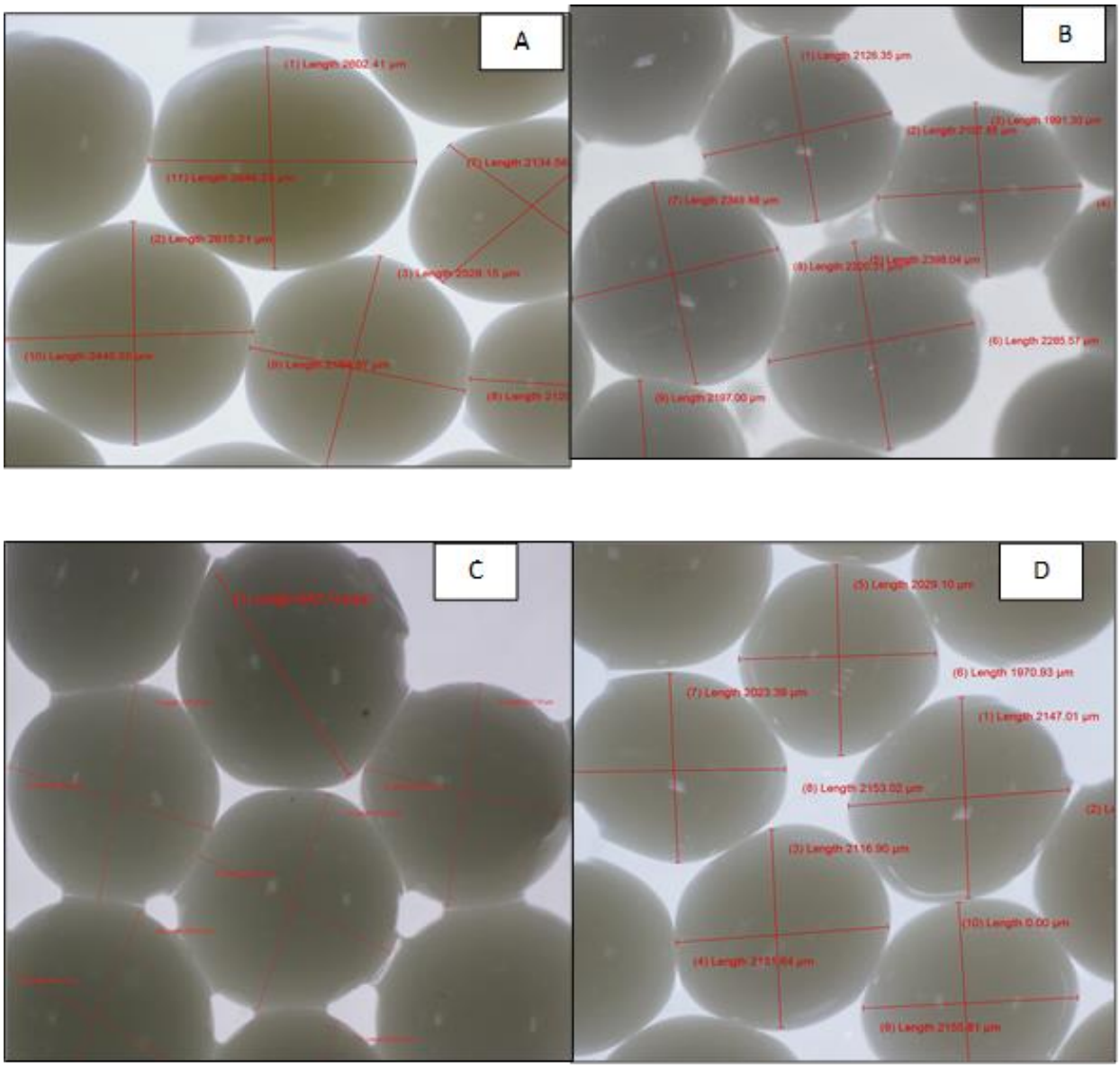


Figure 3. Optical micrograph image using Optical microscope at magnification 40X: (A) Alginate with sodium chloride capsules the dimensions ranged between 2120 and 2646 µm, (B) Alginate capsules coated with canola oil the dimensions ranged between 1991 and 2398 µm, (C) Alginate capsule coated with olive oil the dimensions ranged between 1988 and 2447µm, (D) Alginate capsule coated with chitosan the dimensions ranged between 1970 and 2214 µm.

Figure 5. The stability of encapsulated cells was significantly higher than the free cells after incubation time of 2, 4 and 24 h with 0.3 and 0.5% of bile salts concentrations. It was noted at concentration of 0.3%, bile salts slightly affect the stability of all samples after incubation for 2 h, which arranged in descending order: alginate capsules with sodium chloride, alginate capsules with olive oil, alginate capsules with chitosan, alginate with canola oil, and free cells (99, 87, 67, 54 and 50%), respectively. The results showed that the increase in the concentration of bile salts (0.5%) reduced the stability of all the samples after incubation for 2 h and the stability percentage was arranged in descending order: alginate capsules with olive oil, alginate capsules with chitosan, free cells, alginate capsules with sodium chloride, and alginate with canola oil (82, 67, 48, 43, and 32%), respectively. The results showed that the increasing of incubation period up to 4 h reduced the harmful impact of bile salts (0.3 and 0.5%). Whereas increasing the incubation period up to 24 h diminished the stability for most of the samples except the alginate with canola oil (36%), alginate with olive oil (83%) at the concentration of 0.3%, and alginate with olive oil (41%) at the concentration of 0.5%.

Table 1. Microencapsulation analysis.

Alginate capsules with:	Largest diameter (µm)	Smallest diameter (µm)	Sphericity Factor (SF)	Shape	Surface	Strength
Sodium chloride	2646	2120	*0.169±0.227	Oval	Smooth and uniform round decline	Semi-solid
Canola oil	2398	1991	*0.024±0.023	Spherical	Rough and round decline in the center	Soft
Olive oil	2447	1988	*0.061±0332	Spherical	Rough and round decline in the center	Soft
Chitosan	2214	1970	*0.050±0.069	Drop	Shell-like broken rough	Soft

*The mean difference is significant at the 0.05 level. ±Standard deviation calculated from result of five independent experiments.

Stability at heat treatment

In order to investigate the efficacy of the microcapsules in protecting *L. plantarum* DSM 20174 against heat, the stability of free and microencapsulated cells was evaluated after exposure to temperature of 65°C for 0, 15 and 30 min (Figure 6). The free cells were more sensitive to heat shock than the microencapsulated cells at 65°C, in which free cells were completely lost after 15 min of exposure to heat treatment. The microencapsulated cells varied in their stability after 15 min of incubation. The stability percentage was arranged in descending order: alginate capsules with sodium chloride (7%), alginate capsules with chitosan (0.0000048%), and finally alginate with vegetable oil (canola or olive oil) was 0.00017 and 0.00043%, respectively. Whereas alginate with sodium chloride and chitosan were resistant to heat treatment up to 30 min incubation period with stability percentage of 0.00026 and 0.0018%, respectively.

Stability at cold storage

Cell free *L. plantarum* DSM 20174 and micro-encapsulated cells were stored at refrigerator temperatures (4°C) and their stability was determined over 17 days period (Figure 7). After 14 days of storage at 4°C, the stability of alginate with chitosan was 94% and alginate with olive oil was 74%, while stability of alginate with canola oil reduced to 50%. Whereas stability of free cells and alginate capsules with sodium chloride is almost similar at 63 and 60%, respectively. Stability after 17 days decreased between 40 and 55% for all microencapsulation forms and free cells.

DISCUSSION

Microencapsulation techniques were applied to *L. plantarum* DSM 20174 and extrusion technology was used in all applications of micro-encapsulation. The present study showed the difference between the forms of capsules, size, surface and textures depending on the difference between materials used in the encapsulation process. The wet capsules were smooth texture and ranged in size between 1970 and 2646 µm, while after drying the surface became wrinkled. These results are in agreement with Kim et al. (2008) and Ma et al. (2008) who found out that dry capsules of alginate coated with chitosan were spherical in shape with a curly and wrinkle surface and explained that the drying process have direct role in the formation of curly surface.

In order to exert positive health effects, probiotic should resist the stress conditions of the stomach. The results of the current study showed a significant difference in the stability of the capsules with olive oil compared to free cells and other types of capsules in pH 2, while stability percentage varied at pH 3. In general, the alginate coated with olive oil showed a good stability under low pH conditions (pH 2 and 3) which may be considered as a new application in the field of functional foods industries. The results of this study were supported by Hou et al. (2003) who mentioned that the survival rate of bacteria encapsulated with alginate coated with sesame oil, increased up to 10^4 in pH 2 compared to free cells. Also, microencapsulation technique using alginate with three types of chitosan at different molecular weight improved the survival of *L. bulgaricus* KFRI in acidic media, despite the intensity of sensitivity of this bacterium to acids (Lee et al., 2004).

Moreover, Mokarram et al. (2009) showed that the survival of encapsulated *L. acidophilus* cells with alginate was significantly better than free cells; they explained that the multiplicity of the layers may have a role in the stability of bacterial cells.

They were found to outperform double layers' capsules followed by a single layer then free cell.

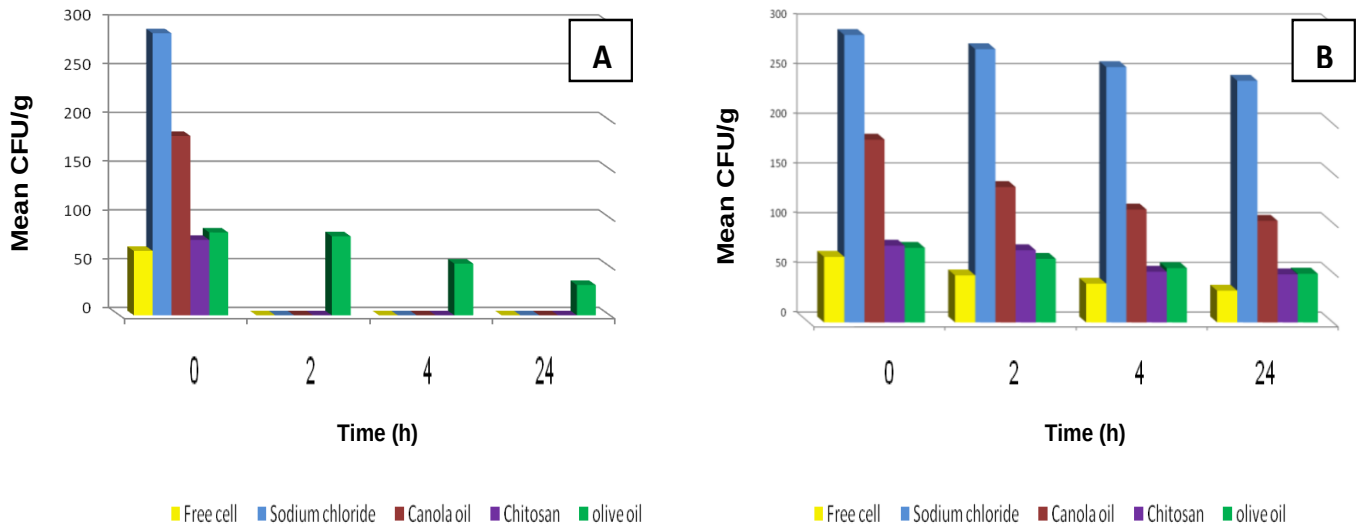


Figure 4. The stability of free and encapsulated *L. plantarum* DSM 20174 at different low pH by using pour plate count method (A) pH 2 and (B) pH 3.

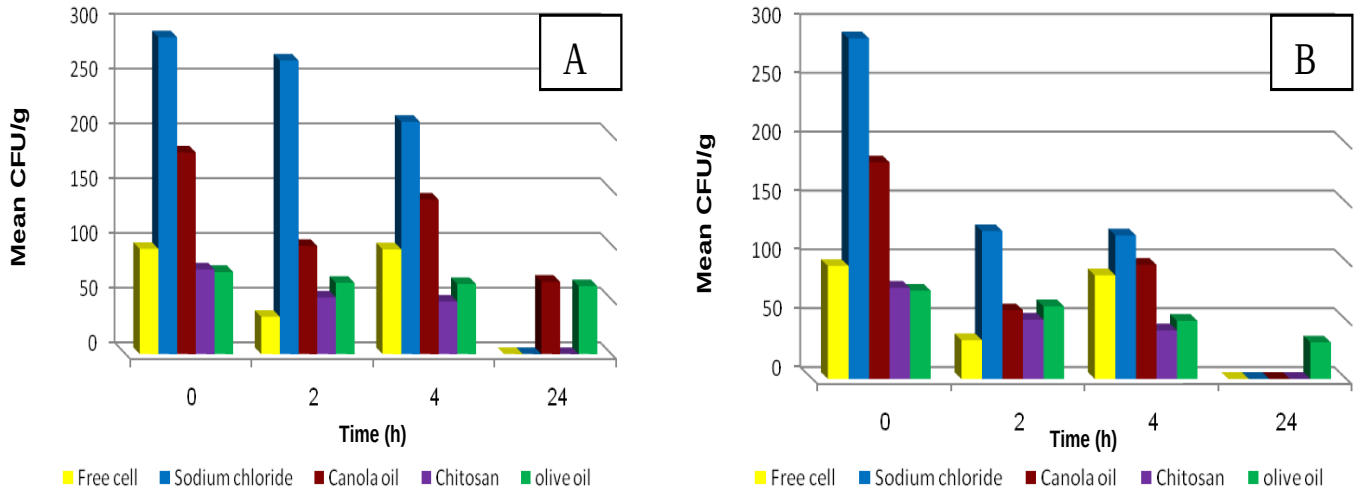


Figure 5. The stability of free and encapsulated *L. plantarum* DSM 20174 at different high concentrations of bile salts by using pour plate count method (A) 0.3% and (B) 0.5%.

Our study supported by a recent foundation of Oana (2014) who found that pH 1.5 negative impacts the stability of free cells of *L. plantarum*, when compared with encapsulated *L. plantarum* cells with alginate coated with chitosan which showed a high survival rate. Annan et al. (2008) also found that alginate capsules of *Bifidobacteria adolescentis* did not give good protection at pH 2, because gelatin were structurally unstable in simulated gastric juice due to degradation by pepsin and completely disintegrated after 45 min.

The present study has shown that there disparity in the stability of capsules at high concentration of bile salt (0.3 and 0.5%). All capsules form high stabile at 0.3 and 0.5% after 2 and 4 h, whereas diminished after 24 h except alginate coated with chitosan and alginate coated with olive oil at 0.3% and alginate coated with olive oil at 0.5%. This finding is in agreement with Kim et al. (2008) who found that alginate coated with chitosan is more stable than the free cells of *L. acidophilus* ATCC 43121 at concentrations 0.3 and 0.5% bile salt.

Due to the importance of heat treatment in the pasteurization of food for the purpose of controlling pathogenic bacteria, which will affect the efficacy of beneficial probiotic, microencapsulation has been proven

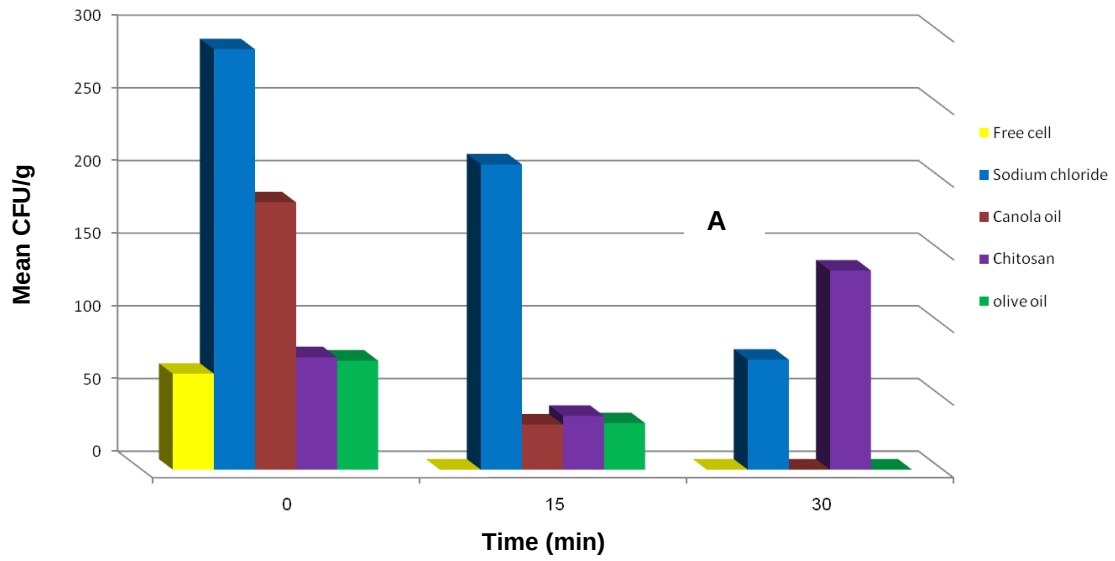


Figure 6. The stability of free and encapsulated *L. plantarum* DSM 20174 at heat treatment at (65ºC) by using pour plate count method.

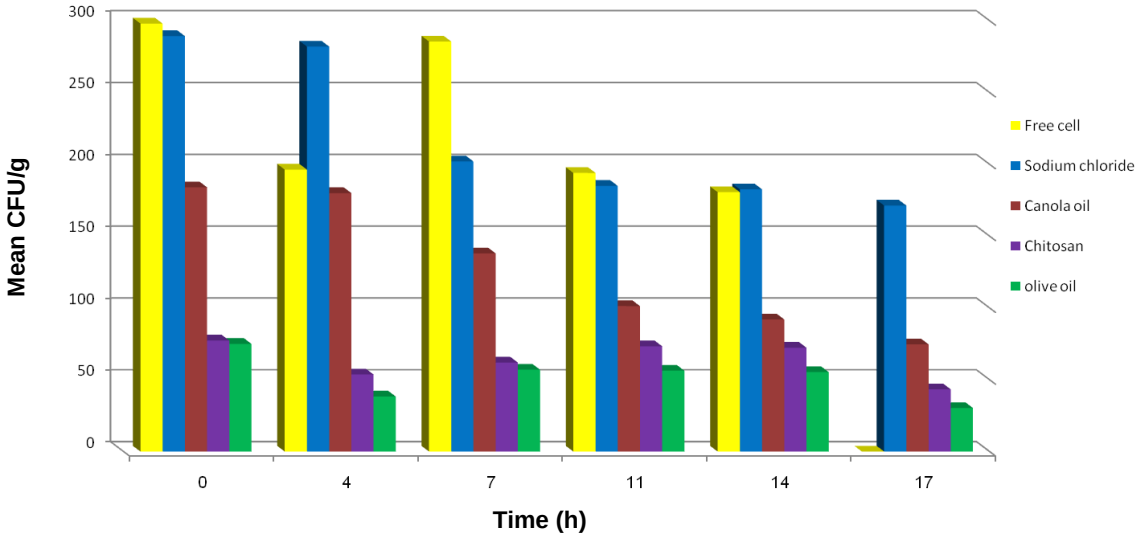


Figure 7. The stability of free and encapsulated *L. plantarum* DSM 20174 during cold storage at (4°C) by using pour plate count method.

to be one of the most efficient methods for maintaining viability and stability of probiotic, as it protects probiotic during food processing (Peck et al., 2011).

The present study showed that high temperature negatively affected free cells and completely lost during exposure to a temperature of 65°C for 15 min. The reason of short duration of thermal resistance may be due to the fact that microencapsulation methods temporarily protect bacterial cells by reducing the heat transfer from the surrounding medium to the inside of the capsule as mentioned by Ding and Shah (2008). The results of the current study are consistent with several researches who found that bacteria encapsulated more stable in heat treatment than free cells (Kim et al., 2008; Chen et al., 2007; Mandal et al., 2006).

Food storage has an important role in the preservation during transportation, and being one of the most important parameters that regulates the activities of microorganisms in food systems (Doleyres and Lacroix, 2005). Therefore, the present study was done to estimate the stability of encapsulated and free cells of *L. plantarum* DSM 20174 when exposed to 4°C for different periods. It

was found out that encapsulation has a role in increasing the stability of the bacterium cells and the results also showed that there is a difference in the stability depending on the material of microencapsulation. Our results were in agreement with Nualkaekul et al. (2012) who found that multiple layers of capsules, chitosan coated alginate, enhanced the stability of *L. plantarum* cells during storage in pomegranate juice at 4°C for 6 weeks. In the study of Woraharn et al. (2010), it was observed that encapsulation of *L. plantarum* with sodium alginate and calcium alginate provided protection after storage for 5 days at 4°C. They also found that sodium alginate capsules were better than calcium alginate as materials for encapsulation. Furthermore, our results showed that the type of oil used in the encapsulation have a direct role in the stability of the capsules under storage conditions. This observation was supported by Hou et al. (2003) who found that using sesame oil encapsulation of *L. delbrueckii bulgaricus* improved the survival of encapsulated cells when stored at a temperature of 4°C for 16 days significantly from 0.023 to 5.45% compared to the free cells. However, the results are inconsistent with the study of Lee et al. (2004) who found that both free and encapsulated cells showed similar stability within 4 weeks of storage at 4°C.

Conclusion

The encapsulation methods of *L. plantarum* DSM 20174 with alginates were carried out using sodium chloride, canola oil, olive oil, and chitosan. Materials for encapsulation impact the form of the resultant capsules which varied in terms of size, texture, shape and hardness. Data showed that the encapsulated probiotic bacterium was more stable and viable compared with free cells and alginate capsules coated with olive oil was the only one recorded stability at pH 2. This material has not been used before, which may be considered as a novel material. The alginate capsules coated with chitosan and olive oil enhanced the stability at concentration 0.5% of bile salts for 2 h of incubation. Alginate capsules with sodium chloride gave the highest stability at 65°C for 15 and 30 min. Moreover, encapsulation may enhance the survival of probiotic bacterium such as *L. plantarum* DSM 20174 in fermented food during cold storage.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank the College of Science, University of Dammam for the opportunity to work in a professional research environment at the research units and for providing facilities for the work. They also thankful to Mr. Badar Alsaqer, coordinator of the Electron Microscope Unit at Institute for Research and Medical Consultations in University of Dammam.

REFERENCES

Alaş A, Öktener A, Solak K (2010). A study on the morphology of Argulus foliaceus Lin., 1758 Crustacea; Branchiura procured from Çavuşcu Lake Central Anatolia-Turkey with scanning electron microscopy. Turk. J. Biol. 34:147-151.

Annan N, Borza A, Hansen LT (2008). Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. Food Res. Int. 41:184-193.

Brinques GB, Ayub MAZ (2011). Effect of microencapsulation on survival of Lactobacillus plantarum in simulated gastrointestinal conditions, refrigeration and yogurt. J. Food Eng. 103:123-128.

Chan ES, Wong SL, Lee PP, Lee JS, Ti TB, Zhang Z, Poncelet D, Ravindra P, Phan SH, Yim ZH (2011). Effects of starch filler on the physical properties of lyophilized calcium-alginate beads and the viability of encapsulated cells. Carbohydr. Polym. 83:225-232.

Chávarri M, Marañón R, Ibáñez FC, Marzo F, Villarán MDC (2008). Nutritional modulation of colon cancer with a microencapsulated symbiotic. International Conference on Bioencapsulation, Dublin, Ireland. Sept 4-6.

Chen MJ, Chen KN, Kuo YT (2007). Optimal thermotolerance of Bifidobacterium bifidum in gellan–alginate microparticles. Biotechnol. Bioeng. 98:411-419.

Dianawati D, Mishra V, Shah NP (2013). Stability of microencapsulated Lactobacillus acidophilus and Lactococcus lactis ssp. cremoris during storage at room temperature at low aw. Food Res. Int. 50:259-265.

Ding WK, Shah NP (2008). Survival of Free and Microencapsulated Probiotic Bacteria in Orange and Apple Juices. Int. Food Res. J. 15:219-232.

Doleyres Y, Lacroix C (2005). Technologies with free and immobilized cells for probiotic bifidobacteria production and protection. Int. Dairy J 15:973-988.

Dong QY, Chen MY, Xin Y, Qin XY, Cheng Z, Shi LE (2013). Alginate-based and protein-based materials for probiotics encapsulation: A review. Int. J. Food Sci. Technol. 48:1339-1351.

FAO/WHO (2002). Guidelines for the Evaluation of Probiotics in Food, Food and Agriculture Organization of the United Nations/World Health Organization, London, UK.

George M, Abraham TE (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan – A review. J. Control Release 114:1-14.

Hou R, Lin M, Wang M, Tzen J (2003). Increase of viability of entrapped cells of Lactobacillus delbrueckii bulgaricus in artificial sesame oil emulsions. J. Dairy Sci. 86:424-428.

Kim SJ, Cho SY, Kim SH, Song OJ, Shin IS, Cha DS, Park, HJ (2008). Effect of microencapsulation on viability and other characteristics in Lactobacillus acidophilus ATCC 43121. Food Sci. Technol. 41:493-500.

Klinkenberg G, Lystad K, Levine D, Dyrset N (2001). pH-controlled cell release and biomass distribution of alginate-immobilized *Lactococcus lactis* subsp. lactis. J. Appl. Meteorol. 91:705-714.

Krasaekoopt W, Bhandari B, Deeth H (2006). Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. Food Sci. Technol. 39:177-183.

Laparra JM, Sanz Y (2010). Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol. Res. 61:219-225.

Lee J, Cha D, Park H (2004). Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. J. Agric. Food Chem. 52:7300-7305.

Lo PR, Yu RC, Chou CC, Huang EC (2004). Determinations of the

antimutagenic activities of several probiotic Bifidobacteria under acidic and bile conditions against benzo pyrene by a modified Ames test. Int. J. Food Microbiol. 93:249-257.
Ma Y, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A, Sabour PM (2008). Microencapsulation of bacteriophage felix O1 into chitosan-alginate microspheres for oral delivery. Appl. Environ. Microbiol. 74:4799-4805.
Mandal S, Puniya A, Singh K (2006). Effect of alginate concentration on survival of microencapsulated *Lactobacillus casei* NCDC. Int. Dairy J. 16:1190-1195.
Martín MJ, Villoslada FL, Ruiz MA, Morales ME (2015). Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. Innov. Food Sci. Emerg. Technol. 27:15-25.
Mokarram R, Mortazavi S, Najafi M, Shahidi F (2009). The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. Food Res. Int. 42:1040-1045.
Nualkaekul S, Lenton D, Cook MT, Khutoryanskiy VV, Charalampopoulos D (2012). Chitosan coated alginate beads for the survival of microencapsulated Lactobacillus plantarum in pomegranate juice. Carbohydr. Polym. 90:1281-1287.
Oana LP (2014). Development of innovative systems for probiotics encapsulation, with applications in biomedicin. PhD Thesis Abstract, University of agricultural sciences and veterinary medicine cluj-napoca.
Peck LT, Mirhosseini SH, Mustafa S, Manap A, Yazid M (2011). Tolerance of free and encapsulated probiotics towards heat treatment and high sodium concentration. J. Food Agric. Environ. 9:69-73.
Rokka S, Rantamäki P (2010). Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. Eur. Food Res. Technol. 231:1-12.
Rowley JA, Madlambayan G, Mooney DJ (1999). Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 20:45-53.
Stukus PE (1997). Investigating microbiology: a laboratory manual for general microbiology: Saunders College Publishing. New York, 143p.
Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K (2000). Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int. J. Food Microbiol. 62:47-55.
Woraharn S, Chaiyasut C, Sirithunyalug B, Sirithunyalug J (2010). Survival enhancement of probiotic *Lactobacillus plantarum* CMU-FP002 by granulation and encapsulation techniques. Afr. J. Microbiol. Res. 4:2086-2093.

academicJournals

Vol. 15(24), pp. 1217-1223, 15 June, 2016
DOI: 10.5897/AJB2015.15131
Article Number: 0AF3F3858866
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Potential biosurfactant producing endophytic and epiphytic fungi, isolated from macrophytes in the Negro River in Manaus, Amazonas, Brazil

João Marcelo Silva Lima[1]*, José Odair Pereira[1,2], Ieda Hortêncio Batista[3], Pedro de Queiroz Costa Neto[2], Jucileuza Conceição dos Santos[1], Solange Pires de Araújo[4], Mozanil Correia Pantoja[2], Adolfo José da Mota[2] and João Lúcio de Azevedo[1,5]

[1]Rede Bionorte de Biodiversidade e Biotecnologia/Universidade Federal do Amazonas (UFAM), Manaus, AM, Brasil.
[2]Faculdade de Ciências Agrárias/UFAM, Manaus, AM, Brasil.
[3]Universidade do Estado do Amazonas, Manaus, AM, Brasil.
[4]Programa Multi-Institucional de Pós-Graduação em Biotecnologia/UFAM, Manaus, AM, Brasil.
[5]Escola Superior de Agricultura "Luiz de Queiroz"/Universidade de São Paulo, Piracicaba, SP, Brasil.

Received 27 November, 2015; Accepted 23 May, 2016

Endophytic and epiphytic fungi isolated from *Eichhornia crassipes* (Mart.) Solms and *Cyperus ligularis* L., macrophytes collected from oil-contaminated waters, were studied to assess their potential for producing biosurfactants; the most promising ones were identified by means of the rDNA region sequencing. In the selection, in the hydrocarbonate biodegradation activity, 2.6-indophenol (DCPIP) in oil-added Bushnell-Haas (BH) medium was the indicator used. The following tests were performed to ascertain the biosurfactant, bioemulsifier activity: emulsification measurement, drop-collapse, surface tension and production slope. Of the twenty fungi isolated, six promoted DCPIP discoloration. The isolate (S31) *Phoma* sp. showed emulsification of diesel (1.5 cm or 52%) and reduction of the surface tension of 51.03 mN/m water identified as *Phoma* sp. The other five fungi were identified as *Rhizopus oryzae* (S24), *Fusarium* sp. (S32, S33, S42, S46), presenting potential for biodegradation of hydrocarbons, as well. New studies on *Phoma* sp. (S31), including its cultivation in different carbon sources will be necessary to improve the production of secondary compounds involved in surface tension bioemulsification and reduction.

Key words: Bioremediation, bioemulsifiers, *Eichhornia*, *Cyperus*, oil, diesel.

INTRODUCTION

Biosurfactants may be of microbial origin and show potential for commercial applications in several fields. These products are shown to be efficient in processes of microbial enhanced oil recovery and bioremediation in

*Corresponding author. E-mail: Jlima873@gmail.com. Tel: 55 92 3237-4672.

hydrocarbon-contaminated environments. They also possess potential applications in agriculture, raw materials for cosmetics, pharmaceutical products, detergents, personal hygiene products and food processing, among others (Sourav et al., 2015). They are more advantageous than the synthetic-derived ones when considering their biodegradability and low toxicity.

Biosurfactants are biologically produced from various substrates, such as waste from tropical agronomic cultures, food processing industries, fruit processing industries, petroleum processing, and coffee processing industries (Bento et al., 2008; Sourav et al., 2015). Given that biosurfactants are produced by bacteria, yeasts and filamentous fungi, they may be produced by endophytic and epiphytic fungi as well. Endophytic microorganisms, according to Azevedo (2008), live inside a plant, at least for one period of their life cycle, as opposed to the epiphytic microorganisms that live on a plant's surface.

Curvularia clavata, *Fusarium proliferatum* and *Phoma* sp. isolated from different hydrocarbon-contaminated environments are the major species of the genera referred to as biosurfactant producers. For the biodegradation of hydrocarbons, genera *Cladophialophora* and *Exophiala* assimilate toluene; *Aspergillus* sp. and *Penicillium* spp. degrade aliphatic hydrocarbons, aliphatic hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons (PAHs), pesticides, synthetic dyes and 2,4,6-trinitrotoluene (TNT) (Harms and Schlosser, 2011; Bhardwaj et al., 2015; Neoh et al., 2015). Fungi are able to grow under environmental stressed conditions: environments with low pH, poor in nutrients, and low water availability media favor their growth (Mollea et al., 2005). They may still, though more rarely, produce biosurfactants (Luz et al., 2011).

This study is an important contribution to identifying fungal strains collected from similar environments in the Amazon region. These strains can further be exploited commercially, both in the production of new bioemulsifiers or biosurfactants, as well as biodegradation of contaminated oil environments, and open a range of new possibilities for research with these microorganisms, notably with regard to their physiology in producing amphiphilic compounds. The purpose of this work was to assess the potential of endophytic and epiphytic fungi, for the biodegradation of hydrocarbons and production of biosurfactants or bioemulsifiers.

MATERIALS AND METHODS

Samples and biological material

The isolates studied are from the collection of cultures of the Biodegradation Laboratory of the Agronomic Sciences College, Federal University of Amazonia. The microorganisms were isolated from the macrophytes: *Cyperus ligularis* L. and *Eichhornia crassipes* (Mart.) Solms collected near the effluent output from a Petrobras/Manaus-AM (REMAN) refinery, and stored according to Castellani (1939). Plant species were identified at the herbarium of the National Research Institute of Amazonia – INPA.

Oil

Crude oil was from the Urucu Oil Base, Amazonas, Brazil, and the diesel was acquired at a gas station, and previously filtered using millipore membrane (0.22 mm) in order to sterilize it. Oil and diesel doses were used according to Jaquiche-Matsuura et al. (2014).

Biodegradability test using the redox 2,6-dichlorophenol indophenol (DCPIP) indicator

The biodegradability test was performed through the DCPIP technique (Hanson et al., 1997). The experiment was carried out in a 96-well polystyrene plate and DCPIP concentration was adjusted to 0.010 g/mL. 200 µL of the DCPIP solution, 10 µL of oil from Urucu and hyphaes of fungi grown in BH + oil, inoculation corresponding to 3 mm of the diameter, were added to each well. The plates were kept at room temperature (27±2°C). Medium discoloring-time measurements were taken following 24 and 48-h. DCPIP with oil and without strain was used as a positive control and DCPIP without oil and without strain was used as negative control.

Specific medium for biosurfactant production

Biosurfactant production was undertaken in 50 mL of culture medium composed of $MgSO_4.7H_2O$ (0.5 g/L), KH_2PO_4 (1 g/L), $NaNO_3$ (3 g/L), yeast (1 g/L) and peptone (0.3 g/L) extract, with pH adjusted at 5 for filamentous fungi, modified by Rapp and Backhaus (1992). Diesel oil or oil at 1.0% v/v was used as carbon source. The diesel was used in biosurfactant production or bioemulsifier because their carbon chains are less complex than the oil initially used.

Microbial culture of isolate (S31) was carried out in 125-mL Erlenmeyer flasks at 30°C in an orbital incubator (New Brunswick Scientific) with 150 rpm steady stirring for 20 days. Erlenmeyer flasks with 50 mL of culture medium and 1% diesel oil (v/v), with no inoculation, was used as control. Every microorganism was cultured in triplicate. Following the incubation period, the culture media were filtered in filtering membrane (TPP, Europe/Switzerland) with 0.45 mm porosity coupled with a 20-mL sterilized syringe.

Oil drop-collapse qualitative test

The test was conducted in 60 x 12 mm Petri dishes containing 3.5 mL of filtered cell-free extract. To carry out the test, an oil drop was added to the cell-free extract in triplicate and observed for 0, 1, 5, 30 min, 1 and 72 h. The result was regarded positive when the oil drop dispersed. A total of 3.5 mL fungus-free extract and 3.5 mL 1 M dodecyl sulfate sodium (DSS) surfactant solution were used as negative and positive control, respectively.

Emulsification activity assessment

The specific medium for the production of biosurfactants was filtered and assessed as the water in oil (W/O) type, emulsification. The test was performed in triplicate. 3 mL of the cell-free culture extracts and 2 mL of diesel were added to test tubes. These were vortexed for 2 min at 70 rpm and kept at rest for 24 h. Following this period, the emulsified oil height (cm) was compared with the total. The emulsification was calculated according to Equation 1:

$$E_{24} = \frac{He}{Ht} \times 100$$

Table 1. Molecular identification of endophytic and epiphytic fungi isolated from macrophytes in the Negro River-Amazonas/Brazil, with the deposition number, National Center for Biotechnology Information (NCBI) data as reference.

Iso*	Microorganisms	NCBI Sequences	Hosts	Endo.*	Epi.*	Id.*(%)
S24	*Rhizopus oryzae*	KU948381	*Cyperus ligularis*		X	99
S31	*Phoma* sp.	KU948382	*C. ligularis*		X	100
S32	*Fusarium* sp.	KU948385	*Eichhornia crassipes*		X	99
S33	*Fusarium* sp.	KU948386	*C. ligularis*		X	98
S42	*Fusarium* sp.	KU948384	*C. ligularis*		X	99
S46	*Fusarium* sp.	KU948383	*C. ligularis*	X		99

*Iso = Isolate; Endo. = endophytic; Epi. = epiphytic; Id. = identity.

Where, E_{24} = Emulsification index following 24 h (%); $E_{mulsion}$ = emulsion height; H_t = total height.

Diesel emulsion production slope

For the production slope, the specific medium for the production of biosurfactants and diesel was used at 1% (v/v) as carbon source, by maintaining pH 5. Into each 50-mL Erlenmeyer flask, was added, as inoculation, five 5-mm disks of the fungus culture through 150 rpm steady stirring in an orbital incubator (New Brunswick Scientific) at 30°C. The emulsification index was measured by using Equation 1 every 48 h for a total of 14 triplicate measurements. The statistical analysis was performed through standard deviation means calculated with the *software* BioEstat 5.3 (Ayres et al., 2007).

Surface tension assessment

Surface tension is a common metric and direct method for monitoring the production of biosurfactants. As the microorganism grows, it synthesizes the biosurfactant and this metabolite is excreted to the metabolic broth, reducing the surface tension. The surface tension was measured in the Kruss model tensiometer (K-6, Germany), by the ring method (Du Noüy). The analyses were performed with the supernatant obtained after the raw sample centrifugation at 25°C. Every time, the analyses began, the ring was sterilized by gas burner and calibrated by checking the distilled water surface tension whose value is about 72.8 mN/m. Three measurements of surface tension were made, considering the arithmetic mean of the results (Jaquiche-Matsuura et al., 2014).

Molecular identification of filamentous fungi

Molecular identification was performed only on samples that presented a positive result in the indicator test with redox 2,6-dichlorophenol indophenol (DCPIP) containing oil and on those with a result above 1 cm for emulsification index. For the extraction of the DNA, the plant/fungi DNA isolation kit (Norgen Biotek Corp) was used according to the manufacturer's instructions.

Primers ITS1 (5' – TCCGTAGGTGAACCTGCG G – 3') and ITS4 (5' – TCCTCCGCTTATTGATAT GC – 3') were used for the amplification of the region kept in specific positions of the 18S and 28S of the rDNA gene. The amplicons were purified with polyethylene glycol 8000 and sequenced in an ABI 3500xL genetic analyzer (Applied Biosystems®). Sequences were aligned and edited in a MEGA program with grouping by neighbor-joining method and, employed for identifying isolates by comparing themselves with the type sequences based on the results found in BLASTn.

Toxicity test using *Artemia salina*

The aqueous extract of *Phoma* sp. selected as the producer of bio emulsifier was used to evaluate the toxicity front larvae *Artemia salina*. Toxicity assay was performed with *A. salina* according to Meyer et al. (1982) by preparing a solution with sea-salt in the concentration of 30 $g.L^{-1}$. The pH was adjusted to 8 with NaOH 0.1 mol/L^{-1} solution. This solution was used in the preparation of the remaining dilutions. The eggs were placed to hatch in a saline solution for 48 h, with steady aeration at 25°C.

The test was conducted in multi-welled plates, by using the cell-free extract concentrations: 100, 50, 25, 13, 7 and 3 µL; ten *A. salina* larvae, with 2000 µL being the final value. After 24 h, mortality was determined with the aid of a stereoscopic binocular microscope (Leica El 224). The LC_{50} was calculated according to Finney (1947).

RESULTS

Isolate molecular identification

Eight fungi selected by growth in oil-added BH medium were identified through sequencing part of the rDNA gene. Its fragments containing the ITS1-5,8S-ITS2 region were presented between 531 and 562 bp. Epiphytic fungi S31 and S36 isolated from *C. ligularis* were identified as *Phoma* sp.; S24 *Rhizopus oryzae*; S32 *Fusarium* sp.; S33 *Fusarium* sp.; and S42 *Fusarium* sp. Among endophytic fungi, S42 was identified as *Fusarium* sp., both originating from *E. crassipes*. The sequences were deposited in the National Center database for Biotechnology Information and are shown (Table 1).

Among the eight analyzed fungi, only *Phoma* sp. (S31), was efficient in the tests undertaken to select biosurfactant producers. Though, they had dispersed an oil drop at different times, the remaining isolates formed no emulsion above 1 cm, according to the method

Table 2. Selection of endophytic and epiphytic fungi isolated from macrophytes in the Negro River– Amazonas/Brazil for the production of biosurfactants.

	Isolates	Oil drop-collapse						Emulsification index	Surface tension (mN/m)
		0	1 min	5 min	30 min	1 h	72 h	H_u/E_{24}	
(S31)	*Phoma* sp.	+	+	+	+	+	+	1.5 cm/52%	51.03
(S24)	*Rhizopus oryzae*	-	-	±	±	±	±	0	-
(S46)	*Fusarium* sp.	-	-	-	-	-	-	0	-
(S42)	*Fusarium* sp.	-	+	+	+	+	+	0	-
(S32)	*Fusarium* sp.	-	-	±	±	±	±	0	-
(S33)	*Fusarium* sp.	-	±	±	±	±	±	0	-
	Control								
	DSS	+	+	+	+	+	+	100%	
	BH+FFO	-	-	-	-	-	-	0	53.03 mN/m
	Water								71.26 mN/m

*DSS = Dodecyl sodium sulfate was used as positive control ; BH+FFO = fundus free oil medium used as negative control; H_u = emulsified height; E_{24} = emulsification index in 24 h. ± = low collapse, + = high collapse.

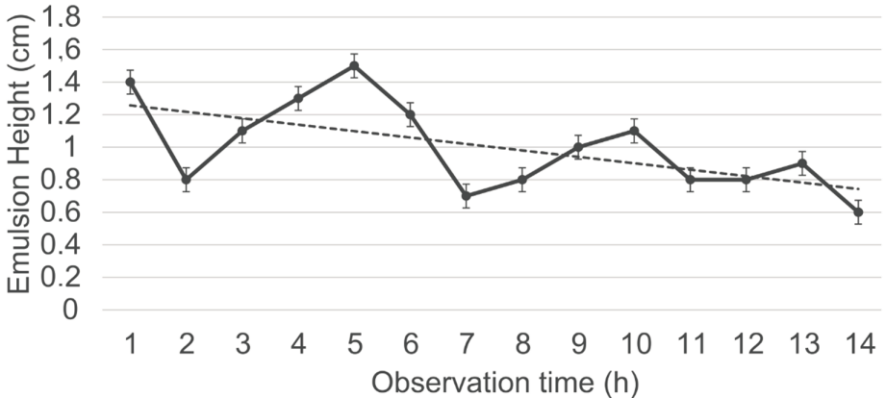


Figure 1. Bioemulsifier production slope of the endophytic fungus *Phoma* sp. (S31) isolated from *Cyperus ligularis* L. occurring in an oil-contaminated area in the Negro River Amazonas State, Brazil.

adopted (Table 2).

Only *Phoma* sp. (S31) was selected for surface tension test, since it was positive in the oil drop-collapse test sooner and its bioemulsifier was above 1 cm. The surface tension initially observed was 51.03 mN/m. The difference between fungus extract and control surface tensions was 2 mN/m, yet when it was added to water, it was 18.23 mN/m, indicating it would be able to break surface tension. To ascertain the fungus extract-promoted diesel emulsification, a production slope was performed, taking emulsified height-fungus growth relativeness into account (Figure 1). The slope shows bioemulsifier production occurred up to the 14th measurement, yet it peaked at the 5th or 10th day following its growth, forming 1.5 cm high emulsion. This variation continued until the very end of the experiment. The cell-free aqueous extract of *Phoma* sp. (S31), showed no toxicity with *A. salina* in the concentrations used (Figure 2).

DISCUSSION

Oil with BH medium-grown isolate behavior may be analyzed according to Jaques et al. (2007), Maciel et al. (2013) and Cruz et al. (2014), wherein, both fungi and bacteria may be hydrocarbon biodecomposers. Micro-organisms shown to be successful in degrading these compounds should produce enzymes able to use complex oil molecules in their metabolic pathways' intermediate products. In this case, fungi had non- and ligneous metabolic pathways, like the fungi *Cunninghamella elegans*, *Pleurotus ostreatus, Aspergillus fumigatos* and *C. lunata*, which have been already studied as effective decomposers of oil and its derivatives (Bhatt et al., 2010).

Junior et al. (2012) identified *Phoma* genus fungi as good producers of laccase, a phenol group enzyme able to biodegrade phenolic compounds and hydrocarbons.

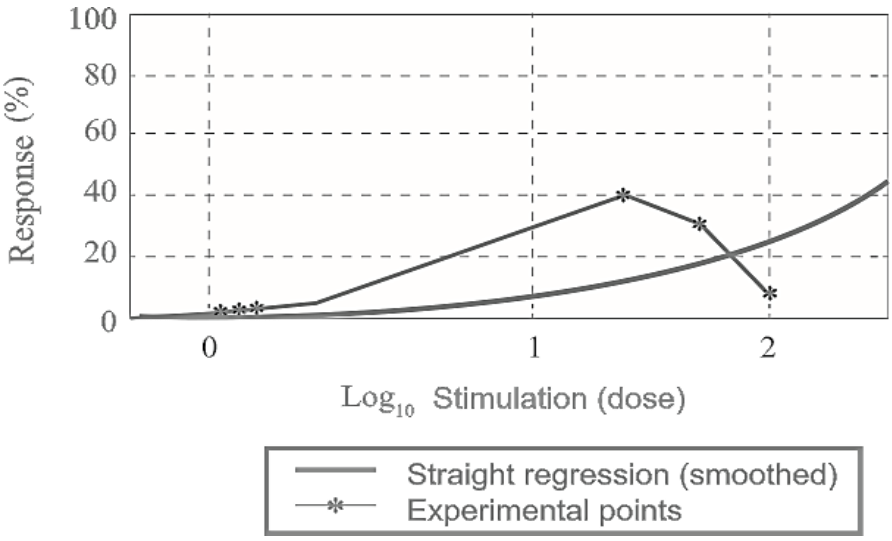


Figure 2. Probit analysis of the six cell-free *Phoma* sp. (S31) fungal extract concentrations: 100, 50, 25, 13, 7 and 3 µL.

Carneiro and Lucas (2010) confirmed this view in an important study on bioremediation, using microorganisms in the metabolization of several compounds, including oil.

Harms and Schlosser (2011) reported *Rhizopus* genus cultures to be potential HPA decomposers; this, in some regard, may account for this genus species presence in selecting tests using oil-added BH medium (Table 1). Balaji et al. (2014), in a study done in India, cited nine genera fungi such as, *Aspergillus*, *Curvulari*a, *Drechslera*, *Fusarium*, *Lasiodiplodia, Mucor, Penicillium, Rhizopus* and *Trichoderma*, isolated from oil-contaminated soil.

The filamentous fungus *C. clavata* was cited by Neoh et al. (2015) to bioremediate palm industry effluents, produce ligneous enzymes, in addition to dramatically reduce polyphenolic compounds. Detoxification of effluent indicates C. *clavata* suitability in the bioremediation of organic effluents, which is important, since it can direct future studies using fungi, identified in the present research in similar processes. With regards to the DCPIP oxidation time, the identified Amazonian fungi findings are consistent with those of Maciel et al. (2013), who found that it takes from 14 to 25 h for these fungi to promote indophenol discoloration. Isolates from macrophytes promoted it within 24 h. In a study by Luz et al. 2011), it took 96 h.

Maciel et al. (2013) pointed out the isolates responsible for degradation to be *Penicillium aurantiogriseum, Penicillium corylophilum* and *Penicillium griseofulvum*. Other species from this genus were identified amongst the isolates from macrophytes as well (Table 2). According to Silva and Esposito (2004), the degradation of pollutants is performed by the intracellular enzyme cytochrome P450 monooxygenase system, which makes water-soluble products less toxic and leads to a detoxification process. These enzymes are likely involved in this process. These authors reported genera *Trichoderma* and *Fusarium* in oil-contaminated soils; *Fusarium* was isolated from macrophytes in an oil-contaminated environment.

Regarding biosurfactant production, though filamentous fungi had grown in oil-added BH medium, as the sole carbon source, most of them were unable to produce it through selective tests such as drop-collapse, emulsification index and surface tension. *Phoma* sp. (S31) was the only one able to produce oil drop-collapse in less than 1 min, showing 1.5 cm or a 52% emulsification index, as compared to oil and diesel; this index is regarded to be moderate. It also decreased surface tension by 51.03 mN/m. The emulsification index of *Phoma* sp. is comparable to the emulsification rate of the bacterium, *Gordonia amicalis* (51%) and *Bacillus licheniformis* (70%) (Dewaliya and Jasodani, 2013). This shows that the crude extract of the selected fungus has potential as an emulsifier.

This fungus has emulsifying properties, yet little ability to reduce surface tension (51.03 mN/m) (Table 2). The low surface tension might be due to the difficulty of using the hydrocarbonate present in diesel oil for the synthesis of biosurfactants (Decesaro et al., 2013). On the other hand, it is likely that, through the tests performed, the biosurfactant present in fungal extract are of high molecular weight, which would indeed account for the diesel oil's low surface tension and stabilizing property (Bento et al., 2008).

The emulsion formed was of the A/O type, suggesting organic compounds present in this emulsifier, possess hydrophilic characteristics due to emulsion formation always occurring between water and diesel oil or oil. This characteristic is paramount in pollutant bioremediation, since it can aid in the bio stimulation or bio augmentation

process, facilitating other microorganisms' physiological growth in the oil hydrocarbonates-contaminated medium (Deon et al., 2012).

Jackisch-Matsuura et al. (2014) cited the difficulty in finding filamentous fungi that are good producers of biosurfactants and have the ability to reduce surface tension, a fact observed in this study. On the other hand, bio emulsifier production by fungi and bacteria should be thoroughly studied, since they may be used in several applications, such as food processing and paint manufacturing industries, among others (Bezerra et al., 2012).

The best bioemulsifier production activity by *Phoma* sp. (S31) occurred on the 10th day. The emulsification index observed in the slope ranged from 0.6 to 1.5 cm. Such emulsification measurements are comparable to those presented by extracts from *Lactobacillus pentosus* used in the bio emulsification of kerosene, gasoline and octane (Moldes et al., 2013) and *F. proliferatum* for n-dodecane compounds (Bhardwaj et al., 2015). From tropical *Phoma* sp., a cyclic-structured lipopeptide compound was identified and classified as *Phomafungy*, which was shown to be efficient as an antifungal in several tests. Apart from the work by Herath et al. (2009), it was not possible to find or cite any other studies of the genus *Phoma* sp. as a biosurfactant or bio emulsifier producer. Consequently, further studies on the *Phoma* sp. (S31) isolate are needed. Through the toxicity test, the aqueous extract from bio emulsifier producing species, showed no toxicity in the different concentrations used. Such findings were also observed by Decesaro et al. (2013), who found no toxic activity for bio emulsifiers from fungi and bacteria tested in their work.

Further studies involving *Phoma* genus species addressing biosurfactant production mainly related to cultivation in different carbon sources are needed to better understand the production of the involved secondary compounds, both on emulsification and reduction of surface tension. The sequence of studies involving *Phoma* sp. (S31) might be promising to different fields of the food, pharmacological and oil industries. The selection of filamentous fungi continues to be a great challenge due to their low biosurfactant yield.

Conclusion

Every identified fungus is shown to be promising in oil and diesel degradation. Thus, further study pertaining to these fungi enzyme yields is necessary, particularly for enzymes of commercial interest, which is related to oil and its derivative biodegradation, as well as on production of biosurfactants by the selected *Phoma* sp. Microorganisms originating from areas with a history of oil contamination and contamination of its derivatives, which possess physiological mechanisms that actually assist them in capturing hydrocarbons used as a carbon source was found in the test carried out in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank CAPES for the help provided in undertaking this project. Academic English Solutions revised the English (http://academicenglishsolutions.com/AES/home.html).

REFERENCES

Ayres M, Ayres Júnior M, Ayres DL, Santos AA (2007). BIOESTAT - Aplicações estatísticas nas áreas das Ciências Bio Médicas. Mamirauá. Belém, PA. P.364

Balaji V, Arulazhagan P, Ebenezer P (2014). Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. J. Environ Biol. 35:3-9.

Bento MF, Camargo FA, Gaylarde CC (2008). Biossurfactantes. In. Melo IS, Azevedo JL. (Ed.) Microbiologia Ambiental. 2. ed. Jaguariúna: Embrapa Meio Ambiente. P.647.

Bezerra MS, Holanda VCD, Amorim JA, Macedo GR, Santos ES (2012). Produção de biotensoativo utilizando *Pseudomonas aeruginosa* (p.a.) e resíduo agroindustrial (manipueira) como substrato. Holos 28:14-27.

Bhardwaj G, Cameotra SS, Chopra HK (2015). Isolation and purification of a new enamide biosurfactant from *Fusarium proliferatum* using rice-bran. RSC Adv. 5:54783-54792.

Bhatt R, Patel K, Trivedi U (2010). Purification and properties of extracellular poly (3-hydroxybutyrate) depolymerase produced by *Aspergillus fumigatus*. J. Polym. Environ.18:141-147.

Carneiro DA, Lucas PG (2010). A biorremediação como ferramenta para a descontaminação de ambientes terrestres e aquáticos. Rev. Tecer 3:82-95.

Castellani A (1939). Viability of some pathogenic fungi in distilled water. J. Trop. Med. Hyg. 24:270-276.

Cruz JM, Tamada IS, Lopes PR (2014). Biodegradation and phytotoxicity of biodiesel, diesel, and petroleum in soil. Water Air Oil Pollut. 225(5):1-9.

Decesaro A, Rigon MR, Thomé A, Colla LM (2013). Produção de biossurfactantes por microrganismos isolados de solo contaminado com óleo diesel. Quim. Nova 36:947-954.

Deon MC, Rossi A, De Magro CD, Reinehr CO, Colla LM (2012). Bioremediation of contaminated soil with oils residuals through bioaugmentation and natural attenuation. Semina: Ciências E. e Tecnológicas 33:73-82.

Dewaliya V, Jasodani R (2013). Isolation and identification of bacillus licheniformis for biosurfactant production. CIBTech. J. Microbiol. 2(4):14-19.

Finney DJ (1947). Probit analysis: A statistical treatment of the sigmoid response curve. Oxford: England. 256 p.

Hanson KG, Desai DJ, Desai AJA (1997). Rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol. Tech. 10:745-748.

Harms H, Schlosser DW (2011). Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. 9:177-192.

Herath K, Harris G, Jayasuriya H, Zink D, Smith S, Vicente F (2009). Isolation, structure and biological activity of phomafungin, a cyclic lipodepsipeptide from a Widespread Tropical *Phoma* sp. Bioorg. Med. Chem. 17:1361-1369.

Jackisch-Matsuura AB, Santos LS, Eberlin MN, Faria AF, Grossman MJ, Durrant LR (2014). Production and characterization of surface-active compounds from *Gordonia amicalis.* Braz. Arch. Biol. Technol. 57:138-144.

Junior NL, Gern RMM, Furlan SA, Schlosseret D (2012). Laccase

production by the aquatic ascomycete *Phoma* sp. UHH 5-1-03 and the white rot basidiomycete *Pleurotus ostreatus* DSM 1833 during submerged cultivation on banana peels and enzyme applicability for the removal of endocrine-disrupting chemicals. Appl. Biochem. Biotechnol. 167:1144-1156.

Luz CC, Santos EA, Santos MOS, Mussy MY, Yamashita M, Bastos WR (2011). Estudos de biodegradação de óleo diesel por consórcio microbiano coletado em Porto Velho – RO, Amazônia. Quim. Nova 34:775-779.

Maciel CCS, Souza CS, Silva PA, Sousa MQ, Gusmão NB (2013). Cinética de degradação de querosene de aviação por *Penicillium* sp. através da bioestimulação. R. Bras. Bioci. 11:39-42.

Meyer BN, Ferrigni NR, Putnan JE, Jacobsen LB, Nichols DE, Aughlin J (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Méd. 5:31-34.

Moldes AB, Paradelo R, Vecino X, Cruz JM, Gudiña E, Rodrigues L, Barral MT (2013). Partial characterization of biosurfactant from *Lactobacillus pentosus* and comparison with sodium dodecyl sulphate for the bioremediation of hydrocarbon contaminated soil. BioMed. Res. Int. 2013, Article ID 961842, 6 pages.

Mollea C, Bosco F, Ruggeri B (2005). Fungal biodegradation of naphthalene: Microcosms studies. Chemosphere 60:636-643.

Neoh CH, Lam CY, Lim CK, Yahya A, Ibrahim Z (2015). Utilization of agro-industrial residues from palm oil industry for production of lignocellulolytic enzymes by *Curvularia clavata*. Waste Biomass Valorization 6:385-390.

Rapp P, Backhaus S (1992). Formation of extracellular lipases by filamentous fungi, yeast, and bacteria. Enzyme Microb. Technol.14:938-943.

Silva M, Espósito E (2004). O papel dos fungos na recuperação ambiental. In: Espósito E, Azevedo JL. (Ed.) Fungos: Uma introdução à biologia, bioquímica e biotecnologia. 2. ed. Caxias do Sul: EDUCS. Pp. 337-375.

Sourav D, Susanta M, Ghosh A, Saha R, Saha B (2015). A review on natural surfactants. RSC Adv. 5:65757-65767.

academicJournals

Vol. 15(24), pp. 1224-1232, 15 June, 2016
DOI: 10.5897/AJB2016.15332
Article Number: 39A0E0F58868
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Foot-and-mouth disease (FMD) prevalence and exposure factors associated with seropositivity of cattle in north-central, Nigeria

Wungak, Y. S.[1,2]*, Olugasa, B. O.[1], Ishola, O. O.[1], Lazarus, D. D.[2] and Ularamu, G. H.[2]

[1]Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria.
[2]Foot-and-Mouth Disease Research Laboratory, National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Received 10 March, 2016, Accepted 29 April, 2016.

This study was designed to determine the seroprevalence and risk factors associated with foot-and-mouth disease (FMD) seropositivity in north central, Nigeria. A cross-sectional study was undertaken from February 2013 to April 2014 using 1206 sera from 150 herds collected by multi-stage and random sampling methods. Pre-tested questionnaire were also administered to participating farmers to collect information on the animal herd structure, movement pattern, management system and herds contact at watering points. Samples collected were tested for evidence of FMD antibodies using the 3ABC non-structural antibodies enzyme-linked immunosorbent assay (ELISA). The overall seroprevalence of FMD in North-Central Nigeria was found to be 70.98% (95% CI: 68.37-73.49). FMD seroprevalence was found to be higher in Niger State 85.4% (95% CI: 83.46-88.03) relative to Plateau State 54.2% (95% CI: 50.12-58.16), which was statistically associated with FMD seropositivity (P<0.05). Risk factors such as sex, management system, trans-boundary crossing and herd mixing at the watering point were found to be statistically associated with FMD seropositivity (p<0.05). This confirms that FMD is enzootic in the study area and control of foot and mouth disease in Nigeria using animal movement control and vaccination is therefore advocated.

Key words: Foot-and-mouth disease (FMD), prevalence, endemic, serotypes, enzyme-linked immunosorbent assay (ELISA), antibodies.

INTRODUCTION

Foot and mouth disease (FMD) has been recognized as an important trans-boundary animal disease impacting negatively on the cattle industry since the sixteenth century (Mahy, 2005). FMD is caused by foot and mouth disease virus (FMDV) of the genus *Aphthovirus,* family *Picornaviridae.* Seven distinct serotypes namely: A, O, C, Asia-1, SAT-1, SAT-2, and SAT-3 have been identified. It is known that infection with one serotype does not confer

*Corresponding author. Email: yiltex2@gmail.com. Tel: +234(0)8064563108.

immune protection against another serotype. Different subtypes can be identified within a serotype by biochemical and immunological tests (OIE, 2012). The disease is known to exhibit high fever, loss of appetite, salivation, and vesicular eruptions on the feet, mouth and teats of lactating cows (Thomson, 1995a). FMD has a broad host range, high degree of infectivity, rapid replication rate and multiple transmission routes, which makes it very difficult and expensive to control and eradicate (Alexandersen and Mowat, 2005). The disease has a high morbidity although mortality is low in adult animals. However, myocarditis may occur in young animals resulting in death. The recovered animals may remain in poor physical condition over long periods of time leading to economic losses for livestock industries (Molla et al., 2010).FMD is endemic in most of sub-Saharan Africa, except in a few countries in southern Africa, where efforts were made to control the disease by the separation of wildlife from susceptible livestock using barrier veterinary cordon fencing in combination with prophylactic vaccination (Vosloo et al., 2002). Furthermore, due to the endemicity of the disease, and the fact that FMD does not normally cause high mortality in adult animals, FMD outbreaks are not often perceived as important and are poorly reported or investigated further to determine the causative serotypes. However, this is now changing, a number of countries are now recognizing FMD as one of the most important trans-boundary animal diseases that should be controlled in order to access profitable international markets for livestock and livestock by-products as well as to maximize the full genetic potential of the animals (Ayelet et al., 2009).

There is a dearth of information on the actual situation of FMD in Nigeria and the neighbouring countries. There are regular outbreaks, no national control strategy, no enforcement of legislation for disease reporting to veterinary authorities, and animal movement control are poor. Since most of the cattle populations in Nigeria are from the neighboring countries of West and Central Africa, the animals are at perpetual risk of infection from the endemic strains as well as antigenic variants prevalent in neighboring countries. Studies have shown that, FMD serotypes O, A, SAT 1 and SAT 2 have circulated in Nigeria between 1924 and 2009 (Lazarus et al., 2012; Fasina et al., 2013; Olabode et al., 2013; Nawathe and Goni, 1976; Owoludun, 1971). However, recent sampling conducted between 2007 and 2009 have indicated that despite the endemicity of FMD in Nigeria with its attendant production losses in livestock, very little is known about the epidemiology of FMD in Nigeria (Fasina et al., 2013). The prevalence of FMD can be determined serologically by measuring the antibody level to the 3ABC nonstructural protein (NSP) (De Diego et al., 1997). The objectives of this study were to determine the seroprevalence of FMD and to identify the risk factors associated with seropositivity of FMD in cattle from the north-central Nigeria.

MATERIALS AND METHODS

Study area

The North Central Nigeria is located at the central point of Nigeria (Middle belt). The zone is populated by mostly minority ethnic groups. It is characterized by Guinea savannah and marked by crystalline rock outcroppings and gently rolling hills such as the Jos Plateau. The major or notable rivers in Nigeria, River Niger and Benue meet at the region precisely at Lokoja town in Kogi State. The two major seasons are the raining season from the mouth of April through October and a dry season from November through March. The temperature is also relative from state to state as it is relatively cold weather in Jos Plateau while other states have predominantly hot weather condition. The zone has six states namely: Plateau, Niger, Nassarawa, Kogi, Benue and Kwara states. The geo-political zone has human population of 20,266,256 (Anon, 2013) and cattle population of 2,363,369 (Kogi 367,754, Kwara 66,905, Nassarawa 88,532, Niger 803,013, Plateau 976,029 and Benue 61,136) (GLIPHA, 2011).

The predominant economic activities are farming and fishing as a result of their fertile nature of land and the presence of river Niger and Benue around Kogi, Benue and part of Niger and some other related areas near the riverine environs, mining amongst Jos Plateau people. Because of the abundance of grassland in the zone, it supports a massive population of livestock and serves as the major cattle treks routes to the Eastern and Southern Nigeria. The region also shares International boundaries to the West with Benin Republic through Niger and Kwara states (Felix, 2009).

Study animals and sampling technique for serum collection

Study animals were selected from the animal population in two states of the region, namely, Plateau and Niger states. The states were selected based on their geographical location, proximity with the livestock market, ruminant population density, movement pattern, as well as cattle trek route and International boundary (Figure 1). Individual animals were randomly selected so that about 10% of animals from each herd were sampled to represent the herd, in total 150 cattle herds were sampled by multi-stage and random sampling method in two states of the North-central Nigeria.

The sample size for the seroprevalence study was determined by assuming a prevalence of 56.3% based on a previous study (Ishola et al., 2011). The sample size was determined using a simple random sampling method of Thrusfield (2005) with 95% confidence interval and desired precision of 0.05. The calculated sample size was 378. However, to improve precision, the sample size was increased by 3-fold and a total of 1250 cattle were sampled in this study.

The potential risks factors for FMD in the study area were assessed by a pre-tested structured questionnaire in all the states. The questionnaire was designed to assess the most important factors that could be associated with FMD such as animal location, management system, mixing at the watering point, animal movement pattern and international boundary crossing.

Whole blood was collected from the jugular vein using a 10 ml sterile plain vacutainer tubes and stored overnight at room temperature for serum separation. Each serum was transferred into a sterile cryovials, bearing the age and sex of sampled animal and was transported in a cold box to FMD Research Laboratory, National Veterinary Research Institute, Vom Nigeria, and stored at 20°C until use. FMD seroprevalence was estimated using 3ABC ELISA (Bronsvoort et al., 2006).

Ethical approval

For this type of study, formal consent is not required. All applicable

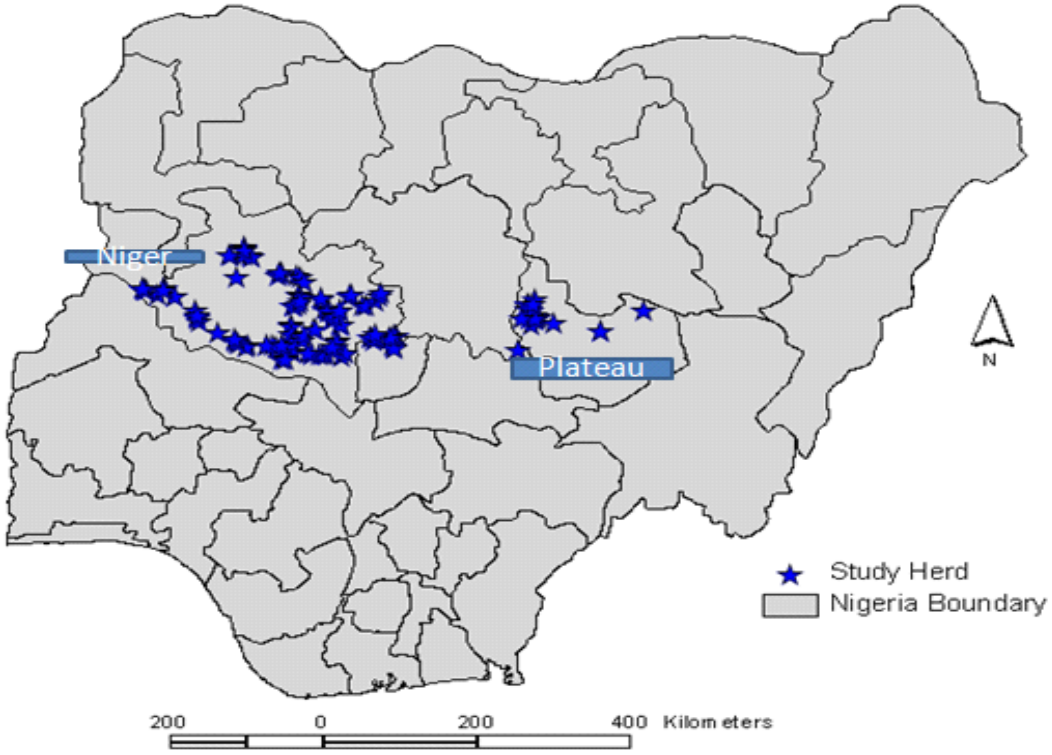


Figure 1. Map of Nigeria showing the distribution of the sampled herds in the North Central Nigeria.

international and national guidelines for the care and use of animals where followed.

Study design

A cross-sectional study was undertaken from February 2013 to April 2014, during which a total of over 1250 blood samples were collected, however, 1206 sera were used for laboratory analysis using 3ABC Non-structural protein ELISA. The questionnaire was designed to collect information on the animal movement pattern, management system and mixing at watering points.

Serum sample collection

Samples were stored overnight at room temperature for serum separation. Each serum was transferred into a sterile cryovial, bearing the age and sex of sampled animal and was transported in an icebox to National Veterinary Research Institute, Vom, Nigeria (NVRI), and stored in freeze at -20°C until analyses. The assay was conducted at the Foot and Mouth Research Centre, National Veterinary Research Institute, Vom, Nigeria.

Detection of antibodies against FMDV non-structural proteins (NSPs) ELISA

All the 1206 sera were subjected to FMD screening using the PRIOCHECK FMD-3ABC NS protein ELISA (NSP-ELISA) (PrioCHECKS® PrionicsLelystad Netherland). The PRIOCHECK FMD-3ABC NS protein ELISA kit is designed to detect FMDV specific antibodies in bovine serum (Sørensen et al., 1998). The test was useful because it was able to discriminate animals that had been infected (wild virus induced antibodies) from those that had been vaccinated with purified vaccine (vaccine induced antibodies). The ELISA serology was performed according to the manufacturer's instructions for (PrioCHECKS® Prionics Lelystad Netherland) (Sørensen et al., 1998). Briefly described, 80 µl of the ELISA buffer and 20 µl of the test sera were added to the 3ABC-antigen coated test plates. Negative, weak positive and strong positive control sera were added to designated wells on each test plate, gently shook and incubated overnight (18 h) at 22°C. The plates were then emptied and washed six times with 200 µl of washing solution and 100 µl of diluted conjugate were added to all the wells. The test plates were sealed and incubated for 60 min at 22°C. The plates were then further washed six times with 200 µl of the washing solution and 100 µl of the chromogen (Tetra-Methyl Benzidine) substrate was dispensed to all wells of the plates and incubated for 20 min at 22°C following which 100 µl of stop solution was added to all the wells and mixed gently. Readings were taken on a spectrophotometer Multiskan® ELISA reader (Thermo Scientific, USA) at 450 nm and the OD450 values of all samples was expressed as percentage inhibition (PI) relative to the OD450 max using the following formula PI = 100 – [OD450 test sample/OD450 max] × 100. Samples with PI = ≥ 50% were classified as positive while those with PI < 50% were declared negative. Since the 3-ABC ELISA for FMD was = 100% specific and > 99% sensitive, the percentage prevalence was taken as true prevalence (Sørensen et al., 1998; Bronsvoort et al., 2006).

Data collection and analysis

The data were stored in Microsoft Excel ® and coded for analysis.

Table 1. Seroprevalence of FMD based on state distribution.

State	Number of sera tested	Number of sera positive	Prevalence (%) (95%CI)
Niger	617	537	85.4 (83.46-88.03)
Plateau	589	319	54.2 (50.12-58.16)
Total	1206	856	70.98

X^2=156.4; p-value= < 0.05.

Table 2. Seroprevalence of FMD based on geographical zones

Geographical zones	Number of sera tested	Seropositivity 3ABC ELISA	Prevalence (%) (95%CI)
Plateau North	215	94	43.12 (37.2-50.4)
Plateau Central	107	59	55.14 (45.64-64.36)
Plateau South	267	166	62.17 (56.24-67.84)
Niger South	195	162	83.08 (77.35-87.86)
Niger East	240	204	85 (80.06-89.1)
Niger North	182	171	93.9 (89.74-96.8)
Total	1206	856	

X^2=184; df = 5; p < 0.05.

Seroprevalence was calculated on the basis of 3ABC ELISA test results. Serological data was subjected to statistical analysis using SPSS (version 13) and Open Epi (Version 2.3.1). Chi-square (χ^2) was used to assess the existence of association with FMD seropositivity. The associations of individual categories of each exposure factor with seropositivity of FMD were analyzed using univariable logistic regression. This univariable analysis assumed all other factors were constant and one category was used as a reference. In all the statistical analysis, confidence interval was set at 95%.

RESULTS

The overall seroprevalence of FMD in north-central Nigeria was found to be 70.98% (95% CI: 68.37-73.49). This was found to be higher in Niger State 85.4% (95% CI: 83.46-88.03) relative to Plateau state 54.2% (95% CI: 50.12-58.16) (Table 1) and the difference in prevalence was statistically associated with FMD seropositivity (P<0.05). Seroprevalence based on different geographical zones revealed that Niger North recorded the highest seroprevalence of 93.9% (95% CI: 89.74-96.8), followed by Niger East 85% (95% CI: 80.06-89.1), Niger South 83.08% (95% CI: 77.35-87.86), Plateau South, 62.17% (95% CI: 56.24-67.84), Plateau Central, 55.14% (95% CI: 45.64-64.36), and the lowest prevalence was recorded in Plateau North, 43.12% (95% CI:37.2-50.4).

The difference in prevalence across geographical zones was statistically associated with seropositivity (χ^2=184, df=5,p-value< 0.05).

Risk factors such as sex, management system, trans-boundary crossing and herd mixing at the watering point were found to be positively associated with FMD seropositivity (p<0.05).

Seroprevalence across geographical zones

Seroprevalence across the six geographical zones are presented in Table 2 and Figure 2 with a higher sero-prevalence of 93.9% (95%CI: 89.74-96.8) for Niger North, while Plateau North recorded the lowest prevalence of 43.12% (95%CI: 37.2-50.4), which was statistically significant (p<0.05).

Seroprevalence based on age category

Age of animals sampled were analyzed in two categories < 2years (Young) and >2 years (Adult). The sero-prevalence in cattle aged >2 years was higher (70.01%) than in cattle aged <2 years (67.7%). The difference in seroprevalence was, however, not statistically associated with the age of the cattle (p<0.05). The odd of FMD seropositivity was relatively more in cattle aged >2 years (1.14) than in cattle aged <2 years old (Table 3).

Seroprevalence based on sex category

Higher disease prevalence was observed more in females (71.9%) relative to the males (30.4%). The difference in prevalence between the two sex groups was found to be statistically significant (χ2 =129.1; p>0.05). The odd ratio of FMD was 4.78% (3.6-6.54) times in females than males (Table 4)

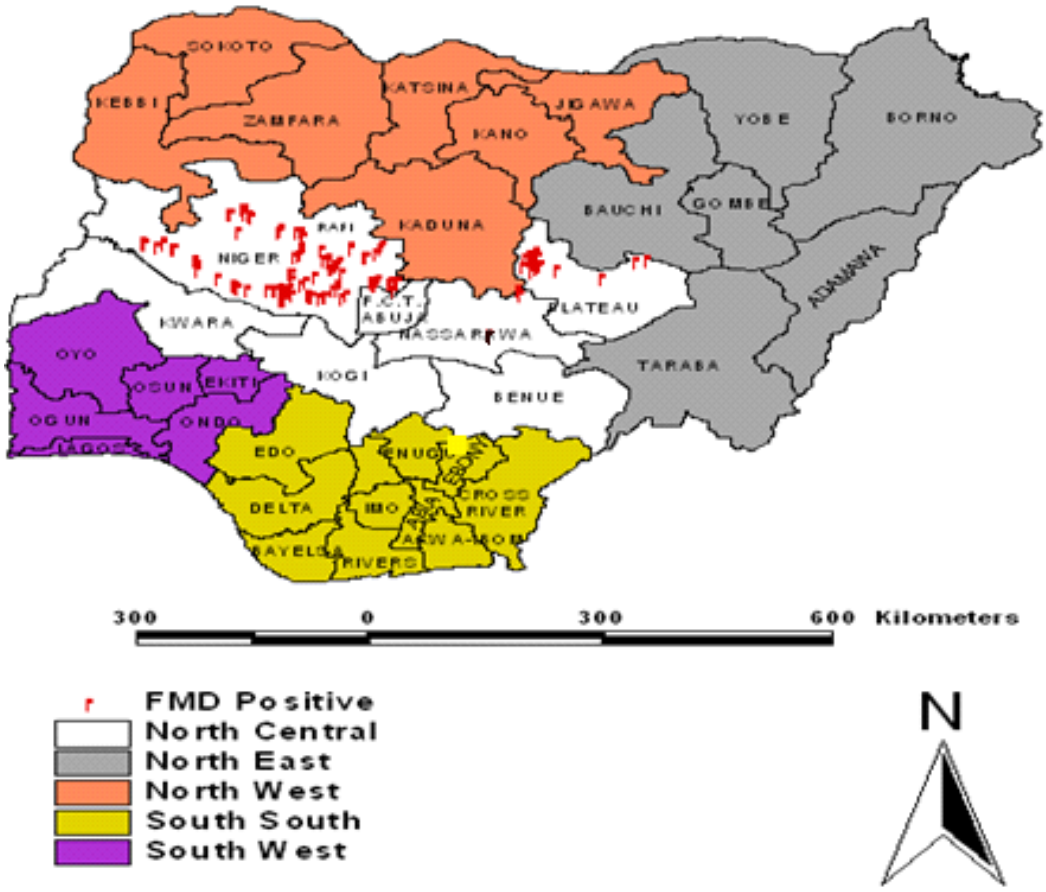


Figure 2. Distribution of herds positives for FMD in the study area.

Table 3. Seroprevalence of FMD based on age category.

Age	Number	%	Serological status		Prevalence (%) (95%CI)	OR
			+ve	-Ve		
Adult	767	72.5	537	230	70.01 (66.7-73.2)	1.14 (0.833-1.489)
Young	291	27.5	197	94	67.7 (62.2-72.9)	1
Total	1058	100				

X^2=0.5324; p>0.05.

Seroprevalence based on management systems

The study revealed higher disease prevalence in nomadic management system (75.8%) followed by sedentary management system (66.3%) and a lower prevalence was observed in the intensive management system (1.8%). The difference in prevalence between the nomadic and sedentary management system was not statistically significant (χ^2=10.79; p>0.05). However, the difference in disease prevalence among the three management systems was statistically associated with FMD sero-positivity (χ^2=123; p<0.05).

The odd of FMD in nomadic and sedentary husbandry systems was 171.9 (23.54-12.56) times more than intensive management system (Table 5). Management system showed a positive association with FMD seropositivity as risk factor.

Seroprevalence based on cattle movement

The study showed a higher prevalence of FMD sero-positivity in cattle that cross national boundary (88.75%) than those that move within the country (57.7%) and the

Table 4. Seroprevalence of FMD based on sex distribution.

Sex	Number	%	Serological status		Prevalence % (95%CI)	OR
			+Ve	- Ve		
Male	322	30.4	112	210	34.8(29.7-40.1)	1
Female	736	69.6	529	207	71.9 (66.5-75)	47.8(3.6-6.54)
Total	1058	100				

χ^2=129.1; p <0.05.

Table 5. Seroprevalence of FMD based on management system.

Management system	Number	%	Serological status		Prevalence % (95%CI)	OR
			+Ve	-Ve		
Sedentary	511	48.3	339	172	66.3 (62.2-70.3)	9.3 (4.885-19.02)
Nomadic	491	46.4	372	119	75.8 (71.8-79.4)	1
Intensive	56	5.3	1	55	1.8 (0.089-8.50)	
Total	1058	100				

χ^2=125.4; Df=2 p-value <0.05.

Table 6. Seroprevalence of FMD based on trans-boundary border crossing.

Trans-boundary crossing	Number	%	Serological status			OR
			+Ve	-Ve	Prevalence % (95% CI)	
Yes	417	39.4	370	47	88.7 (85.4-91.5)	5.184(3.696-7.369)
No	641	60.6	370	244	57.7(53.8-61.5)	1
Total	1058	100				

χ^2= 99.35; p< 0.05.

difference in FMD seropositivity was statistically significant (χ^2=99.35; P<0.05). The odd of FMD in cattle crossing the national boundary is 5.184 (3.699-7.369) times greater than those that move within the country (Table 6).

Seroprevalence based on herd mixing at the watering point

The study revealed higher FMD seropositivity in herds that indicated mixing at the watering points (75.8%) than those that do not mix at watering point (1.8%). The difference in seropositivity was statistically significant (χ^2=143.9; p<0.05). The odd of FMD is 171.8 (23.8-1253) times greater in herds that mixed at water points than those that do not (Table 7).

A survey to determine the seroprevalence of FMD in abattoir and cattle market as FMD hot spots was also conducted. The prevalence in abattoir samples was 65.7% and for the cattle market sample was 69.1%, respectively. The findings revealed that seroprevalence of FMD in these study areas were statistically insignificant. This could be attributed to the fact that most of the cattle population slaughtered in Nigeria abattoirs are directly purchased from the local cattle markets (Table 8).

DISCUSSION

FMD is one important trans-boundary animal disease (TAD) that limits prospects in local livestock production in Nigeria, with outbreaks occurring seasonally. In this study, nomadic and sedentary cattle in North-Central Nigeria were investigated for antibodies to FMD and risk factors for seropositivity evaluated. The overall seroprevalence of the disease was found to be 70.98% (95% CI: 68.37-73.49). This is consistent with the results of previous surveys conducted in Nigeria, in which a seroprevalence of 75.11% was reported by Olabode *et* al. (2013) in a study conducted in Kwara State, 64.7% in a

Table 7. Seroprevalence of FMD based on cattle herd mixing a watering point.

Mixing at the watering point	Number	%	Serological status		Prevalence (%) (95% CI)	OR
			+Ve	-Ve		
Yes	1001	94.7	759	242	75.8 (73.1-78.5)	171.8(23.8-1253)
No	56	5.3	1	55	1.8 (0.09-8.5)	
Total	1057			100		

χ^2=143.9; p-value <0.05.

Table 8. Seroprevalence of FMD hot spots (abattoir and cattle markets).

FMD hot spots	Number	Serological status		Prevalence % (95% CI)
		+Ve	-Ve	
Cattle Market	81	43	11	69.1 (58.5-78.5)
Abattoir	67	44	13	65.7 (51.4-76.3)
Total	148			

χ^2= 1.088; p-value > 0.05.

study conducted at the border states in Nigeria (Lazarus et al., 2012), 64.3 and 56.3% (Ehizibolo et al., 2010; Ishola et al., 2011), respectively, in studies carried out in Plateau State. The consistence of these findings confirmed that FMD is still an enzootic disease in Nigeria and this could be attributed to the fact that there has been no complementing vaccination campaign programme in the region, there is unrestricted herds mobility, continuous contact and intermingling of different herds at water points, communal grazing areas and porous borders. In addition, clinical diseases are usually underreported. This prevalence represents a higher prevalence than the 55% national prevalence reported by Abegunde et al. (1988).

Higher seroprevalence was recorded in Niger State (85.4%) than in Plateau State (54.2%). This could be attributed to the fact that many of the herds sampled indicated trans-boundary animal movement between Nigeria and the Republic of Benin. Niger State shares international boundary with the Republic of Benin, consequently, the animal population moves freely across the border in search of feed and drinking water. In most parts of West and Central Africa, the role of wildlife in the epidemiology of FMD has not been fully studied (Hedger and Condy, 1985; Thomson, 1995b; Alexandersen et al., 2002). However, the presence of wildlife population along the national park in Borgu might be a probable exposure factor that may have contributed to high FMD sero-positivity observed in this area. It has been established that countries like Nigeria with less developed livestock industries; the presence of many species of cloven-hoofed animals provides a possibility of reservoirs of the infectious viruses being established. It is believed that these free roaming species may normally come in contact with domesticated livestock, providing an opportunity for disease transmission. In comparison with the high seroprevalence observed in Niger North, Plateau North had the lowest seroprevalence which might be attributable to the fact that most of the cattle sampled in this area strictly practice intensive and sedentary management system contrary to the nomadism and extensive systems observed in most parts of Niger North.

Age category seropositivity revealed a higher sero-prevalence in cattle aged >2 years than in young cattle aged <2 years old. However, there was no association in seropositivity to age groups. The relative low seropositivity in young animals might be due to low exposure to risk factors. This is as a result of the practice of keeping young animals around the homestead and around areas separate from adult animals. Radostits et al. (2000), has indicated that young animals are relatively more susceptible than the adults, even though the present study showed that seroprevalence of FMD in adult cattle is slightly higher than that of the young cattle. This might be due to the fact that, adult cattle have repeated exposure and close contacts with other animals due to free animal movement. Generally, mortality is higher in young animals (over 20%) compared to 2% in adults. It has been observed that during outbreaks, morbidity rate in cattle can be up to 100% while mortality in young animals is up to 40% (Fiebre, 2015).

Furthermore, exposure factor to FMD seropositivity indicated both age groups had equal odds of FMD infection. Age association with FMD seropositivity was consistent with the previous study by Olabode et al. (2013) and Ishola et al. (2011) which reported higher prevalence of FMD in adult cattle than in young ones.

The higher seropositivity observed in female cattle was

consistent with the findings of Olabode et al. (2013), who reported a risk difference in association with sex in Kwara State, Nigeria. Also, Mazengia et al. (2010) reported higher incidence of FMD in females in Northwest Ethiopia. However, more of the animals sampled were female as oppose to male cattle, therefore, the significant association in seropositivity in sex could be attributed to a small number of males sampled as both male and female animals are equally at risk.

Nomadic and sedentary management systems revealed a higher prevalence respectively, whereas, a lower seropositivity was recorded in the intensive management system. The higher seroprevalence recorded in nomadic and sedentary management systems might be as a result of unrestricted cattle movement, contact with the different herd and mixing at watering point, whereas the lowest prevalence recorded in the intensive management system could be attributed to restricted movement, less contacts with other herds and mixing at watering points. The study further revealed that the odd of FMD infection is 171.9% times more in nomadic and sedentary management than in intensive management system. This finding is in agreement with a study conducted in Southern Ethiopia by Megersa et al. (2009) where pastoral system was identified as one of the major risk factors for FMD transmission.

The seropositivity due to herd movement had indicated that the herds that reported movement across national borders recorded higher seropositivity relative to herds that reported movement within the country. This might be attributed to contacts with wildlife reservoirs which are continuous source of infection, as well as contact with different herds and different locations. All the herds that indicated national border crossing were in Niger State.

Cattle herd mixing at watering point had higher likelihood of being classified as FMD seropositive than those that do not mix at watering points, infection was observed to be 5.2 times higher in animals crossing national borders than those that do not. This study is in agreement with other studies which reported that the movement of herds in search of pasture and water from one area to another is a significant risk factor for the occurrence of FMD (Habiela et al., 2010; Molla et al., 2009; Megersa et al., 2009).Herds that reported mixing at the watering point with other herds recorded the highest seroprevalence relative to those that do not mix with other herds. Watering point was observed to be a common place where cattle of different herds meet in search of water, thereby serving as foci of FMD transmission. The odd of FMD infection at watering point was observed to be 17.8 times in herds mixing than those that do not mix at all. A similar observation was made in Thailand by Cleland et al. (1996) where the odds of FMD increased by 1.6 for every additional village that shared a water source (and village equates with the herd in our study).

This correlation might be due to either an increase in potential for transmission or from higher virus survival in a more humid microclimate around water sources (Donaldson and Ferris, 1975; Dawe et al., 1994).

The equal distribution of prevalence in abattoir and cattle market samples from study area is insignificant, which could be attributable to the fact that most of the cattle population being slaughtered in Nigeria abattoirs are directly purchased from the local cattle markets.

Conclusion

Identifying the risk factors of FMD is the first step towards progressive control pathway for FMD control. This study has established that FMD is enzootic in north central Nigeria, and it has also been able to identify some of the risk factors associated with FMD seropositivity in the study area. Further study to determine the possible role of wildlife and small ruminants in the epidemiology of FMD in the study area is strongly recommended. This will help in the implementation of the effective control programme.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors wish to thank Mr Agom Danmarwa and Mrs Monica Onoja for the role they played in the analyses of the sample. Dr Pius Ekong is also acknowledged for plotting the map, lastly the authors also thank all the farmers for the use of their animals.

REFERENCES

Abegunde AA, Ezeokoli CD, Umoh JU, APB (1988). Epidemiology of Foot and mouth disease in Nigerian cattle. Viral Diseases of animals in Africa. OAU/STRC Scientific Publication Lagos Nigeria. pp. 203-212.

Alexandersen S, Mowat N (2005). Foot-and-mouth disease virus: host range and pathogenesis. Curr. Top. Microbiol. Immunol. 288:9-42.

Alexandersen S, Zhang Z, Donaldson AI (2002). Aspects of the persistence of foot-and-mouth disease virus in animals--the carrier problem. Microbes Infect. 4(10):1099-1110.

Nigeria Population Statistics. [Online]. (2013). Available from: www.geohive.com/cntry/nigeria.aspx. [Accessed: 1 January 2015].

Ayelet G, Mahapatra M, Gelaye E, Egziabher BG, Rufeal T, Sahle M, Ferris NP, Wadsworth J, Hutchings GH, Knowles N J,(2009). Genetic characterization of foot-and-mouth disease viruses, Ethiopia, 1981-2007. Emerg. Infect. Dis. 15(9):1409-1417.

Bronsvoort BM, Toft N, Bergmann IE, Sørensen K-J, Anderson J, Malirat V, Tanya VN, Morgan KL (2006). Evaluation of three 3ABC ELISAs for foot-and-mouth disease non-structural antibodies using latent class analysis. Br. Mountaineering Council Vet. Res. 2(1):1-30.

Cleland PC, Baldock FC, Chamnanpood P, Gleeson LJ (1996). Village level risk factors for foot-and-mouth disease in northern Thailand. Prev. Vet. Med. 26:253-261.

Dawe PS, Sorensen K, Ferris N P, Barnett IT, Armstrong RM, Knowles NJ (1994). Experimental transmission of foot-and-mouth disease

virus from carrier African buffalo (*Syncerus caffer*) to cattle in Zimbabwe. Vet. Rec. 134(9):211-215.
De Diego M, Brocchi E, Mackay D, De Simone F (1997). The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch. Virol. 142:2021-2033.
Donaldson AI, Ferris NP (1975). The survival of foot-and-mouth disease virus in open air conditions. J. Hyg. (Lond) . 74(3):409-416.
Ehizibolo DO, Ajogi I, Umoh JU, Kazeem HM, Ehizibolo PO, Perez AM, Metwally S (2010). Serological survey of Foot and Mouth Disease (FMD) 3d Non-structural Proteins using virus-infection associated (VIA) Antigen assay in livestock animals from Plateau State, Nigeria. In: Book of Abstract 47th Annual Congress of the Nigerian Veterinary Medical Association, Benue, P 75.
Fasina FO, Connell DR, Talabi OA, Lazarus DD, Adeleke GA, Olusanya TP, Hernandez JA (2013). Foot-and-mouth disease virus strains and examination of exposure factors associated with seropositivity of cattle herds in Nigeria during 2007 – 2009. Prev. Vet. Med. 109(3-4):334-342.
Felix J (2009). Nature, Problems and Prospects of Middle Belt Region of Nigeria. [Online]. 2009. Available from: http://wwwjimoh4bunuland.blogspot.com/2009/03/nature-proplems-and-prospects-of-middle.html accessed. [Accessed: 20 February 2015].
Fiebre A (2015). Foot and mouth disease. [Online] 2015. The Foot and Mouth Disease (FMD). The Center for Food Security & Public Health. Iow a State University, College Veterinary Medicinee British Journal of General Practice. Available from: http://www.cfsph.iastate.edu/Factsheets/pdfs/foot_and_mouth_disease.pdf. [Accessed: 29 December 2015].
GLIPHA (2011). Nigeria- Livestock population. [Online]. 2011. Available from: http://kids.fao.org/glipha/.
Habiela M, Ferris NP, Hutchings GH, Wadsworth J, Reid SM, Madi M, Ebert K, Sumption KJ, Knowles NJ, King DP, Paton DJ (2010). Molecular Characterization of Foot-and-Mouth Disease Viruses Collected from Sudan. Transbound. Emerg. Dis. 57:305-314.
Hedger RS Condy J (1985). Transmission of foot-and-mouth disease from African buffalo virus carriers to bovines. Vet. Rec. 9(117):205-205.
Ishola OO, Wungak YS, Olugasa BO, David LD, Ekong P (2011). Serological Survey of Foot and mouth Disease in Cattle in Jos South Local Government Area of Plateau State. Vom. J. Vet. Sci. 8:16-20.
Lazarus DD, Schielen WJG, WungakY, Kwange D, Fasina FO (2012). Sero-epidemiology of foot-and-mouth disease in some Border States of Nigeria. Afr. J. Microbiol. Res. 6(8):1756-1761
Mahy BWJ (2005). Introduction and history of foot-and-mouth disease virus. Curr. Top. Microbiol. Immunol. 288:1-8.
Mazengia H, Taye M, Negussie H, Alemu S, Tassew A (2010). Incidence of foot and mouth disease and its effect on milk yield in dairy cattle at Andassa dairy farm, Northwest Ethiopia. Agric. Biol. J. N. Am. 1(5): 969-973.
Megersa B, Beyene B, Abunna F, Regassa A, Amenu K, Rufael T (2009). Risk factors for foot and mouth disease seroprevalence in indigenous cattle in Southern Ethiopia: The effect of production system. Trop. Anim. Health Prod. 41(6):891-898.
Molla B, Ayelet G, Asfaw Y, Jibril Y, Ganga G, Gelaye E (2009). Epidemiological Study on Foot-and-Mouth Disease in Cattle: Seroprevalence and Risk Factor Assessment in South Omo Zone, South-western Ethiopia. Transbound. Emerg. Dis. pp. 1-8.
Nawathe DR, Goni M (1976). Foot and mouth disease in Nigeria. Bull. Anim. Health Prod. Afr. 24:(1)1-4.
OIE (2012). Foot and Mouth Disease Chapter 2.1.5 in: OIE Terrestrial Manual 2012, 1-29. In: [Online]. 1–29. Available from: Http://http//www.Oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FM.
Olabode OH, Kazeem HM, Raji M, Ibrahim ND (2013). Seroprevalence of foot and mouth disease virus antibodies in trade cattle (Bos indicus) in Kwara state of Nigeria. Vet. World 6(10):828-832.
Owoludun BY (1971). Foot and mouth disease and virus type distribution in Nigeria. Bull epizoot Dis. Afr. 8:152-158.
Radostits OM, Gay CC, Blood DCKWH (2000). Veterinary Medicine a Textbook of the disease of Cattle, Sheep, Pigs, Goats and Horses, Foot and Mouth Disease. 9th Ed. California, Elservier.
Sørensen K J, Madsen KG, Madsen ES, Salt JS, Nqindi J, Mackay DK (1998). Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus Arch. Virol. 143:1461-1476.
Thomson GR (1995a). Overview of foot and mouth disease in southern Africa. Rev. Sci. Technol. 14(3):503-520.
Thrusfield M (2005). Veterinary Epidemiology, 2nd edn. Blackwell Science Ltd, UK.
Vosloo W, Bastos ADS, Sangare O, Hargreaves SK, Thomson GR (2002). Review of the status and control of foot and mouth disease in sub-Saharan Africa. Rev. Sci. Tech. Int. Des. Epizoot. 21(3):437-449.

academicJournals

Vol. 15(24), pp. 1233 -1245, 15 June, 2016
DOI: 10.5897/AJB2016.15224
Article Number: 628FA0058870
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

GhNAC18, a novel cotton *(Gossypium hirsutum L.)* NAC gene, is involved in leaf senescence and diverse stress responses

Ondati Evans[1], Lingling Dou[1,2], Yaning Guo[1,2], Chaoyou Pang[1], Hengling Wei[1], Meizhen Song[1], Shuli Fan[1] and Shuxun Yu[1]*

[1]State Key Laboratory of Cotton Biology, Institute of Cotton Research of CAAS, Anyang, 455000, Henan, P. R. China.
[2]College of Agronomy, Northwest A&F University, Yangling, 712100, Shaanxi, P. R. China.

Received 12 January, 2016; Accepted 23 May, 2016

GhNAC18 **is a novel NAC gene that was isolated from cotton (*Gossypium hirsutum* L.). The full-length cDNA was 1511 bp including an open reading frame of 1260 bp in length and encodes a protein of 419 amino acids. With qRT-PCR analysis, *GhNAC18* was downregulated during natural and dark-induced senescence, implicating this gene as anti-aging gene in cotton. Analysis of its promoter revealed a group of putative *cis*-acting elements especially, the light and stress responsive elements, indicating that it may have a potential role in leaf development. Additionally, *GhNAC18* was found to have transcriptional activation activities on its C-terminal region and by bioinformatics analysis, *GhNAC18* was localized in the nucleus. Tissue specific expression analysis indicated that *GhNAC18* is constitutively expressed in roots, stems, earlier stages of senescing leaves, fibers and flower parts with high expression levels registered in the young leaves and cotyledon leaves. *GhNAC18* was up-regulated by exogenous application of various phytohormones including salicylic acid (SA), methyl Jasmonate (MeJA) and ethylene (ET) but downregulated with abscisic acid (ABA). Moreover, the gene was induced by drought (PEG6000), H_2O_2, cold (4°C) and wounding but was inhibited by high salinity. These results indicated that *GhNAC18* is a transcriptional activator that is involved in leaf development, especially inhibition of leaf senescence and plant stress responses in cotton. This study provides fundamental information on understanding the function of *GhNAC18* gene in cotton leaf senescence and stress tolerance and thereafter its manipulation for breeding of late-aging and stress-tolerant cultivars.**

Key words: *GhNAC18*, stress response, senescence, *Gossypium hirsutum* L. NAC.

INTRODUCTION

Plants face survival challenges posed by ever varying adverse environmental conditions which include but not limited to abiotic stress such as cold, high salinity, drought and extreme temperatures (Fujita et al., 2004; Ning et al., 2010; Nakashima et al., 2011). Biotic attacks such as infectious pathogens also complicate the sessile habit of plants. In this regard, plants adapt to these dynamic conditions by evoking responses at physio-logical, biochemical and molecular levels (Nakashima et al., 2011; Fan et al., 2015) including regulation of genes enhancing survivability (Nuruzzaman et al., 2013). The immune response in plants is triggered by pathogen

infection that is characterized by activation of multiple defense responses including expression of defense-related genes, regulated by different types of transcription factors (TFs).

TFs play important roles in regulating plant development and stress responses. They can be grouped into different families on the basis of conserved structural domains involved in DNA binding to *cis*- acting elements in the promoters of target genes, or other functional modular structures. Many TFs belong to NAC (Puranik et al., 2012), ERF, MYB/MYC (Christian et al., 2010), WRKY (Eulgem and Somssich, 2007), DREB/CBF, AP2/EREBP (Dietz and Viehhauser, 2010) and bZIP families.

NAC (for NAM, ATAF1,2 and CUC2) is a plant-specific family of transcription factors which share the N-terminal DNA-domain with a varying C-terminus that regulates transcription (Hao et al., 2011). The diverse C-terminal sequences among NACs are putative transcriptional activation domains which either activate or repress downstream of target genes. NACs are widely distributed in land plants and comprise one of the largest transcription factor families (Olsen et al., 2005). Since the first NAC gene denoted as NAM for no apical meristem was isolated from petunia (Yamasaki et al., 2013), many NACs have been reported to contribute to various developmental processes such as shoot apical meristem development (Nuruzzaman et al., 2013; Yamasaki et al., 2013), lateral root development, senescence, flowering and secondary wall formation. Moreover, NACs have also been associated with plant responses to biotic and abiotic stresses such as fungus infection, drought, cold, and high salinity (Sefyan et al., 2013; Hao et al., 2011; Xingwang et al., 2014). For instance, *OsNAC6* improves stress tolerance to dehydration and salinity in rice (Nakashima et al., 2007).

GhNAP regulate leaf senescence via the ABA-mediated pathways and has been associated with improved yield and quality in cotton (Fan et al., 2015; Mauch-Mani and Mauch, 2005). Age mediated senescence genes have been reported to be upregulated during the process (Zhao et al., 2015). Weaver et al. (1998) demonstrated that several SAGs are internally induced while others are elicited by external factors, however some SAGs may inhibit senescence (Weaver et al., 1998). Worthy to note is that some NACs simultaneously play multiple roles in regulating plant development and responses to exogenous stimuli (Shah et al., 2014). In arabidopsis *AtNAC2*, a transcription factor in the downstream of ethylene and auxin signaling pathways is simultaneously involved in salt stress response and lateral root development (Cao et al., 2005). Other NAC genes have been found to be upregulated during senescence (Shah et al., 2014) or by wounding and bacterial infection (Boller et al., 2001). Further, NAC proteins have been shown to mediate viral resistance. Apparently NAC family members play various roles not only in plant development but also in the recognition of environmental stimuli.

Upland cotton (*Gossypium hirsutum* L.) is the most important and widely cultivated crop in the world because of its fiber. The challenges posed by environmental stress and competition for land area by food crops call for short season stress tolerant varieties to tackle these challenges. Cotton short-season varieties are accompanied by premature leaf senescence which affects yield quality and quantity. In order to understand the molecular mechanism of cotton leaf senescence and stress responses, we selected a short-season variety CCRI-10 for this study. CCRI-10 exhibit early aging traits. Although, some *GhNAC* genes have been isolated and classified, there is no information available for specific functions of *GhNAC* genes in cotton stress responses and leaf senescence (Shah et al., 2013, 2014). This study reports the characterization of a novel *GhNAC18* gene that could play crucial role in cotton leaf senescence and stress responses. *GhNAC18* as transcriptional activator is downregulated by both natural and dark-induced senescence. Its rapid response to abiotic stress and induction by signal molecules validate *GhNAC18* as novel gene which could be involved in developmental processes and stress responses in cotton. These results taken together, demonstrate that *GhNAC18* could be involved in the regulation of leaf senescence and stress response in cotton.

METHODS AND MATERIALS

Plant materials and growth conditions

Cotton (*G. hirsutum* L. cv CCRI-10) seedlings were grown in a growth chamber at 25°C under a 16 h light and 8 h dark photoperiod. Seedling leaves were harvested at three and four leaf stages, frozen in liquid nitrogen and stored at -80°C for RNA extraction. For tissue specific expression analysis, cotyledon leaves, true leaves, stems, roots, flower parts and fiber tissues were collected from field plants and stored at -80°C for later use. Seven-day-old cotton seedlings were used for the various treatments.

For exogenous application of hormone treatments, leaves of uniformly developed seedlings were irrigated with 2 mM salicylic acid (SA), 100 µM abscisic acid (ABA), 100 µM methyl jasmonate (MeJA) and 100 µM ethylene (ET), respectively.

For salinity and drought treatments, the seedlings were treated with 200 mM NaCl or 15% (w/v) PEG6000, respectively. For hydrogen peroxide (H_2O_2) and wounding, seedlings were sprayed with 20 mM H_2O_2 and wounds inflicted by injuring three leaves from the top. Control plants were sprayed with sterile distilled water. After each treatment, samples from treated and control were frozen in liquid nitrogen and stored at -80°C for further analysis.

For analysis of natural leaf senescence, CCRI10 seeds were field

*Corresponding author. E-mail: yu@cricaas.com.cn. Tel: +8603722525363; Fax: +8603722525363.

grown under natural conditions during the summer of 2015 in Anyang (Henan province, China). Leaves from these plants were harvested at five leaf senescence stages defined by severity of visible symptoms, from non-senescent leaf stage (NS) to completely senescent stage (CS) approximately 90% yellowing of the leaf surface (Shah et al., 2014). For dark-induced senescence, detached flag leaves, submerged in water were incubated in dark for three days at room temperature. At each senescence stage and time, RNA was isolated for qRT-PCR analysis. Further, natural senescence was monitored on the cotyledon leaves from one week after germination. Samples of cotyledon leaves for 8 weeks were collected from the field, noting their morphological changes and then frozen in liquid nitrogen for transcript measurement. To enhance reliability of results, three repeats for each experiment mentioned above was conducted.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using hot borate method described by Wan and Wilkins (1994), and treated with DNase I digestion using RNAprep Pure Plant Kit (Tiangen, China) to eliminate potential genomic DNA contamination. The RNA concentration and purity were determined. Only those that met the criterion (260/280 ratio of 1.8-2.1, 260/230 ratio ≥ 2.0) were used for further analyses and stored at -80°C. The cDNA for cloning work was synthesized by using 5X All-In-One RT MasterMix (ABM. Canada) according to manufacturer's protocol .The RT-PCR was set as follows; 25°C for 10 min, 42°C for 50 min and 85°C for 5 min then put in ice for a few minutes. The newly synthesized strand cDNA was stored at -20°C.

Expression analysis of *GhNAC18*

Total RNA extraction from different tissues was performed by using RNAprep Pure Plant Kit (Tiangen China). The cDNA was synthesized from 2 µg of RNA in a 20 reaction volume using ReverTra Ace qPCR RT kit (TOYOBO, Japan) according to the manufacturer's manual. Relative expression levels of genes in each sample were normalized to the expression level of *GhActin1.* For real-time quantitative PCR, the gene-specific primer pairs (Table 2) were used for *GhNAC18, GhCAB*, *GhNAP* and *GhActin1*. PCR products were detected by SYBR Green I fluorescence dye (Takara, China) in the Applied Biosystems 7500/7500 Faster Real-Time PCR system machine. The following thermal cycle conditions were used: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, products collected at 60°C for 34 s. All reactions were performed in triplicate. Following the PCR, a melting curve analysis was performed. Ct or threshold cycle was used for relative quantification of the input target number. Relative expression fold difference (N) is the number of treated target gene transcript copies relative to that of the untreated gene transcript copies, and is calculated according to Schmittgen and Livak (2001) as follows:

$$N= 2^{\Delta\Delta Ct}=2^{(\Delta Ct\ treated-\Delta Ct\ control)}$$

Where $\Delta\Delta Ct = \Delta Ct$ of the treated sample minus ΔCt of the untreated control sample, and ΔCt is the difference in threshold cycles for *GhNAC18* target and the *GhActin1* internal reference.

Multiple sequence alignment and phylogenetic analysis

The nucleotide sequences from cDNAs were downloaded from NCBI Blast program (http://www.ncbi.nlm.nih.gov/BLAST). Translation of nucleotide sequences was done using Expasy online program (http://www.web.expasy.org/translate/) and alignment was conducted using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic analysis was employed to investigate the evolutionary relationships between *GhNAC18* and NAC proteins from other plants. A neighbor joining tree was generated by MEGA6. A bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology.

Bioinformatics analysis

Open reading frame (ORF) and protein prediction were made using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The theoretical isoelectric point (pI) and mass values for mature peptides were obtained using the Peptide-Mass program (http://us.expasy.org/tools/peptide-mass.html). Protein subcellular localization was predicted using WoLF PSORT (http://wolfpsort.org/). In order to investigate the Genomic structure of *GhNAC18*, gene structure display server (GSDS) program (http://gsds.cbi.pku.edu.cn/) was used to illustrate exon/intron organization by comparing the cDNA with their corresponding genomic DNA sequences obtained from cotton genome project database (http://cgp.genomics.org.cn/). The structure of the cotton *GhNAC18* protein was analyzed by Motifscan (http://myhits.isbsib.ch/cgi-bin/motif_scan) and (http://www.ebi.ac.uk).

Cloning of *GhNAC18* gene

The RT products were used to amplify the full length of *GhNAC18* using gene specific primer designed by Oligo 7.0 based on the cDNA (Accession no. *KC847195)*. A pair of primers (Table 1) was used with MightyAmp polymerase enzyme (Takara, China) and the PCR products were gel purified. They were then linked to simple T-vector (pMD18-T Vector). The clones were confirmed by sequencing.

Promoter analysis

Total genomic DNA was extracted from cotton leaf using the cetyl trimethylammonium bromide (CTAB) method (Permingeat et al., 1998). *GhNAC18* promoter of approximately 1.5 kb upstream of ATG was amplified by PCR. The gene specific primes were designed based on the known upstream sequence region of the coding region of *GhNAC18*. The PCR products were purified, linked to T-vector and sequenced. The promoter sequence was then searched in the PLACE and PLANT CARE databases to investigate the putative *cis*- acting elements.

Transcriptional activation activity of *GhNAC18*

To investigate whether *GhNAC18* has transcriptional activities, the entire or partial coding regions of *GhNAC18* were obtained by PCR using fragment specific primers (Table 1). The PCR products were inserted into the *Eco*R1 and *Bam*H1 site of pGBKT7 vector, containing the GAL4 DNA binding domain to obtain pGBKT7:*GhNAC18*-F, pGBKT7:*GhNAC18*-N, and pGBKT7:*GhNAC18*-C. Three constructs and pGBKT7 vector (negative control) were transformed into the yeast strain Y187 (clontech China), plated and incubated for three days.

RESULTS

Characterization and cloning of *GhNAC18*

To date, there are 77 cotton *GhNACs* (Zhao et al., 2015).

Table 1. Primers used for isolating corresponding sequences of *GhNAC18*

Prime name	Primer sequence (5'-3')	Gene/Region
GhNAC18-F	GGAGCTGACATAGTTTCTGGTTAGT	GhNAC18
GhNAC18-R	GTAGGCGCCGCAGTATTTCTTATAT	GhNAC18
pGhNAC18F	TTTATTTCTTCTCGAGTACGCATGG	Promoter
pGhNAC18F	GTTGGTATTATCGTTGGGGTCGTTG	promoter
GhNAC18NF	CGGAATTCCG AAACTAAGAGGGTAGGAGCTCGG	N- terminus
GhNAC18NR	CGGGATCCCG CTCACCACGAAACTGAACGCTAC	N- terminus
FGhNAC18F	CGGAATTCCGGGAGCTGACATAGTTTCTGGTTAGT	Full length
FGhNAC18R	CGGGATCCCGATATAAGAAATACTGCGGCGCCTAC	Full length
GhNAC18CF	CGGAATTCCG ACCACGAAACTGAACGCTACCAA	C-terminus
GhNAC18CR	CGGGATCCCG CCTATTTGGTTCTGGGATTGGGT	C-terminus

Table 2. Primers used for expression analysis by qRT-PCR.

Prime name	Primer sequence (5'-3')	Gene
qPCRGhNAC18-F	CGACGACCTCCACAGACTAGT	GhNAC18
qPCRGhNAC18-R	ACTTGGAATTGCGCTGGGTAG	GhNAC18
qGhNAP-F	GCCCCAATTCACATGACACAGT	GhNAP
qGhNAP-R	TCTCAACATGGTCACCTGTGGT	GhNAP
qGhCAB-F	TGTCCCCGGAAAATGAACAAC	GhCAB
qGhCAB-R	TATGTGCTGCAGAAAATCATGCT	GhCAB
GhACTIN-F	ATCCTCCGTCTTGACCTTG	Actin
GhACTIN-R	TGTCCGTCAGGCAACTCAT	Actin

Genome-wide analysis of these genes as demonstrated by the expression levels in cotton, predict their potential role they play in cotton growth and development (Meng et al., 2007; Shah et al., 2013, 2014; Puranik et al., 2012). *GhNAC12* and *GhNAP* are reported to promote senescence and yield improvement (Zhao et al., 2015; Fan et al., 2015). Our study points out to one of *GhNACs, GhNAC18,* which, unlike others previously reported, is downregulated during leaf senescence and could be involved in cotton stress responses. *GhNAC18* was isolated from upland cotton using gene specific primers (Table 1). The full length of cDNA (GenBank Accession Number KC847195.) was 1511 bp with an open reading frame (ORF) of 1260 bp, encoding 419 amino acids. The relative molecular weight and theoretical isoelectric point of the predicted protein were 48.23 kDa and 6.94, respectively. Using WoLF PSORT program *GhNAC18* was predicted to be located in the nucleus, confirming its role as nuclear transcription factor.

Sequence alignment, phylogenetic analysis and genomic structure

Multiple sequence alignments of the full-length protein sequences from cotton and other known NACs from other plant species, including the highly conserved N-terminal NAM domain and the more divergent C-terminal activation domain, were performed by ClustalW program. To investigate the evolutionary relationship of *GhNAC18* and these other proteins, an unrooted phylogenetic tree was constructed with MEGA 6.0 using the neighbor joining (NJ), minimal evolution (ME) and maximum parsimony (MP) methods and the bootstrap test was carried out with 1000 iterations. Pair wise gap deletion mode was used to ensure that the more divergent C-terminal domains could contribute to the topology of the NJ tree. In this regard, *GhNAC18* belongs to *NAM* subfamily with the NAM domain stretching from 45-192aa of its protein (Figure 1a). *GhNAC18* showed homology with *TaNAC67, ANA036* and *NAM* (Figure 1d). *TaNAC67* is reported to be involved in chlorophyll retention, photosynthetic efficiency and enhanced water retention (Mao et al., 2013). *ANAC036* is highly expressed in the leaf and it is involved in the growth of leaf cells (Kato et al., 2010). This identity with other proteins, imply that *GhNAC18* would have similar functions like these proteins. Moreover multiple sequence alignment resulting to phylogenetic relationship, showed that all the members used, contained A-E subdomains (Figure 1c) (Ooka et al., 2003). This is consistent with previously reported work (Puranik et al., 2012) confirming that *GhNAC18* is a

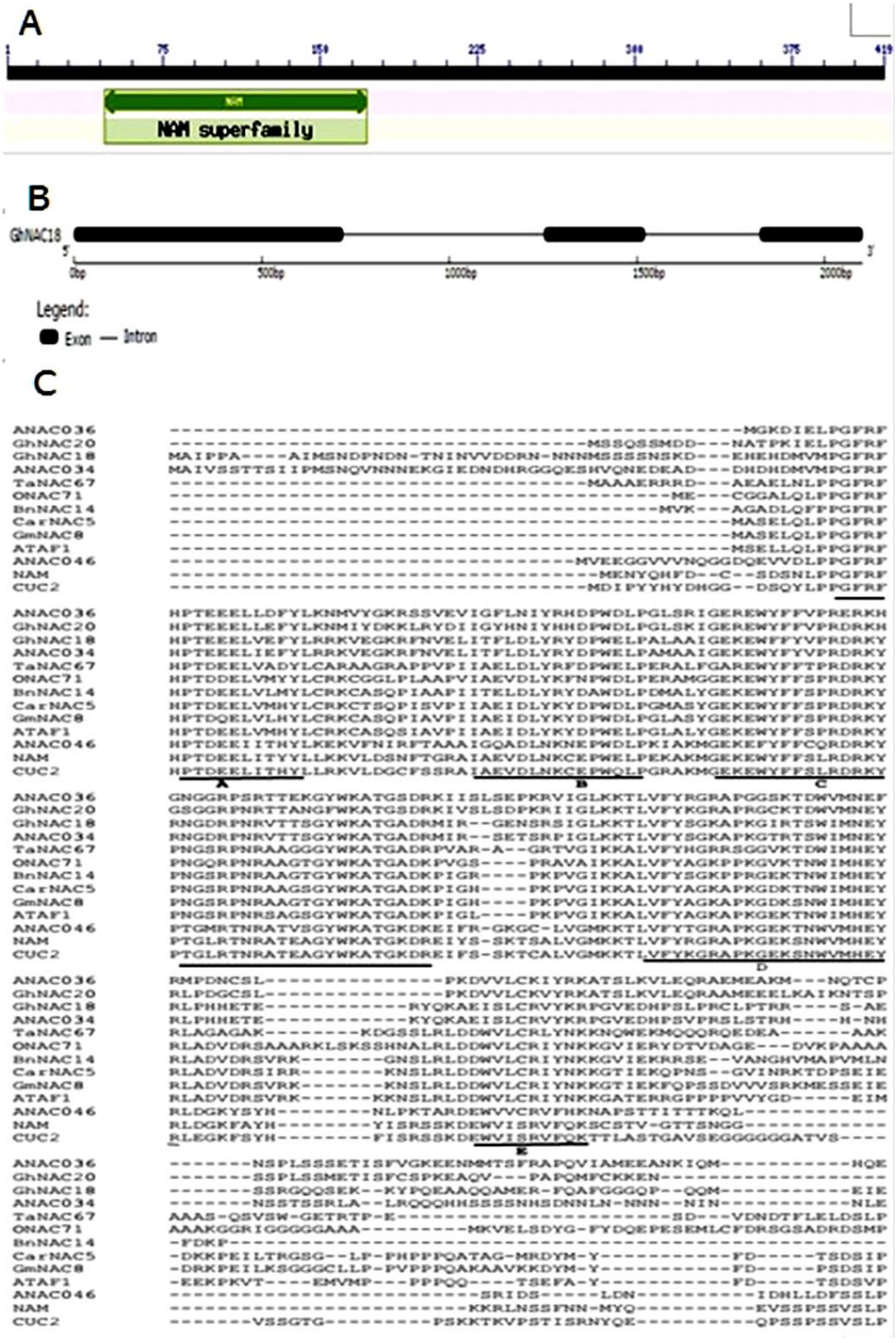


Figure 1. NAM subdomain of NAC domain, genomic organization of *GhNAC18*, Alignment with other related proteins and Phylogenetic tree. ***A***. is a NAC domain (*45-192aa*) of *GhNAC18*, ***B.*** Genomic structure showing exons (*in solid blocks*)and introns (*lines*) of *GhNAC18*, ***C.*** Multiple alignment of putative amino acids sequence of *GhNAC18* and other NAC protein. The location of conserved motifs are underlined and labeled *A-E*. ***D.*** A Phylogenetic tree with *GhNAC18* marked with black circle.

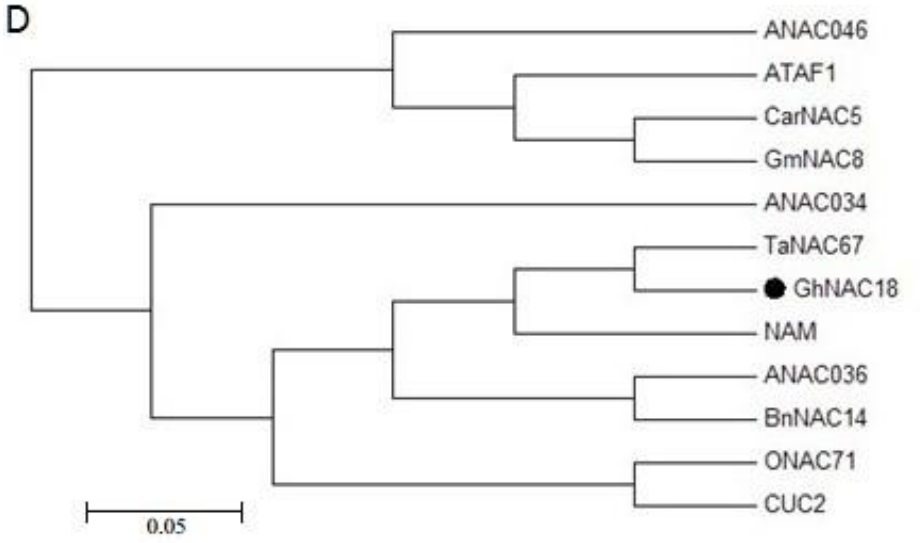


Figure 1. Contd.

member of *NAC* TF family.

Additionally, genomic structure of *GhNAC18* was determined by comparing the genomic sequence and the cDNA sequence. Like most of the NAC family members, *GhNAC18* has two introns and three exons (Meng et al., 2007; Yu et al., 2014). The first two exons encoded the N-terminal domain while the last exon encoded the highly divergent C-terminal region (Figure 1b).

Promoter analysis

Expression of stress-responsive NACs may be tightly regulated by several stress-responsive regulatory elements contained in the promoter region (Puranik et al., 2012). The presences of these cis-acting elements predict some of the roles played by the target gene. *GhNAC18* showed several *cis*- acting elements including 5UTR Py-rich stretch ACE,G-box, MRE(MYB) TC-rich repeats TCA-element, WUN-motif, DREs and LTREs among others. The existence of numerous elements suggested that *GhNAC18* could be involved in regulation of stress responses.

GhNAC18 transcriptional activation activity

To examine whether *GhNAC18* has transcriptional activation activity, the N- and C-terminal fragments as well as the full-length *GhNAC18* were fused to the GAL4 DBD of the pGBKT7 vector. The resulting constructs and the negative vector control (pGBKT7) were transformed into Y187 yeast strain. After three days all of the transformants grew well on SD/–Trp/medium, but only the yeast cells containing pGBKT7-*GhNAC18* and pGBKT7-*GhNAC18*-C plasmid grew and turned blue on SD/–Trp/X-α-Gal/ medium (Figure 2) These results indicated that *GhNAC18*, has trans-activation activity in the C-terminus region.

Tissue-specific expression of *GhNAC18*

To investigate how *GhNAC18* is expressed in cotton, various tissues were harvested from the field at specific period of cotton development (Figure 3). *GhNAC18* was constitutively expressed in all parts investigated except 10 days post anthesis (DPA) fiber and pistil. Strong expression was observed in cotyledon leaves and young leaves. Moderate expression was exhibited in the stem and stamen. Low expression level was detected in the roots, sepal, petal, ovule and senescing leaves (Figure 3). The significant abundance in both cotyledon and young leaf suggested that *GhNAC18* may play an important role in leaf development.

Expression of *GhNAC18* during leaf senescence

With high expression level of *GhNA18* in the young leaves and cotyledon leaves, we decided to further investigate its role in these tissues during natural and dark-induced leaf senescence. For leaf senescence in cotyledons, cotyledon leaves were collected weekly after germination for eight weeks. By qRT-PCR analysis, *GhNAC18* was gradually expressed from week 1 up to week 4 after which there was a steady decline in expression level. The decline in expression level corresponded to the onset of senescence due to aging of the cotyledon leaves (Figure 4a).This indicated that *GhNAC18* is down regulated as senescence is initiated. Morphologically, some cotyledon leaves started to yellow

Figure 2. Transactivation activity of *GhNAC18.* Three constructs: pGBKT7:*GhNAC18*-F, pGBKT7:*GhNAC18*-N and GBKT7-C.) and GBKT7 vector (negative control) were transformed into the yeast strain Y187 plated and incubated for three days. All yeast cells containing the plasmids grew well into white in SD/-Trip medium, but only yeast cell containing pGBKT7:*GhNAC18*-F and pGBKT7:*GhNAC18*-C turned blue in SD/-Trp/-α-Gal medium.

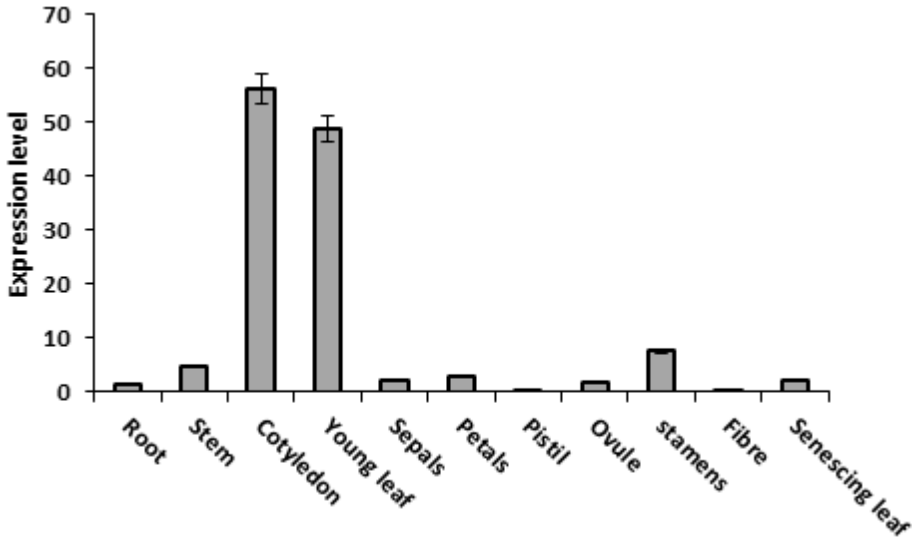


Figure 3. Expression profiling of *GhNAC18*. Tissue-specific expression level of GhNAC18 in cotton (*Gossypium hirsutum* L. CCRI-10).

at 8th week, an indication of senescence (Weaver et al., 1998). Progression of natural senescence on mature leaves was marked by the severity of yellowing in the leaf (Figure 4c).The stages of leaf senescence start from NS leaf to CS leaf, where *NS* is non-senescent leaf, *IS* is initial senescent leaf (15% yellow) *ES* is early senescent leaf (30% yellow), *LS* is late senescent leaf (50% yellow) and *CS* is complete senescent leaf (90% yellow) (Shah et al., 2013). By qRT-PCR analysis, *GhNAC18* expression level decreased from NS to CS (Figure 4b). To verify that senescence took place in these leaves, the expression level of positive and negative marker genes for senescence, *GhNAP* and *GhCAB* respectively for senescence were used on the same leaves as shown in Figure 4b. Chlorophyll content, a measure of senescence, was also measured in these five senescence stages (Figure 4d). For dark-induced senescence, *GhNAC18* was down regulated (Figure 4e). Contrary to early reports

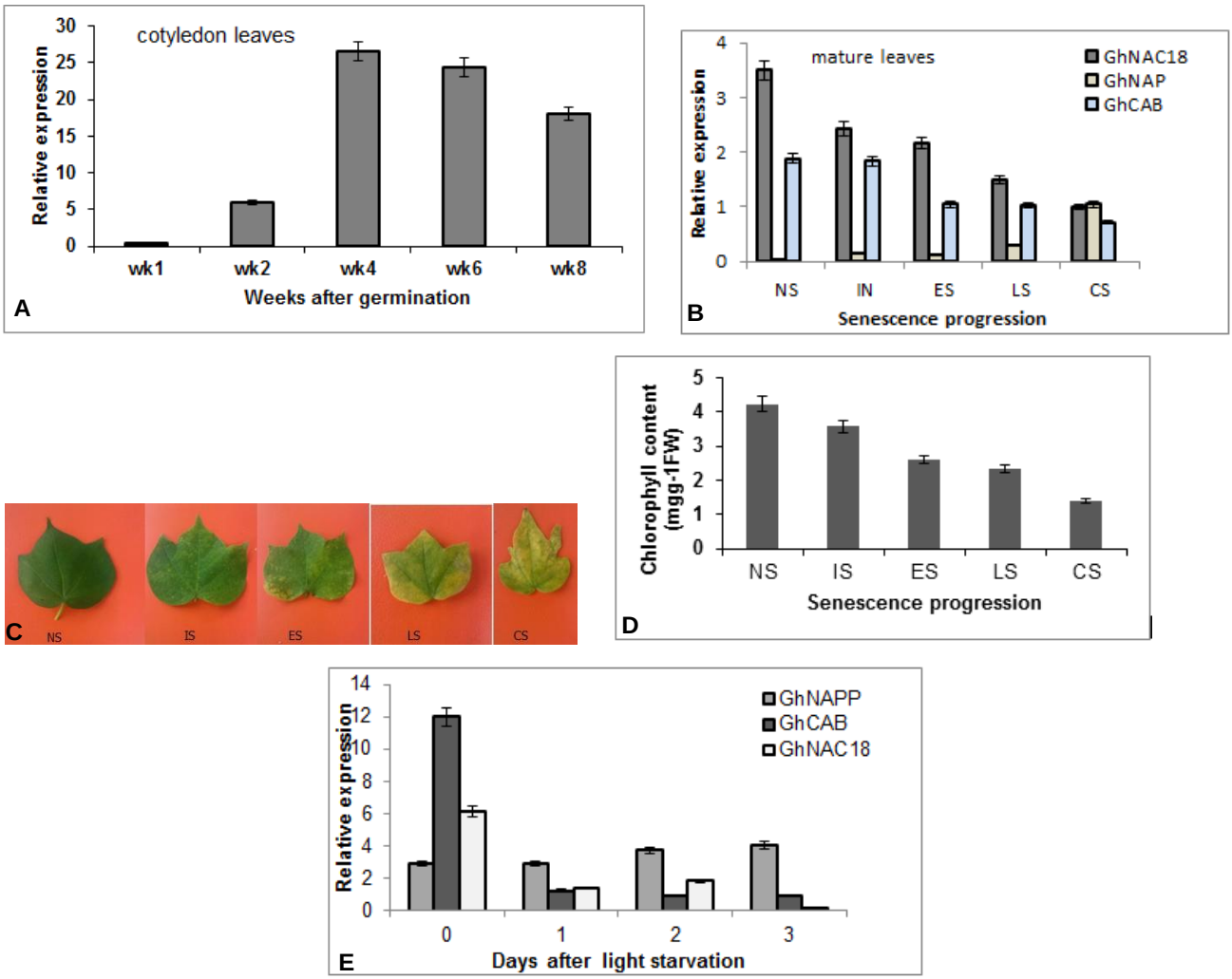


Figure 4. Progression of natural senescence and dark-induce senescence. **A**). Expression level of *GhNAC18* during cotyledon leaves senescence. **B**). Relative expression level of *GhNAC18 GhNAP* and *GhCAB* during progression of natural senescence in mature leaves. **C**) Morphological changes from *NS* leaf to *CS* leaf, where *NS* non-senescent leaf, IS initial senescent leaf (15%yellow) *ES* early senescent leaf(30%yellow), *LS* later senescent leaf (50%yellow) ,and *CS* complete senescent leaf(90%yellow). **D**) Chlorophyll content decrease from *NS* leaf to *CS* leaf. **E**) Relative expression level of *GhNAC18 GhNAP* and *GhCAB* during dark-induced leaf senescence. (*GhNAP* was used as appositive marker gene and *GhCAB* as negative marker gene for senescence during 3days of incubation). Error bars indicate SD.

on senescence associated genes (SAGs), *GhNAC18* was apparently inhibited by dark-induced senescence. Previous studies have generally indicated that *GhNAC*s are involved in leaf senescence, however, this studies show that *GhNAC18* might be required at the onset of senescence (IS-ES), but not on progression of this process.

Effects of phytohormones on *GhNAC18* expression

Plant hormones are implicated in complex signaling pathways and play crucial roles in regulating plant responses to a variety of environmental stresses and developmental processes (Bari and Jones, 2009). In the present study, the effect of phytohormones on *GhNAC18* expression were investigated. ABA, ET, JA and SA solutions were sprayed on cotton leaves and *GhNAC18* transcript levels measured by qRT-PCR. The application of MeJA and ET induced the expression level of *GhNAC18* reaching its maximum at 24 h (Figure 5). SA as a signaling molecule plays a significant task in plant defence and generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens (Lamb and Grant, 2006). Further, SA level accumulates in the pathogen infected tissues of

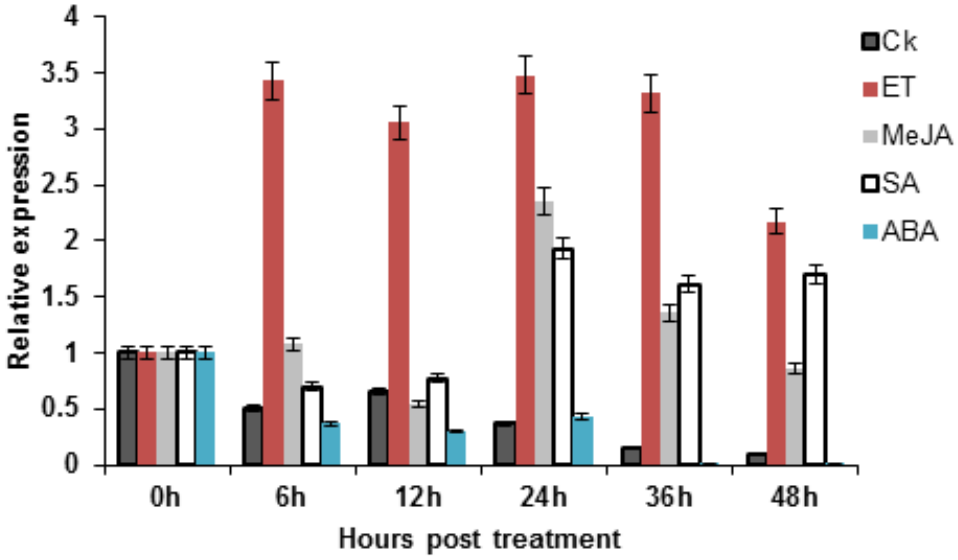


Figure 5. Expression patterns of *GhNAC18* in response to chemical stress during 48h of treatment. Uniformly grown two-week old cotton seedlings were treated with 2mM SA, 100µM ABA, 100µM MeJA, and 100µM ET. Total RNA was isolated at indicated times after treatment for qPCR analysis. *GhActin* was used as standard control in the experiments. Error bars indicate Standard deviations.

plants and exogenous application results to expression of pathogen related genes enhancing tolerance to infections (Bari and Jones, 2009). In this study, application of SA induced the expression of *GhNAC18* suggesting that *GhNAC18* could be involved in early detection of biotic stress triggering stress response network (Figure 5). ABA regulates many aspects of plant growth and development such as leaf senescence, seed germination, embryo maturation, stomatal aperture and adaption to environmental stress. Earlier studies have shown that ABA induces senescence in plants (Becker et al., 1993; Oh et al., 1996). In the present experiment, it was demonstrated that treatment of cotton seedlings with ABA lowered the expression of *GhNAC18* (Figure 5) indicating that *GhNAC18* is antagonistically regulated by ABA pathways that lead to senescence. Overall, activation of GhNAC18 by these phytohormones, indicated that GhNAC18 could be involved in abiotic and biotic responses through signaling pathways.

Expression of *GhNAC18* under abiotic stress

To further examine the expression pattern of *GhNAC18* in cotton under various stress conditions, cotton seedlings were subjected to drought (PEG6000), salt (NaCl), cold (4°C), H_2O_2 and wounding. In the analysis, *GhNAC18* was induced by all these treatments, however salt and cold treatments did not have a significant change on its expression (Figure 6a-b). There was steady increase of *GhNAC18* transcripts levels when seedlings were wounded, peaking at 8 h (Figure 6b). *GhNAC18* was sensitive to H_2O_2 treatment at 2 h (Figure 6b) followed by decline in transcript level. Drought treatment upregulated the expression level of *GhNAC18* (Figure 6a). Based on these results, *GhNAC18* might be involved in regulation of abiotic responses in cotton.

DISCUSSION

NAC proteins form the largest transcription factors in plants (Ooka et al., 2003). Their role in regulating plant responses to stresses and plant development cannot be underestimated. To date numerous *NAC* family genes have been isolated and characterized revealing a wide range of functions they play in plant species. This family of genes share common NAC domain (Ooka et al., 2003; Nakashima et ., 2007; Ma et al., 2010; Christiansen et al., 2011; Saad et al., 2013). Like other known NACs, *GhNAC18* has a conserved NAC domain at N-terminal region which can be divided into five subdomains namely A-E (Figure 1a and c). By bioinformatics analysis, *GhNAC18* was located in the nucleus while their transcriptional activation activity was located in the C-terminal region (Figure 2). The results taken together indicated that *GhNAC18* is a nuclear protein with a NAC domain and may function as transcription activator in cotton.

Although, the functions of NAC genes have been analyzed in various plants, not much information is available on the specific function of *GhNACs* in cotton (*G.*

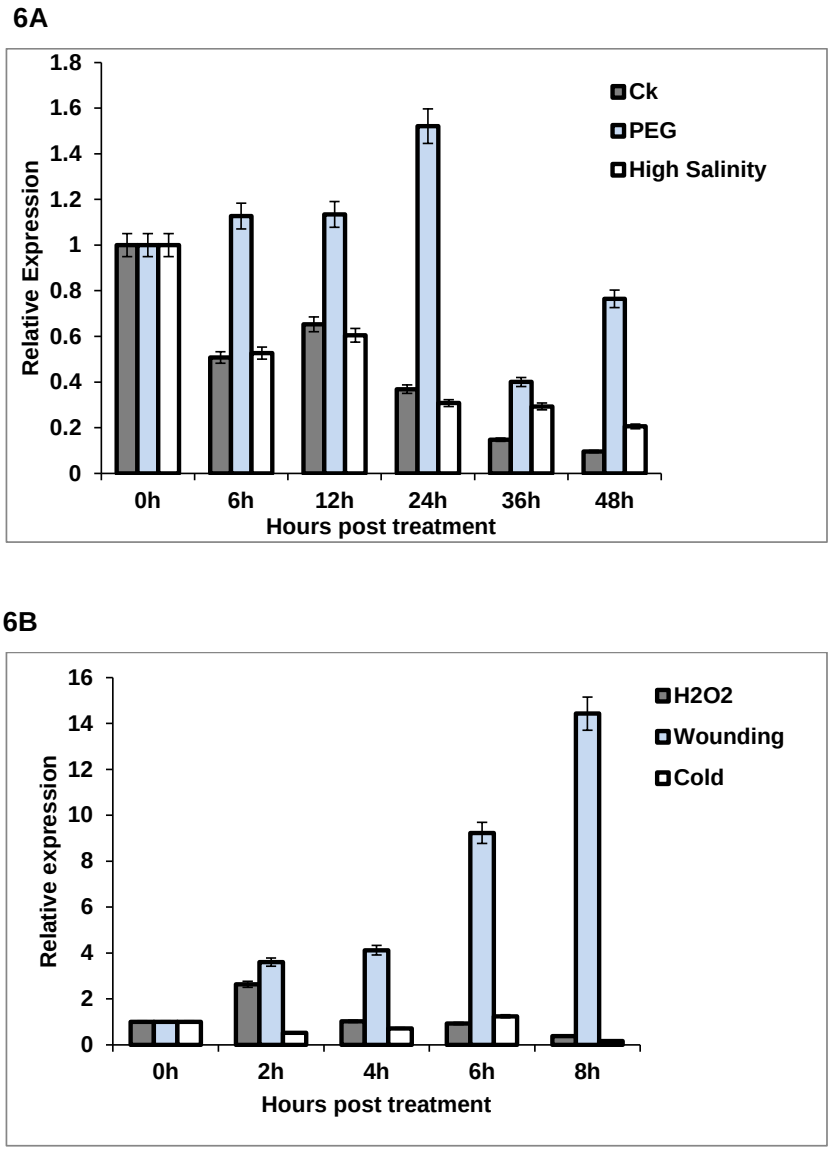


Figure 6. Expression pattern of GhNAC18 under abiotic stress. **A**. Drought stress with15% PEG 6000, Salinity stress with 200 mM NaCl and control (Ck). **B**. Hydrogen peroxide (20mMH2O2), Wounding and cold (4^{o}C) treatments.

hirsutum L) (Meng et al., 2009; Nuruzzaman et al., 2013; Xu et al., 2014). In cotton, two *NAC* genes (*GhNAC12* and *GhNAP*) have been fully functionally characterized, however, many more are yet to be reported (Zhao et al., 2015). In the current study, *GhNAC18* was isolated and characterized during senescence and under stress conditions. During natural and dark-induced senescence, *GhNAC18* was down regulated (Figure 4). Its expression level markedly decreased as the leaf matured and as senescence sets in, suggesting that senescence inhibits the expression of this gene. It has been reported that most SAGs are induced by darkness however, *GhNAC18* was apparently downregulated by absence of light while *GhNAP*, a positive regulator for senescence was upregulated implying that *GhANAC18* could act as a negative regulator for this process. This was also confirmed by comparing it with another known negative regulator gene for senescence (*GhCAB*) (Figure 4e).

Many NACs have been reported to be expressed in specific tissues of plants. For example, *ANAC036* gene is highly expressed in rosette leaves and slightly expressed in seedlings and inflorescence. It is believed to function in leaf cell development due to its abundance but its overexpression caused semi-dwarfism in arabidopsis (Kato et al., 2010). The expression of *GhNAC18* was found to have varied expression levels in different tissues. There was high expression on the young and cotyledon leaves than other parts (Figure 3). This implies

that *GhNAC18* may have a significant role in the young leaves. Although, *NAC*s have been reported to be involved in early and late senescence in upland cotton (Kong et al., 2013), *GhNAC18* seemed to have a different trend during aging process because, its transcript abudance decreases as aging advances. Premature leaf senescence causes poor or low yield in cotton in early maturing cultivars (Wright, 1998). During this process, many SAGs are upregulated with possible regulatory roles. On the contrary, *GhNAC18* was found to be downregulated during leaf senescence, however low expression was observed on the onset of senescence implying that it may not be involved in progression of senescence.

GhNAC18 shared high identity with *TaNAC67* as indicated by phylogenetic analysis. *TaNAC67* is reported to improve abiotic stress tolerances and enhances high chlorophyll content retention in wheat (Mao et al., 2014). Because proteins which align together may have similar functions, there is a possibility that *GhNAC18* may have the same functions like *TaNAC67* in cotton leaves. Promoter analysis also revealed presence of light responsive elements which function in the leaf to enhance photosynthesis, probably a reason why *GhNAC18* was downregulated in the absence of light. These results taken together indicate that *GhNAC18* is non-aging gene that could be important in delaying senescence and increasing the life span of cotton.

Plant defense mechanism triggers molecular, biochemical and morphological changes such as oxidative burst, expression of defence-related genes, production of antimicrobial compounds, and/or damage-limiting mechanisms (Collinge and Bollar, 2001;Van Loon et al., 2006) boosting adaptability. In the current study *GhNAC18* was induced by SA, ET and MeJA. These signaling molecules are reported to be involved in regulating plant defense responses against various biotic and abiotic stresses (Glazebrook, 2001; McGrath et al., 2005). Upon treatment of cotton seedlings with ET and MeJA, transcript level of *GhNAC18* significantly accumulated indicating its sensetivity to responding to these stresses (Oh et al., 2005) therefore it could be involved in cotton defense response, possibly through the ET/MeJA-dependent signal transduction pathway. *OsNAC19* and *CarNAC1* which belong to NAC family are involved in stress tolerance at the same time induced by exogenous application of ET, MeJA and ABA (Lin et al., 2007). The induction of *GhNAC18* by SA and ET further confirm its involvement in biotic stress responses (Figure 5) since both SA and ET are important signaling mediators in biotic stress pathways (Fujita et al., 2004; Peng et al., 2009; Xia et al., 2010). Moreover, transcripts of *GhNAC18* showed significant increase under H_2O_2 treatments (Figure 6b), suggesting that there exists across-talk between abiotic stress and signal transduction pathways. Interestingly, *GhNAC18* was rapidly and transiently induced by wounding (Figure 6b), indicating its potential as an early regulator in the biotic stress response (Tran et al., 2004; Hao et al., 2011). Additionally it has been reported that NAC transcription factors can regulate drought stress response through both ABA-dependent and ABA-independent pathways (Fujita et al., 2004). In this study, the expression of *GhNAC18* is induced by dehydration, but not by ABA (Figure 5), suggesting that this protein may be associated with drought response in an ABA-independent manner.

The functions of some transcription factors are generally involved in plant development and stress responses at one time. For example, the Arabidopsis *ATAF1* gene was induced by wounding, pathogen attack, drought and ABA (Boller, 2001; Jensen et al., 2007; Lu et al., 2007). *AtNAC2,* as a transcription factor downstream of ET and auxin signaling pathways, was simultaneously involved in salt stress (Cao et al., 2005). Five *GhNACs* (*GhNAC2-GhNAC6*) genes were upregulated by drought, cold and salt (Meng et al., 2009). Reactive oxygen species, especially H_2O_2 are important signal transduction molecules, mediating the acquisition of tolerance to various stress. H_2O_2 induced the expression of *RD26* gene which regulates genes involved in defense and senescence (Fujita et al., 2004). This results have shown that *GhNAC18* is responsive to not only plant developmental processes, such as leaf senescence, but also to exogenous stimuli, such as drought and wounding (Figure 6), indicating that the *GhNAC18* transcription factor may be a common regulator of the molecular mechanisms of special plant development and stress responses.

In conclusion, this result suggests that *GhNAC18*, as a transcription activator, is possibly involved in developmental processes and stress responses in cotton. We are currently investigating what effect *GhNAC18* will have on transgenic plants and how *GhNAC18* is integrated into special phytohormone signaling pathway.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors appreciate China Agriculture Research System (Grant No. CARS-18) for providing financial support for this work. They also thank Mohammed Asaad, for helping to prepare experimental materials.

Abbreviations

SA, Salicylic acid; **MeJA,** methyl Jasmonate; **ET,** ethylene; **ABA,** abscisic acid; **DNA,** deoxyribonucleic acid; **cDNA,** complementary DNA; **PCR,** polymerase chain reaction; **qRT-PCR,** quantitative reverse

transcription PCR; **CTAB,** cetyl trimethylammonium bromide; **NJ,** neighbor joining; **ME,** minimal evolution; **MP,** maximum parsimony; **NS,** non-senescent leaf; **IS,** initial senescent leaf; **ES,** early senescent leaf; **LS,** late senescent leaf; **CS,** complete senescent leaf; **TFs,** transcription factors; **NAC,** a family of transcription factors comprising NAM, ATAF1,2 and CUC; **DREB,** dehydration-responsive element-binding protein; **ERF,** ethylene-responsive factor; ***GhNAC18,*** **NAC gene of *Gossypium hirsutum* L.**

REFERENCES

Bari R, Jones JD (2009).Role of plant hormones in plant defence responses.Plant Mol. Biol. 69(4):473-488.

Boller M (2001). Accumulating evidence demonstrates that the NAC proteins play critical roles in regulation of plant defense responses against different types of pathogens. Plant Mol. Biol. 46:521-529.

Cao H, Zhang J, Chen S (2005). AtNAC2, a transcription factor downstream of ethylene andauxin signaling pathways, is involved in salt stress responseand lateral root development. Plant J. 44(6):903-916.

Christian D, Stracke R, Erich G, Bernd W, Cathie M, Lepiniec L (2010). MYB transcription factors in Arabidopsis. Trends Plant Sci. 15(10):573-581.

Christiansen MW, Holm PB, Gregersen PL (2011). Characterization of barley (*Hordeum vulgare* L.) NAC transcription factors suggest conserved functions compared to both monocots and dicots. BMC Res. Notes 4(1):302.

Collinge M, Bollar T (2001). Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by Phytophthora infestans and to wounding. Plant Mol. Biol. 46(5):521-529.

Dietz K, Viehhauser A (2010). AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and etrograde signalling. Protoplasma 245(1-4):3-14.

Eulgem T, Somssich IE (2007). Networks of WRKY transcription factors in defense signaling. Curr. Opin. Plant Biol. 10:366-371.

Fan K, Bibi N, Gan S, Li F, Yuan S, Ni M, Wang M, Shen H, Wang X (2015). A novel NAP member GhNAP is involved in leaf senescence in Gossypium hirsutum. J. Exp. Bot. 66(15):4669-4682.

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LSP, Yamaguchi K, Shinozak K (2004). A dehydration-induced NAC protein, RD26, is involvedin a novel ABA-dependent stress-signaling pathway. Plant J. 39(6):863-876.

Glazebrook J (2001). Genes controlling expression of defense responses in Arabidopsis. Curr. Opin. Plant Biol. 4(4):301-308.

Hao Y, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY (2011).Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. Plant J. 68:302-313.

Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, Joehnk N, Lyngkjae MF Collinge B (2007). The HvNAC6 transcription factor: a positive regulatorof penetration resistance in barley and Arabidopsis. Plant Mol. Biol. 65(1-2):137-150.

Kato H, Komeda Y, Saito T, Kato A (2010). Overexpression of the NAC transcription factor family gene ANAC036 resultsin a dwarf phenotype in Arabidopsis thaliana. J. Plant Physiol. 167(7):571-577.

Kong X, Dong H, Eneji E, Li W, Lu H (2013). Gene Expression Profiles Deciphering Leaf Senescence Variation between Early- and Late-Senescence Cotton Lines. Plos One 8(7):e69847.

Lamb C, Grant M (2006). Systemic immunity. Curr. Opin. Plant Biol. 9(4):414-420.

Lin R, Zhao W, Meng X, Wang M, Peng Y (2007). Rice gene OsNAC19 encodes a novel NAC-domain transcription factor and responds to infection by Magnaporthe grisea. Plant Sci. 172:120-130.

Lu PL, An R, Su Z, Qi BS, Ren F, Chen J, Wang XC (2007). A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. Plant Mol. Biol. 63(2):289-305.

Mao X, Li A, Zhai C, Jing R (2014). Novel NAC Transcription Factor TaNAC67 Confers Enhanced Multi-Abiotic Stress Tolerances in Arabidopsis. Plos One 9(1):e84359

Mauch-Mani B, Mauch F (2005).The role of abscisic acid in plant–pathogen interactions Curr. Opin. Plant Biol. 8(4):409-414.

McGrath KC, John BD, Manners M, Schenk P M, Edgar CI, Maclean DJ, Scheible R, Udvardi MK, Kazan K (2005). Repressor- and Activator-Type Ethylene Response Factors Functioning in Jasmonate Signaling and Disease Resistance Identified via a Genome-Wide Screen of Arabidopsis transcription Factor Gene Expression. Plant Physiol. 139(2):949-959.

Meng C, Cai C, Zhang T, Guo W (2009). Characterization of six novel NAC genes and their responses to abiotic stresses in *Gossypium hirsutum* L. Plant Sci.176:352-359.

Meng Q, Gai J, Yu D (2007). Molecular cloning, sequence characterization and tissue-specific expression of six NAC-like genes in soybean (*Glycine max* (L.) Merr.). J. Plant Physiol.164(8):1002-1012.

Nakashima K, Junya HT, Shinozaki K, Yamaguchi-Shinozaki K (2011). NAC transcription factors in plant abiotic stress responses. Biochim. Biophys. Acta 1819(2):97-103.

Nakashima K, Tran L, Van D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki (2007). Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 51(4):617-630.

Nuruzzaman M, Sharoni AM, Kikuchi S (2013). Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front. Microbiol. 4(9):1-16.

Oh SK, Yu SH, Choi D (2005). Expression of a novel NAC domain-containing transcription factor (CaNAC1) is preferentially associated with incompatible interactions between chili pepper and pathogens. Planta 222(5):876-887.

Olsen AN, Ernst H, Leggio L, Skrive K (2005). NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci. 2(10):79-87.

Ooka HK, Doi SK, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K,Kikuchi S (2003). Comprehensive Analysis of NAC Family Genes in Oryza sativa and Arabidopsis thaliana. DNA Res. 247(10):239-247.

Peng H, Yu X, Cheng H Shi ,Q Zhang H, Li J, Ma H (2010). Cloning and Characterization of a Novel NAC Family Gene CarNAC1 from Chickpea (*Cicer arietinum* L.). Mol. Biotechnol. 44 (1):30-40.

Penga H, Chenga H, Chena C, Yua X, Yanga J, Gaoa W, Shic Q, Zhangc H, Lic J, Maa H (2009). A NAC transcription factor gene of Chickpea (*Cicer arietinum*), CarNAC3, is involved in drought stress response and various developmental processes. J. Plant Physiol. 166(17):1934-1945.

Permingeat HR, Romagnoli MV, Vallejos RH (1998). A Simple Method for Isolating High Yield and Quality DNA from Cotton (*Gossypium hirsutum* L.) Leaves. Plant Mol. Biol. Rep. 6(1):1-6.

Puranik S, Sahu PP, Srivastava PS, Prasad M (2012). NAC proteins: regulation and role in stress tolerance. Trends Plant Sci. 17(6):369-381.

Schmittgen T, Livak KJ (2001). Analysis of Relative Gene Expression Data Using RealTime Quantitative PCR and the 22DDCT Method. Methods 25(4):402-408.

Sefyan A, Saad XL, He-Ping L, Tao H, Guo CM, Wei C, Zhao G, Liao Y (2013). A rice stress-responsive NAC gene enhances tolerance of transgenic wheat to drought and salt stresses. Plant Sci. 203-204: 33-40.

Shah ST, Peng C, Anwar H, Song FM, Roshan Z, Shuxun Y (2013). Isolation and expression profiling of GhNAC transcription factor genes in cotton (*Gossypium hirsutum* L.) during leaf senescence and in response to stresses. Gene 531(2):220-234.

Shah ST, Peng C, Anwar H, Song FM, Roshan Z, Shuxun Y (2014). Molecular cloning and functional analysis of NAC family genes associated with leaf senescence and stresses in *Gossypium hirsutum* L. Plant Cell Tissue Organ Cult. 117(2):167-186.

Van Loon LC, Rep M, Pieterse CM (2006). Significance of Inducible Defense-related Proteins in Infected Plants. Annu. Rev. Phytopathol. 44:135-162.

Wan CY, Wilkins TA (1994). A Modified Hot Borate Method Significantly Enhances the Yield of High-Quality RNA from Cotton (*Gossypium hirsutum* L.). Anal. Biochem. 223(1):7-12.

Weaver L, Gan M, Quirino S, Amasino RM (1998). A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol. Biol. 37(3):455-469.

Wright P (1998). Research into Early Senescence Syndrome in Cotton. Better Crops Int. 12(2):14-16.

Xia N , Zhang G, Sun Y, Zhu L, Xu L, Chen X , Liu B , Yu Y , Wang X, Huang L, Kang Z (2010). TaNAC8, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. Physiol. Mol. Plant Pathol. 74(8):3703-3712.

Xu Q, Li S, Tian Z (2014). Molecular characterization of StNAC2 in potato and its overexpression confers drought and salt tolerance. Acta Physiol. Plant. 36(7):1841-1851.

Yamasaki K, Kigawa T, Seki M, Shinozak K, Yokoyama S (2013). DNA-binding domains of plant-specific transcription factors: structure, function and evolution. Trends Plant Sci. 18(5):267-276.

Yu X, Peng H, Liu Y, Zhang Y, Shu Y, Chen Q, Shi S, Ma L, Ma H, Zhang H (2014). CarNAC2, a novel NAC Transcription Factor in Chickpea , is Associated with Drought-response and Various Developmental Processes in Transgenic Arabidopsis. J. Plant Biol. 57:55-66.

Zhao F, Ma J, Li L, Fan S, Guo Y, Song M, Yu, S (2015). GhNAC12, a neutral candidate gene, leads to early aging in cotton (*Gossypium hirsutum* L). Gene 576(1):268-274.

academicJournals

Vol. 15(24), pp. 1246-1252, 15 June, 2016
DOI: 10.5897/AJB2016.15344
Article Number: 233B98958872
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fatty acids and amino acids contents in *Scomber scombrus* fillets from the South East of Tunisia

EL Oudiani Salma*, Darej Cyrine and Moujahed Nizar

National Institute of Agricultural Sciences of Tunisia, University of Carthage, 43 Av. Charles Nicolle 1082 Tunis Mahrajéne, Tunisia.

Received 15 March, 2016; Accepted 13 May, 2016

Proximate composition, fats and amino acids of Atlantic Mackerel fillets (*Scomber scombrus*) from the South East of Tunisia in different seasons, were analyzed in order to assess nutritive characteristics of this species. Samples were collected monthly from Zarzis fishing port located in the South-East of Tunisia. Total fats and protein contents varied significantly (P<0.05) according to the season. The highest values were obtained in spring (11.53 and 24.1% DM, respectively). Gas chromatography analysis showed the existence of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids. Palmitic and stearic acids were the major fatty acids in SFA family. Palmitoleic and oleic acids were the predominant in MUFA family. Arachidonic acid was the dominant in n-6 PUFA family. While eicosapentanoic (EPA) and docosahexanoic (DHA) were the most abundant in n-3 PUFA series. We observed that high PUFAs percentages were related to those of n-3 PUFA family, mainly DHA which was present at a high level and varied significantly with season (P< 0.01) with the highest value in winter (40%). The n-6 PUFA series were present at low rates comparatively with those of n-3 PUFA series ranging between 4.5 and 5.7%. The highest level of n-6 PUFAs was observed for arachidonic acid in autumn 3.71%. The n-3/n-6 ratio exhibited the highest level in spring (11.02). The Atlantic Mackerel fillets were high in essential amino acids (34.59 g/100 g of proteins). The highest rates were noted for phenylalanine, valine, threonine, isoleucine, leucine and methionine. It was concluded that Atlantic Mackerel was high in interesting human feeding nutriments, mainly PUFA and essential amino Acids. Even when significant, differences between seasons were not drastic and *S. scombrus* could be consumed during all the year.

Key words: *Scomber scombrus*, lipids, fatty acids, amino acids, seasonal variation.

INTRODUCTION

Currently, a growing interest is given to marine resources, mainly fishes as high nutritive and dietetic value foods (Ackman et al., 2002). These nutritive qualities are related to the high quality of the nutriments they include and the high digestibility of their protein and fat. Fishes are known to have an advantageous composition of fatty acids particularly rich in polyunsaturated essential fatty acids (PUFAs).

*Corresponding author. E-mail: salma.inat@yahoo.fr.

Unsaturated fatty acids may be synthesized by animals or humans only to a limited extent and must be supplemented by the diet (Steffens, 1997). These acids, mainly those from n-3 family interest consumers and scientists since they could have beneficial effects on human health by preventing some diseases such as asthma, arteriosclerosis, and joint inflammation as well as preventing cardiovascular diseases and delaying the development of some cancers (Kinsella, 1988; Sidhu, 2003; Chen et al., 2007; Calviello et al., 2007). Fish fats included also large amounts of saturated fatty acids mostly associated with triacylglycerols and minor's amounts of phospholipids (Shahidi and Wanasundara, 1998; Qari et al., 2014) and the HMSO (1994) recommend a ratio of PUFA/SFA less than 0.45 in consumed diets. The PUFAs are known by their different biological effects (James and Cleland, 1996) and include two main series consisting on n-3 and n-6 families. Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, the most important PUFA from n-3 family, play the most important role in the prevention of cardiovascular diseases and the daily prophylactic dose of DHA and EPA for adults and patients with circulatory disorders is 1 to 4 g (Kris-Etherton et al., 2002). Fishes are also high in PUFA from the family of n-6, but in lower proportions than those of n-3 PUFA family. The n-6 family is mostly represented by arachidonic acid and could have an antagonist effect with n-3 PUFAs. That is why it is recommended to have a maximum n-6/n-3 ratio of 4. In addition, fishes are an advantageous source of amino acids which are considered as the major constituent of proteins content, known to play an important role in the synthesis of metabolic molecules, enzymes and hormones (Oluwaniyi et al., 2010).

In Tunisa, Atlantic Mackerel are produced in important quantities, during the last year's production reached an amount of 4725 tones (DGPA, 2014). This species, associated to some other blue ones such as *Sardina Pilichardus*, *Sardinelle aurita* and, *Trachurus trachurus* is considered as one of the most popular fish consumed by the population and largely included in several local gastronomical habits. *Scomber Scombrus* is known to be high in protein and fat and is characterized by its high content of long chain of PUFAs (Ackman, 1990). In Tunisia, some studies focused on Mackerel eco-biology investigations (Hattour, 1979; Hattour, 2000; Cherif et al., 2008), but there was few or not available details on their flesh biochemical composition as related to the variation factors.

The current study aimed to assess seasonal impact on proximate composition and fatty acid contents of *S. scombrus* fillets and to characterize its amino acid profile.

MATERIALS AND METHODS

Sample collection and preparation

The studied samples were collected monthly and randomly from Zarzis fishing port located in the south east of Tunisia during the period between June 2010 and May 2011. Samples were taken from the fishers at landing. A case of Mackerel samples (30 specimens) was transferred in ice, in polystyrene boxes and transported to the laboratory, where they were measured, beheaded, washed, filleted, bag packed and frozen at -20°C. Mean length and weight of analyzed samples were about 23.7 cm and 120.65 g, respectively.

Laboratory analysis

Chemical analysis

Moisture content of flesh fish samples was determined by oven drying (5 g, at 105°C until constant weight, ISO 6496-1999). Ash content was determined by calcinations of 5 g samples in a furnace at 550°C according to the AOAC (2000) and results were expressed as percentage of dry matter (DM). Crude protein (N×6.25) was determined by the Kjeldahl method (ISO 5983-1997) and results were expressed as percentage of DM. Total lipid quantification was carried out using the Soxhlet method. A sample of 5 g of flesh powder were added to 200 ml of petroleum ether and heated for 6 h (AOCS, Ba 3-38). The extracted oil was evaporated under vacuum at 65°C using a rotary evaporator, and then placed in an oven at 45°C for 1 h before being transferred into desiccators and reweighed. All the analysis were performed in 6 repetitions.

Amino acids analysis

Amino acids were analyzed according to the method described by Cohen and Michaud (1993). Flesh fish (5 g) was hydrolyzed in 6 M HCl for 22 h at 110°C and then the identification of amino acids (AAs) was made by Gas-Liquid Chromatography (L1100 Waters) equipped with a quaternary pump, a 20 µl injection valve, a diode array fluorescence detectors and a C18 column (internal diameterof: 250 × 4.6 mm) 5 µm. The mobile phase A was composed of 10% of acetonitrile/methanol/water (45:45:10; v/v/v) and the mobile phase B was composed of 90% of sodium phosphate buffer Na_2HPO_4 (pH= 6.5). The flow rate was constant (1 ml/min), and the column temperature was set at 25°C. The fluorescence excitation and emission wavelengths were 340 and 450 nm respectively. Amino acids were identified by comparison of their retention times with those of standards and quantified with the software EZChrom Elite™ CDS Chromatography. Analysis was made in duplicate and results were expressed in g of amino acid per 100 g of protein.

Fatty acids analysis

Methyl esters preparation was carried out using a direct transesterification according to the procedure described by Mosers (1991). A sample of 0.5 g of fish flesh was mixed with 2 ml of methanol/methylene chloride (3:1) and 50 µmol of 17:0 free fatty acid as an internal standard (in 50 µl of hexane) was added to the mixture. Under continuous vortexing, 200 µl of acetyl chloride was added and the mixture was incubated in the oven at 75°C for one hour. After cooling during 15 min at room temperature, 4 ml of 7% potassium carbonate was added, vortexed and then 2 ml of hexane was added. The mixture was vortexed for 60 s and then centrifuged for 5 min. The hexane layer was removed, dried under nitrogen to a final volume of 100µl approximately and 1 µl of the sample was used for gas chromatography analysis. Fatty acid identification was performed using a gas chromatograph (HP series 6890) with a split/splitless injector, and a flame ionization detector was used for the analysis. The device includes a 30 m long HP Innowax capillary

Table 1. Proximate composition of Atlantic Mackerel Fillets.

Parameter	Autumn	Winter	Spring	Summer	Significance
Moisture (%)	70.61 ± 0.41^{b}	71.09 ± 0.69^{b}	71.03 ± 0.73^{b}	72.5 ± 1.04^{a}	**
CP (%)	18.71±1.52^{a}	19.94 ± 0.90^{a}	24.1 ± 0.90^{b}	23.5 ± 2.52^{a}	**
Fat (%)	6.32 ± 0.91^{c}	7.55 ± 0.29^{b}	11.53 ±1.93^{a}	4.35 ± 0.11^{d}	***
Ash (%)	2.3 ± 0.75^{a}	1.97 ± 0.72^{b}	1.80 ± 0.28^{b}	1.95 ± 0.32^{b}	***

Means with the same letter in the raw are not significantly different.

column with an internal diameter of 250 µm and a 0.25 µm film. The stationary polar phase of the column was composed of polyethylene glycol. Comparison of the retention times of the studied fatty acids and those of standard fatty acid methyl esters (Supelco PUFA-3) allowed to identify the different fatty acids contained in mackerel oil extract. All the analysis were performed in 6 repetitions.

Statistical analysis

Statistical analysis was performed using analysis of variance according to GLM procedure (SAS software, version 9.1). The model included season effect and each month of sampling was considered as replication. Means were compared using Student Newman and Kull tests.

RESULTS

Chemical composition

S. scombrus proximate chemical composition is presented in Table 1. Moisture content varied significantly (P<0.01) with season, a maximum value was obtained in summer 72.5% DM and minimum one in autumn (70.61% DM). Ash contents varied significantly (P<0.01) with seasons, reaching a maximum value of 2.3% DM in autumn and minimum value in spring (1.80% DM). Fat seasonal variation was highly significant (P<0.001). The highest level was noted in spring (11.53% of DM), whereas the lowest one was observed in summer (4.35% of DM). Also, crude protein contents varied significantly with seasons (P<0.001) with highest content in spring (24.1% DM) and a lowest one in autumn (18.71%DM).

Fatty acids contents

Results relative to FAs composition are presented in Table 2. Mackerel lipid extract is composed mainly of PUFAs followed by SFAs and MUFAs. The SFAs group was represented by palmitic acid (C16:0) as the most important one, reaching in autumn 25.2%, followed by stearic acid (C18:0) with a maximum level in winter (11.52%) and the myristic acid (C14:0) showing a highest content in summer (2.24%). The season effect of the last FAs was significant (P<0.01). Total SFAs seasonal variation was highly significant (P<0.001) and rates ranged between 33 and 38%.

The MUFAs group was represented by palmitoleic (C16:1) n-7 and oleic (C18:1) n-9 acids, for these two FAs the highest levels were registered in autumn (3.01 and 11.20% respectively). The effect of season was significant (P<0.01).

The PUFAs represented the most important group of FAs in *S. scombrus* lipids. The seasonal variation for these FAs was significant (P<0.01). High PUFA levels are related to those of n-3 family, mostly represented by the EPA and the DHA. The highest levels were registered in spring (7.03%) for EPA and in winter for the DHA (40%). In the group of the n-6 PUFAs, the highest proportion was noted for the arachidonic fatty acid (C20:4). The values varied significantly with season (P<0.01) and reached the maximum level of 3.71% in winter. The highest n-3/n-6 ratio is obtained in spring 11. 02%.

Amino acid contents

Amino acids results are exhibited in Table 3. The highest values were noted for glutamic and aspartic acids 11.76 and 9.84 g/100 g CP, respectively. The highest EAA concentrations were observed for Lysine, followed by Threonine, Tyrosine, Isoleucine and then Leucine. The lowest concentration corresponded to Cystine (1 g/100 g CP). Total EAA was about (34.59 g/100 g of CP). The other identified amino acids are non-essentials (NEAA) and their total content was about 55.15 g/100 g of CP. The established TEAA/TNEAA ratio was 0.62.

DISCUSSION

Chemical composition

The fat contents in *S. scombrus* fillets varied significantly (P<0.01) with season. This finding is in agreement with those of Caponio et al. (2004), Ennouri et al. (2013), and Ben Rebah et al., (2014) in their studies on *Sardina pilchardus, Sarinella aurita, and Liza aurata*, from the Ionian Sea, the Gulf of Tunis and the Gulf of Gabès, respectively. However, according to Wallace (1991) mackerel fat fillets content values are ranging between 25 and 30% in December and are around 5% in May, when

Table 2. Atlantic Mackerel Fatty Acids (%TFAs).

Fatty acids	Autumn	Winter	Spring	Summer	Signifiance	SEM
C14:0	1.81±0.79[a]	1.20±0.37[a]	1.53±0.48[a]	2.24±0.20[b]	**	0.144
C16:0	25.25±4.78[a]	23.62±5.14[a]	21.48±0.77[a]	22.26±1.94[a]	ns	0.445
C18:0	10.94±2.22[a]	11.52±3.49[a]	9.99±1.43[a]	11.02±2.04[a]	ns	0.278
Total SFA	38 [a]	36,3[b]	33[b]	35[b]	***	0.648
C16:1	3.07±0.50[a]	2.45±0.74[a]	2.71±0.95[a]	2±0.31[b]	**	0.137
C18:1	11.20±1.6[a]	9±2.91[a]	9,62±2,28[a]	9 ± 0.25[a]	**	0.477
Total MUFA	13.8[b]	11.4[a]	12.33[a]	10.4[c]	***	0.566
C18:2 n-6	1.45±0.21[a]	1.31±0.14[a]	1.18±0.16[b]	1.42±0.15[a]	**	0.03
C18:3 n-6	0.20±0.08[a]	0.20±0.12[a]	0.16±0.05[a]	0.20±0.11[a]	ns	0.009
C20:2 n-6	0.34±0.47[a]	0.13±0.07[b]	0.29±0.13[a]	0.39±0.3[a]	**	0.025
C20:4 n-6	2.69±0.35[b]	3.71±0,55[a]	2.83±0,45[b]	3.7±0.49[a]	**	0.028
Total PUFA n-6	5.3[a]	5.3[a]	4.5[b]	5.7[a]	**	0.021
C18:3 n-3	0.37±0.11[a]	0.32±0.06[a]	0.54±0.13[a]	0.7±0.42[b]	***	0.084
C20:3 n-3	0.38±0.15[b]	0.29±0.17[a]	0.24±0.16[a]	0.44±0.17[b]	**	0.095
C20:5 n-3	5.04±0,78[a]	5.43±0.62[a]	7.03±0.85[b]	5.61±0.38[a]	**	0.185
C22:5 n-3	1.08±0.23[a]	1.15±0.25[a]	1.51±0.15[a]	1.13±0.05[a]	ns	0.043
C22:6 n-3	36±7.10[a]	40±10.23[b]	33±2.64[a]	36.01±3.36[a]	**	0.822
TotalPUFAn-3	42.7[b]	46.8[a]	50[a]	48[a]	**	0.831
PUFA/SFA	1.26a	1.43a	1.65a	1.53a	ns	0.042
n-3/n-6	8.08[a]	8.75[a]	11.02[a]	8.57[a]	ns	0.217

*, P < 0.05; **, P < 0.01; ***, P< 0.001; ns, not significant; SEM, standard error of the mean; SFA, Saturated fatty acids, MUFA,: monounsaturated, PUFA, polyunsaturated fatty acids.

Table 3. Mackerel flesh amino acid composition (g/100 g of crude proteins).

Amino acids	Concentration (g/100 g of crude proteins)
Aspartic acid	9.84 ± 0.7
Glutamic acid	11.76 ± 0.9
Lysine [a]	5.90 ± 1
Histidine	4.56 ± 0.67
Arginine	5.86 ± 0.24
Threonine [a]	5.00 ± 0.36
serine	4.88 ± 0.2
Proline	6.02 ± 0.59
Glycine	5.30 ± 0.04
Alanine	6.93 ± 0.95
Cystine [a]	1.00 ± 0.01
Valine [a]	3.94 ± 0.50
Methionine [a]	3.20 ± 0.08
Isoleucine [a]	4.02 ± 0.39
Leucine [a]	3.89 ± 0.48
Tyrosine [a]	5.00 ± 0.78
Phenylalanine [a]	2.64 ± 0.11
Total amino acids (TAA)	96.67
Total essential amino acids (TEAA)	34.59
Total non-essential amino acids (TNEAA)	55.15
(TEAA)/(TNEAA)	0.62

[a]Essential amino acids according to FAO/WHO(1975); Values are expressed as means ±SD with (n=2).

fish spawns. These variations are the result of the impact of changes of some factors such as temperature, salinity, food availability and fish life cycle (Zaboukas et al., 2006; Pirini et al., 2010).

Crude protein content varied significantly with season ($P<0.01$) with a maximum value in spring 24.1% and a minimum value in autumn 18.71%.This finding is in line with those in other marine species as reported by Kacem et al. (2011) in their study on *Sardinella aurita*, *Sarpa salpa*, *Sepia officinalis* from Tunisia and by Orban et al. (2011) in their study on horse mackerel from the Southern Adriatic coast of Italy. This variation is the result of the impact of environmental parameters such as temperature, fluctuation in food availability and essentially fish life cycle. In fact, during the spawning period, lipids and proteins contents are mobilized from mussels and transferred to the gonads (Love, 1997).

Fatty acids composition

Fatty acid analysis indicated the presence of different categories of fatty acids, mainly SFAs, MUFAs and PUFAs. It was noted that the predominant fatty acid in SFA family was Palmitic acid followed by the stearic acid. However, Myristic acid (C14:0) exhibited the lowest proportion. This result is in line with those of Rioux and Legrand (2001) who claimed that the lowest proportion in the animal body is represented by Myristic acid (ranging between 0.5 and 2% of TFAs). Palmitic acid was the most represented SFA but the season effect was not significant (averaged 35.5%). Similar trend was found by Bouriga et al. (2010) on *Atherina boyeri, Atherina lagunae, Atherina* sp. and and by Ben Smida et al. (2010) on *Xiphias gladius* respectively.

Palmitoleic and oleic acids were the two main MUFAs identified in Mackerel lipid extract. The MUFAs highest proportions were those of oleic acid. This result is in accordance with those of Ben Smida et al. (2010) in *X. gladius* red and white mussels; those of Ben Rebah et al. (2014) in the males of *liza aurata* from the Tunisian coasts, and those of Kacem et al. (2011) in *Sardinella aurita* and *Sarpa salpa* from the Gabès Gulf. However, Soriguer et al. (1997) registered high MUFAs level in winter in Atlantic mackerel from Spain, this may be explained by the impact of environmental parameters essentially temperature. The Oleic acid is the characteristic of fish tissue (Steffens 1997) and is actively synthesized by cells (Legrand, 2007). Under the action of ACAT (acyl CoA-cholesterol acyltransferase), oleic acid binds to cholesterol (Legrand, 2007).The formed Cholesterol esters represents the form of the transport of cholesterol in lipoproteins (Steffens, 1997). As reported by Dalsgaard et al. (2003), high MUFAs level is an indicator of high degree of carnivory of this species. Indeed, according to the classification of fishes in functional groups based on their TROPH relation, *S. scombrus* is a pelagic and carnivorous fish species, fed only on the bases of animal species, mainly fish such as *Sardina pilchardus* and Crustaceans and Gasteropods (Stergiou and Karpouzi, 2002). This was confirmed by our observation on digestive tract content of *S. scombrus* (unpublished).

The PUFAs group was the most abundant in Mackerel flesh lipids. The content ranged from 54.5% in spring to 48% in autumn. The highest observed PUFAs levels are linked to the high content of n-3 FA series, mainly represented by EPA and DHA. The DHA represented the highest proportion in winter (40 %). The n-6 family high values are related to those of arachidonic acid, reaching 3.71% in winter. This result is in line with the findings of Özogul et al. (2007) on Mackerel from Turkey. Fishes are generally rich in n-3 FAs and low in n-6 fatty acids. These groups of FAs are known to have beneficial effects for human health (Pigott and Tucker, 1990). An increase in n-3/n-6 ratio is essential to help the body use of fatty acids since n-6 FAs could have an antagonist effect with n-3 FAs (Polak-Juszczak and Komar-Szymczak, 2009). The proportions of EPA and DHA are responsible of variation in n-3/n-6 ratio (Hossain, 2011). In our study the n-3/n-6 ratio was the highest in spring (11.02). This result is in concordance with those of Özogul et al. (2007). The PUFAs /SFAs ratio reached the highest level in spring 1.65. This value is higher than the minimum recommended value (0.45) as claimed by HMSO (1994) for human nutrition. Seasonal variation in PUFAs may be explained by genetic factors, fishing period, sexual maturity stage, reproduction activity, and nutritional factors. Moreover, water temperature is the most important factor influencing PUFA synthesis in fishes. Temperature variations influence desaturase and synthesize enzyme activities with a direct effect, or by long term adaptative process- leading to the synthesis of omega-3 fatty acids (De Torrengo and Brenner, 1976; Farkas and Csengeri, 1976; Caponio et al., 2004).

Amino acids contents

Results showed that Scomber scombrus was high in TAA (96.67 g/100 g protein). This result is in line with which of Oluwaniyi et al. (2010) found in Atlantic Mackerel from Nigeria. It was found that Glutamic acid had the highest concentration (11.76 g/100 g CP). This finding is similar to which of Selmi et al. (2009) study on *Sardina Pilchardus*. The established TEAA/ TNEAA ratio was about 0.62 which is close to the finding of Kaya et al. (2014) in *Sardina melonosticta* (0.69).

Amino acids are the basis of all life processes, as they are necessary for all metabolic processes. Their main task is to ensure optimal transport and to optimize the storage of all nutrients. Their composition in fish and in human tissues is similar. The essential amino acids cannot be synthesized in human body, so they are

required from fish consumption as reported by Osibona et al. (2009). In our study, we did not consider AA variation according to the season. It seems that the type and the amount of amino acids are related to fishing season, locality, feeding habit and fish life cycle (Wesselinova, 2000; Kaya et al., 2014). This aspect needs further investigation in *S. scombrus*.

Conclusions

It was concluded that *S. scombrus* flesh from the region of Zarzis (Tunisia) is rich in unsaturated fatty acids, mainly from the n-3 family, especially DHA. The studied samples had high n-3/n-6 ratio and the PUFAs/SFAs coefficient exceeded the recommended minimum value by HMSO. This represents an advantageous impact when consumed by human. Associated to high level of protein and essential amino acid, our results indicate that the studied species is of a high nutritive value and could be healthy compound in human diets. Even when significant, differences between seasons were not drastic and *S. scombrus* could be consumed beneficially during all the year.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

AA, Amino acids; **TAA,** total amino acids; **TEAA,** total essential amino acids; **TNEAA,** total non-essential amino acids; **FA,** fatty acid; **SFAs,** saturated fatty acids; **MUFAs,** monounsaturated fatty acids; **PUFAs,** polyunsaturated fatty acids; **EPA,** eicosapentanoic acid; **DHA,** docosahexanoic acid; **ACAT,** acyl CoA-cholesterol acyltransferase.

REFERENCES

Ackman RG (1990). Sea food Lipids and Fatty Acids. Food Rev. Int. 6:617-646.

Ackman RG, Mcleod C, Rakshit S, Misra KK (2002). Lipids and fatty acids of five freshwater fishes of India. J. Food. Lipids 9:127-145.

Ben Rebah F, Ketata Khitouni I, Boudhrioua Mihoubi N, Bouain A (2014). Seasonal variations in proximate and fatty acid composition of golden grey mullet Liza aurata (R, 1810) from the Tunisian coast. Int. J. Agric. Pol. Res. 2(7):273-280.

Ben Smida MA, Brahim M, El Cafsi M (2010). Seasonal Variation of Fatty Acids in Muscles of Tunisian Swordfish (*Xiphias gladius* L.1758). J. Soc. Chim. Tun. 12:129-137.

Bouriga N, Selmi S, Faure E, Trabelsi M (2010). Biochemical composition of three Tunisian silverside (fish) populations caught in open sea, lagoon and island coasts. Afr. J. Biotechnol. 9(26):4114-4119.

Calviello G, Serini S, Piccioni E (2007). n−3 polyunsaturated fatty acids and the prevention of colorectal cancer: Molecular mechanisms involved. Curr. Med. Chem. 14(29):3059-3069.

Caponio F, Lestingi A, Summo CMT, Bilancia1 MT, Laudadio V (2004). Chemical characteristics and lipid fraction quality of sardines (Sardinapilchardus W.): influence of sex and length. J. Appl. Ichthyol. 20:530-535

Chen YQ, Edwards IJ, Kridel SJ, Thornburg T, Berquin IM (2007). Dietary fat-Gene interaction in cancer. Cancer Metastasis Rev. 26:535-551.

Cherif M, Zarrad R, Gharbi H, Missaoui H, Jarboui O (2008). Length-weight relationship for 11 fish species from the Gulf of Tunis (SW Mediterranean Sea, Tunisia). Pan. Am. J. Aquat. Sci. 3(1):1-5.

Cohen SA, Michaud KE (1993). Synthesis of fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimildyl carbamate and its application for the analysis of hydrolysate aminoacids via high-performance liquid chromatography. Anal. Biochem. 211:279-287.

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003). Fatty acid trophic markers in the pelagic marine environment. Adv. Mar. Biol. 46:225-340.

De Torrengo MP, Brenner RR (1976). Influence of environmental temperature on the fatty acid desaturation and elongation activity of fish (*Pimelodus maculatus*) liver microsomes. Biochem. Biophys. Acta 424:36-44.

DGPA (2014). Direction Générale de la Pêche et de l'Aquaculture (DGPA). Annuaires des statistiques des produits de la pêche en Tunisie. Ministere de l'Agriculture et des Ressources Hydrauliques (M.A.R.H).

Ennouri R, Mili S, Chouba L (2013). Evaluation de la qualité nutritionnelle des la Sardinelle Ronde Sardinella aurita (Valenciennes, 1947) du Golfe de Tunis.Bull.Ins.Natn, Scien. Tech. Mer. 40:87-96.

Farkas T, Csengeri I (1976). Biosynthesis of fatty acids by the carp *Cyprinus carpio* L., in relation to environmental temperature. Lipids 11:401-407.

Hattour A (1979). Contribution a l'étude des scombridés des eaux tunisiennes, *DEA Biologie marine et océanographie, Fac. Scien. Tunis.* 168 p.

Hattour A (2000). Contribution à l'étude des poissons pélagiques des eaux tunisiennes. *Thèse de Doctorat d'Etat de la Fac des Scien de Tunis.* 343 p.

HMSO, UK (1994). Nutritional aspects of cardiovascular disease (report on health and social subjects No. 46). London: HMSO.

Hossain MA (2011). Fish as Source of n-3 Polyunsaturated fatty acids (PUFAs), Which One Is Better- Farmed or Wild? Adv. J. Food Sci. Technol. 3:455-466.

James MJ, Cleland LG (1996). Dietary polyunsaturated fats and inflammation. Proc. Nutr. Soc. Aust. 20:71-77.

Kacem M, Sellami M, Kammoun W, Frikha F, Miled N (2011). Seasonal variation in proximate and fatty acids composition of Viscera of *Sardinella aurita*, *Sarpa salpa*, and *Sepia officinalis* from Tunisia. J. Aquat. Food Prod. Technol. 20:233-246.

Kaya Y, Erdem ME, Turan H (2014). Monthly Differentiation in Meat Yield, Chemical and Amino Acid Composition of Wild and Cultured Brown Trout (*Salmo Trutta Forma Fario*Linneaus, 1758). Turk. J. Fish. Aquat. Sci. 14:479-486.

Kinsella JE (1988). Fish and sea food: Nutritional implications and quality issues. Food Technol. 42:146-150.

Kris-Etherton PM, Harris WS, Appel LJ (2002). Fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. Circulation 106:2747-2757.

Legrand P (2007). Les acides gras: Structures, fonctions Apports nutritionnels conseillés. Cah. Nutr. Diét. 42:7-12.

Love RM (1997). Biochemical dynamics and the quality of frech and frozen fish. Fish. Process. Technol. pp. 1-31.

Moser HW, Moser AB (1991). Measurement of saturated very long chain fatty acids in plasma. Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual. Willey-Liss, Inc. pp. 177-191.

Oluwaniyi OO, Dosumu OO, Awolola GV (2010). Effect of local processing methods (boiling, frying and roasting) on the amino acid composition of four marine fishes commonly consumed in Nigeria. Food Chem. 123(4):1000-1006.

Orban E, Dilena G, Nevigato T, Masci M Casini I (2011). Proximate unsaponifiable lipid and fatty acid composition of bogue (*Boops*

boops) and horse mackerl (*Trachurus trachurus*) from the Italian trawl fishery. J. Food. Comp. Anal. 24:1110-1116.
Osibona AO, Kusemiju K, Akande GR (2009). Fatty acid composition and amino acid profile of two freshwater species, African Catfish (*Clariasgariepinus*) and Tilapia (*Tilapia zillii*). Afr. J. Food Agric. Nutr. Dev. 9(1):608-621.
Özogul Y, Özogul F, Alagoz S (2007). Fatty acid profiles and fat contents of commercially important seawater and freshwater fish species of Turkey: A comparative study. Food Chem. 103:217-223
Pigott GM, Tucker BW (1990). Seafood: Effects of Technology on Nutrition. Marcel Dekker Inc., New York.
Pirini M, Testi S, Ventrella V, Pagliarani A, Badiani A (2010). Blue-back fish: Fatty acid profile in selected seasons and retention upon baking. Food Chem.123:306-314.
Polak-Juszczak L, Komar-Szymczak K (2009). Fatty acid Profiles and Fat contents of Commercially Important Fish from Vistula Lagoon. Pol. J. Food Nutr. Sci. 59(3):225-229.
Qari SA, Moharram SG, Alowaidi SA (2014). Fatty acids Composition in Male's of the Red Sea fish Rhabdosargus sarba during the spawning season. Am. J. Life. Sci. 2(2):103-107.
Rioux V, Legrand P (2001). Métabolisme et fonctions de l'acide myristique. *Oléagineux*, Corps Gras Lipides 8:161-166.
Selmi S, Batista I, Sadok S, Bandarra NM, Numes ML (2009). Changes in biochemical composition and developpement of lipid oxidation during Sardine *Sardina pilchardus* mince preparation. J. Food Process. Eng. In Press.
Shahidi F, Wanasundara MN (1998). Omega-3 fatty acids concentrate nutritional aspect and production technologies. Trends Food Sci. Technol. 9:230-240.
Sidhu KS (2003). Health benefits and potential risks related to consumption of fish or fish oil. Regul. Toxicol. Pharm. 38:336-344.
Soriguer F, Serna S, Valverde E, Hernando A Martín-Reyes H, Soriguer M, Parejal A, Tinahones A, Esteval I (1997). Lipid, protein, and calorie content of different Atlantic and Mediterranean fish, shellfish, and molluscs commonly eaten in the south of Spain. Eur. J. Epidemiol. 13:451-463.
Steffens W (2006). Freshwater fish – wholesome foodstuffs. Bulg. J. Agric. Sci. 12:320-328.
Stergiou KI, Karpouzi VS (2002). Feeding habit and trophic levels of Mediterranean Fish. Rev. Fish Biol. Fish. 11:217-254.
Wallace PD (1991). Seasonal variation in fat content of mackerel (*Scomber scombrus* L.) caught in the English Channel. Fish. Res. Tech. Rep. 91.
Wesselinova D (2000). Amino acid composition of fish meat after different frozen storage periods. J. Aquat. Food Prod. Technol. 9(4):41-48.
Zaboukas N, Milou H, Megalofonou P, and Moraitou-Apostolopoulou M (2006). Biochemical composition of the Atlantico bonito *Sarda sarda* from the Aegean Sea (eastern Mediterranean Sea) in different stages of sexual maturity. J. Fish Biol. 69:347-362.

academicJournals

Vol. 15(24), pp. 1253-1257, 15 June, 2016
DOI: 10.5897/AJB2016.15362
Article Number: B66366D58874
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biochemical and hematological profile of different breeds of goat maintained under intensive production system

Samira Ahmed Mohammed[1]*, Mohammed Abdur Razzaque[1], Anaam E. Omar[2], Sheeba Albert[1], and Waleed M. Al-Gallaf[1]

[1]Desert Agriculture and Ecosystems Program, Kuwait Institute for Scientific Research (KISR), P. O. Box 24885, Safat 13109, Kuwait, Kuwait.
[2]Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt.

Received 23 March, 2016; Accepted 6 May, 2016

The aim of the study was to monitor the health and nutritional status of Kuwaiti's Aradi and exotic Damascus and Barbari goat breeds raised under an intensive system of production through the determination of biochemical parameters and hematology profiles. The study was conducted during the summer season and blood samples were collected from lactating does and Damascus bucks. According to the findings of the present study, concentrations for glucose ranged from 60.75 to 71.76 mg/dl, for blood urea nitrogen (BUN) between 25 and 60 mg/dl, for creatinine between 1.2 and 1.9 mg/dl, for albumin between 3.7 g/dl and 4.05 g/dl, and total protein between 6.0 and 6.98 g/dl and in general were within the normal ranges for goats. The hematology profiles of Damascus bucks showed normal range for the most contents of white blood cells, apart from monocytes (M) %. At the same time, number of red blood cells was within the normal range (11.20-11.90 × 10^6/µL). However, hematocrit (HCT) % was lower (12.1-14.0) and mean corpuscular volume (MCV) content was higher (11.30 fL) compared to values reported for healthy goats in previous studies. The hematology profiles of lactating does of all breeds were within the normal range for white blood cells (8.05-12.88 × 10^3/µL) and red blood cell (10.44-12.65 × 10^6/µL). Low hemoglobin levels and a significantly (P<0.05) lower mean platelet volume (MPV), procalcitonin (PCT) and platelet distribution width (PDW) were found in Damascus does compared to the other examined breeds. The data of the present study could serve as reference values, since this is the first time that biochemical and hematological parameters were determined in Aardi, Damascus and Barbari goats managed under an intensive production system in Kuwait. These data can be used in the realistic evaluation and improving the management practices, nutrition, and monitoring health status and diagnosis of diseases.

Key words: Blood biochemical parameters, hematology profile, Damascus, Barbari, Aardi, intensive production system.

INTRODUCTION

Biochemical and hematological variables of blood are generally used to monitor and evaluate health, nutritional

and physiological status of ruminants (Chapple et al., 1991; Al-Eissa et al., 2012; Gupta et al., 2007). The evaluation of blood constituents has been widely used as a marker to determine the efficacy of feed nutrient content and supplements (Belewu and Ogunsola, 2010; Belewu et al., 2009; Akingbade et al., 2002) but also an index of transportation stress (Ambore et al., 2009). The biochemical and hematology profiles can also be used to assess the immunity status in goats (Al-Seaf and Al-Harbi, 2012). These profiles could be altered during pregnancy (Waziri et al., 2010) and seasonal variations had also an effect on these profiles (Abdelatif et al., 2009). Finally, nutrition, stress, reproductive status, age, sex, genetics, management, housing, and other environmental factors (temperature, relative humidity etc.) are known to have a profound effect on the hematological and biochemical profiles of small ruminants (Balikci et al., 2007; Olayemi et al., 2009).

According to the existing literature, there is a great variation among goat breeds concerning their hematological and biochemical profile (Azab and Abde-Maksoud, 1999; Tambuwal et al., 2002; Tibbo et al., 2004). Therefore, there is a need for establishing a reference value data for the physiological variables of goat breeds raised under a feedlot management system (zero grazing) with the intension of further evaluation of their nutrition, management and health. The objective of the present experiment was therefore to investigate the biochemical and hematological parameters of the lactating does of local Aardi, exotic Damascus, and Barbari breeds and the hematology profile of Damascus bucks managed under an intensive system in Kuwait.

MATERIALS AND METHODS

The study was conducted at the Kuwait Institute for Scientific Research, in the Agricultural Research Station in Sulaibiyia, about 40 km southwest of the Kuwait City during summer. The climate conditions were typical of the Arabian desert, temperature was high (up to 52°C) and extreme variation existed between day and night. Relative humidity was 12 to 20% in mid-summer (June to August). All goats were housed in partially enclosed sheds.

A total of 16 lactating Does from Syrian Damascus, Indian origin Barbari and two strains of Local Aardi does and four bucks from Syrian Damascus were obtained from local goat breeders in Kuwaiti. The does were 22 to 24 months old, the mean live weight were 52.00 ±2.83, 43.33 ±5.5, 56.08 ±5.58 and 32.79 ±2.97 kg for White Aardi, Black Aardi, Damascus and Barbari does respectively. The does were in their first parity and in second third of their lactation period. The bucks were around three years old and their live weight was 61.5 ±1.44 kg. After purchasing the goats, they maintained in quarantine and were adapted to an intensive production system and through the provision of feedlot diets containing 60% concentrates and 40% roughages (alfalfa straw) with 12% of crude protein and medium level of energy (2.8 Mcal/kg) (Table 1). Health status of the animals was checked, and they were then weighed and vaccinated for endemic diseases. Each group of the same goat breed was housed in separate pen providing free access to water and diets that were formed feeds according to the NRC standards (NRC, 1981).

Table 1. Composition (%) and analysis of concentrate mixture offered to goats.

Ingredients	Percentage
Barley (*Hordeum vulgare* L.)	85
Wheat bran (*Triticum boeoticum* Boiss)	12
Ground limestone	1.6
Common salt	0.7
Vitamins and minerals	0.7
Analysis (% DM)	
Ash	3.21
CP	11.9
EE	2.3
ADF	6.88
NDF	28.03

CP, Crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber.

Blood samples were collected once during month of July between 6:00 and 7:00 am. Blood samples were collected with 18-ga needle by jugular venipuncture. The blood was collected into two tubes, one in a plain tube for serum analysis and another with heparin for whole blood analysis. Blood samples were centrifuged within two hours of collection and serum was harvested and stored at -20°C for biochemical analysis. Two methods of serum total protein analysis were used for comparative purposes; Biuret and Refractometry. The blood serums were analyzed for glucose, creatinine, albumin, chloride, urea and hemoglobin by a spectrophotometer (Perkin Elmer Lambda 25).

Hematological profiles were also examined in the lactating does of the four breeds and in the bucks of Damascus breed. Blood samples were collected with an 18-ga needle by jugular venipuncture. Ethylenediaminetetraacetic acid (EDTA) tubes were analyzed within two hours of collection for the determination of the following parameters: white blood cell (WBC), neutrophils (N), lymphocytes (L), monocyte (M), eosinophil (E), basophil (B) and red blood cells (RBC), hemoglobin (Hb), mean corpuscular volume (MCV), hematocrit (HCT), mean corpuscular hemoglobin (MCH), corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV), procalcitonin (PCT) and platelet distribution width (PDW).

Statistical analysis

All results are expressed as means ± standard deviation (SD). The effects of breed on biochemical parameters and hematology profiles of does were analyzed by one-way analysis of variance (ANOVA). If ANOVA showed an acceptable level of significance ($P<0.05$), Duncan Multiple Range Test was applied for post hoc

*Corresponding author. E-mail: smohamad@kisr.edu.kw.

Table 2. Biochemical parameters of blood serum in four breeds of lactating does (Mean ±SD)

Breed	Glucose (mg/dl)	BUN (mg/dl)	Creatinine (mg/dl)	Albumin (g/dl)	Total protein (g/dl) (Biuret)	Total protein (g/dl) (Refractometry)	Refractive Index
Damascus	71.74± 8.23[b]	30.49±8.71[a]	1.19±0.34[ab]	3.83±0.16[a]	6.42±0.80[a]	6.68±0.55[a]	1.35±0.0[a]
Black Aardi	71.76± 3.69[b]	33.16±13.45[a]	1.02±0.32[b]	3.70±0.38[a]	6.76±0.34[a]	6.56±0.33[a]	1.35±0.0[a]
White Aardi	98.07± 4.72[a]	27.12±7.35[a]	1.57±0.11[a]	4.05±0.21[a]	6.17±0.52[a]	6.00±0.57[a]	1.35±0.0[a]
Barbari	60.75± 10.05[b]	37.28±10.57[a]	1.11±0.30[ab]	3.72±0.19[a]	6.98±1.07[a]	6.30±0.87[a]	1.35±0.0[a]
Normal ranges*	50-75	25-60	1.2-1.9	2.7-3.8	6.0-7.0	N/A	N/A

[abc]Means in the same row bearing different superscripts differed significantly (P<0.05); BUN: Blood urea nitrogen; N/A: Not available .*From http://goat-link.com.

comparison by using the General Linear Model (GLM), procedures of the SPSS Statistical Package (SPSS, 1999).

RESULTS AND DISCUSSION

The mean concentration value of glucose for Damascus, Black Aardi, and Barbari does were 71.74, 71.76 and 60.75 mg/dl, respectively (Table 2). These values were within the normal range for goats (50-75 mg/dl). However, White Aardi goat showed significantly (p<0.01) higher glucose levels than the other breeds. The concentration of glucose in the blood serum is regulated by the nutritional regime and hormones; however, it could be influenced by many other factors, such as age, sex, breed, and environment (Sakha et al., 2009). The high glucose levels could be a result of stressful situation, or the administration of some drugs like steroids (Amer et al., 1989).

The levels of BUN for the examined does were within the normal range (Table 2) for goats (25-60 mg/dl). Studies reported varied values of BUN in different goat breeds. Benjamin (1989) suggested an average BUN concentration of 21.4 mg/dl; while Behera et al. (1993) found that BUN concentration ranged between 34.6 and 57.4 mg/dl in Black Bengal goats. Many reports suggested that BUN concentration was lower in kids than in adults with no significant differences between the sexes (Castro et al., 1977). Turner et al. (2005) reported a breed by diet interactions effect on BUN values in a study where three goat breeds, that is, Boer, Nubian, and meat Spanish goat were offered lespedezq (*Lespedeza cuneate*) or alfalfa hay (*Medicago sativa* L). In general, lower concentration of BUN is an indication of low dietary protein level or hepatic chronic disease. On the other hand, the increase of BUN could be the result of renal failure and body dehydration (Mishra et al., 2013).

In study, the creatinine levels of Black Aardi, Damascus and Barbari were within the normal range for the goats (1.2-1.9 mg/dl). However, White Aardi showed significantly higher (p≤0.01) value (1.57 mg/dl) compared to Black Aardi. Belewu and Ogunsola (2010) reported different low creatinine concentrations as a result of the diet fed to goats, 0.7 mg/dl for un-treated kernel cake (*Jatropha curcas*) and 1.2 mg/dl for goats fed with fungi-treated Kernel cake. The level of creatinine in the blood serum could be altered due to differences in energy and protein content of feeds. Solaiman et al. (2009) showed that creatinine levels in male kids increased linearly when cotton seed (*Gosspium hirsutum*) ration was offered to them. Moreover, a study on goat grazed on rangeland showed that the level of creatinine had varied values depended on certain roughages available in wet and dry periods of grazing (Mellado et al., 2006). The level of creatinine could also be elevated in the serum of goats after water deprivation (Abdelatif et al., 2010).

The albumin content ranged between 3.7 to 4.05 g/dl in the four breeds of doe (Table 2). These values were within the normal range of albumin for goat serum of (2.7-3.8 g/dl) except the White Aardi which had insignificantly high value (4.05 ±0.21 g/dl). Seasonal variation had been reported for albumin in blood serum of Nubian goats (Abdelatif et al., 2009). Zubcic (2001) also reported albumin level of 3.3 g/dl in grazing German fawn goats.

The total protein for the four breeds of goats was between 6.0 and 6.98 g/dl (Table 2). These values were within the normal range value of 6.0 to 7.0 g/dl. High serum protein levels may result from high intake of grains, dehydration, and high temperature (Sandabe and Chaudhary, 2000). Zubcic (2001) reported that total protein value in goat serum could be increased till 7.5 g/dl in extensively raised animals. Sakha et al. (2009) also reported a normal value of 7.0 g/dl; however, they also found significant differences between sexes. Deangelino et al. (1990) also showed that the total protein concentration in young goats was significantly lower than that of adult.

The white blood cells count was within the normal range reported for goats (Table 3). WBC of Damascus does was apparently lower than the other breeds, however this difference was not significant. M and B counts of Damascus does were slightly higher than that of the normal range. Low levels of MCV was found for all goat breeds in the study; whereas MCHC levels were higher (P>0.05) than the reference values. The reason for these discrepancies in MCV and MCHV values is not

Table 3. Hematological profiles of different breeds of does (Mean ±SD).

Parameter	Barbari	Black Aardi	White Aardi	Damascus	Reference value*
WBC (x 10^3/µL)	12.88±4.83[a]	12.20±2.97[a]	12.10±2.12[a]	8.05±2.06[a]	4-13
N%	56.80±5.23[a]	42.90±5.80[a]	29.95±7.28[a]	39.90±38.18[a]	30-48
L%	36.35±4.45[a]	49.70±1.70[a]	64.25±8.13[a]	47.15±27.36[a]	50-70
M%	4.52±2.11[a]	3.73±2.56[a]	4.10±1.12[a]	5.61±4.99[a]	0-4
E%	2.06±1.51[a]	2.56±1.14[a]	0.93±0.04[a]	6.04±4.33[a]	1-8
B%	0.27±0.15[a]	1.08±0.40[a]	0.78±0.30[a]	1.36±1.52[a]	0-1
RBC (x 10^6/µL)	12.15±1.06[a]	11.20±0.42[a]	12.65±1.20[a]	10.44±0.93[a]	8-18
Hb (g/dL)	9.97±2.73[a]	8.43±0.38[a]	10.65±0.35[a]	6.97±0.26[a]	8-12
HCT (%)	16.80±2.26[a]	14.55±0.64[a]	18.25±3.61[a]	12.70±1.55[a]	22-38
MCV (fL)	13.80±0.56[a]	12.95±0.07[a]	14.40±1.41[a]	12.15±0.49[a]	16-25
MCH (pg)	8.14±1.53[a]	7.53±0.63[a]	8.44±0.56[a]	6.70±0.33[a]	5.20-8
MCHC(g/dL)	58.90±8.63[a]	58.05±5.16[a]	59.20±9.76[a]	55.25±4.88[a]	30-36
RDW (%)	34.05±3.04[a]	35.25±6.29[a]	34.45±4.31[a]	31.35±3.32[a]	N/A
MPV(fL)	10.40±0.00[a]	10.36±0.62[a]	8.53±0.00[ab]	8.12±1.19[b]	N/A
PCT (ng/m)	70.70±0.00[a]	45.40±5.23[b]	44.50±0.00[b]	30.00±8.48[c]	N/A
PDW (%)	20.20±0.00[a]	20.30±1.27[a]	20.50±0.00[a]	18.10±0.14[b]	N/A

[abc] Means in the same row bearing different superscripts differed significantly (P<0.05). WBC, White blood cell; N, neutrophils; L, lymphocytes; M, monocyte; E, eosinophil; B, basophil; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet Volume; PCT, procalcitonin; PDW, platelet distribution width; N/A, not available. *According to Feldman et al. (2002), from Schalm's *Veterinary Hemetology*. Philadelphia. Baltimore, New York, London, Buenos Aires, Hong Kong, Sidney, Tokyo: Lippincott Williams and Wilkins.

clear. MCH concentrations for Black Aardi and Damascus were within normal range, whereas, White Aardi and Barbari had insignificantly higher than normal range. No significant difference was observed for RDW (31.35-35.25%). Significantly lower MPV, PCT and PDW values were found in Damascus does compared to three other breeds (P<0.05). Erythrocyte values were observed different in present study than those previously reported (Piccione et al., 2010; Olayemi et al., 2009; Rice and Hall, 2007). Age and sex have been reported to influence the hematological values of goat in the arid zone Egbe-Nwiyi et al. (2000); Zumbo et al. (2011) and Zamfirescu et al. (2009). Disease could also influence the hematological parameters in goats (Sulaiman et al., 2010).

Table 4 shows the means (±SD) of the hematological parameters for of the Damascus bucks. Values were within the normal range for goat apart from M% that was higher than the reference values (0-4%). The high level could be attributed to an inflammation, since only one animal showed a very high value (19.5%) resulting in an overall high mean of M%. The mean RBC value was 11.55 which were within the normal range. The HCT% and MCV content were lower than the normal range, which could indicate anemia, caused by vitamin B12 or folate deficiency.

The data obtained in the present study are the first reference value's obtained from Aardi, Damascus and Barbari goats raised under Kuwait's intensive production system. It may be concluded that the biochemical and

Table 4. Hematological profile of Damascus bucks (Mean ±SD).

Parameter	Mean ±SD	Reference Value*
WBC (x 10^3/µL)	11.80 ± 2.26	4.00-13.00
N%	42.15 ± 8.56	30-48
L%	35.70 ± 0.14	50-70
M%	13.45 ± 8.56	0-4
E%	8.17 ± 0.23	1-8
B%	0.56 ± 0.99	0-1
RBC (x 10^6/µL)	11.55 ± 0.49	8.00-18.00
Hb (g/dL)	8.60 ± 0.66	8.00-12.00
HCT (%)	13.05 ± 1.34	22.00-38.00
MCV (fL)	11.30 ± 0.71	16.00-25.00
MCH (pg)	7.45 ± 0.28	5.20-8.00
MCHC(g/dL)	65.85 ± 1.77	30.00-36.00
RDW (%)	28.75 ± 3.04	N/A
MPV(fL)	11.33 ± 1.94	N/A
PCT (ng/m)	57.15 ± 15.06	N/A
PDW (%)	17.55 ± 0.64	N/A

WBC, White blood cell; N, neutrophils; L, lymphocytes; M, monocyte; E, eosinophil; B, basophil; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet Volume; PCT, procalcitonin; PDW, platelet distribution width; N/A, not available. *According to Feldman et al. (2002), from Schalm's *Veterinary Hemetology*. Philadelphia. Baltimore, New York, London, Buenos Aires, Hong Kong, Sidney, Tokyo: Lippincott Williams and Wilkins.

hematological parameters were mostly within the physiological range for goats as reported from previous studies. These data can contribute to our knowledge for monitoring health status, diagnosis of diseases and management in these four breeds in Kuwait.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The project team members express their appreciations to the Kuwait Foundation for the Advancement of Sciences (KFAS) for the partial funding of the project.

REFERENCES

Abdelatif AM, Elsayed SA, Hassan YM (2010). Effect of state of hydration on body weight, blood constituents and urine excretion in Nubian goats (*Capra hircus*). World J. Agric. Sci. 6(2):178-188.

Abdelatif, AM, Ibrahim MY, Hassan YY (2009). Seasonal variation in erythrocytic and leukocytic indices and serum proteins of female Nubian goats. Middle East J. Sci. Res. 4(3):168-174.

Akingbade AA, Nsahlai IV, Morris CD, Iji PA (2002). Field activities and blood profile of pregnant South African indigenous goats after receiving dihydroxy pyridine-degrading rumen bacteria and grazing *Leucaena leucocephala* grass or natural pastures. J. Agric. Sci. 138:103-113.

Al-Eissa MS, Alkahtani S, Al-Farraj SA, Alarifi SA, Al-Dahmash B, Al-Yahya H (2012). Seasonal variation effects on the composition of blood in Nubian ibex (*Capra nubiana*) in Saudi Arabia. Afr. J. Biotechnol. 11(5):1283-1286.

Al-Seaf AM, Al-Harbi KB (2012). Variability of disease resistance, hematological parameters and lymphocyte proliferation in two goat breeds and their F1 and F2 crosses. Int. J. Food Agric. Vet. Sci. 2(1):47-53.

Ambore B, Ravikanth K, Maini S, Rekhe DS (2009). Haematological profile and growth performance of goats under transportation stress. Vet. World 2(5):195-198.

Amer HA, Ahmed AS, Gohar HM, Abdel Mamid MA (1989). Effects of steroid anaesthesia on some liver function tests in goats. J. Steroid Biochem. 32(3):475-476.

Azab ME, Abde-Maksoud HA (1999). Changes in some hematological and biochemical parameters during pregnancy and post-partum periods in female Baladi goats. Small Rumin. Res. 34:77-85.

Balikci E, Yildiz A, Gurdogan F (2007). Blood metabolite concentrations during pregnancy and post-partum in Akkaraman ewes. Small Rumin. Res. 67:247-251.

Behera PC, Bisoi PC, Mohanty BP, Panda GM (1993). Clinically important serum constituents of black Bengal goats. Ind. Vet. J. 70(8):713-717.

Belewu MA, Muhammed NO, Ajayi FT, Abdulgafar DT (2009). Performance characteristics of goat fed *Trichoderma* treated feather meal-rice husk mixture. Anim. Nutr. Feed Technol. 9:203-208.

Belewu MA, Ogunsola FO (2010). Haematological and serum indices of goat fed fungi treated *Jatropha curcas* kernel cake in a mixed ration. J. Agric. Biotechnol. Sustain. Dev. 2(3):35-38.

Benjamin MM (1989). Outline of Veterinary Clinical Pathology. 3rd edition. USA: Iowa State University Press, pp. 55-75.

Castro A, Dhinsa DS, Hoversland AS, Malkus H, Rosenthiel C, Metcalf J (1977). Serum biochemistry values in normal Pygmy goats. Am. J. Vet. Res. 38(12):2085-2087.

Chapple RS, English AW, Mulley RC, Lepherd EE (1991). Haematology and serum biochemistry of captive unsedated chital deer (*Axis axis*) in Australia. J. Wildl. Dis. 27(3):396-406.

Deangelino JL, Ishizuka MM, Ribeiro L, Tucci TV, Birgel EH (1990). Standard serum biochemical values of healthy goats reared in Saopaulo state. Braz. J. Vet. Res. Anim. Sci. 27(1)91-97.

Egbe-Nwiyi TN, Nwaosu SC, Salami HA (2000). Haematological values of apparently healthy sheep and goats as influenced by age and sex in arid zone of Nigeria. Afr. J. Biomed. Res. 3:109-115.

Feldman BF, Zink JG, Jain NC (2002). Schalm's Veterinary Hemetology. Philadelphia. Baltimore, New York, London, Buenos Aires, Hong Kong, Sidney, Tokyo: Lippincott Williams and Wilkins.

Gupta AR, Putra RC, Saini M, Sawrup D (2007). Haematology and serum biochemistry of Chital (*Axis axis*) and barking deer (*Muntiacus muntjak*) reared in semi-captivity. Vet. Res. Commun. 31:801-808. http://goat-link.com. Goat blood values. Accessed March 31, 2016..

Mellado M, Olivares I, Rodriguez A, Mellado J (2006). Relation among blood profiles and goat diets on rangeland. J. Appl. Anim. Res. 30:93-98.

Mishra, A, Chatterjee US, Mandal, TK (2013). Induction of Chronic Renal Failure in Goats Using Cisplatin: A New Animal Model. Toxicol. Int. 20(1):56-60.

NRC (1981). The National Research Council. Nutrient Requirements of goats. National Academy Press, Washington DC, USA.

Olayemi FO, Oboye OO, Azeez IO, Oyagbemi AA, Soetan KO (2009). Influence of management systems and sex on haematology of West African dwarf goat. Afr. J. Agric. Res. 4(11):1199-1202.

Piccione G, Casella S, Lutri L, Vazzana I, Ferrantelli V, Caola G (2010). Reference values for some haematological, haematochemical and electrophoretic parameters in the Girgentana goat. Turk. J. Vet. Anim. Sci. 34(2):197-204.

Rice CG, Hall B (2007). Hematologic and biochemical reference intervals for mountain goats (*Oreamnos americanus*): effects of capture conditions. Northwest Sci. 81(3):206-214.

Sakha M, Shamesdini M, Mohamad-zadeh F (2009). Serum biochemistry values in Raini goat of Iran. Internet J. Vet. Med. 6:1-6.

Sandabe UK, Chaudhary SUR (2000). Effect of environmental temperature on some biochemical values in female Sahel goats. Pak. Vet. J. 20(1):10-12.

Solaiman SG, Gurung NK, McCrary Q, Goyal H, McElhenney WH (2009). Feeding performance and blood parameters of male goat kids fed Easiflo cottonseed. Small Rumin. Res. 81(2-3):137-145.

SPSS (1999). Statistical Package for the Social Sciences, release 10.0. SPSS Inc. IL, Chicago, USA.

Sulaiman EG, Arslan SH, Al-Obaidi QT, Daham E (2010). Clinical, haematological and biochemical studies of babesiosis in native goats in Mosul. Iraqi J. Vet. Sci. 24(1):31-35.

Tambuwal FM, Agale BM, Bangana A (2002). Haematological and biochemical values of apparently healthy Red Sokoto goats. Proceedings of 27th Annual Conference. Nigerian Society for Animal Production (NSAP), FUT, Akure, Nigeria, pp. 50-53.

Tibbo M, Jibril Y, Woldemeskel, M, Dawo F, Aragaw K, Rege JEO (2004). Factors affecting hematological profiles in three Ethiopian indigenous goat breeds. Internet J. Appl. Res. Vet. Med. 2(4):297-305.

Turner KE, Wildeus S, Collins JR (2005). Intake, performance and blood parameters in young goats offered high forage diets of lespedeza or alfalfa hay. Small Rumin. Res. 59:15-23.

Waziri MA, Ribadu AY, Sivachelvan N (2010). Changes in the serum proteins, hematological and some serum biochemical profiles in the gestation period in the Sahel goats. Vet. Arhiv. 80(2):215-224.

Zamfirescu S, Topoleanu I, Nadolu D (2009). Observations concerning haematological profile in goat. Lucrari Seria Stiintifice 52:86-91.

Zubcic D (2001). Some biochemical parameters in the blood of grazing German improved fawn goats from Istria, Croatia. Vet. Arhiv. 71(5):237-244.

Zumbo A, Sciano S, Messina V, Casella S, Rita di Rosa A, Piccione, G (2011). Haematological profile of messinese goat kids and their dams during the first month post-partum. Anim. Sci. Pap. Rep. 29(3):223-230.

academicJournals

Vol. 15(24), pp. 1258 -1268, 15 June, 2016
DOI: 10.5897/AJB2015.15138
Article Number: 98A77DC58876
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Chitosan from shrimp shell (*Crangon crangon*) and fish scales (*Labeorohita*): Extraction and characterization

Suneeta Kumari[1], Rath P.[1] and Sri Hari Kumar A.[2]*

[1]Department of Chemical Engineering, NIT, Rourkela-769008, India.
[2]Department of Chemical Engineering, Defence Engineering College, Bishoftu, Ethiopia.

Received 30 November, 2015; Accepted 10 May, 2016

Chitosan is a naturally available biopolymer. It has been prepared by alkaline N-deacetylation process of shrimp (*Crangon crangon*) chitin and fish (*Labeorohita*) chitin. The physico-chemical properties such as the degree of deacetylation (DD), solubility, water binding capacity, fat binding capacity and chitosan yield have indicated that shrimp shell and fish scale waste are good sources of chitosan. The deacetylation value of shrimp shell chitosan, fish scales and commercial chitosan was found to be 76, 80 and 84%, respectively. The crystalline index (CrI) of fish and shrimp shell was 84 and 82%. Fat binding capacity of fish chitosan, shrimp chitosan and commercial chitosan was found to be 226, 246 and 446%, respectively. Fourier transforms infrared spectroscopy (FTIR) spectra presented a detailed structure of α-chitin with O-H, N-H and CO stretching movements. Structural differences between shrimp chitosan and fish chitosan were studied by using FTIR, thermo-gravimetric analysis (TGA), X-ray powder diffraction (XRD) and scanning electron microscopy (SEM). FTIR spectra were used to determine the chitosan degree of deacetylation (DD). Characteristic properties of extracted chitosan were found to depend upon the source of origin and degree of deacetylation.

Key words: Chitosan, fish scales, shrimp shell.

INTRODUCTION

All fish processing industries generate different types of wastes. Fish processing plants produce solid waste such as bones, shells, skin, head and meat. These waste materials generate pollution in coastal areas and contaminate the environment. Fishery wastes tend to get spoiled quickly by enzymatic and bacteriological processes which accumulate flies, rodents and other vermins. The Fish processing industry produces 30-40% of solid waste (Islam et al., 2004). Fishery waste is very useful and it contains high amount of proteins, fats, minerals, oil and chitin. Chitin and chitosan are polysaccharide polymeric materials; chitin is the second most abundant renewable polysaccharide after cellulose (Salaberria et al., 2014). Shrimp, crab, squid, lobster, insect cuticle, fungi and yeast are the best naturally occurring sources of chitin (Figure 1). Chitin and its derivatives are biomolecules of great importance, having versatile biological actions, and they exhibit

*Corresponding author. E-mail: annamareddy360@gmail.com. Tel: +91-8978600061.

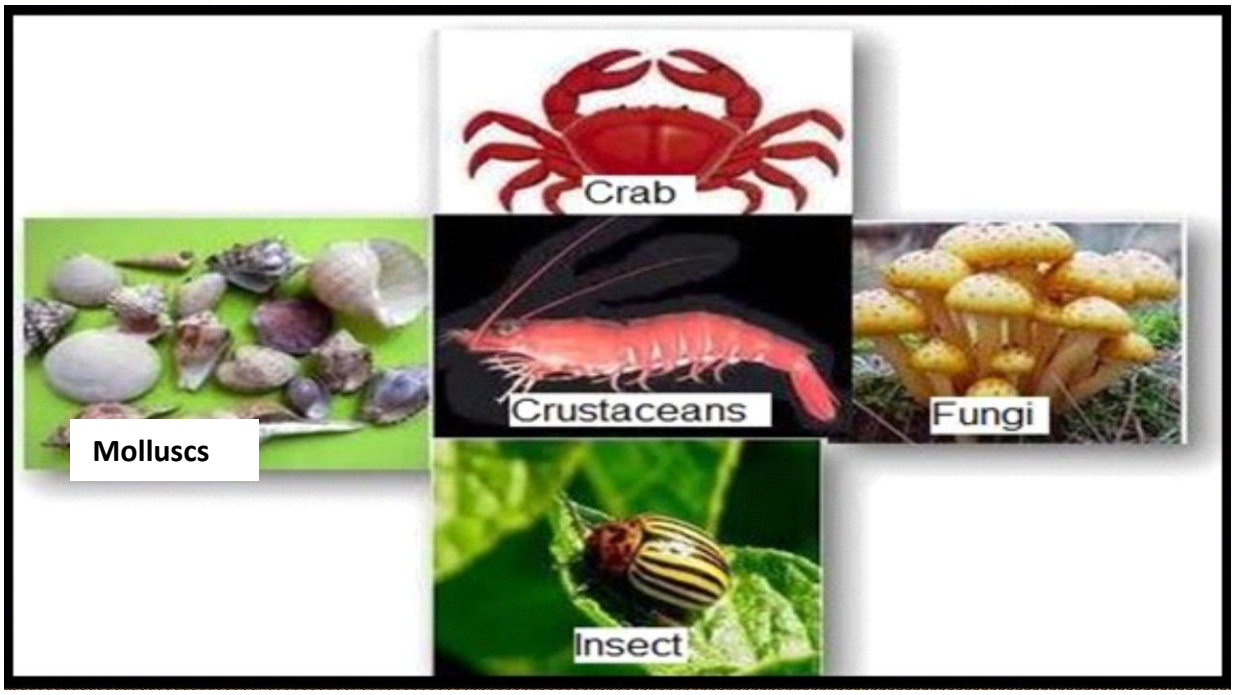


Figure 1. Sources of chitin.

biocompatibility and biodegradability. As a result, chitin and its derivatives are extensively used in pharmaceutical, cosmetics, feed additives, agriculture, semi-permeable membranes, food and textile industries and waste water treatment (Zeng et al., 2012).

Chitosan is prepared by deacetylation of chitin. During this reaction, the acetamide groups (-$NHCOCH_3$) of chitin are converted into amino groups (-NH_2) leading to chitosan formation. Depending on the source from where it is extracted, chitin can be found in three polymorphic conformations (α, β and γ) as shown in the Figure 2. Chitosan and chitin have become materials of great interest not only as an under-utilized resource but also as a new functional biomaterial of high potential in various fields. Recent progress in chitin chemistry is quite significant. In India chitin, solid waste fraction has ranged from 60,000 to 80,000 tons per year. Chitin and chitosan are now produced commercially in India, Poland, Japan, US, Norway and Australia (Eijsink et al., 2010).

Naturally occurring polysaccharides such as cellulose, pectin, alginic acid and carrageenans are neutral or acidic in nature. Chitin and chitosan are examples of highly basic polysaccharides (des Rieux et al., 2006; Vinsova and Vavrikova, 2008). Chitosan is known for its properties such as being nontoxic, odorless, biocompatible in animal tissues and enzymatically biodegradable. The most important properties of chitin and chitosan include polyoxysalt formation, ability to form films, chelation with metal ions and optical, structural characteristics (Dash et al., 2011). Degree of deacetylation and molecular weight of chitosan have a strong impact on its physical

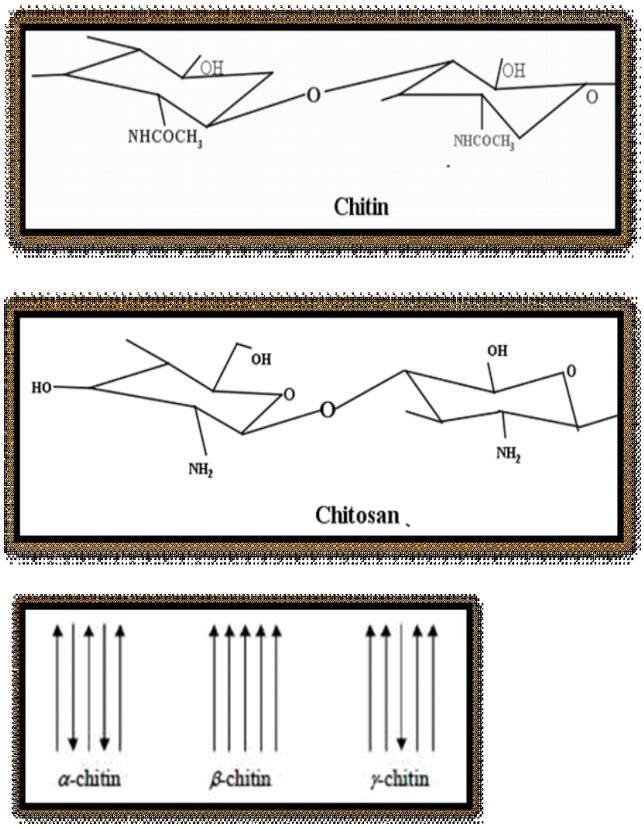


Figure 2. Chemical structure of the polysaccharide, (a) chitin (b) chitosan and (c) the polymer chains in different forms of chitin [3].

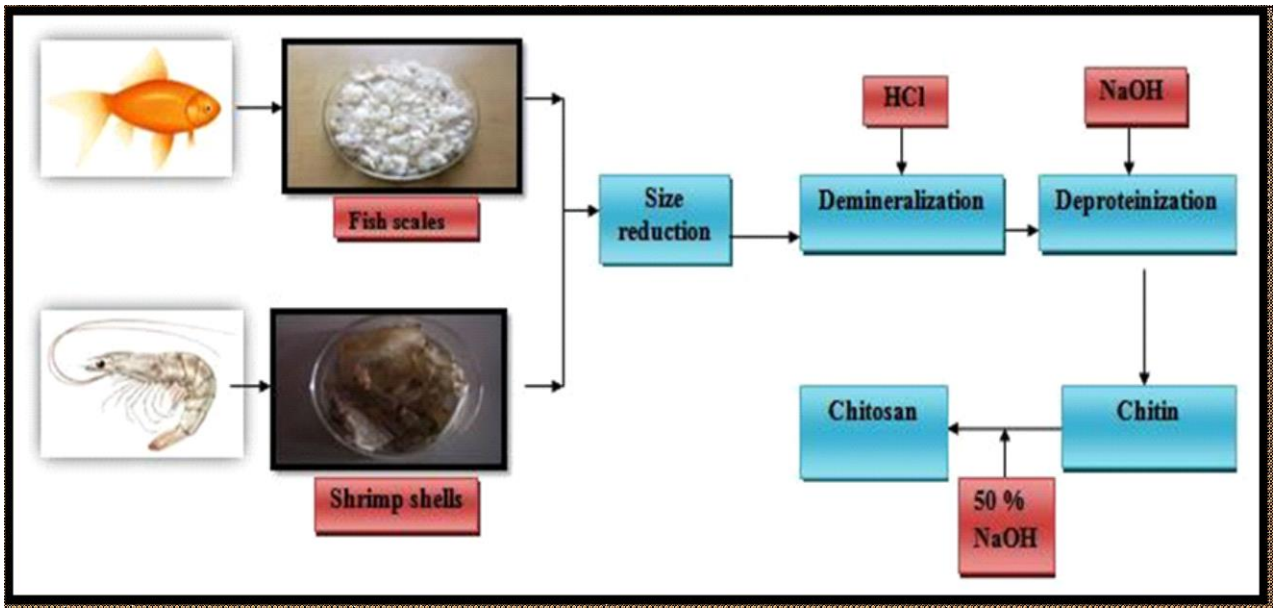


Figure 3. Isolation of chitosan from marine sources.

properties (Kumirska et al., 2010). Chitin is inimitable material for different applications because it has a high percentage of nitrogen (6.89%) as compared to that of synthetically modified cellulose (1.25%) (Hayes, 2012). Chitin has already found applications in various products that have reached the market. The industrial production and usage of chitin have been gradually increasing since the 1970's. The worldwide chitin production is estimated at approximately 10^{10} -10^{12} tons per annum (María and Roque, 2013). The major applications of chitin are focused on water treatment, food processing and metal ion chelation.

Previous studies have reported that molecular weight of chitosan affects its solubility (solubility decreases with increasing molecular weight), tensile strength, bacterio-logical properties, coagulant-flocculant performance of chitosan and crystallinity (Shukla et al., 2013). Chitosan is insoluble in water, and it is soluble in acidic solutions due to the protonation of its amine groups (Aranaz et al., 2009; Lee et al., 2011). Due to the presence of NH_2 group, chitosan potentially has high attraction properties to absorb pollutants such as heavy metals and dyes (Peng et al., 2013). Crystallinity and availability of amine groups affect the adsorption capacity of chitosan (Miretzky and Cirelli, 2009).

The objective of the present work is to synthesize chitin from shrimp shell and fish scales using acid and alkaline treatments followed by decolorization with potassium permanganate and to prepare chitosan by further N-deactylation treatment with concentrated sodium hydroxide solution. The percentage yields, degrees of N-deacetylation (DD) and molecular weights (Mw) have been determined. The physico-chemical properties of chitosan thus prepared have been determined by using the techniques such as scanning electron microscopy (SEM), fourier transforms infrared spectroscopy (FTIR), X-ray powder diffraction (XRD) and thermo-gravimetric analysis (TGA). Physico-chemical properties of the prepared chitosan have been also determined.

MATERIALS AND METHODS

Preparation of raw material

Shrimp shells (*Crangon crangon*) and fish (Labeorohita) scales were obtained in fresh conditions from a local fish market and thoroughly washed with tap water, desiccated at room temperature and subjected to size reduction followed by drying at room temperature. Hydrochloric acid (analytical reagents, Rankem), glacial acetic acid 100% (Merck) and sodium hydroxides pellets (Rankem) were purchased from Rankem and Merck chemicals. Commercial chitosan (86% deacetylated) was purchased from India Sea Foods, Kerala in India.

Demineralization

Shells contain many inorganic components out of which calcium carbonate is the main. Dilute hydrochloric acid was used to remove calcium carbonate and to prevent the hydrolysis of chitin. The hydrochloric acid concentrations ranging from 0.25 to 2 N was used and the reaction was carried out for 36 h at 30°C under constant stirring (150 rpm) (Figure 3). The dry shell to acid ratio was maintained in the range of 1/10 to 1/30 (w/v). The product mass was washed with distilled water to attain neutrality and then oven-dried at 80°C for 12 h.

$$\text{Dried shells} \xrightarrow[\text{Demineralization}]{\text{HCl}} \text{demineralized solid chitin} + CO_2\ (\uparrow)$$

Deproteinization

Deproteinization of chitin was carried out using 2 N NaOH (1:10 (W/V) ratio of chitin to NaOH solution) at 40°C. The treatment was repeated several times. The synthesized chitin was filtered and washed to neutrality using distilled water. The solid mass was dried in oven at 110°C.

$$\text{Chitin} \xrightarrow[\text{at } 40°C]{2N\ NaOH} \text{Deprotinized chitin} \rightarrow \text{washed and dried} \quad (2)$$

Deacetylation

The conversion of chitin to chitosan involves deacetylation and it was carried out using the process suggested by Kurita (2006). The prepared chitin was refluxed in aqueous sodium hydroxide (50% by weight) at 90 to 100°C temperature with constant stirring. After 6 h of reflux, the solid mass was filtered, washed with both water and ethanol (80% v/v) till the filtrate reached neutrality and then the material was oven dried at 80°C for 12 h (Sânia et al., 2012).

Physico-chemical properties of chitosan

Viscosity average molar mass of chitosan

The viscosity measurements were done using an Ubbelohde Viscometer and the efflux time of the solution was recorded at constant bath temperature (25 ± 0.1°C). Chitosan samples were dissolved in a solvent system of 0.3 M acetic acid/0.1 M sodium acetate. The intrinsic viscosity (η) was obtained from linear plots of reduced viscosity (ηsp/C) against concentration (C, g/ml), by extrapolating the plot to zero concentration. The viscosity average molar mass (MW) of chitosan was estimated using the Mark-Houwink relationship (Brugnerotto et al., 2001):

$$[\eta] = \mathrm{K}(MW)^{a} \quad (3)$$

Where K= 1.81 x 10^{-3} cm^3/g and a = 0.93. The mean of four replicates was taken from the viscosity measurements.

Solubility

0.1 g chitosan powder (sample taken in triplicate) was placed in a centrifuge tube of known weight, and then dissolved in 10 ml of 1% acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25°C. The mixture in the centrifuge tube was heated for 10 min using boiling water bath and then cooled to 25°C and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and undissolved particles were washed in distilled water (25 ml) and then undissolved matter was dried at 60°C for 24 h and by weighing the mass of particles, the percentage solubility was determined using the following equation:

$$\text{Solubility}(\%) = \frac{(\text{initial weight of (tube+chitosan)}) - (\text{final weight of (tube+chitosan)})}{(\text{initail weight of (tube+chitosan)}) - (\text{weight of empty tube})} \times 100 \quad (4)$$

Fat binding capacity (FBC)

FBC of chitosan was measured using the method suggested by Wang and Kinsella (1976). 10 ml of soya bean oil was added to a centrifuge tube with 0.5 g of prepared chitosan and thoroughly mixed. The contents were left at ambient temperature for 30 min with intermittent shaking (5 s) for every 10 min and centrifuged for 25 min (3500 rpm). The supernatant was decanted, and the tube was weighed. FBC was calculated as follows:

$$FBC(\%) = \frac{(fat\ bound)\,g}{(initial\ sample\ weight)\,g} \times 100 \quad (5)$$

Water binding capacity (WBC)

WBC of chitosan was measured using a modified method of Wang and Kinsella (1976). 10 ml of water was added to a centrifuge tube with 0.5 g of prepared chitosan and thoroughly mixed. The sample contents were left at ambient temperature for 30 min with intermittent shaking (5 s) for every 10 min and centrifuged at 3,500 rpm for 25 min. The supernatant was decanted, and the tube was weighed.

$$WBC(\%) = \frac{(water\ bound)g}{(initail\ sample)\,g} \times 100 \quad (6)$$

Chitosan characterization

X-ray powder diffraction (XRD)

The X-ray diffraction (XRD) powder patterns were recorded in transmission geometry with CuKα radiation in the 2θ range of 10 to 80° on a Rigaku D max 2000 machine at 40 kV, 30 mA. Crystalline index (CrI) values were calculated by using the formula given below:

$$CrI = [\mathrm{I}_{110} - \mathrm{I}_{am}] \times 100/\mathrm{I}_{110} \quad (7)$$

I_{110} = The highest intensity at 2θ value of 20°; I_{am} = the amorphous diffraction intensity at 2θ value of 13°.

Thermo-gravimetric analysis (TGA)

During TGA, 1 mg of sample was weighed and a warm-up operation was conducted from 10 to 600°C by ramping temperature input of 10°C per min. For TGA analyses, Shimadzu DTG-60H machine was used.

Fourier transforms infrared spectroscopy (FTIR)

Infrared spectra were obtained using a Perkin-Elmer type FTIR 1000 spectrometer at room temperature and using KBr pellet scanning method. Pellets were scanned at room temperature (25°C) in the spectral range of 400 – 4000 cm^{-1}. FTIR was used to confirm the formation of chitin and chitosan (synthesized from the fish and shrimp shells). The KBr pellets were prepared by thoroughly mixing KBr (200 mg) and sample (4 mg) and made into pellets. The degree of deacetylation (DD) of chitosan was calculated according to the method proposed by Yeul and Rayalu (2013) as follows:

$$DD(\%) = [A_{1650} - A_{3450}]/1.33 \times 100 \quad (8)$$

Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDX)

Scanning electron microscopy coupled with energy dispersive X-ray

Table 1. Physico-chemical properties of fish chitosan, shrimp chitosan and commercial chitosan

Sample	Solubility (%)	FBC (%)	WBC (%)
Fish chitosan	75	226	492
Shrimp chitosan	70	246	358
Commercial chitosan	90	446	520

spectroscopy is the best known and most widely-used methods of the surface analytical techniques. High resolution images of surface topography, with excellent depth of field, are produced using a highly-focused, scanning (primary) electron beam. The primary electrons enter the surface with an energy of 0.5 to 30 kV (Shimadzu SSX-550 EDX) and generate many low-energy secondary electrons.

RESULTS AND DISCUSSION

Solubility

Chitosan is a semi-crystalline biopolymer; because of its rigid crystalline structure, it is not soluble in most of the solvents like water, alkali or aqueous solution (pH ≈7) and common organic solvents. At certain pH values under continued stirring, chitosan is soluble in few acids such as hydrochloric, lactic, propionic, phosphoric, tartaric, citric, succinic, acetic and formic acids (Chung et al., 2005; Krajewska, 2004; Qin et al., 2006). Brine and Austin (1981) have stated that lower solubility values suggest incomplete removal of protein. In the present work, it was found that fish chitosan has more solubility as compared to shrimp shell chitosan (Table 1). Solubility values (Equation 4) of fish chitosan, shrimp shells chitosan and commercial chitosan are found to be 75, 70 and 90%, respectively.

Fat binding capacity (FBC)

Fat binding capacity (FBC) of fish chitosan, shrimp chitosan and commercial chitosan samples were measured using soybean oil. FBC value depends on chitosan produced and its sources. Rout (2001) has observed that the average FBC of commercial crab chitosan and crawfish chitosan for soybean oil was 587% and 706% respectively. The FBC values were calculated by following the procedure explained earlier and it was found that fish chitosan (226%) had lower fat binding capacity as compared to shrimp shell chitosan (246%) and commercial chitosan (446%). Conducting demineralization prior to deproteinization and deacetylation results in increase in FBC than deproteinization followed by demineralization and deacetylation (Moorjani et al., 1975).

Water binding capacity (WBC)

The WBC is generally involved with the molecular weight, DD and degree of crystallinity of chitosan. The surface area increases due to the decomposition of chitosan; as a result, the area for binding with the –OH groups, $–NH_2$ groups and end groups also increases. The increased DD provides more $–NH_2$ groups to bind water, and the decrease in crystallinity increases the penetration of the water molecules (Rout, 2001). The FBC value of shrimp chitosan (358%) was found to be lower than that of fish scale chitosan (492%) and commercial chitosan (520%).

Table 2. Crystalline index (%), (DD %) and molecular weight (MW) of fish chitosan, shrimp chitosan and commercial chitosan.

Sample	Crystallinity index (%)	DD (%)	MW (g/mol)
Fish chitosan	84	80	5200.96
Shrimp chitosan	82	76	1263.11
Commercial chitosan	96	84	17,046.39

Viscosity average molar mass of chitosan

Viscosity average molar mass (Table 2) strongly depends on the sample polydispersity, especially on the constant K. For a given set of constants (K and a) and [η]° values, it is known that the constant (K) is underestimated when the polydispersity of the sample increases, thus leading to overestimated molar mass. Viscosity average molar mass of chitosan varied with the sources, the extraction method and the residual aggregates in solution. In the present study, fish chitosan showed the higher viscosity average molar mass (5200.96 g/mol) whereas shrimp chitosan showed the lower viscosity average molar mass (1263.11 g/mol). For better comparisons, viscosity average molar mass of commercial chitosan sample, was also determined and it was found that commercial chitosan exhibited significantly high viscosity average molar mass (17,046.39 g/mol) as compared to fish and shrimp chitosan. According to Jia et al. (2001), chitosan viscosity average molecular mass decreases with increase in the hydrolysis time (demineralization, deproteinization and deacetylation). Due to a higher temperature (90-100°C) deacetylation process employed in the present study, viscosity average molar mass of prepared fish and shrimp chitosan is found to be significantly lower than that of commercial chitosan.

X-Ray powder diffraction (XRD)

X-Ray diffraction is normally used to determine the polymorphic forms of a compound which has different crystalline structures for which distinct powered X-ray diffraction patterns are obtained. The XRD patterns of chitosan samples extracted from two sources (shrimp shell and fish scales) exhibited strong reflections at 2θ

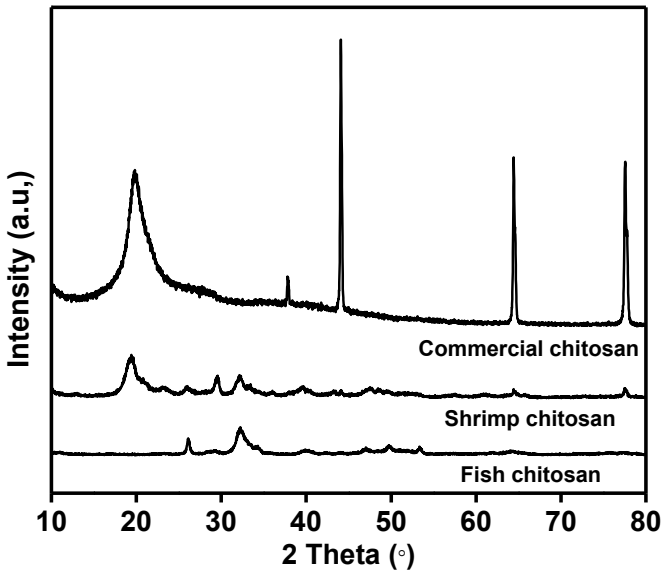


Figure 4. X-ray diffraction patterns of commercial chitosan, fish chitosan and shrimp chitosan.

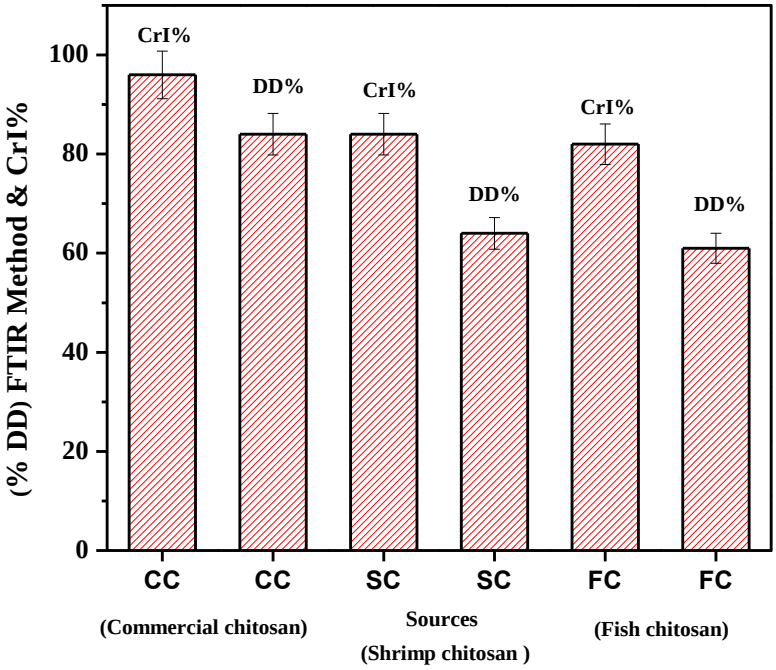


Figure 5. CrI% and DD%. The values represent means ± SD n=3.

values around 19-25 and 29-32°, respectively (Figure 4). The XRD analysis of chitosan from shrimp shells displayed different characteristic peaks at 2θ values of 19.50, 29.52 and 32.21°, whereas chitosan from fish scales exhibited peaks at 25.79 and 32.02°. XRD pattern of commercial chitosan sample has also been recorded and presented for better comparison, where the characteristic peaks were observed at 2θ values of 19.74, 37.82, 44.06, 64.43 and 77.92 (Kaya et al., 2014).

The crystallinity (Figure 5) was calculated on the basis of X-ray diffractogram. The two sharp peaks were observed for chitosan synthesized from fish scales chitosan at 25.0 and 32.9° whereas for chitosan synthesized from shrimp shell intense peaks were

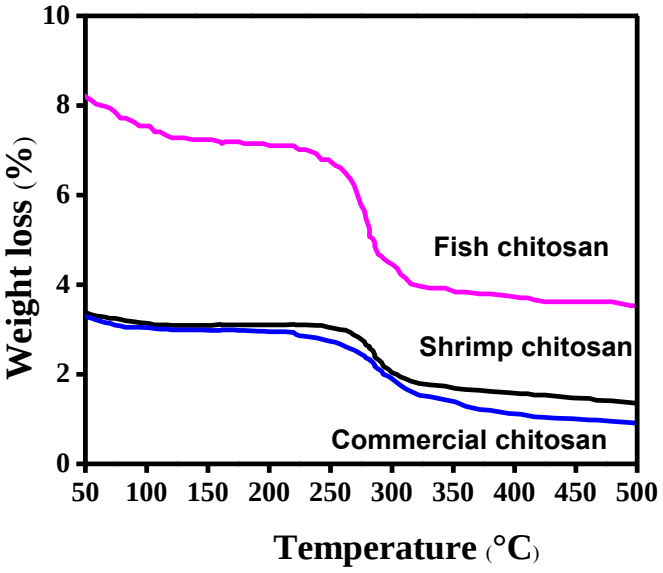


Figure 6. TGA thermograms of fish chitosan, shrimp chitosan and commercial chitosan.

observed at 10.57 and 20.72°. The intense peak at 32.9° for fish chitosan sample indicates the presence of hydroxyapatite mineral content. A similar observation was made by Allison et al. (2014). Two peaks of chitosan (extracted from shrimp shell) at 10.57 and 20.72° are in good match with the chitosan XRD patterns (chitosan isolated from organisms such as shrimp, crab and insects) reported by Yen et al. (2009) and Krajewska (2004).

Zhang et al. (2005) have proposed a method to determine CrI % using XRD peak intensity (Equation 7). CrI value of the shrimp shell chitosan was calculated and it was found to be 82%, whereas the CrI values of chitosan extracted from fish scale and commercial chitosan sample were found to be 84 and 96% respectively (Table 2). Kaya et al. (2014) and Shaofang et al. (2012) have estimated the CrI values of chitosan isolated from organisms such as crab and insects and the CrI values were reported to be in the range 54 and 91%. Zhang et al. (2005) observed the linear relationship between CrI_{020} and DD, and suggested a possibility for XRD to determine DD of macromolecular chitin.

Thermo-gravimetric analysis (TGA)

The thermogravimetric curves were obtained at a heating rate of 10°C min^{-1} under a dynamic atmosphere of nitrogen in the temperature range of 10 – 600°C. The profiles of the thermal decomposition of chitosan samples are depicted in Figure 6. It is observed from the thermograms that fish chitosan has stage wise weight loss in the range of 50-150 and 250 – 300°C, whereas in the case of shrimp shell chitosan and commercial sample, decomposition occurred in single stage (250 - 300°C). The initial weight loss in the range of 50-150°C corresponds to the removal of moisture content. It was observed from a TGA curve in the figure and the decomposition stage of chitosan occurred between temperatures of 250 – 300°C, which suggests that chitosan had a lower thermal stability (Sânia et al., 2012).

Fourier transforms infrared spectroscopy (FTIR)

FTIR analysis of shrimp chitin and fish chitin are depicted in the Figure 7. Formation of two separate bands in the region of 1662 - 1630 cm^{-1} (shown in inset for better visibility) confirms the presence of α chitin in both shrimp and fish chitin. The two separate bands exhibited by α chitin in the range of 1662 - 1630 cm^{-1} correspond to the occurrence of the intermolecular hydrogen bond CO• • •HN and the intra molecular hydrogen bond CO• • •$HOCH_2$, respectively (Focher et al., 1992). In the case of β chitin, only a single peak at 1659 cm^{-1} could be observed, which corresponds to the stretching of CO group hydrogen bonded to amide group of the neighboring intra-sheet chain (Hajji et al., 2014).

The vibrational modes involved in intermolecular hydrogen bonding CO• • •HN and the intramolecular bonds of NH groups exhibit characteristics bands at 3264 and 3110 cm^{-1}, respectively. These bands can be seen clearly in the α-chitin spectra, whereas these bands are not usually observed in β chitin. Sagheer et al. (2009) have observed the presence of a specific band at 1429 cm^{-1} in the case of α chitin and a strong well-defined band at 1436 cm^{-1} (CH_2) could be seen in β chitin. The nonexistence of bands at 1436 and 1659 cm^{-1} conforms the absence of β chitin phase in both shrimp and fish chitin samples.

The FTIR spectra of chitosan samples are shown in the Figure 8. The peak noticed at 1555 cm^{-1} corresponds to N–H bending of the secondary amide II band of –CONH– whereas, the amide I band is generally observed at 1655 cm^{-1}. In the present case, the amide 1 band is not found. Further bands that are observed in the region of 1380–1460 cm^{-1} are attributed to the symmetric and asymmetric bending vibrations of the methyl groups. Li et al. (1998) also found that the peak at 1415 cm^{-1} indicates the C–H bending vibrations of $–CH_2$.

A small peak around 2900 cm^{-1} relates to $–CH_2–$, $–CH_3$ functional groups. The C–O stretching vibrations of the structure are observed at 1075 cm^{-1}. The peak near 3300 cm^{-1} is usually attributed to intermolecular –H bands. Choi et al. (2007) have observed similar characteristic peaks for chitosan at 2940 ($–CH_3$, $–CH_2$), 1655 (C=O stretch vibration of secondary amide I band), 1555 (N–H bending vibration of amide II band), 1570 (N–H bending vibration of primary amides) and 1070 cm^{-1} (C–O stretching). Strong amide II bands are not present in this case, due to the high degree of deacetylation of the produced chitosan. The present results are completely in line with

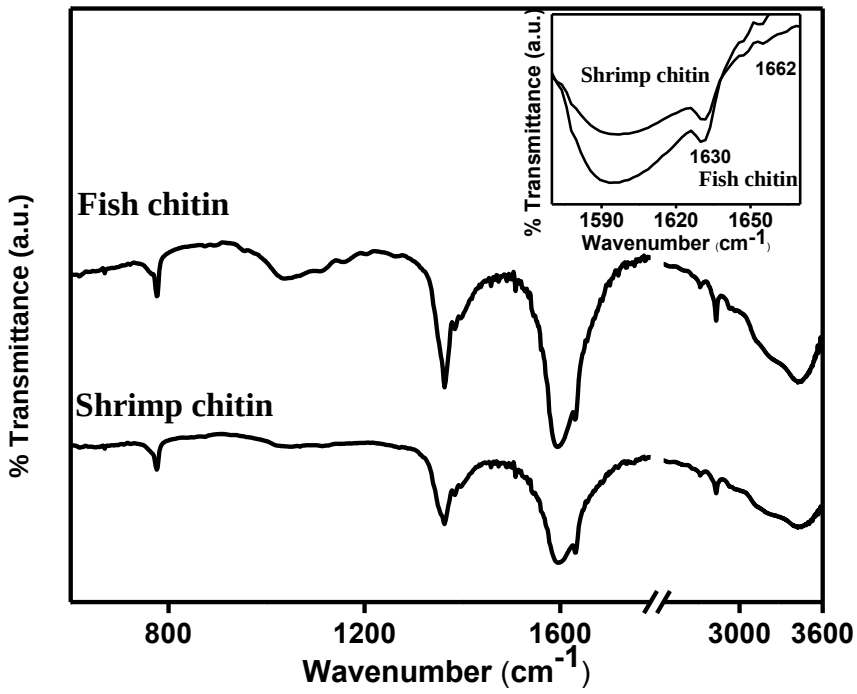


Figure 7. FTIR spectra of shrimp chitin and fish chitin.

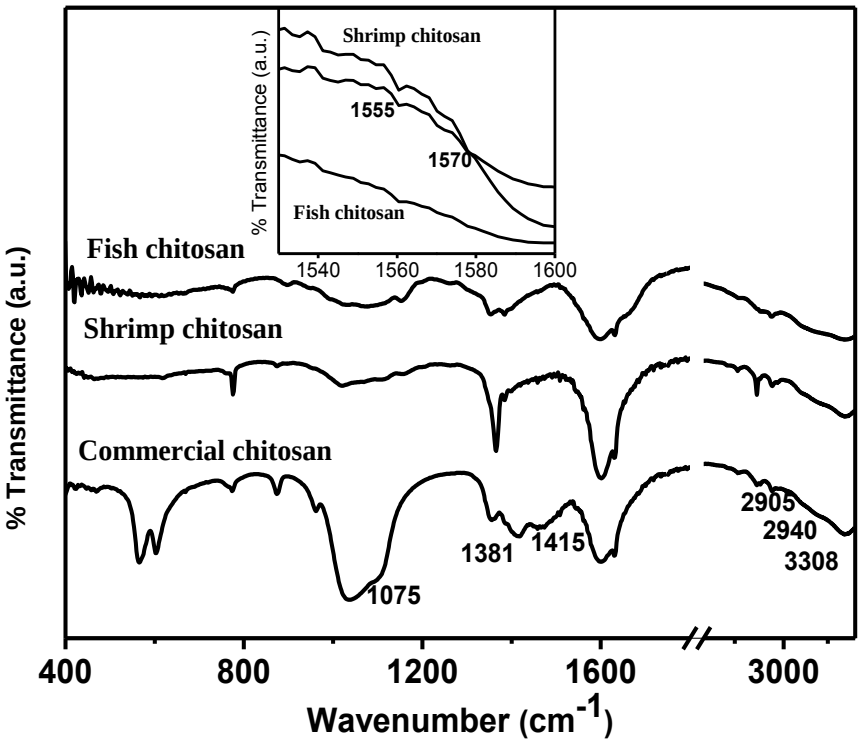


Figure 8. FTIR spectra of fish chitosan, shrimp chitosan and commercial chitosan.

reported literature and from FTIR patterns the formation of chitosan can be confirmed.

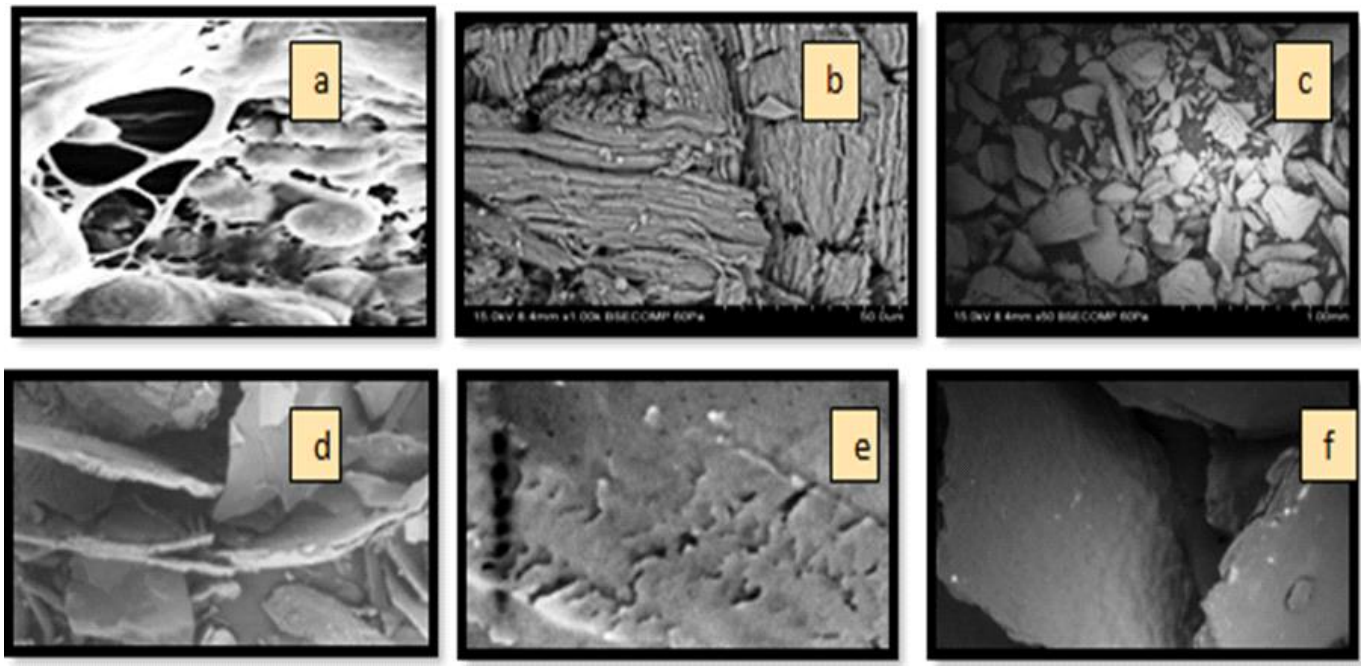


Figure 9. SEM image of (a) raw fish scales (b) fish chitin (c) fish chitosan (d) raw shrimp shells (e) shrimp chitin and (f) shrimp chitosan.

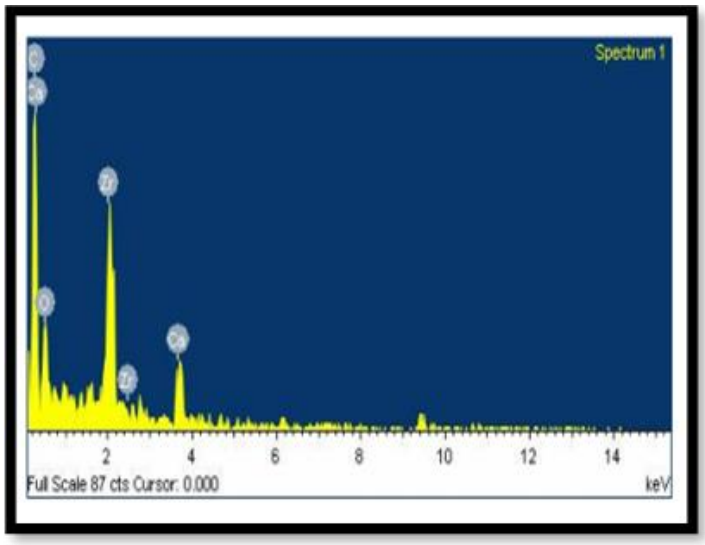
Scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/EDX)

The chitin and chitosan produced from fish scales and shrimp shell were selected for examination by SEM (Figure 9). SEM images of raw fish scales and shrimp shell showed fibrillary structure (Figure 9a and d) (Ikoma et al., 2003). The chitin demonstrated a noticeable organized microfibrillar crystalline structure in FESEM (Figure 9b and e) which was truant in the chitosan (Figure 9c and f), similar observation was reported by Yen et al. (2009), Arbia et al. (2013) and Muzzarelli et al. (2014). The extracted shrimp shell chitosan was observed to have layers of flakes, and porous nature could be seen in some areas. In some parts of chitosan, fibril structures can easily be distinguished. With the increased magnification, crumbling flakes were observed with fibril structures in some portions of chitosan, as in the study of Yen et al. (2009).

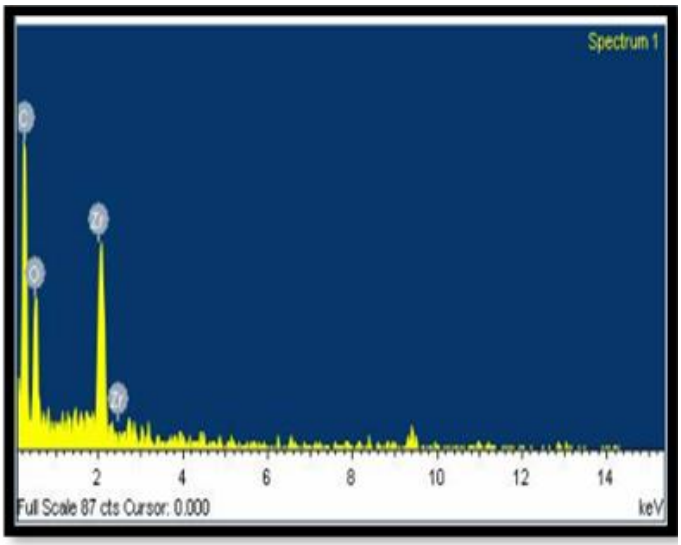
EDX analysis for fish chitosan and shrimp chitosan has been carried out and depicted in Figures 10a and b, respectively. The EDX results confirmed that the fish chitosan (Figure 10a) has small amount of Ca, whereas similar mineral contents could not be seen in the case of shrimp chitosan (Figure 10b). XRD patterns have also suggested the presence of hydroxyapatite mineral in the fish chitosan sample. However, shrimp chitosan is free of mineral content. Recent studies by Li et al. (2011) and Guan et al. (2015) suggested that purity of extracted chitosan depends on its original source, treatment method (demineralization and deproteinization) and treatment time.

Conclusion

Chitin has been extracted from local sources, fish scales and shrimp shells. Chitin preparation involves demineralization, followed by deproteinization and deacetylation. Prepared chitosan was used to investigate the physicochemical properties such as viscosity average molar mass, solubility, fat binding capacity and water binding capacity. The physicochemical properties of prepared chitosan from fish scales (water-binding capacity (492%) and fat-binding capacity (226%)) and shrimp chitosan (water-binding capacity (358%) and fat binding capacity (246%)) are in total concurrence with commercially available chitosan. Solubility of chitosan from fish and shrimp shell was 75 and 70%, respectively. Using FTIR method, DD % of fish chitosan, shrimp chitosan and commercial chitosan was estimated and DD % was found to be 80, 76 and 84%, respectively. The XRD analysis has shown that commercial chitosan was more crystalline as compared to that of shrimp and fish scales. XRD analysis has also indicated that fish chitosan was more crystalline than shrimp chitosan. The CrI % of fish and shrimp scales was found to be 84 and 82%, respectively and commercial chitosan exhibited 96%. The results suggest that shrimp waste and fishery waste are most remarkable and good sources of chitosan.



Element	Weight %	Atomic %
C K	59.04	78.47
O K	16.09	16.06
Ca K	5.01	1.99
Zr L	19.85	3.47
Total	100	



Element	Weight %	Atomic %
C K	57.60	69.97
O K	30.92	28.19
Zr L	11.48	1.84
Total	100	

Figure 10. EDX spectra of shrimp chitosan and fish chitosan.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are thankful to Prof. Sunil Kumar Sarangi (Director), National Institute of Technology, Rourkela, India, for providing the facilities to conduct the present research work.

REFERENCES

Allison PG, Rodriguez RI, Moser RD, Williams BA, Poda AR, Seiter JM, Lafferty BJ, Kennedy AJ, Chandler MQ (2014). Characterization of multi-layered fish scales (Atractosteus spatula) using nanoindentation, X-ray CT, FTIR, and SEM. J. Vis. Exp. 89:1-9.

Aranaz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N, Galed G, Heras A (2009). Functional Characterization of Chitin and Chitosan. Curr. Chem. Biol. 3(2):203-230.

Arbia W, Adour L, Amrane A, Lounici H (2013). Optimization of medium composition for enhanced chitin extraction from Parapenaeus longirostris by Lactobacillus helveticus using response surface methodology. Food Hydrocoll. 31(2):392-403.

Brine CJ, Austin PR (1981). Chitin variability with species and method of preparation. Comp. Biochem. Physiol. 69(2):283-286.

Brugnerotto J, Desbrières J, Roberts G, Rinaudo M (2001). Characterization of chitosan by steric exclusion chromatography. Polymer 42(25):9921-9927.

Choi CY, Kim SB, Pak PK, Yoo DI, Chung YS (2007). Effect of N-acylation on structure and properties of chitosan fibers. Carbohydr. Polym. 68(1):122-127.

Chung Y-C, Kuo C-L, Chen C-C (2005). Preparation and important functional properties of water-soluble chitosan produced through Maillard reaction. Bioresour. Technol. 96(13):1473-1482.

Dash M, Chiellini F, Ottenbrite R M, Chiellini E (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. Prog. Polym. Sci 36(8):981-1014.

des Rieux A, Fievez V, Garinot M, Schneider Y-J, Préat V (2006). Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. J. Control. Release 116(1):1-27.

Eijsink V, Hoell I, Vaaje-Kolstada G (2010). Structure and function of enzymes acting on chitin and chitosan. Biotechnol. Genet. Eng. Rev. 27:331-366.

Focher B, Naggi A, Torri G, Cosani A, Terbojevich M (1992). Chitosans from Euphausia superba. 2: Characterization of solid state structure. Carbohydr. Polym. 18(1):43-49.

Guan J-J, Tian B, Tang S, Ke Q-F, Zhang C-Q, Zhu Z-A, Guo Y-P (2015). Hydroxyapatite coatings with oriented nanoplate arrays: synthesis, formation mechanism and cytocompatibility. J. Mater. Chem. B3(8):1655-1666.

Hajji S, Younes I, Ghorbel-Bellaaj O, Hajji R, Rinaudo M, Nasri M, Jellouli K (2014). Structural differences between chitin and chitosan extracted from three different marine sources. Int. J. Biol. Macromol. 65:298-306.

Hayes M (2012). Chitin, chitosan and their derivatives from marine rest raw materials: Potential food and pharmaceutical applications in: Hayes M (Ed.), Marine Bioactive Compounds. Springer US. pp. 115-128.

Ikoma T, Kobayashi H, Tanaka J, Walsh D, Mann S (2003). Microstructure, mechanical, and biomimetic properties of fish scales from Pagrus major. J. Struct. Biol. 142(3):327-333.

Islam MS, Khan S, Tanaka M (2004). Waste loading in shrimp and fish

processing effluents: potential source of hazards to the coastal and nearshore environments. Mar. Pollut. Bull. 49(1-2):103-110.
Jia Z, shen D, Xu W (2001). Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. Carbohydr. Res. 333(1):1-6.
Kaya M, Seyyar O, Baran T, Turkes T (2014). Bat guano as new and attractive chitin and chitosan source. Front. Zool. 11(1):1-10.
Krajewska B (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme Microb. Technol. 35(2-3):126-139.
Kumirska J, Czerwicka M, Kaczyński Z, Bychowska A, Brzozowski K, Thöming J, Stepnowski P (2010). Application of Spectroscopic Methods for Structural Analysis of Chitin and Chitosan. Mar. Drugs 8(5):1567-1636.
Kurita K (2006). Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans. Mar. Biotechnol. 8(3):203-226.
Lee H-S, Eckmann DM, Lee D, Hickok NJ, Composto RJ (2011). Symmetric pH-Dependent Swelling and Antibacterial Properties of Chitosan Brushes. Langmuir 27(20):12458-12465.
Li LH, Zhao MY, Ding S, Zhou CR (2011). Rapid biomimetic mineralization of chitosan scaffolds with a precursor sacrificed method in ethanol/water mixed solution. Express Polym. Lett. 5(6):545-554.
Li Q, Weng S, Wu J, Zhou N (1998). Comparative study on structure of solubilized water in reversed micelles. 1. FT-IR spectroscopic evidence of Water/AOT/n-Heptane and Water/NaDEHP/n-Heptane systems. J. Phys. Chem. B 102(17):3168-3174.
María CG, Roque AH (2013). Biotechnological processes for chitin recovery out of crustacean waste: A mini-review. Electron. J. Biotechnol. 16(3):1-14.
Miretzky P, Cirelli A F (2009). Hg(II) removal from water by chitosan and chitosan derivatives: a review. J. Hazard. Mater. 167(1-3):10-23.
Moorjani M N, Achyuta V, Khasim T (1975). Parameters affecting the viscosity of chitosan from prawn waste. J. Food. Sci. Technol. 12:187-189.
Muzzarelli R, Mehtedi M, Mattioli-Belmonte M (2014). Emerging Biomedical Applications of Nano-Chitins and Nano-Chitosans Obtained via Advanced Eco-Friendly Technologies from Marine Resources. Mar. Drugs 12(11):5468-5502.
Peng Y, Chen D, Ji J, Kong Y, Wan H, Yao C (2013). Chitosan-modified palygorskite: Preparation, characterization and reactive dye removal. Appl. Clay Sci. 74:81-86.
Qin C, Li H, Xiao Q, Liu Y, Zhu J, Du Y (2006). Water-solubility of chitosan and its antimicrobial activity. Carbohydr. Polym. 63(3):367-374.
Rout SK (2001). Physicochemical, functional, and spectroscopic analysis of crawfish chitin and chitosan as affected by process modification. Louisiana State University: Baton Rouge, LA, USA.
Sagheer FAA, Al-Sughayer MA, Muslim S, Elsabee MZ (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. Carbohydr. Polym. 77(2):410-419.
Salaberria AM, Labidi J, Fernandes SCM (2014). Chitin nanocrystals and nanofibers as nano-sized fillers into thermoplastic starch-based biocomposites processed by melt-mixing. Chem. Eng. J. 256:356-364.
Sânia MBdA, Rasiah L, Brismak G d R, Débora D B, Alcione O G (2012). The Use of Exoskeletons of Shrimp (Litopenaeus vanammei) and Crab (Ucides cordatus) for the Extraction of Chitosan and Production of Nanomembrane. Mater. Sci. Appl. 3(7):495-508.
Shaofang L, Jie S, Lina Y, Chushu Z, Jie B, Feng Z, Mingjing Q, Chen J, Qingli Y (2012). Extraction and Characterization of Chitin from the Beetle Holotrichia parallela Motschulsky. Molecules 17(4):4604-4611.
Shukla SK, Mishra AK, Arotiba OA, Mamba BB (2013). Chitosan-based nanomaterials: A state-of-the-art review. Int. J. Biol. Macromol. 59:46-58.
Vinsova J, Vavrikova E (2008). Recent Advances in Drugs and Prodrugs Design of Chitosan. Curr. Pharm. Des. 14(13):1311-1326.
Wang JC, Kinsella J E (1976). Functional properties of novel proteins: Alfalfa leaf protein. J. Food Sci. 41(2):286-292.
Yen M-T, Yang J-H, Mau J-L (2009). Physicochemical characterization of chitin and chitosan from crab shells. Carbohydr. Polym. 75(1):15-21.
Yeul V, Rayalu S (2013). Unprecedented Chitin and Chitosan: A Chemical Overview. J. Polym. Environ. 21(2):606-614.
Zeng J-B, He Y-S, Li S-L, Wang Y-Z (2012). Chitin Whiskers: An Overview. Biomacromolecules 13(1):1-11.
Zhang Y, Xue C, Xue Y, Gao R, Zhang X (2005). Determination of the degree of deacetylation of chitin and chitosan by X-ray powder diffraction. Carbohydr. Res. 340(11):1914-1917.

academicJournals

Vol. 15(24), pp. 1269 -1275, 15 June, 2016
DOI: 10.5897/AJB2015.15200
Article Number: 46D3B5D58878
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro regeneration of a common medicinal plant, *Ocimum sanctum* L. for mass propagation

Mohammad Abu Hena Mostofa Jamal[1,2]*, Imdadul Hoque Sharif[1], Md. Mostofa Shakil[1], A. N. M. Rubaiyath-Bin Rahman[1], Nilufa Akhter Banu[1], Md. Rezuanul Islam[1] and Md. Nazmuzzaman[1]

[1]Department of Biotechnology and Genetic Engineering, Faculty of Applied Science and Technology, Islamic university, Kushtia-7003, Bangladesh.
[2]Department of Biomedical Sciences, Chonbuk National University Medical School, Chonbuk National University, Jeonju, South Korea.

Received 31 December 2015, Accepted 23 May, 2016.

Embracing micro-propagation method for large scale production of plantlets and also for protection of appropriate germplasm is a prerequisite that needs to be undertaken in order to develop a rapid *in vitro* regeneration protocol for *Ocimum sanctum* L. Shoot tips as well as nodal segments were subjected to numerous shoots inducement. Explants were cultured on Murashige and Skoog Basal Medium (MS) supplemented for different plants' development controllers. $HgCl_2$ was utilized as a surface disinfecting agent. Nowadays, many researchers do not use $HgCl_2$, so 1% sodium hypochlorite can be used. Cleaned explants were chiseled to 3-4cm length at right edges. The explants were inoculated vertically on the culture medium. The cultures were incubated at 25±2°C under cool fluorescent light. The photoperiod was set at 16 h light and 8 h darkness by automated timer. Data on shoot induction and expansion and root induction were recorded following three weeks of inoculation and utilized for figuring. Built up plantlets were transplanted in earthen pots under circumstances and outliving degree was recited. The practically viable surface sanitization medication for explants of *O. sanctum* was discovered at 0.1% $HgCl_2$ for 7 min. 1% sodium hypochlorite also showed same result. Maximum number of shoots per culture was recorded in MS medium containing 2.0 mg/l BAP in a mixture of 0.5 mg/l NAA. Regenerated shoots of *O. sanctum* were rooted most effectively in full MS medium supplemented with 1.0 mg/l NAA. It was observed that nodal segments are more responsive to micro-propagation than shoot tips. This protocol is used to explore the opportunities of utilizing *O. sanctum* L., as important medicinal plant of Bangladesh, in modern medical health care system by rapid clonal propagation, and germplasm conservation. The developed plants were acclimatized in pot successfully and also maintained in normal environment.

Key words: *Ocimum sanctum,* micro-propagation, explants, nodal segments, medicinal plant, regeneration.

INTRODUCTION

Bangladesh is a country which investigates sumptuously in medicamentary hereditary contrivance by virtue of its favorable agro-climatic condition and seasonal diversity. Almost 500 medicinal plants have medicinal applications.

80% rustic people depend on unregenerate explants (e.g. medicinal plants) for their chief healthcare (Rahman et al., 2013). When they are malignant medicinal plants, Bangladesh people trust on imported raw materials of pharmaceuticals. The most sacred plant in Bangladesh, *Ocimum sanctum* L. (Lamiaceae) is an aromatic herb; it is an under shrub or shrub (Saha et al., 2013); it is up to 45 cm tall, grows in a low bush and commonly known as holy basil, Tulsi or Tulasi. Within Ayurveda, tulsi is known as “The Incomparable One,” “Mother Medicine of Nature” and “The Queen of Herbs,” and is respected as an “elixir of life" that is without equivalent for both its restorative and profound properties (Singh et al., 2010).The hydro-refining of the aeronautical parts of *O. sanctum* is at the vegetative, botanical maturing stages. Furthermore, full blooming phases yield 0. 98, 0.92 and 1.1% (w/w) essential oil, individually. Concoction parts of the fundamental oils refined starting with three developmental phases of *O. sanctum* are ethyl isovalerate, α-pinene, sabinen, β-pinene, myrcene, 1,8-cineole, linalool, terpinen-4-ol, α-terpineol, estragol, eugenol, α-cis-bergamotene, α-humlene, β-bisabolen, γ-elemene and methyl chavicol (Saharkhiz et al., 2015).

The fundamental properties of *O. sanctum are: it* brings down glucose levels, is antispasmodic, a pain relief, brings down circulatory strain, mitigates cardiovascular depressant, is antiulcer, fights against fruitfulness, prevents cancer, is hostile to stretch marks, an invulnerable stimulant, and a smooth muscle relaxant. It may also be able to keep early occurences of carcinogenesis and different concentrates; additionally it provides evidence of anti HIV-I (Kayastha, 2014). It is hostile to gonorrheal, is against multi-resistant strains of *Neisseria gonorrhea* and clinical confines of beta lactamase-delivering methicillin-safe *Staphylococcus aureus* (Gupta et al., 2014). The oil from *O. sanctum* likewise has radio-protective applications (Ramesh and Satakopan, 2010). A consultative body cautioned against potential anti-fertility impacts of *O. sanctum* when devoured in considerably high amounts (Narayana et al., 2014). Micropropagation of medicinal plants is of great importance for mass propagation of high yielding clones. *In vitro* technologies offer a tremendous potential for obtaining raw material for the pharmaceuticals (Tasheva and Kosturkova, 2013). Utilization of the rising line of genomic innovations can overhaul our concern about the relationship between genomic assorted qualities and metabolite differences and the feasible use of plant pharmaceutical assets (Hao and Xiao, 2015). Considering the therapeutic and odoriferous importance of *O. sanctum*, not very many endeavors have been made to institutionalize micropropagation technique for cloning this plant. *O. sanctum* is not an endangered plant, but a valuable medicinal plant; so if we develop a method for *in vitro* regeneration of this plant, that can open many areas for further research and germplasm preservation.

In this paper, our point is to portray a straightforward and solid convention to increase this pharmaceutically important plant through high-recurrence axillary shoot multiplication.

MATERIALS AND METHODS

O. sanctum plants, gathered from an habitation of Hindu religious people in Palpara, Jugia, Kushtia District, Bangladesh were utilized as explants hot spots for micro-propagation. Plant development controllers, that is, BAP (6-benzyl amino purine), zeatin of cytokinin group and naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D) of auxin group were utilized for this analysis. $HgCl_2$ was utilized as a surface cleaning specialists and Tween-20 and Savlon (an antiseptic, ACI Pharma, Bangladesh) were used as detergent and surfactant.

Murashige and Skoog (MS) medium composed of nutrient basal salts was used, which contains macro, micro elements and vitamins. It is necessary to utilize ideal supplement for accomplishing high development rates (Murashige and Skoog, 1962; Saad and Elshahed, 2012). The explants were washed together under flowing faucet water and treated with antiseptic (4-5) drops savlon along with 2-3 drops of Tween-20; and then washed with distilled water. They were then suspended in 0.1% (w/v) $HgCl_2$ solution for 7 min to ensure contaminant free cultures; this was followed by five rinses with sterile distilled water to distance all hints of $HgCl_2$. Sterilized explants were incised to 3-4 cm length at right edges. At present $HgCl_2$ is not used by most of researchers, so 1% sodium hypochlorite can be used.

For shoot initiation, BAP was used at different concentrations (0.5, 1, 2, 3 and 4 mg/L) as well as BAP in combination with NAA, IAA, 2,4-D at different concentrations (0.1 and 0.5 mg/L) in full strength of MS medium. NAA and IAA (0.1, 0.5, 1 and 2 mg/L) were used for root initiation in full and half strength of MS medium. The prepared melted medium was dispensed into test tubes (150 × 25 mm). For carbon source 3% sugar was used and the medium was solidified with 1.0% agar. The pH of the media was adjusted to 5.8 before autoclaving at 15-lbs/inch^2 pressure at 121°C temperature for 15 min. The explants were inoculated vertically on the culture medium. The cultures were incubated at 25±2°C under the cool fluorescent light. The photoperiod was set at 16 h light and 8 h darkness via computerized clock. The regenerated shoots of 1-3 cm in length were removed aseptically and transferred into test tubes containing the same or different hormones supplemented media for the shoot and root induction. Optic notice of culture was made every week. Data on shoot induction and expansion and root induction were recorded following three weeks of inoculation and utilized for count. The healthy plantlets that attained 5-8 cm heights were taken out from the test tubes; and the roots were washed under running tap water to remove the medium. At that point the plantlets were prepared for transplantation to plastic pots containing soil, sand and fertilizer (1:1:1) for solidifying. Built up plantlets

*Corresponding author. E-mail: jamalbtg@gmail.com. Tel: +821064670862

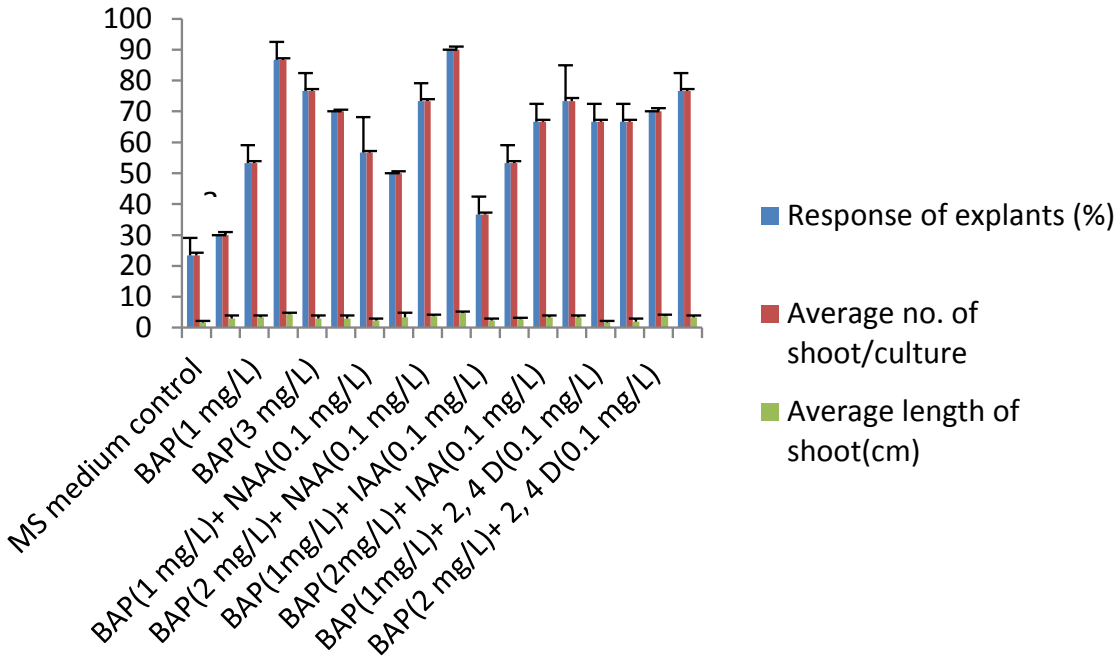


Figure 1. Effect of cytokinins and cytokinins in combination with different Auxin on micropropagation of *Osmium sanctum* using shoot tips isolated from field grown plant for Multiple shoot development

were transplanted in earthen pots under certain conditions and outliving rate was recorded.

RESULTS

It was observed that, 86% explants of *O. sanctum* were found free of contamination and healthy when treated with 0.1% $HgCl_2$ for 7 min; they were the best. At the point when BAP was utilized as a part of distinctive fixation, the most elevated quantities of shoots and most noteworthy length of the shoot per explants were recorded from shoot tips and nodal fragments in media having 2.0 mg/L BAP. In this investigation, explants from field grown plants of 2 mg/L BAP used in combination with 0.5 mg/L NAA are proved suitable for culture of shoot tip and nodal segment. Different concentrations and combinations of cytokinin or NAA, IAA and 2,4-D were used to test multiple shoots proliferation potentiality from shoot tip and nodal segment. The Highest number of shoots 7 was obtained in media having 2.0 mg/L BAP in combination with 0.5 mg/l NAA from shoot tip (Figures 1 and 2) and in case of nodal segment the highest number of shoots was 9 and was obtained in media having 2.0 mg/L BAP in combination with 0.5 mg/l NAA (Figures 3 and 4) respectively. Nodal explants were discovered superior to anything shoot, for there was high recurrence of shoots in *O. sanctum* most likely due to their more herbaceous nature (Figure 3). Shoot tips were refined on MS medium supplemented with BAP and NAA,IAA and 2,4 D separately in distinctive fixations (Figure 2), whereas nodal sections were cultured on MS medium supplemented with BAP and NAA, IAA and 2,4 D at different concentrations. It was discovered that NAA is superior to other auxins (IAA) because 90% shoot developed root in different concentrations of NAA (0.1, 0.5 and 2.0 mg/L) and IAA (1.0 mg/L) containing medium. NAA produced highest average number of root/culture (6) and highest length of root was 2.4 cm observed in medium having 1.0 mg/l NAA with full strength of MS medium. Different concentrations of NAA and IAA on full strength MS medium are superior to half strength MS medium for root actuation (Figures 5, 2D and 3).

In the free examination, 90% of plants adapt for long, and the apex quantities of shoots per explants were found from shoot tips and nodal portions in media having 2.0 mg/l BAP in combination with 0.5 mg/l NAA. The most astounding mean length of the shoot was additionally considered in these media. It was established well in full MS containing 1.0 mg/l NAA. Along these lines, for high productivity of micro propagation, it is suggested that nodal explants can be refined for 6-7 weeks in 2.0 mg/l BAP in combination with 0.5 mg/L NAA.

DISCUSSION

Proficient regeneration systems are vital for hereditary

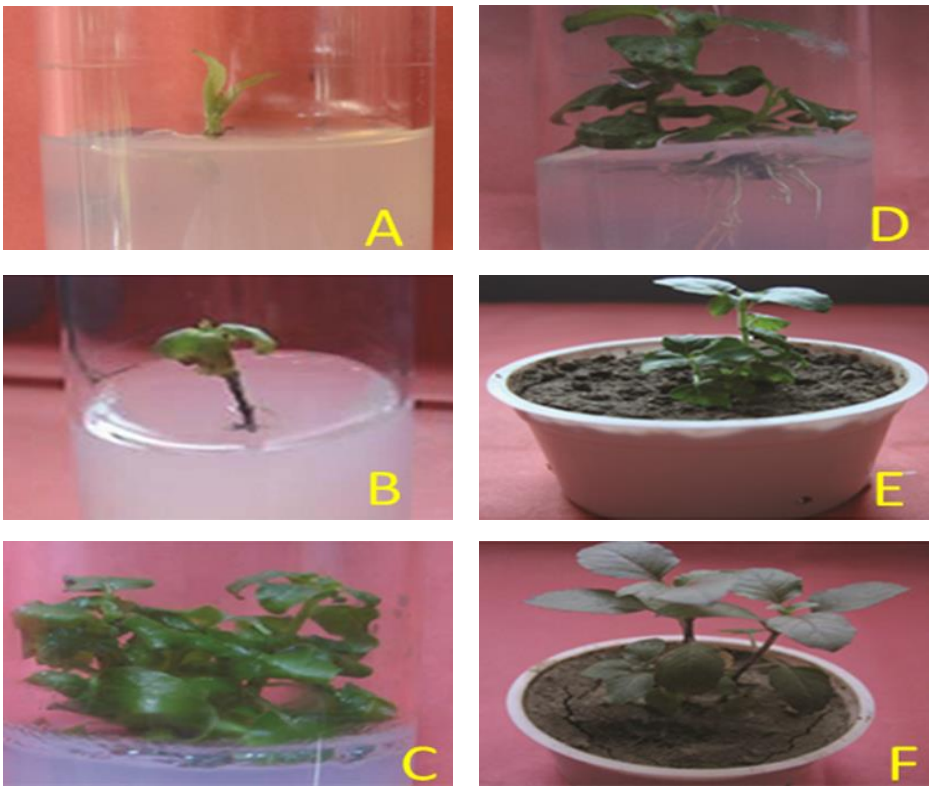


Figure 2. *In vitro* regeneration of *Ocimum sanctum* from shoot tips. **A.** Explants inoculation, **B.** Proliferation initiation, **C.** multiple shoots proliferation, **D.** Root initiation, **E.** Acclimatization, **F.** Acclimatized plant.

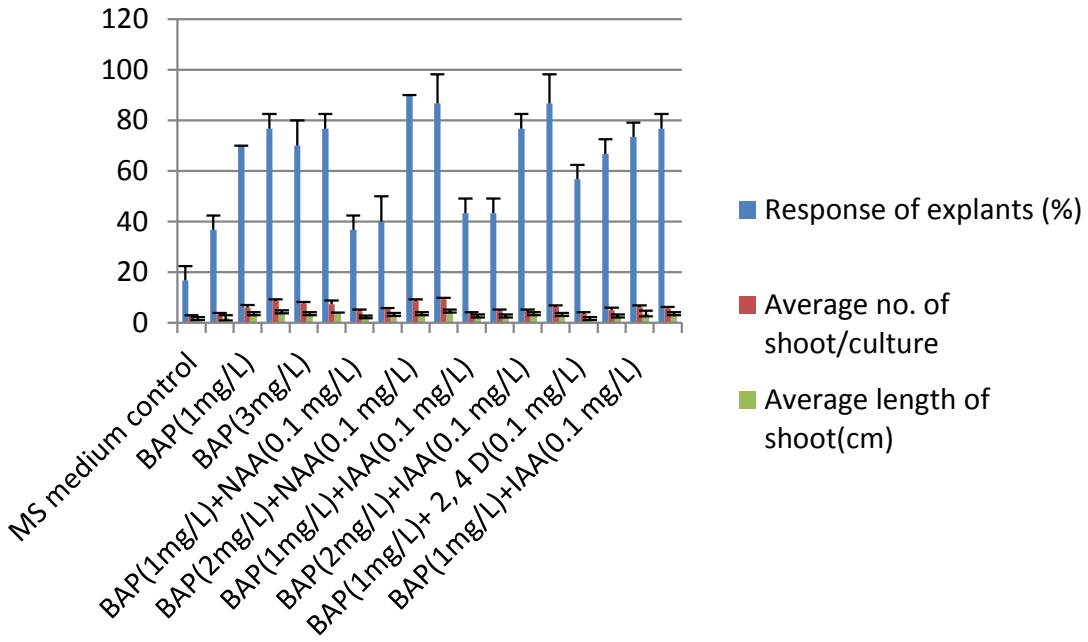


Figure 3. Effect of cytokinins and cytokinins with different combination on auxins for micropropagation of *Osmium sanctum* using nodal segments isolated from field grown plant for multiple shoot development.

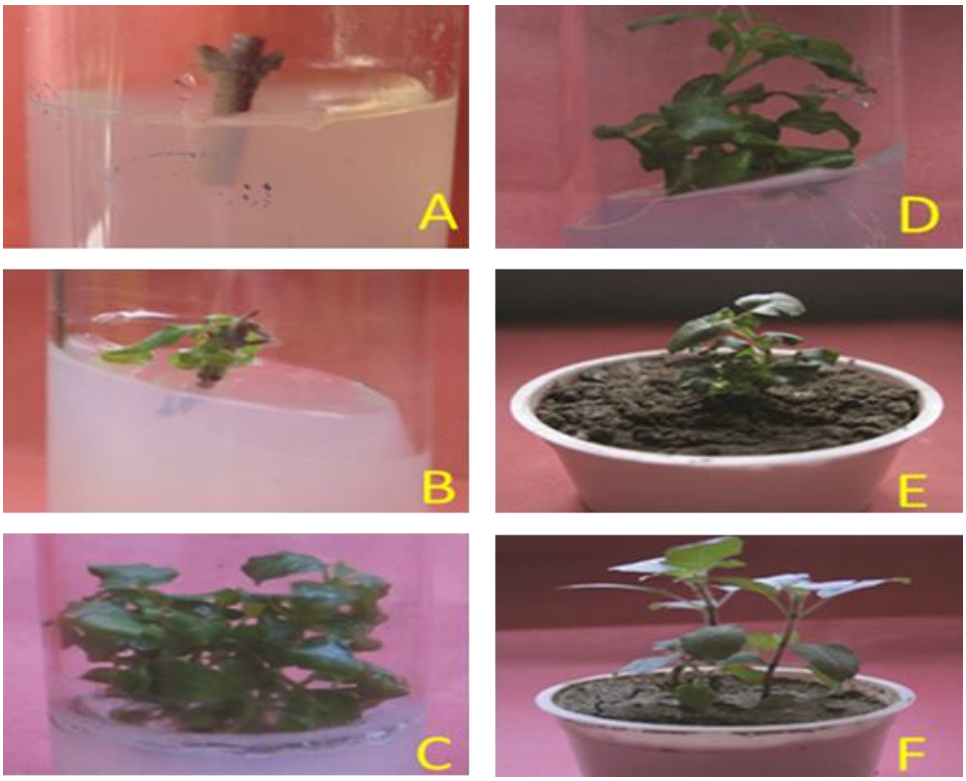


Figure 4. *In vitro* regeneration of *Ocimum sanctum* from nodal segment. **A.** Explants inoculation, B. Proliferation initiation, **C.** multiple shoots proliferation, **D.** Root initiation, **E.** Acclimatization, **F.** Acclimatized plant.

designing, mass-propagation studies and enhanced plant generation (Murch and Saxena, 2004). Distinctive concentrations of cytokinin alone or in combination with auxin were utilized to observe the response of shoot regeneration from shoot tip and nodal explants. Cytokinin promotes cell division and shoots induction (KyteL, 1987). Various investigations have demonstrated that plant root improvement may be interceded by the deliberate activity of auxin (Moriwaki et al., 2011).

Different concentrations of cytokinin or in combination with NAA, IAA and 2, 4-D were used to test multiple shoots proliferation potentiality from shoot tip and nodal explants (Ndoye et al., 2003). In the present investigation, combination of BAP with NAA was also considered effective. Higher concentrations of cytokinin (BAP) have a positive effect on shoot multiplication. Multiple shoot formation was recorded with higher concentration of BAP (Susila et al., 2013).

It is shown that the regeneration protocol improved by this experiment is more proper for commercial use than established report (Singh et al., 2009) because of minimum use of hormone concentration. An efficient shoot proliferation is made possible with a well-developed rooting system for successful acclimatization and finally it can be established in field (Abdulmalik et al., 2012). Mostly, NAA and IAA were used in different concentrations in full and 1/2 strength of MS basal medium (Fotopoulos and Sotiropoulos, 2005). Roots were formed even within 2 weeks of culture in media having 1.0mg/L NAA. The plantlets that improved from separate *in vitro* culture were proficiently rooted in field. So, the plants can luxuriantly grow in the field (Sharma et al., 2014). In the bestow scrutiny, the outliving of seasonal plant is 85%, which has been clearly proved better than previous report (Begum et al., 2002).

Conclusion

In conclusion, this study describes an efficient procedure for *in vitro* micro-propagation and a successful acclimatization of *O. sanctum* L. This protocol can be completely used in this species for variegated purposes such as *in vitro* conservation, cryopreservation, substantial scale augmentation and hereditary transformation. This enhances the opportunities to employ *O. sanctum* L, an important medicinal plant of Bangladesh, for modern medical health care.

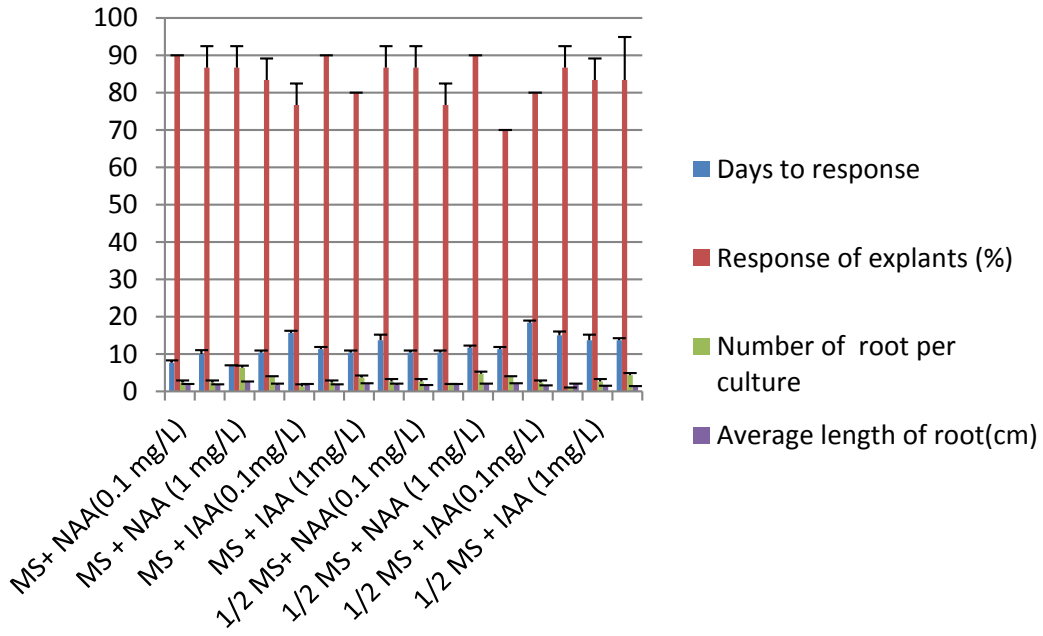


Figure 5. Effect of different concentration of NAA and IAA in full and half strength of MS medium on root induction of the elongated micro shoots of *Osmium sanctum*.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors are very much grateful to the authority of Islamic University, Kushtia, Bangladesh for their financial support from IUBT 0756, and also to the Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh for their help in using the Laboratory of Plant Tissue Culture and Molecular Biology Laboratory of the Department.

REFERENCES

Abdulmalik MM, Usman SI, Olarewaju DJ, Aba AD(2012). Effect of Naphthalene Acetic Acid (NAA) on *In Vitro* Rooting of Regenarated Microshoots of Groundnut (*Arachishypogaea* L.). Bayero J. Pure Appl. Sci. 5(2):128-131.

Begum F, Amin MN, Azad MAK (2002). *In vitro* Rapid Clonal Propagation of *Ocimumbasilicum* L. Plant Tissue Cult. 12(1): 27-35.s.

Fotopoulos S ,Sotiropoulos T.E (2005) *In vitro* rooting of PR 204/84 rootstock (*Prunus persicax P. amygdalus*) as influenced by mineral concentration of the culture medium and exposure to darkness for a period. *Agron. Res.* **3**(1):3-8.

Gupta D, Bhaskar JD, GuptaKR, Karim B, Jain A, Singh R. Karim W(2014). A randomized controlled clinical trial of *Ocimum sanctum* and chlorhexidine mouthwash on dental plaque and gingival inflammation. J. Ayurveda Integr. Med. 5(2):109-116.

Hao D, Xiao P (2015). Genomics and Evolution in Traditional Medicinal Plants: Road to a Healthier Life. Evol. Bioinform. Online 11:197-212.

Kayastha LB (2014). Queen of herbs tulsi (*Ocimum sanctum*) removes impurities from water and plays disinfectant role. J. Med. Plants Stud. 2(2):1-8

Kyte L (1987). Plants from test tubes: An introduction to micropropagation. (Rev. Ed.) Portland. Oreg. Timber Press pp. 43-44.

Moriwaki T, Miyazawa Y, Kobayashi A, Uchida M, Watanabe C, Fujii N, Takahashi H (2011). Hormonal Regulation of Lateral Root Development in Arabidopsis Modulated by *MIZ1* and Requirement of GNOM Activity for *MIZ1*Function. Plant Physiol. 157(3):1209-1220.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio-assays with tobacco tissue culture. Physolplantarum. 15:473-497.

Murch JS, Saxena KP (2004). Role of Indoleamines in Regulation of Morphogenesis in *In Vitro* Cultures of St. John's wort (*Hypericumperforatum* L.) *Acta Horticulturae, Congress:* The Future for Medicinal and Aromatic Plants 629:425-432.

Narayana ABD, Manohar R, Mahapatra A, Sujithra MR, Aramya RA (2014). Posological Considerations of *Ocimum sanctum (Tulasi)* as per Ayurvedic Science and Pharmaceutical Sciences. Indian J. Pharm. Sci. 76(3):240-245.

Ndoye M, Diallo I, Gassama YK (2003). *In vitro* multiplication of the semi- arid forest tree, *Balanitesaegyptiaca* (L.) Del. Am. J. Bot. 2(11):421-424.

Rahman HM, Atangana AR, Tigabu M, Zhang J (2013). A Study on Exploration of Ethnobotanical Knowledge of Rural Community in Bangladesh: Basis for Biodiversity Conservation. Int. Sch. Res. Notices Biodivers.

Ramesh B, SatakopanNV(2010). Antioxidant Activities of Hydroalcoholic Extract of *Ocimum sanctum* Against Cadmium Induced Toxicity in Rats. Indian J Clin. Biochem. 25(3):307-310.

Saad MIA, and ElshahedME(2012). Plant Tissue Culture Media, Recent Advances in Plant in vitro Culture, Dr. AnnaritaLeva (Ed.). ISBN: 978-953-51-0787-3, InTech

Saha S, Dhar TN, Sengupta C, Ghosh PD (2013). Biological activities of essential oils and methanol extracts of five *Ocimum*species against pathogenic bacteria. Czech J. Food Sci. 31(2):194-202.

Saharkhiz JM, Kamyab AA, Kazerani KN, Zomorodian K, Pakshir K, Rahimi JM.(2015) Chemical Compositions and Antimicrobial Activities of *Ocimum sanctum* L. Essential Oils at Different Harvest Stages. Jundishapur J. Microbiol. 8(1):e13720.

Sharma A, Kaur R, SharmaN(2014). *In vitro* morphogenic response of different explants of *Gentianakurroo*Royle from Western Himalayas-an endangered medicinal plant. Physiol. Mol. Biol. Plants 20(2):249-256.

Singh N, Hoette Y, Miller R (2010). Tulsi. The Mother Medicine of Nature 2nd ed. Lucknow: Int. Institute Herb. Med. pp 28-47.

Singh P, Singh A, Shukla KA, Singh L, Pande V, Nailwal KT (2009). Somatic Embryogenesis and *In Vitro* Regeneration of An Endangered Medicinal Plant Sarpgandha (*Rauvolfiaserpentina*. *L*). Life Sci. J. 6(2):57-62.

Susila T, Reddy SG, Jyothsna D (2013). Standardization of protocol for *in vitro* propagation of an endangered medicinal plant *Rauwolfia serpentine* Benth. J. Med. Plants Res. 7(29):2150-2153.

Tasheva K, Kosturkova G (2013). Role of Biotechnology for Protection of Endangered Medicinal Plants, Environmental Biotechnology - New Approaches and Prospective Applications pp. 235-238.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals