ISSN: 2513 - 2695


# CONFERENCE PROCEEDINGS

# CONFERENCE PROCEEDINGS


12th – 13th January, 2016

Colombo, Sri Lanka

Committee of the BioTech- 2016

The International Institute of Knowledge Management (TIIKM)

Tel: +94(0) 11 3132827

info@tiikm.com

**Disclaimer**

The responsibility for opinions expressed, in articles, studies and other contributions in this publication rests solely with their authors, and this publication does not constitute an endorsement by the BioTech or TIIKM of the opinions so expressed in them.

Official website of the conference

www.bioscienceconference.com

Conference Proceedings of the International Conference on Bioscience and Biotechnology, 2016

Edited by Emeritus Prof. Eric H Karunanayake and Udayangani Premarathne

ISSN: 2513 - 2695



Published by The International Institute of Knowledge Management (TIIKM)

Tel: +94(0) 11 3132827

Fax: +94(0) 11 2835571

**Hosted by:**

Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka

**Organized by:**

The International Institute of Knowledge Management (TIIKM)

## BioTech 2016 Committee

| | |
|---|---|
| EMERITUS PROF. ERIC H KARUNANAYAKE | (Chair, BioTech 2016)<br><br>*Emeritus Professor of Biochemistry, Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.* |
| PROF. ATTA-UR-RAHMAN, FRS | (Key-Note Speaker/ Session Chair, BioTech 2016)<br><br>*UNESCO Science Laureate/ Professor Emeritus, International Centre for Chemical & Biological Sciences, University of Karachi, Pakistan.* |
| PROF. K.M. NALIN DE SILVA | (Key-Note Speaker/ Session Chair, BioTech 2016)<br><br>*Professor of Chemistry, University of Colombo and Science Team Leader, Sri Lanka Institute of Nanotechnology (SLINTEC), Sri Lanka.* |
| PROF. WEI ZHANG | (Key-Note Speaker/ Session Chair, BioTech 2016)<br><br>*Director of Flinders Centre for Marine Bioproducts Development and Head of Molecular Bioprocess Engineering Laboratory, President of Australia, New Zealand Marine Biotechnology Society.* |
| PROF. KAMANI TENNEKOON | (Session Chair, BioTech 2016)<br><br>*Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.* |

| | |
|---|---|
| DR. DINITHI PEIRIS | (Session Chair, BioTech 2016)<br><br>*Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.* |
| DR. ASHISH WADHWANI | (Session Chair, BioTech 2016)<br><br>*Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, India.* |
| DR. DILANTHA GUNAWARDANA | (Session Chair, BioTech 2016)<br><br>*Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.* |
| MR. ISANKA. P. GAMAGE | (Conference Convener, BioTech 2016)<br><br>*The International Institute of Knowledge Management* |
| MR. OSHADEE WITHANAWASAM | (Conference Publication Chair, BioTech 2016)<br><br>*The International Institute of Knowledge Management* |
| MS. SUVINIE RAJAPAKSHA | (Conference Coordinator, BioTech 2016)<br><br>*The International Institute of Knowledge Management* |

## Editorial Board - BioTech - 2016

## Scientific Committee - BioTech - 2016

Prof.Kamani Tennekoon, *University Colombo, Sri Lanka.*

Dr.Sumadee De Silva, *University Colombo, Sri Lanka.*

Dr.Nimal Punyasiri, *University Colombo, Sri Lanka.*

Dr.Sameera R Samarakoon, *University Colombo, Sri Lanka.*

Dr. Ashish Wadhwani, *JSS College of Pharmacy, India*

Dr. Shamim Ahmed, *Shahjalal University of Science and Technology (SUST), Bangledesh.*

Dr Narayan Saha, *Shahjalal University of Science and Technology (SUST), Bangledesh.*

Dr. Anugya Bhatt, *Sree Chitra Tirunal Institute for Medical Sciences and Technology, India.*

Dr A.Sabareeswaran, *Sree Chitra Tirunal Institute for Medical Sciences and Technology, India.*

Dr. Md. Mohiuddin Kabir, *East West University, Bangladesh*

.Dr. A Maya Nandkumar, *Sree Chitra Tirunal Institute for Medical Sciences & Technology, India*

Assoc. Prof. Bishwajit Kundu, *School of Biological Sciences, IIT Delhi, India*

## MESSAGE FROM PROF. ATTA-UR-RAHMAN, FRS

### KEYNOTE SPEAKER - BioTech 2016

Biotechnology has emerged in recent years as a powerful medium for socio-economic development. A large number of biotechnologies are being developed which include recombinant vaccines, vaccines in drug delivery, molecular diagnostics, bioremediation, sequencing of genomes, bioinformatics development, genetically modified crops, recombinant therapeutic proteins etc. Multibillion dollar industries are being developed in many of these fields and countries such as Cuba, which are investing massively in such technologies, have begun to benefit in a major way.

The three major players in the development of a knowledge economy are universities, industry and the government. All three thrive on the extent of merit-based competiveness that should be in-built into the systems and on the efficiency of interaction among all three players. The development of a knowledge economy requires a thorough understanding of the dynamic interplay between research, invention, innovation, and economic growth. Such an understanding allows them to be modulated according to national needs and challenges.

The global trends in manufacturing and exports over the last 3 decades clearly show a sharp rise in high and medium technology goods and a sharp consistent decline in low technology goods and in natural resources. The big money lies in high technology and countries that have realized this have invested massively in knowledge-based economies leading to the manufacture and exports of electronics, pharmaceuticals, engineering goods, biotech products, computers, software, automobiles, aircraft, defense equipment, alternative energy products and many other such items requiring highly specialized skills and world class research centres. That is why the annual exports of a tiny country which has a population only of about 5 million and no significant natural resources, Singapore, are a stupendous US $ 458 billion.

Pakistan made remarkable progress in science and higher education during the 9 years (2000-2008) when I was Federal Minister for Science & Technology as well as the Federal Minister responsible for Higher Education that resulted in over 1000% increase in international publications in high impact journals and even greater increases in citations and PhD output. This laid the foundations of a strong knowledge economy.

I hope such issues as well as the exciting recent advances in biotechnology will be discussed in depth at the conference.

I wish Biotech-2016 all success.

**Prof. Atta-Ur-Rahman. FRS**
UNESCO Science Laureate/ Professor Emeritus, International Centre for Chemical & Biological Sciences, University of Karachi, Pakistan.

## MESSAGE FROM PROF. SAMPATH AMARATUNGE

It is with great pleasure I warmly welcome all participants to the First Annual International Conference on Bioscience and Biotechnology 2016 on behalf of University of Sri Jayewardenepura, the hosting partner of the Conference which is organized by The International Institute of Knowledge Management (TIIKM) during 12-13 January at Colombo, Sri Lanka under the theme of "Molecular Life Sciences for the Development in the 21st Century".

Bioscience and Biotechnology, in both developed and developing countries are increasingly important fields in the modern era. The innovations and researches in this field are already impacting the society positively and hence we could expect more innovations that will contribute to the sustainability of human beings in this century. Main conference tracks being Bioprocess and Biological Engineering, Agriculture and Food Biotechnology, Bionanotechnology, Biopharmaceutics and Medical Biotechnology, and Biodiversity and Natural products. I have no doubt that this conference can promote such developments and innovations in the field of Bioscience and Biotechnology in this country and at the global level.

I wish the Conference every success and wish to see the knowledge and expertise derived from the 1st Conference will go a long way enriching the fields of Bioscience and Biotechnology and thereby contribute to the developmental activities both local and abroad.

**Prof. Sampath Amaratunge**
Vice Chancellor,
University of Sri Jayewardenepura,
Sri Lanka

Table of Contents Page No

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 1-4

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1101

# POTENCY OF AGRICULTURAL WASTE FOR FORAGE IN TOBASA REGENCY

[1]M. Adelina and [2]S. Kisno

[1, 2] *Del Institute of Technology, North Sumatera, Indonesia*

**Abstract**

Forage, which is provided by environment, is one of the important factors to consider in animal husbandry since the productivity of the livestock is 70% affected by environment rather than genetic factor whilst 60 up to 80% of cost production is allocated for producing and providing forage. In addition, the grazing land which simultaneously functions as the green resources for animal feed is getting lesser and lesser. In Tobasa regency, North Sumatera, Indonesia, the land used for farming is about 22,685 hectares which are used by the local people for growing paddy, cacao, corn, coffee, and other agricultural products. The waste produced as the result of these agricultural and farming activities can be used as animal feed or forage. This research aims to explore the efforts in reducing agricultural waste and utilize it as forage through fermentation process (ammonization and molasses) as the solution to the forage issues such as low nutrition and rough-fibred substance. The result of this research is expected to fulfill the needs of alternative animal feed and protecting the environment through diminishing agriculture waste.

**Keywords:** forage, agricultural waste, fermentation, livestock

## INTRODUCTION

Referring to the national long term development plan of Indonesia 2005-2025 and to maintain the continuance of the projects have been developed five years before, the development of information and technology is aimed at supporting some sectors. One of the sectors is food which aims to realize the independent, prosperous, and justice people of Indonesia. Nowadays, the utilization of technology in the community is still limited due to some factors. One of the factors is the lack of efficient technological use which can be implemented in managing natural resources. This problem has effect on the waste handling which are as the result of the natural waste. The continual increase amount of waste without proper handling will lead to new environmental issues and indeed it will affect the living thing.

Sitoluama village is situated on Laguboti district in Toba Samosir regency in North Sumatera. Most of the people here works in agricultural sector and one of the favorable dominant commodity here is coffee. There are two types of coffee here; they are Robusta and Arabica.


*Figure 1. Implementation Area in Sitoluama Village – Laguboti District (*www.tobasamosirkab.go.id, *2016)*

The problem aroused from this agricultural sector is the waste produced since most people only use the coffee bean while other parts of coffee are only waste. The waste is getting much and much everyday due to the people knowledge limitation in processing the waste to be useful materials in life.

The potency of the wet process waste rind has big opportunity since the waste rind of the coffecy is physically has 48% mass of the total weight of the wet fruit. This leads to do more efforts on utilizing the coffee waste rind as an effort to benefit the people and to preserve the environment. One of these efforts is by utilizing the waste as the materials for forage since this effort will help the agricultural sector in providing more resources for forage due to the green area as the raw materials have ben lesser and lesser and this will provide alternative materials for feeding the animal. This project aims to overcome the problem of overloading coffee rind waste amount through processing it as the raw material for forage. Moreover, this project objective is to help the cattleman in providing food for their cattle.

## EXPERIMENTAL

To solve the existing problem as the result of increasing amount of coffee rind waste, the researchers try to provide a solution by utilizing the waste as the forage. The result of the implementation will help the coffee farmers and cattleman in increasing the life of the people in Sitoluama.

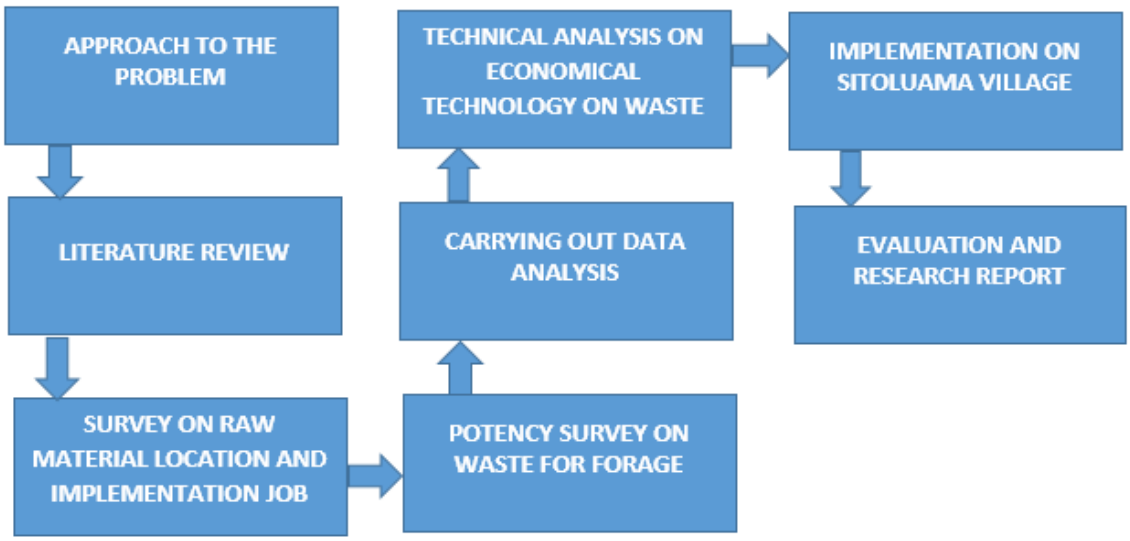



*Figure 2. Project Scope Scheme*

At this time, the coffee waste is left intake and it pollutes the environment. Seeing the big chance of the amount of coffee rind waste, a technology is needed to process it. There are some drawbacks if the waste is directly given to feed the animal such as the content of the protein is relatively low, the highly raw fiber content which contains the substance obstructing the animals' growth and the high content of water inside the waste which makes it easy to decay.

*Figure 3. Coffee Rind Waste (www.kompasiana.com, 2015)*

To overcome those issues, some processes can be conducted to increase the quality of the nutrition and to lengthen the storage period.

Some steps in the production process are: fermentation, drying, drilling, packaging, and safekeeping. Fermentation aims to produce the quality of the nutrition and reduce the substance level which obstructs the digestive process in the cattle (Enari, 1983). Guntoro *et al* (2006) declared that fermentation with *Aspergillus niger* could increase the protein content from 7.9% up to 12.41% and it can be used as the proper animal food. This result can replace bran as the important component in forage both for ruminant and non-ruminant animals.

First, *Aspergillus niger* is activated by sterilized clean water, added by sugar, urea, and NPK. The composition to make 10 liters of *Aspergillus niger* are 10 liters water 100 grams sugar, 100 gr urea, 50 grams NPK and 100 grams *Aspergillus niger*. All these materials are dissolved in the clean container and they are well stirred. Then, aeration process is conducted with aerator for 24-36 hours. The ready-fermented coffee rind waste is spread on a 5-10 cm thickness medium and poured with *Aspergillus niger* solvent. Flushing can be carried out with sprayer and the stack of the waste is covered with a clean plastic cover for 4-5 days. Next, the drying process can be done through under-sun drying or using dryer aiming

to stop fermentation process and to ease the drilling process and to extend storage period. Drying is carried out until the water level comes at 12-14% which marked by texture change becoming hard and the color becomes blackened. Drilling is carried out to obtain softer texture (like powder) to make it easily consumed by the animals. The efficient drilling can be done through drilling machine so that the form and size of the powder is adjustable. This powder as the result of waste processing can be directly given to feed the animal or stored in the period of 6-10 months. To keep the quality in good condition and to avoid early decay, this powder must be well packaged. Packaging can be carried out using a well-sealed plastic container to avoid insects or harmful microorganism. All these processing must be implemented through training for the farmers both in class and practical session. The effectivity of the training can measured through conducting pre-test and post-test.

*Figure 4. Forage form Coffee Rind Waste (www.litbang.pertanian.go.id, 2015)*

## REFERENCES

Anonymous. Kecamatan Laguboti. http://www.tobasamosirkab.go.id/wilayah. Accessed in 2015

Anonymous. Limbah Kulit Buah Kopi. www.kompasiana.com. Accessed in 2015.

Enari, T.M. (1983). Microbial Cellulase. In Microbial Enzyme and Biotechnology. Edited W.M. Fogarty. Applied Science Publishing. New York.

Guntoro, S. (2006). Petunjuk Teknis Pengolahan Limbah Perkebunan Untuk Pakan. Balai Pengkajian Teknologi Pertanian (BPTP). Bali. Denpasar.

Guntoro, S. (2008). Membuat Pakan Ternak Dari Limbah Perkebunan. Agromedia Pustaka. Jakarta.

Guntoro, et al. (2006). Pengaruh Pemberian Limbah Mete Olahan terhadap Pertumbuhan Kambing. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner Puslitbang. Badan Litbang Pertanian. Bogor.

Anonymous. Pakan Ternak dari Limbah Kulit Buah Kopi. www.litbang.pertanian.go.id. Accessed in 2015

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 5-10

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1102

# ANALYSIS OF BLOOD TRANSCRIPTOME IN PATIENTS WITH CHRONIC KIDNEY DISEASE OF UNCERTAIN AETIOLOGY

[1]S. Sayanthooran, [1*]D. N. Magana-Arachchi, [2]L. Gunarathne, [3]T. Abeysekera and [4]S. D. S. S. Sooriyapathirana

*[1]National Institute of Fundamental Studies, Sri Lanka*
*[2]Renal Care & Research Centre, District Hospital, Girandurukotte, Sri Lanka*
*[3] Department of Pharmacology, Faculty of Medicine, University of Peradeniya, Sri Lanka*
*[4]Department of Molecular Biology and Biotechnology, University of Peradeniya, Sri Lanka*

**Abstract**

Chronic kidney disease of uncertain aetiology (CKDu) is an increasing health problem in certain agricultural regions of the tropical world including certain parts of Sri Lanka, Latin America, and India. This form of chronic kidney disease (CKD) does not have common causative factors such as diabetes or hypertension. The purpose of this study was to test the hypothesis that patients suffering with CKDu have differential expression patterns of genes in blood that will help differentiate them from healthy individuals, and to identify such differentially expressed genes as possible biomarkers of CKDu. Briefly, total RNA was isolated from peripheral whole blood of Stage 2, 3 and 4 CKDu patients in three different pools and compared to the expression pattern of a pooled sample of RNA extracted from healthy individuals. The RNA was amplified, reverse transcribed and hybridized to Illumina HumanHT-12 v4 Expression BeadChip arrays and scanned with an Illumina BeadArray Reader confocal scanner. Seven genes were identified that were commonly differentially expressed (fold change $\geq 2$ or $\leq 0.5$) in the three stages of CKDu population compared to healthy group. The genes identified included those involved in hypertensive response (ADM), gap junction channel activity (GJB4) and infectious/immune response (IFIT1, PI3, DEFA1, HBZ, RN7SK). These genes have the potential to be used as diagnostic markers of CKDu as they are differentially regulated in the studied stages of CKDu and can diagnose disease patients from the healthy individuals.

**Keywords:** Biomarkers, CKD, CKDu, gene expression analysis, peripheral whole blood, RT-qPCR

## INTRODUCTION

Over a million people worldwide die every year due to end stage renal disease. The disease is an equal threat to both the developed and developing countries. Increased age contributes to the disease in developed countries whereas the lack of proper treatment and renal replacement and dialysis facilities are the major factor in developing countries [1]. Diagnosis of the disease is also complicated as symptoms become apparent only with worsening stages and it is usually too late for preventive measures.

The major concern in Sri Lanka is the rise of chronic kidney disease which is of unknown etiology (CKDu), and is currently threatening to reach epidemic proportions. This disease is mainly clustered in the North Central region of the country among the dry zones of the country. High incidence has been noted in the divisions of Medawachchiya, Girandurukotte, Mahiyanganaya, Padaviya, Medirigiriya, Dehiattakandiya, Nikawewa and Kebithigollewa [2].

CKDu is not only limited to Sri Lanka, but has been observed in other agricultural regions in the world. It has especially been noted in the cotton and sugarcane plantation workers in Central America and has been linked to the geographical location, where excessive heat and volume depletion are hypothesized to be the cause [3].

Corresponding Author Email: *cellbio@ifs.ac.lk

The disease is chronic tubulointerstitial in nature with the proximal tubules being mostly affected. There are multiple sources that have been implicated in other tubulointerstitial diseases including, but not limited to, toxins, drugs, cystic diseases, vascular conditions, infection, immunological conditions and mechanical damage. These conditions could also be playing a role in the tubulointerstitial pathology seen in chronic kidney disease of unknown etiology in Sri Lanka [2, 4].

Although the search for the possible cause of CKDu has been done from various different angles, few studies have looked at it from a molecular/genetic standpoint. Genome wide association studies and exome sequencing have been recently carried out and point to genetic susceptibility of patients [5, 6]. Gene expression studies have also been carried out using selected panel of genes and have pointed towards environmentally induced oxidative stress and genetic susceptibility of individuals [7].

Gene expression analysis is a quantitative way of studying the expression of genes from the mRNA levels. Although all the genes are present in the genome, only some are expressed; the expression of which is regulated by complex mechanisms, both genetically and environmentally stimulated. The human gene expression varies significantly from individual to individual and the expression levels directly match with the underlying phenotype of the cells (Cheung & Spielman, 2009). Expression patterns of genes can be both inherited and also modified by external environmental factors. It is found that factors such as diet or medication of individuals can have a significant influence on gene expression and therefore can even override the genetic influence on gene expression [8].

The research aimed to determine the gene expression patterns in blood of CKDu patients, to identify possible causative factor/s and biomarkers for the disease. By identifying commonly differentially expressed genes in the different stages of disease, a common biomarker for the disease can be identified.

## METHODOLOGY

### Ethical clearance and informed consent

Ethical clearance for the study was obtained from the relevant hospital authorities and the Postgraduate Institute of Science, University of Peradeniya, Sri Lanka. Written informed consent was obtained from each subject.

### Study Population

Samples were collected in three batches for the three different stages of CKDu; stage 2, stage 3 and stage 4, from August 2014 to June 2015. Six patients were recruited for the study from each of stage 2, stage 3 and stage 4 of CKDu as diagnosed by the attending nephrologist from the Renal Clinic, District Hospital, Girandurukotte, a region of Sri Lanka endemic to CKDu. Six healthy volunteers were also recruited for the study from the Kandy District, an area not endemic to CKDu. Blood samples of 1 mL each was collected from the patients during routine blood collection.

### RNA Preparation

Total RNA was extracted from whole blood using Qiazol (Qiagen, USA), purified using RNeasy columns (Qiagen, USA) according to the manufacturers' protocol. After processing with DNase digestion and clean-up procedures, RNA samples were checked for integrity on Agarose gel electrophoresis and quantified, aliquot and stored at -80°C until it was shipped to an external facility, Macrogen Inc., South Korea for microarray analysis.

### Microarray Procedures

#### *Quality control*

For quality control of samples after shipment, RNA purity and integrity were evaluated by Macrogen Inc. (South Korea) using denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

#### *Labeling and purification*

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total

RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, USA).

***Hybridization and data export***

750 ng of labeled cRNA samples were hybridized to each human HT-12 expression v.4 bead array for 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc., USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions

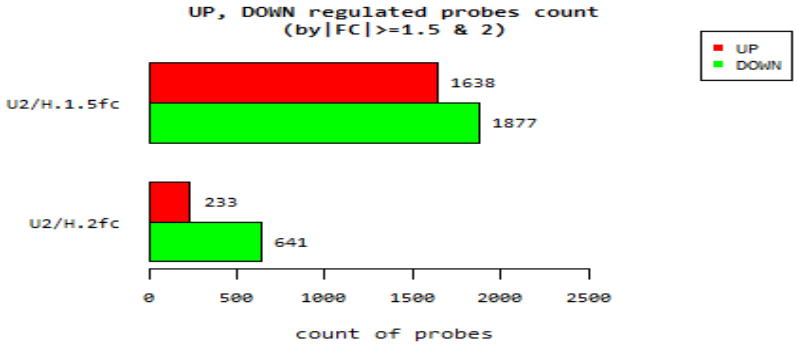
**Analysis of Microarray Data**

The results obtained from the primary microarray analysis were further analyzed. The genes that were differentially expressed (fold change $\geq 2$ or $\leq 0.5$) were selected from each of the three stages; stage 2, stage 3 and stage 4 CKDu, and the genes common to all three stages were identified.

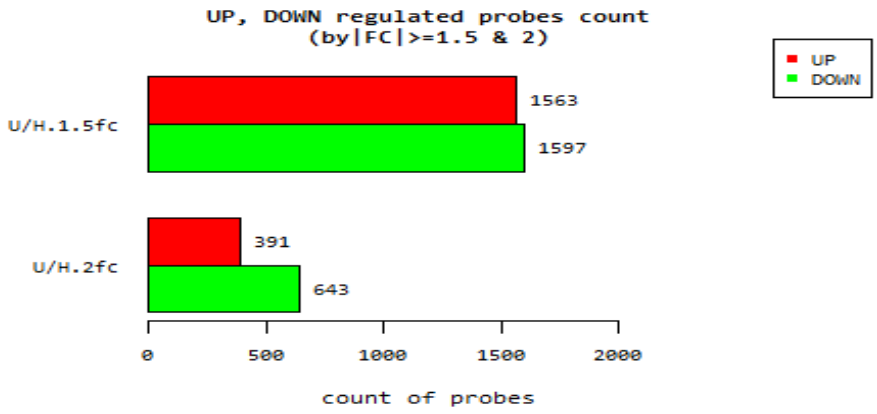
**RESULTS**

Differentially expressed genes were obtained for each of the three stages relative to healthy individuals (Figure 1). The results obtained from the primary microarray analysis were further analyzed. Genes were identified that are differentially expressed (fold change $\geq 2$ or $\leq 0.5$) specifically in each of Stage 2 (403 genes), Stage 3 (612 genes) and Stage 4 (31 genes) CKDu. Genes that are commonly differentially expressed in stage 2 and 3 (303 genes), stage 3 and 4 (18 genes), and all the three stages (7 genes) were also identified. The seven genes commonly differentially expressed in all of the three stages have functions related to hypertensive response (ADM), gap junction channel activity (GJB4) and infectious/immune response (IFIT1, PI3, DEFA1, HBZ, RN7SK).

A)



B)




C)

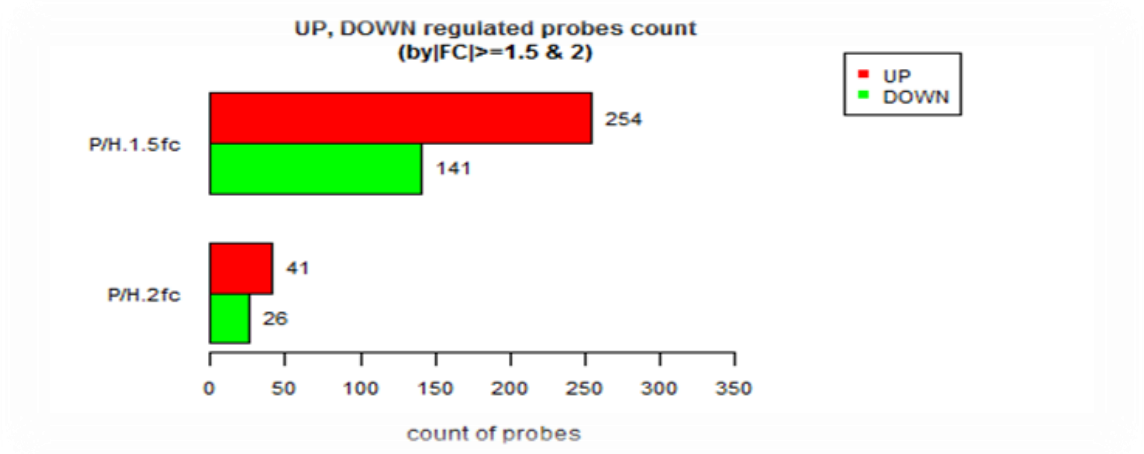



*Figure 1. Differentially expressed genes with fold change (FC) ≥ 1.5 & 2 for A) Stage 2 B) Stage 3 and C) Stage 4 CKDu*

## DISCUSSION

From the transcriptome analysis of the three stages of CKDu, seven genes from different functional groups were identified, that were commonly expressed in the diseased patients when compared to healthy individuals of a CKDu non-endemic area.

Adrenomedullin (ADM) is a potent hypotensive peptide having main functions of vasodilation, hypotension, angiogenesis, and regulation of fluid and electrolyte homeostasis [9, 10]. The protein has been linked to considerable numbers of diseases, such as hypertension, congestive heart failure, ischemic heart injury, pulmonary hypertension, sepsis, cancers, renal impairment, and diabetes. Elevated plasma levels of ADM have been useful in assessing the progression of these diseases and circulating ADM was also seen to be increased after tissue transplantation, suggesting its protective role against oxidative damage. ADM holds high potential and is currently undergoing clinical studies to be used in diagnosis and treatment [10].

Gap junction protein beta 4 (GJB4) is coding for the protein Connexin 30.3 (Cx30.3) which is crucial for epidermal differentiation. Connexins are membrane-spanning proteins that allow for the formation of cell-to-cell channels and cell-to–extracellular space hemi channels. Many connexin subtypes are expressed in kidney cells. Some mutations in connexin genes have been linked to various human pathologies, including cardiovascular, neurodegenerative, lung, and skin diseases, but the exact role of connexins in kidney disease remains unclear [11, 12]. Mutations of the GJB4 gene have been implicated in erythrokeratoderma variabilis with erythema gyratem repens [12, 13]. Interestingly keratosis is a common symptom in CKDu patients and has been linked to arsenic poisoning [5].

Phosphatidylinositol 3-kinases (PI3K's), a family of enzymes which catalyze the phosphorylation of the 30–OH of the inositol ring, play a central role in regulating a wide range of cellular processes including metabolism, survival, motility and cell activation. There is currently an intense focus by pharmaceutical companies to develop PI3K inhibitors as therapy for cancer, cardiovascular, respiratory, autoimmune and inflammatory diseases [14].

A few genes that were differentially expressed in the three stages of CKDu have previously been associated with viral and bacterial infections. The human Interferon-induced protein with Tetratricopeptide Repeats 1 (IFIT1) blocks West Nile virus (WNV), Japanese encephalitis virus (JEV), and coronavirus mutants [15, 16]. The 7SK small nuclear RNA (RN7SK) functions as a negative regulator of HIV-1 transcription by interacting with the positive elongation factor b (p-TEFb) [17] . The upregulation of the Human T-cell leukemia virus type 1 (HTLV-1) basic leucine zipper factor gene (HBZ) has been associated with HTLV-1, the cause of Adult T-cell leukemia/lymphoma (ATL) [18, 19]. The HBZ gene was also suggested to possibly have a functional role in cellular transformation and leukemogenesis [19]. Human alpha-defensins like the alpha defensin 1 (DEFA1) have been reported as natural antimicrobial peptides of neutrophils which have evolved in host defense reactions. The circulating non stressed alpha-defensins have also been associated with serum lipid levels [20].

From the results it can be seen that CKDu shares certain molecular level similarities to CKD of known origin and other metabolic disorders as is seen with the differential regulation of the ADM, GJB4, PI3K and DEFA1 genes. There also is a possibility of infections in this population as seen by the differential expression of the IFIT1, RN7SK, and HBZ genes. Real time quantitative PCRs are currently being carried out to verify findings of the microarrays. The regulation patterns of these genes need to be further looked into in detail in each of the stages to understand progression of the disease and for further biomarker development.

## CONCLUSION

The seven identified genes; ADM, GJB4, PI3K, DEFA1, IFIT1, RN7SK and HBZ, together as a panel have potential as biomarkers of CKDu, however need to undergo further validation studies prior.

## ACKNOWLEDGMENT

The study was supported by the grant 11-059 received from the National Research Council, Sri Lanka. The corresponding author for the paper, D. N.

Magana-Arachchi can be contacted via e mail at cellbio@ifs.ac.lk .

## REFERENCES

[1]. Hamer RA, El Nahas AM: The burden of chronic kidney disease: Is rising rapidly worldwide. *BMJ : British Medical Journal* 2006, 332(7541):563-564.

[2]. Johnson S, Misra S, Sahu R, Saxena P: Environmental contamination and its association with CKDu in North Central Region of Sri Lanka. In. New Delhi, India: Centre for Science and Environment; 2012.

[3]. Weiner DE, McClean MD, Kaufman JS, Brooks DR: The Central American epidemic of CKD. *Clinical journal of the American Society of Nephrology : CJASN* 2013, 8(3):504-511.

[4]. Nanayakkara S, Komiya T, Ratnatunga N, Senevirathna ST, Harada KH, Hitomi T, Gobe G, Muso E, Abeysekera T, Koizumi A: Tubulointerstitial damage as the major pathological lesion in endemic chronic kidney disease among farmers in North Central Province of Sri Lanka. *Environmental health and preventive medicine* 2012, 17(3):213-221.

[5]. Nanayakkara S, Senevirathna ST, Abeysekera T, Chandrajith R, Ratnatunga N, Gunarathne ED, Yan J, Hitomi T, Muso E, Komiya T *et al*: An integrative study of the genetic, social and environmental determinants of chronic kidney disease characterized by tubulointerstitial damages in the North Central Region of Sri Lanka. *Journal of occupational health* 2014, 56(1):28-38.

[6]. Nanayakkara S, Senevirathna ST, Parahitiyawa NB, Abeysekera T, Chandrajith R, Ratnatunga N, Hitomi T, Kobayashi H, Harada KH, Koizumi A: Whole-exome sequencing reveals genetic variants associated with chronic kidney disease characterized by tubulointerstitial damages in North Central Region, Sri Lanka. *Environmental health and preventive medicine* 2015, 20(5):354-359.

[7]. Sayanthooran S, Magana-Arachchi D, Sooriyapathirana S, Abeysekera T, Gunarathne L: environmentally Influenced Gene Expression in Patients with Chronic Kidny Disease Living in the Dry Zone of Sri Lanka. 2014.

[8]. Cheung VG, Spielman RS: Genetics of human gene expression: mapping DNA variants that influence gene expression. *Nature reviews Genetics* 2009, 10(9):595-604.

[9]. Geny B, Ellero B, Charloux A, Brandenberger G, Doutreleau S, Piquard F: Circulating adrenomedullin is increased in relation with increased creatinine and atrial natriuretic peptide in liver-transplant recipients. *Regulatory Peptides* 2003, 114(1):61-66.

[10]. Ogoshi M: Adrenomedullin. 2016:240-e227D-244.

[11]. Lai-Cheong JE, Arita K, McGrath JA: Genetic diseases of junctions. *The Journal of investigative dermatology* 2007, 127(12):2713-2725.

[12]. Veenstra RD: Biology of Gap Junctions. 2012:409-429.

[13]. Richard G, Brown N, Rouan F, Van der Schroeff JG, Bijlsma E, Eichenfield LF, Sybert VP, Greer KE, Hogan P, Campanelli C *et al*: Genetic heterogeneity in erythrokeratodermia variabilis: novel mutations in the connexin gene GJB4 (Cx30.3) and genotype-phenotype correlations. *The Journal of investigative dermatology* 2003, 120(4):601-609.

[14]. Foster JG, Blunt MD, Carter E, Ward SG: Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies. *Pharmacological reviews* 2012, 64(4):1027-1054.

[15]. Danish H, Goyal S, Taunk NK, Wu H, Moran MS, Haffty BG: Interferon-induced Protein with Tetratricopeptide Repeats 1 (IFIT1) as a Prognostic Marker for Local Control in T1-2 N0 Breast Cancer Treated with Breast Conserving Surgery and Radiation Therapy (BCS+RT). *International Journal of Radiation Oncology*Biology*Physics* 2011, 81(2):S214.

[16]. Roby JA, Clarke BD, Khromykh AA: Loop de loop: viral RNA evades IFIT1 targeting. *Trends in microbiology* 2014, 22(4):171-173.

[17]. Krueger BJ, Varzavand K, Cooper JJ, Price DH: The mechanism of release of P-TEFb and HEXIM1 from the 7SK snRNP by viral and cellular activators includes a conformational change in 7SK. *PloS one* 2010, 5(8):e12335.

[18]. Landry S, Halin M, Vargas A, Lemasson I, Mesnard JM, Barbeau B: Upregulation of human T-cell leukemia virus type 1 antisense transcription by the viral tax protein. *Journal of virology* 2009, 83(4):2048-2054.

[19]. Matsuoka M, Green PL: The HBZ gene, a key player in HTLV-1 pathogenesis. *Retrovirology* 2009, 6:71-71.

[20]. Li YX, Lin CQ, Shi DY, Zeng SY, Li WS: Upregulated expression of human alpha-defensins 1, 2 and 3 in hypercholesteremia and its relationship with serum lipid levels. *Human immunology* 2014, 75(11):1104-1109.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 11-14

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1103

# *IN VITRO* MICROPROPAGATION OF *Gyrinops walla* (GAERTH.) USING LEAF DISC EXPLANTS

A.N. Buddhapriya and W.T.P.S.K. Senarath*

*Department of Botany, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka*

**Abstract**

*Gyrinops walla* (Gaertn.) (Thymelaeaceae) is a slender tree which grows in wet zone regions in Sri Lanka. Potentiality of production of resinous natural product, agarwood, which used in perfumery and medicine manufacturing, grant a great demand for this species in worldwide. In vitro micropropagation technique may be a practicable solution to provide healthy planting materials in commercial scale, in order to overcome the problems caused to the natural population due to overexploitation. Thus, using leaf disc explant, possibilities of mass propagation through in vitro techniques was explored in in vitro study. Explants were surface sterilized using 0.2% carbendazim, 10% sodium hypochlorite (Clorox) and 70% ethanol each followed by two successive washings in sterile distilled water. Murashige and Skoog (MS) basal medium supplemented with varied concentrations of different plant growth regulators, 6-benzylaminopurine (BA) and naphthaleneacetic acid (NAA) were tested for induction of calli from leaf discs. Out of different growth regulator combinations, rapid callus growth was observed in MS medium supplemented with 1.0 mg/L BA and 3.0 mg/L NAA within six weeks. Calli obtained were yellowish, compact and callus induction was mainly observed along the midrib. Calli obtain could either be used for mass propagation, through indirect organogenesis or for establishment of plant cell culture to obtain secondary metabolites. From the results obtain, it could be concluded that callus initiation is feasible through leaf disc explant.

**Keywords:** *Gyrinops walla*, in vitro micropropagation, callus formation, plant growth regulators, leaf disc explant

## INTRODUCTION

*Gyrinops* is a genus of eight species belongs to family Thymelaeaceae and native to South and South East Asia [12]. The species *Gyrinops walla* (Gaertn.) is native to Sri Lanka and India. The plants grow in scrub jungles, woodlands and small or large forests in wetlands. Also can be found in home gardens as an ornamental plant. Habit of *G. walla* is a medium tall tree that grows up to 15 m in height with straight, slender trunk and also with a small rounded crown [4]. It has dark green thin leaves with small white flowers about one centimetre wide. *G. walla* is closely related to *Aquilaria* sp., which may produce a resinous substance called agarwood resin. Agarwood is reputed to be the most expensive wood in the world. There are many names for the resinous, fragrant heartwood produced primarily by trees in the genus *Aquilaria* and *Gyrinops*. Most commonly, the resin is known as agarwood, aloeswood, eaglewood, gaharu etc. As a self-defence mechanism, *G. walla* also produces a resin similar to agarwood which has been well recognized as a primary raw material in perfumery industry worldwide [2]. It is also used pharmaceutically as an anti-emetic, sedative and digestive in oriental medical treatments.

Worldwide trading of agarwood is facing a serious shortage of resources because of its disordered collection in forests and the decrease in the tropical rainforest area. *G. walla* has been listed as a potentially threatened species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) due to overexploitation of natural population [1]. Efforts have been undertaken to increase the population of *G. walla*. and variety of methods are used towards this matter. Some recently developed techniques have proven to be more

Corresponding Author Email: * wtpsk2011@yahoo.com

effective than previous methods. Developing *in vitro* micropropagation protocol is one such technique, which could be an applicable solution to implement healthy planting material to produce stocks for commercial scale plantations.

Plant tissue culture is referred to as an *in vitro*, sterile culture, which is an important tool in basic studies as well as in commercial scale plant propagation [11]. For the present study, isolated pieces of a selected leaf disc explants are obtained aseptically from a second fully opened leaves and cultured on a nutrient medium. However, growth induced after transfer of the explants to the nutrient medium supplemented with different plant growth regulators, results in an unorganized mass of calli [7]. This mass propagation techniques, then used to generate *in vitro* multiples shoots or for establishment of plant cell culture to obtain secondary metabolites.

## MATERIALS AND METHODS

A stock of six months to one year old *G. walla* seedlings were obtained from a forest reserve at Kamburupitiya, which belongs to the low country wet zone in Sri Lanka. All the seedlings were maintained under controlled environment at the Botanical Garden of the Department of Botany, University of Sri Jayewardenepura. Plants were watered regularly and well maintained to protect from insects and fungal attacks. Healthy, young plants were used as the mother stock.

Mother stock was sprayed with 0.1% Carbendazim® solution a week before collection of explants and then 24 hours prior to collection of explants. Leaf discs from fresh leaves (second and third fully opened) were used as explants in the present study. Explants collected from mother stock was initially washed in water containing 0.01% w/v Teepol and a few drops of Tween 20, then washed under running tap water for 1 hour and finally dipped in a 0.2% Carbendazim® solution for 10 minutes prior to be taken into the laminar flow cabinet.

The explants were surface sterilized in 10% Clorox for 10 min and then in 70% Ethanol for 1 min, each followed by three successive washings in sterile distilled water [3]. Leaf discs (5.0 $mm^2$) were prepared centring the midrib and cultured on Murashige and Skoog (MS) medium [6] supplemented with different concentrations of BA (1.0 - 2.0 mg/L) and NAA (1.0 - 3.0 mg/L) [8]. Sucrose (30.0 g/L) was added to the medium and the pH of the medium was adjusted to 5.8. Growth regulator free MS medium was used as the control. Cultures were incubated under a 16 h light in 25 ± 1˚C. There were 30 replicates per treatment and Cultures were closely observed for any contaminations. The survival rate of explants, effect of plant growth regulators on callus induction and nature of calli were observed over a period of twelve weeks.

## RESULTS AND DISCUSSION

### Percentage Contamination and Survival

Low rate of contamination (10%) was observed when leaf discs of *G. walla* were treated with 0.01% w/v Teepol solution with a few drops of Tween 20 followed by 10% Clorox for 10 min. and 70% ethanol for 1 min each followed by three successive washings in sterile distilled water despite the fact that explants were collected from the plants grown in the field. Use of optimum concentrations of surface sterilants and exposure period greatly determines the success of the mass propagation technique by means of low contamination percentage and the considerably high leaf disc explant survival. Plants growing in the field are invariably contaminated with microorganisms. These two step, two reagent procedure [5], found to be effective for the surface sterilization of *G. walla* leaf disc explants. It was observed, out of 90% healthy leaf disc explants, 78% of them survived yet rest died after browning in a few days after inoculation.

### Callus Induction from Leaf Disc Explants

Callus initiation was observed from leaf disc explants cultured on MS medium supplemented with BA (1.0 – 2.0 mg/L) and NAA (1.0 – 3.0 mg/L) over a period of twelve weeks. MS medium supplemented with 1.0 mg/L BA and 3.0 mg/L NAA was effective in callus induction in *G. walla.* which produced callus within 43 days of incubation (Table 1). Growth regulator free MS basal medium (Control) did not promote the callus induction.

*Table 1: Effect of Plant Growth Regulators on Callus Induction*

| BA (mg/L) | NAA (mg/L) | Growth* (No. of Days) |
|---|---|---|
| 1.0 | 1.0 | 55 |
| 1.5 | 1.0 | 59 |
| 2.0 | 1.0 | 63 |
| 1.0 | 2.0 | 57 |
| 1.5 | 2.0 | 60 |
| 2.0 | 2.0 | 64 |
| 1.0 | 3.0 | 43 |
| 1.5 | 3.0 | 48 |
| 2.0 | 3.0 | 49 |

* Mean number of days taken to initiate callus induction

Exogenous application of auxin and cytokinin induces callus in various plant species. Generally, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin to cytokinin or low ratio of auxin to cytokinin, induces root and shoot regeneration, respectively [5]. In contrast to that, in the present study, it was observed that high auxin to cytokinin ratios tends to induce calli from the *G. walla* leaf disc explants. NAA - a synthetic auxin and BA - a synthetic cytokinin both induces the cell growth and division. The combination of auxin and cytokinin play an important role on callus growth. However, the activity of auxin and cytokinin varies with the metabolic activities in the plant cells. Due to the presence of high amount of secondary metabolites in leaf disc explants used, their accumulation may affect the optimum activity of plant growth regulators and their ratios. *G. walla* is a slow growing plant species and with the maturity of the explants, response in *in vitro* culture may reduce due to the presence of high levels of secondary metabolites in tissues. This might be the reason for slow growth in callus production by explants from mature plants [13].

**Nature of Calli**

A number of different expressions of physiological and morphological changes have been reported in callus including loss of exogenous requirement for some growth regulators, changes in biochemical sensitivity and requirements, alterations of growth habit and modifications of cellular components [9]. Variations in the colour and quality of calli changes during its growth were observed (Figure 1).

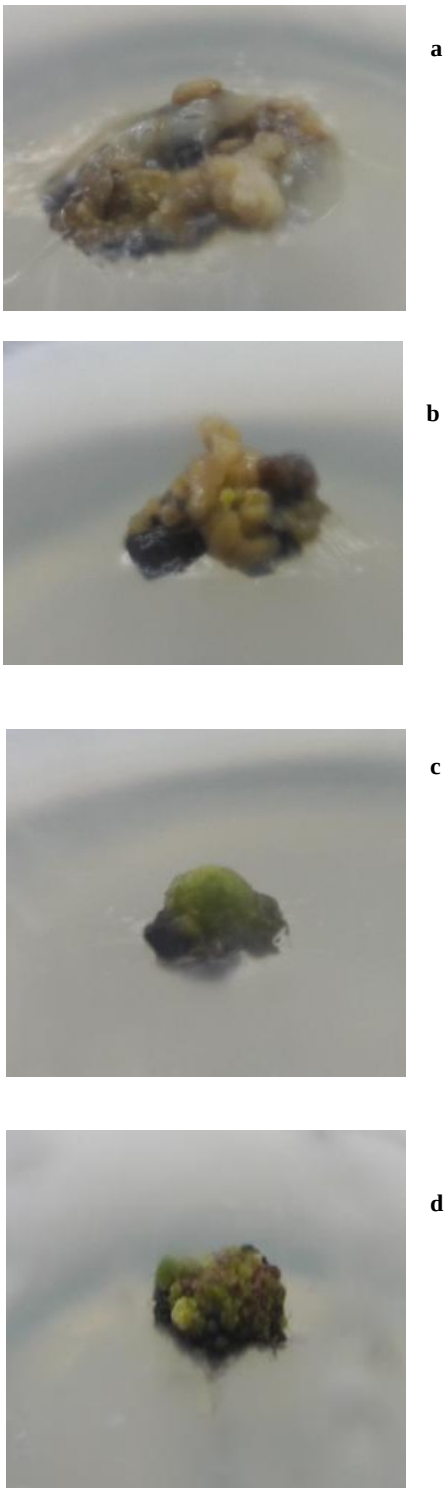



*Figure 1. Stages of callus induction and growth of G. walla leaf disc explants*

At the initial stage of the callus (Figure 1a), cell clusters were white-opaque in colour and fragile in

nature. During the callus growth with time they become yellow in colour and compact in nature (Figure 1b). This stage is observed as the best to transfer the calli to liquid medium for cell culture to obtain secondary metabolites. When callus become mature on the same medium, then turns to green in colour and become hard due to the production of chlorophyll in the cells (Figure 1c). Greenish callus could be transferred to the multiplication medium for multiple shoot induction. The further accumulation of secondary metabolites, give rise brown colour to the callus (Figure 1d). This does not mean that the decay of the callus, but accumulation of high levels of metabolites. Analysis of the chemical constitutes in the cell clusters at this stage, may provide a qualitative and quantitative measure for the commercially important chemicals.

## CONCLUSIONS AND RECOMMENDATIONS

Surface sterilization using 10% sodium hypochlorite (Clorox) for 10 min and 70% ethanol for 1 min each followed by two successive washings in sterile distilled water gave the lowest percentage of contamination and highest survival rate for leaf disc explants. Out of different growth regulator combinations, rapid callus growth was observed in MS medium supplemented with 1.0 mg/L BA and 3.0 mg/L NAA within six weeks. Calli obtained were yellowish compact and callus induction was mainly observed along the midrib. Calli obtain could either be used for mass propagation, through indirect organogenesis or for establishment of plant cell culture to obtain secondary metabolites. From the results obtain, it could be concluded that callus initiation is feasible through leaf disc explant.

## ACKNOWLEDGMENT

This research was granted by the University of Sri Jayewardenepura (USJP), Gangodawila, Nugegoda, Sri Lanka. We are thankful to our colleagues who provided expertise that greatly assisted the research. We have to express our appreciation to the academic staff, Department of Botany, USJP for sharing their pearls of wisdom with us during the course of this research. Principle researcher for this project is Prof. (Mrs) W.T.P.S.K. Senarath, Head/Department of Botany, USJP.

## REFERENCES

Barden, A., Anak, N. A., Mulliken, T. and Song, M. (2000) Heart of the matter: agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. portals.iucn.org

Brechbill, G. O. (2007) *The fragrance industry – profiles*. New Jersey: Creative Endeavour Books.

Buddhapriya, A. N. and Senarath, W. T. P. S. K. (2015) *In vitro* Seed Germination and Callus Induction of *Gyrinops walla* Gaertn. International Conference on Multidisciplinary Approaches, 192. (ISSN: 2386-1509).

Dassanayake, M. D., Fosberg, F. R. and Trimen, H. (1981) *A revised handbook to the flora of Ceylon*, New Delhi: Amerind Publishing.

Matthews, R. and Duncan, E. J. (1993) A method to reduce microbial contamination in *in vitro* culture. Third Caribbean Biotechnology Conference, 160-166.

Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15* (3), 473-497.

Neumann, K. H., Kumar, A. and Imani, J. (2009) *Plant Cell and Tissue Culture – A Tool in Biotechnology*. Berlin: Springer.

Saikia, M., Shrivastava, K. and Singh, S. S. (2012) An Efficient Protocol for Callus Induction in *Aquilaria malaccensis* Lam. Using Leaf Explants at Varied Concentrations of Sucrose. *International Journal of Plant Research*, *2* (6), 188-194.

Skirvin, R. M. (1978) Natural and Induced Variation in Tissue Culture. *Euphytica*, *27*, 241-266.

Skoog F. and Miller C. O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symposia of the Society for Experimental Biology, *11*, 118–130.

Smith, H. R. (2013) *Plant Tissue Culture Techniques and Experiments*. San Diego: Elsevier Inc.

Subasinghe, S. M. C. U. P., Hettiarachchi, D. S. & Rathnamalala, E. (2012) Agarwood-type Resin from *Gyrinops walla* Gaertn: A New Discovery. *Journal of Tropical Forestry and Environment*, *2* (2), 43-48.

Talukdar, T. (2012) *In vitro* Regeneration of an Endangered Ornamental Plant Impala Lily (Adenium multiflorum Klotzsch), I*ndian Journal of Fundamental and Applied Life Sciences*, *2* (3), 42-50.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 15-20

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1104

# *IN-VITRO* MULTIPLICATION OF GINGER (*Zingiber officinale* ROSC.) CULTIVARS LOCAL, CHINESE AND RANGUN

[1*]D.B.R. Swarnathilaka, [2]N.S. Kottearachchi and [2]W.J.S.K.Weerakkody

*[1]Plant Tissue Culture Research Station, Dept of Export Agriculture, Walpita, Sri Lanka*
*[2]Department of Biotechnology, Faculty of Agriculture and Plantation Management, University of Wayamba, Sri Lanka*

**Abstract**

In Ginger, it is necessary to find an alternative method for production of disease free planting materials as the conventional propagation technique through rhizomes transmit many diseases. The present study summarizes an efficient micropropagation method developed for *Zingiber officinale* Rosc. (Ginger) cultivar, Local, Chinese and Rangun using sprouted bud explants from fresh rhizomes.

Newly sprouted rhizome buds with the size of 0.5-1.0 cm were surface sterilized with 30% Clorox for 30 minutes prior to culture and this treatment resulted 60%-70% healthy pure explants for all three cultivars. Murashige and Skoog (1962) medium has shown the best performance in culture establishment for three cultivars. Highest survival rate of the explants was observed in the cultivar, Chinese while lowest was observed in Local ginger. MS medium supplemented with 2mgl$^{-1}$ BA and 0.25 mg l$^{-1}$ NAA was shown the best performance for initial multiplication of ginger buds (8.6 shoots per explants) in the cultivar, Local. However buds of Rangun and Chinese were shown the highest rate of multiplication (respectively 7.33 and 7.20 shoots per explants) in the MS supplemented with 4.0 mg l$^{-1}$ BAP and 0.25 mg l$^{-1}$ NAA. Half strength of MS medium supplemented with 1.5 -2.0 mg l$^{-1}$ NAA has shown induction of roots in ginger cultivars tested. Of the combination tested ½ strength MS with 1.50 mg l$^{-1}$ NAA elicited optimal rooting response in which an average of 5.5 roots with 3.64 cm. root length for cultivar Local. But highest value of root length 4.95 (cm) and 4.12 (cm) were recorded in plantlets of Rangun and Chinese respectively in the half strength MS medium with 2.0 mgl$^{-1}$ NAA. Further experiments are being continued for acclimatization of in-vitro raised plants.

**Keywords:** Ginger, explants of sprouted buds, micropropagation, *Zingiber officinale*

## INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is a well known oriental spice crop having significant commercial value for its use in various medicinal and culinary preparations. It is usually propagated through the underground rhizomes with a low multiplication rate. Several local and imported ginger cultivars namely Local, Rangun and Chinese are grown in Sri Lanka in large scale. Rhizomes of local ginger are small and the fibrous flesh is somewhat ash white in color. Its' taste and aroma are comparatively higher than Rangun and Chinese. It is largely used as a spice, a beverage and a basic material in pharmaceutical industry. Rhizomes of Chinese are large with watery flesh which is pale yellow in color. It is largely used for pickles because of the low taste and aroma. Rhizome of Rangun is medium in size and with well spread finger rhizomes and the taste and the aroma exists in between local and Chinese. In order to increase the production of ginger, high quality planting materials are required. As ginger is propagated exclusively through underground rhizome with low proliferation rate (10-15 buds from one unit per year), a significant fraction of the total production is utilized as seed material for the subsequent season. Furthermore, conventional/traditional vegetative propagation technique of ginger has high risk of

Corresponding Author Email: *thilakaswarna@yahoo.com

spreading pest and systemic infections. The germplasm collections of clonal repositories are also seriously affected by fungal and bacterial diseases. Therefore, the traditional propagation method does not ensure the supply of disease-free high quality planting materials for commercial ginger growers. A three-fold increase in the production of rhizomes could be possible by the effective control of the diseases (Balacharandran *et al*., 1990).

Crop improvement through the breeding of ginger is seriously handicapped by poor flowering and seed set. Genetic transformation is one of the options for crop improvement in ginger. A major pre-requisite for genetic transformation is the availability of an in-vitro regeneration system (Akter.2001). Therefore, regeneration of plants from the tissue culture technique is an important and essential component of biotechnological research and the production of quality planting materials. The present study is an attempt to develop a successful protocol for regeneration of plantlets from newly sprouted buds. This protocol is important for obtaining large scale disease-free quality planting materials for year-round cultivation, conservation of germplasm, and finally to crop improvement program.

## MATERIALS AND METHODS

Newly sprouted rhizome buds with the size of 0.5-1.0 cm were dissected from dried scales, and they were washed thoroughly in running tap water and immersed in 70 % ethanol for one min. Then they were surface sterilized with different concentrations (0%,10%,20%,30%,40% and 50%) of Clorox (5.25 % sodium hypochlorite) solution and washed 4 to 5 times in sterile distilled water prior to culture in basal medium of Murashige and Skoog (MS) (1962). The cultures were incubated at 26 °C with 16 hr photo period (20 explants were used for one treatment and the experiment was repeated 3 times). After four weeks, data on the amount of healthy surviving cultures percentage were recorded.

The sterilized explants were trimmed and inoculated into basal media of Murashige and Skoog (MS) (1962), Woody plant medium (WPM) (Llyod and McCown (1981) and Schenk- Hilderbrandt medium (1972) to select the suitable basal medium. To evaluate the hormonal combinations on multiplication, ginger buds established in basal MS medium for 3-4 weeks were introduced to a basal MS medium fortified with sixteen treatments structuring factorial combination with BAP (Benzyl aminopurine) (0, 2.0, 4.0 and 6.0 mg $L^{-1}$) and NAA (Naphthalene acetic acid ) (0, 0.1, 0.25 and 0.5 mg $L^{-1}$).

For root induction, *in-vitro* raised well developed plantlets (3-5 cm shoot length) were cultured on full and half strength MS medium supplemented with either NAA or IBA (Indole 3 butyric acid) in concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mg $L^{-1}$. The cultures were incubated at 26 °C with 16 hr photo period. Twenty replicates were used for each treatment. The number of roots per shoots, length of roots and percentage of cultures with induced roots was recorded.

## RESULTS AND DISCUSSION

Highest count of uninfected healthy cultures was observed in 30% Clorox (5.25 % sodium hypochlorite) for 30 minutes and this treatment resulted 60%-70% healthy pure explants for all three cultivars (Figure 1). The contamination of explants may be due to fungi, bacteria, etc., present on the surface or in the scales and damages. Researchers have argued that the contamination rates of underground rhizomes were high and the establishment of contamination free cultures was difficult (Islam *et al*. 2004). However, similar results to the present study, with less contamination were obtained by Hamirah *et al*. (2010) in micropropagation of red ginger (*Zingiber montanum*).

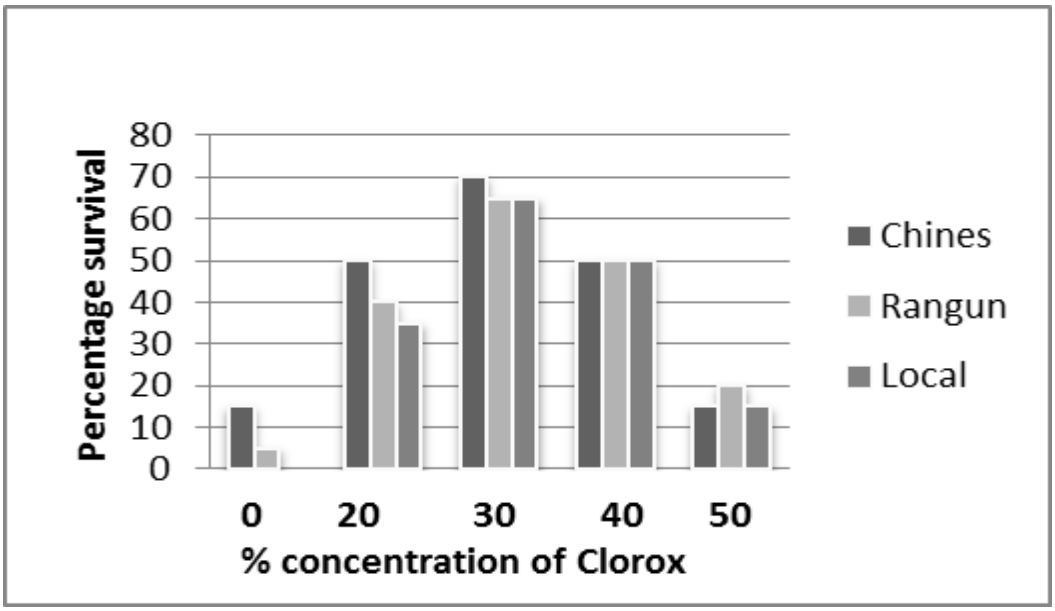



*Figure1.Survival of ginger ex-plants after sterilization*

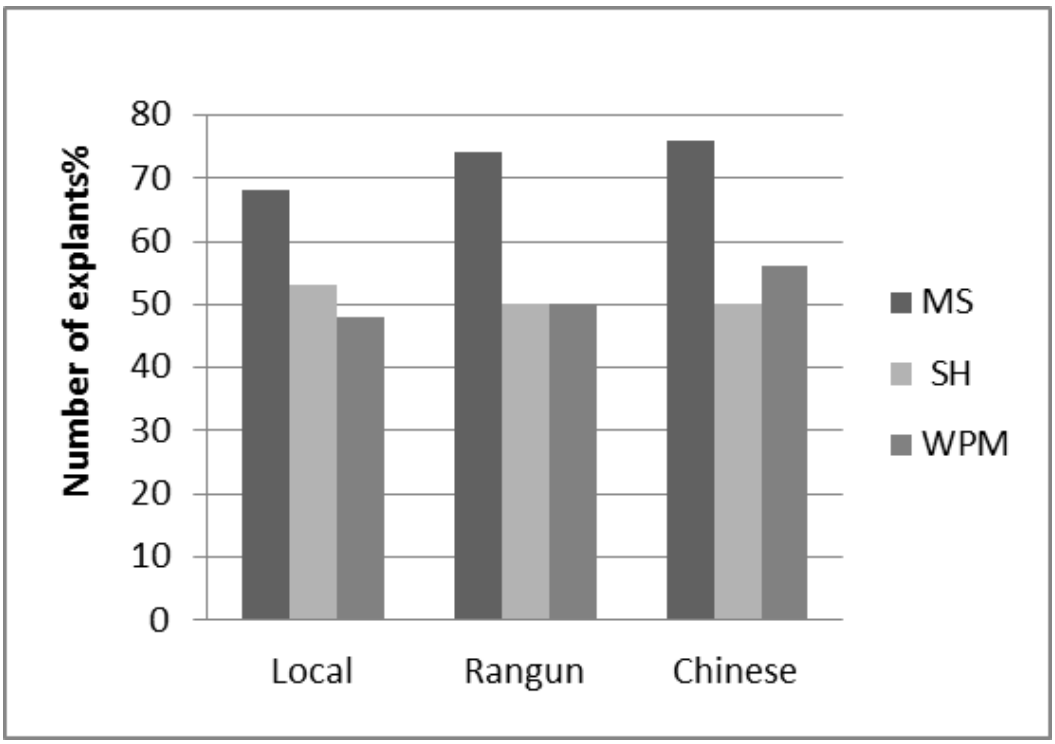



*Figure 2: Survival of ginger ex-plants in different culture media*

The buds on MS medium showed very satisfactory results with around 70% survival compare to WPM and SH on establishment of explants. Explants of Chinese ginger have shown the highest survival rate compare to Rangun and Local (Figure 2). The lowest death rate was observed in explants grown in MS medium. The percentage of contaminated cultures in all treatments was around 20- 25 percent.MS is the most commonly used and many others have been developed medium. Madke *et al.* (2014) have shown that the highest rate of establishment and regeneration in MS compare to SH and WPM in *G. arborea* Roxb.

*Table1:Bud formation of ginger ex-plants on MS medium supplemented with different concentration of BA and NAA*

| Treatment | Concentration of NAA mg mgl-1 | Concentration of BA mgl-1 | No of Buds Local | No of Buds Chinese | No of Buds Rangun |
|---|---|---|---|---|---|
| 1 | 0.00 | 0.00 | 1.20 h | 1.4 h | 1.46 h |
| 2 | 0.00 | 2.00 | 3.46 f | 3.26 g | 2.93 e |
| 3 | 0.00 | 4.00 | 3.86 f | 3.86 f | 3.33 e |
| 4 | 0.00 | 6.00 | 1.60 gh | 3.80 f | 1.73 gh |
| 5 | 0.10 | 0.00 | 1.93 g | 1.86 h | 2.46 fg |
| 6 | 0.10 | 2.00 | 6.20 e | 5.73 de | 6.20 cd |
| 7 | 0.10 | 4.00 | 7.60b | 6.80 ab | 6.06 cd |
| 8 | 0.10 | 6.00 | 6.66 cde | 6.06 cd | 6.40 cd |
| 9 | 0.25 | 0.00 | 1.53 gh | 1.40 h | 1.33 h |
| 10 | 0.25 | 2.00 | 8.60 a | 6.53 bc | 6.80 bc |
| 11 | 0.25 | 4.00 | 7.06 bcd | 7.20 a | 7.33 a |
| 12 | 0.25 | 6.00 | 6.33 e | 6.13 d | 6.66bcd |
| 13 | 0.50 | 0.00 | 1.93 g | 1.66 h | 1.80 gh |
| 14 | 0.50 | 2.00 | 7.13 bc | 6.53 bc | 6.73 bc |
| 15 | 0.50 | 4.00 | 7.26 bc | 6.26 c | 7.20 ab |
| 16 | 0.50 | 6.00 | 6.46 de | 5.40 e | 5.93 d |

Within a column, means followed by the same letter are not significantly different at p=0.05

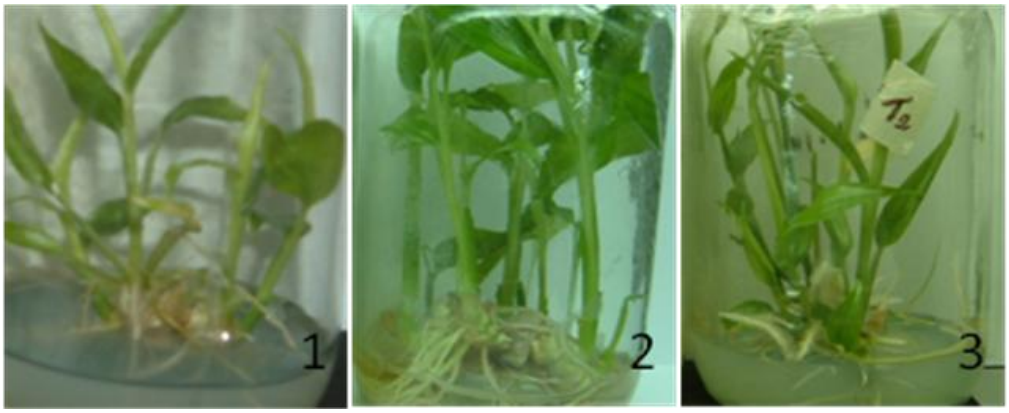



*Figure 3: Multiplying ginger explants in MS media supplemented with BAP& NAA 1-Local 2-Chinese 3-Rangun*

MS medium supplemented with 2mgl$^{-1}$ BA and 0.25mgl$^{-1}$ NAA has shown the best performance of initial multiplication of local ginger buds. However same basal medium supplemented with 4mgl$^{-1}$ BA and 0.25mgl$^{-1}$ NAA has shown the highest rate of multiplication for ginger cultivars of Chinese and Rangun (Table1and Figure3).

According to previous reports, ginger explants cultured in MS basal medium supplemented with 2.0 mg L-1 BAP +0.5 mg L-1 NAA showed the highest

rate of shoot multiplication (Kambaska *et.al.* 2009). Dipti *et al.* (2005) reported that the highest number of multiple shoots from rhizome buds of turmeric in MS media supplemented with 3 mg L-1 BAP, indicating the necessity of similar quantities of cytokinin.

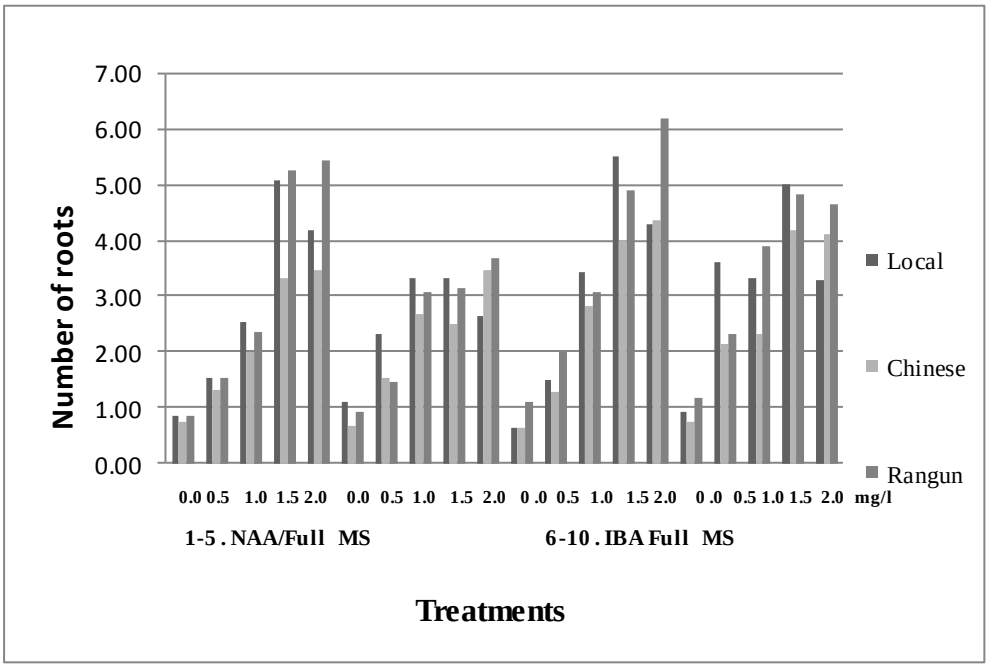



*Figure 4: Root induction of in-vitro produced*

Rooting was induced in the plantlets on MS medium without plant growth regulator within 35-45 days. About 1-2 roots were observed in plantlets in medium with low concentration of NAA /IBA (0 -0.5mg $L^{-1}$) and without growth regulator. Kambaska and Santilata, 2009 reported that the accumulation of endogenous auxin promote ginger rooting even though it is insufficient for establishment in the field. In the present study has shown that the enhancement of root induction of the plantlets in the basal medium supplemented with growth hormones. Accordingly higher concentrations of NAA (1.0-2.0 mg $L^{1}$) and IBA (0.5-2.0 mg $L^{-1}$) have shown better root induction and elongation. The highest root formation of local ginger was observed in ½ strength MS medium with 1.5 mg $l^{-1}$ NAA with an average number of 5.5 roots per plantlet and 3.79 cm mean root length. However similar medium with 2.0mg $L^{-1}$ NAA has shown the highest root induction in Chinese and Rangun 4.36 and 6.18 number of roots and with 4.12cm and 4.95cm of average root length respectively (Figure.4). Proving our results, Rahman *et al.* (2004) also reported that ½ strength of MS medium supplemented with 0.1-1.0 $mgL^{-1}$ of any auxin (NAA, IAA and IBA) was effective for rooting in Curcuma longa. Further experiments are being continued for acclimatization of *in-vitro* raised plants.

## ACKNOWLEDGMENT

The financial assistance and all facilities received from Department of Export Agriculture in Sri Lanka is gratefully acknowledge.

## REFERENCES

[1]Akter, N. (2001).Effect of different explants and concentrations of NAA on callus induction and plant regeneration of brinjal cv Uttara M.S thesis dept. of Hort.Bangladesh agricultural University,Mymensingh. 48-50.

[2].Balacharandran, S.M., Bhat, S.R., Chandel, K.P.S. (1990). In-vitro clonal multiplication of Turmeric (Curcuma spp.) and ginger (Zingiber officinale Rosc.). Plant cell reports, 8:521-524.

[3].Dipti,T,, Ghorade,R.B., Swati,M., Pawar, B.V.Ekta,S. (2005) Rapid multiplication of turmeric by micro propagation . Annual Plant Physiology, 9: 35-37.

[4].Hamirah M.N, Sani H.B, Boyce P.C, Sim S.L.(2010).Micropropagation of red ginger (Zingiber

montanum) a medicinal plant. As Pac J. Mol Biol Biotechnol.; 18: 127-130.

[5].Islam, M.A., Kloppstech, K., Jacobsen, J., (2004). Efficient procedure for in-vitro microrhizome induction in Curcuma longa (Zingiberaceae) - a medicinal plant of tropical Asia. Plant tissue culture, 14: 123-!34.

[6].Kambaska, K.B, Santila, S, (2009). Effect of plant growth regulator on micropropagation of ginger (Zingiber officinale Rosc.) Journal of Agricultural Technology. 5: 271- 280.

[7].Lloyd, G., McCown, B. (1981). Commercially feasible micropropagation of Mountain Laurel, Kalmia latifolia, by use of shoot tip culture. Combined Proceedings – International Plant Propagator's Society, 30: 421-427.

[8].Madke,S.S., Cherian,K.J., Badere, R.S.(2014). A modified Murashige and Skoog media for efficient multiple shoot induction in G. arborea Roxb. Journal of Forestry Research 25, Issue 3, 557-564.

[9]Murashige, T,and Skoog, F. ( 1962). A revised medium for rapid growth and bio assays with tobacco tissue culture, Physiol. Plan.; 15: 473-497.

[10].Rahman, M.M., Amin, M.N.M., Jahan, H.S., Ahmed, R. ( 2004). In- vitro regeneration of plantlets of Curcuma longa L. A valuable spice in Bangladesh, Asian J. Plant Sci.; 3: 306 -309.

[11].Schenk, R.U., Hildebrandt, A.C. 1972. Medium and teqniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can.J. Bot. 50: 199-204.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 21-26

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1105

# ESTABLISHMENT OF A MOLECULAR BASED METHOD FOR THE IDENTIFICATION OF SKIPJACK TUNA (*Katsuwonus pelamis*) IN LARGE SCALE FISH PROCESSING INDUSTRY

[1]D.R.C. Perera, [2]W.W.P.Rodrigo, [2]A.M.M.H. Athapaththu and

[2*]P.A.D.H.N. Gunathilaka

*[1]Faculty of Animal Science and Export Agriculture, Uva Wellassa University, Sri Lanka*
*[2]Biotechnology Unit, Industrial Technology Institute, Colombo 7, Sri Lanka*

**Abstract**

Skipjack tuna (*Katsuwonus pelamis*) is most often sold as canned light tuna and is the most common species found in tuna cans. In Sri Lanka differentiation of tuna species prior to processing is achieved through morphological identification, which is not a reliable method. Since the quality and market value of tuna products differ from species to species, a fraudulent replacement of valuable species with less valuable ones may occur. This has become a major limitation in fishery industry in order to reach products to the international market. Therefore, the objective of the current study was to establish a molecular based diagnostic method to differentiate skipjack tuna from other tuna species commonly found in Sri Lanka. Genomic DNA of skipjack tuna (*K. pelamis*), yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) were extracted from the muscle tissues. Amplification of DNA from tuna samples were carried using genus specific primers which flank at 558 bp region of Cytochrome b gene. The amplified DNA products of tuna species were digested with ScaI restriction enzyme. The pattern restriction fragments evidence that products having band sizes of 215 bp and 343 bp were detected only from *T. albacares* (n= 10) and *T. obesus* (n= 10) while, *K. pelamis* (n= 10) was remained as an indigestive product (558 bp). Therefore, this can be used to differentiate *K. pelamis* from the other tuna species which are commonly found in Sri Lanka.

**Keywords:** Tuna species, DNA extraction, polymerase chain reaction, restriction enzyme digestion

## INTRODUCTION

Tuna fishery industry is a valuable global trade in which is becoming species of special economic interest to many countries bordering the Western Indian Ocean including Sri Lanka. Tunas enjoy a very good export market as sashimi or loins (Subasinghe, 2004). In Sri Lanka, this industry has been identified as a rapid growing area which has a great potential to earn for earning foreign exchange to the country by reaching the export market. There are three major species such as yellowfin tuna (*Thunnus albacares*), bigeye tuna (*Thunnus obesus*) and skipjack tuna (*Katsuwonus pelamis*) are highly abundant in the Sri Lankan sea. Skipjack is the most common species of canned and pouched light meat tuna available on the US market. Skipjack is the best choice among these light tuna options for lowering the risk of mercury exposure. Skipjack tuna identification is traditionally based on external morphological features, including body shape, pattern of colors, scale size and count, number and relative position of fins, number and type of fin rays, or various relative measurements of body parts (Fabrice, 2009).

The authenticity of food labeling is a serious issue that involves not only the consumer but also the manufacturers and everyone involved in the food chain (Michelini *et al.*, 2007). There is no proper method to confirm the identification of tuna species after it had been processed. It has become a limitation to reach the international markets, which is on high demand. In Sri Lanka, differentiation of tuna species before the processing is done through morphological

Corresponding Author Email: *hasnayana@yahoo.com

identification, which is not a reliable method. The main difficulties in fish species identification especially in processed fish derive from many factors, such as the absence of morphological features (e.g., fins, head, and skin) in the processed product, the denaturation of proteins or other material important for the analysis during heat treatments and finally the presence of contaminants (e.g., olive and other vegetal oils), which may interfere with the analysis (Michelini *et al.,* 2007). Frozen tuna are far more difficult to distinguish due to the fin damage, discoloration, skin abrasion and distortion or crushing during the storage process. But even at small sizes, each species has distinct coloration, body markings and body morphologies that allow rapid visual keys to positive identification (Itano, 2005).

Therefore, the current study will focus on developing a molecular based diagnostic method to detect species identity of tuna species in order to facilitate the fish processing industries and fish exporters by making the test available. The development of analytical methods for fish species identification may help detecting and avoiding unintentional/intentional substitution of different fish species and thus enforce labeling regulations. Thus the objective of the present study was to develop a PCR based assay to differentiate *Katsuwonus pelamis* (skipjack tuna) from other tuna species.

## MATERIALS AND METHODS

### Collection of Samples

Raw samples belong to *T. albacares* (yellowfin), *T. obesus* (bigeye) and *K. pelamis* (skipjack) tuna, which were certified, by the National Aquatic Resources Research and Development Agency (NARA), Sri Lanka were obtained as positive controls. Processed samples of *K. pelamis* were obtained from leading fish processing industries in Sri Lanka. To avoid contaminations, all the tissue samples were collected using sterile blades and packed in labeled sealed polythene. All these samples were kept at 4 °C until those are taken to the research laboratory. Then all the muscle samples were stored at -20 °C until used for DNA extraction.

### Extraction of Genomic DNA from Tuna Samples

A tissue sample (200 mg) of each species was taken in to a micro-centrifuge tube. The tissues were incubated with 500 µL of STE buffer and 500 µL of 5M Ammonium Acetate at 65 °C for one hour. The incubated samples were homogenized using sterile mortars and pestles followed by incubated at 55 °C for one hour and centrifugation, at 14000 rpm for 5 minutes at 4 °C. The DNA was extracted using 500 µL of the supernatant by phenol-chloroform method (Coen *et al.,* 1982). The extracted DNA was re-suspended in TE buffer and kept at -20 °C.

### Primer Selection

The sequences of the mitochondrial *cytochrome b* gene for genus *Thunnus* (Table 1) were targeted for the PCR amplification (Michelini *et al.,* 2007).

*Table 1: Sequences genus specific primers used for PCR protocol*

| Name of the primer | Sequence (5' – 3') |
|---|---|
| Forward (TnaF) | CAGGACTATTCCTCGCAA TACA |
| Reverse (TnaR) | CGAAACCAAGGAGGTCT TTGTA |

### Amplification of the Genomic DNA Using Polymerase Chain Reaction (PCR)

The PCR amplifications were conducted using family specific primers for *Scombridae spp* (TnaF/TnaR) in 15 µL of solution containing 0.6 µL each of Forward (TnaF- 5 µM) and Reverse primer (TnaR -5 µM), 4.5 µL of Master mix [10× PCR buffer + $MgCl_2$ (50 mM) + dNTPs- all four types (100 mM)], 0.6 µL of Taq polymerase (2 U/µL), 7.7 µL PCR water and 1.0 µL of DNA template. The cycling conditions were hot start at 95 °C for 60 seconds followed by 30 cycles each of denaturation at 95 °C for 60 seconds, annealing for 52 °C for 60 seconds, and extension at 68 °C for 60 seconds, followed by the final extension at 68 °C for 5 minutes.

### Validation of Results by Restriction Enzyme Digestion

Restriction enzymes digestions were performed to differentiate the two tuna species using purified PCR product. The following restriction enzyme was selected for the digestion by analyzing the genomic sequences of *K. pelamis*, *T. obesus* and *T. albacares* (Table 2).

*Table 2: Restriction enzymes used for restriction enzyme digestion*

| Name of the restriction enzyme | Restriction site (5' – 3') | Accession number | Species differentiated |
|---|---|---|---|
| *Sac*I | AGT▼ACT<br>TCA▲TGA | NC_014059.1 | *T. albacares*<br>*T. obesus*<br>*K. pelamis* |

### Agarose Gel Electrophoresis

Agarose powder (Promega) was used for the preparation of gels. The 1 % agarose gels which stained with Ethidium Bromide (0.5 µg/mL) were prepared. Amplified PCR products were loaded with Promega 100 bp DNA ladder and gel electrophoresis was carried out at 70 V for 60 minutes to get a good separation in the amplified products. Following the gel electrophoresis, the migrated DNA was visualized and documented using gel documentation apparatus and Image Lab 3.0 software protocol (GelDoc ™XR+). The results obtained from the gel electrophoresis were compared with the 100 bp DNA ladder.

## RESULTS

#### Amplification of Tuna Samples

As depicted in the gel photograph, a specific band of 558 bp was identified which is the genus specific band size for *Scombrida* species.

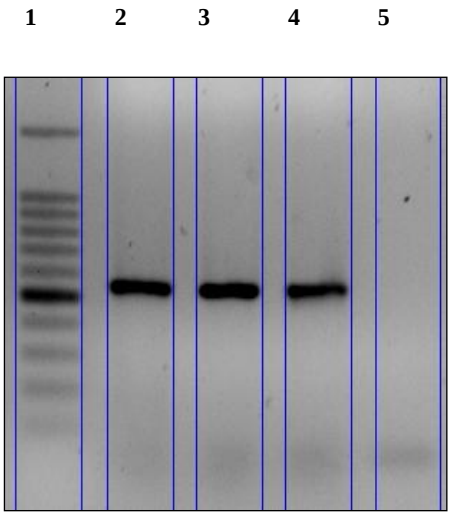



*Figure 1. Amplified of tuna samples using family specific primer. (Lane 1: 100 bp DNA ladder; Lane 2: T. albacares; Lane 3: T. obesus; Lane 4: K. pelamis; Lane 5: Negative control - PCR water)*

### Digestion of the PCR Product of Tuna Samples with *Sca*I Restriction Enzymes

The pattern restriction fragments evidence that products having band sizes of 215 bp and 343 bp were detected only from T. albacares and T. obesus while, K. pelamis was remained as an indigestive product (558 bp). Therefore, restriction enzyme digestion was successful achieved and *K. pelamis* species were differentiated according to the specific sizes of bands (Figure 2).

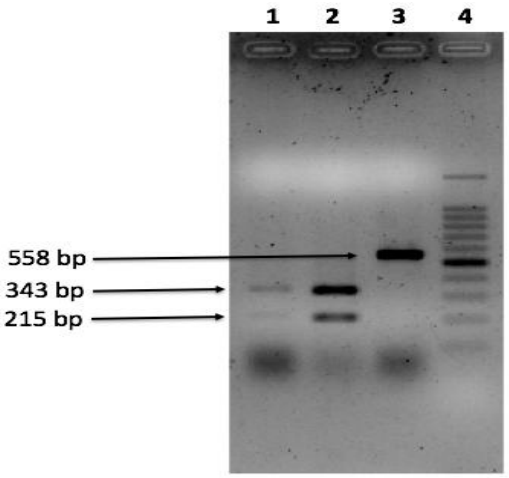



*Figure 2. Restriction enzyme digestion of positive tuna samples using ScaI enzyme (Lane 1: T. albacares; Lane 2: T. obesus; Lane 3: K. pelamis; Lane 4: 100 bp DNA ladder Lane).*

## DISCUSSION

A PCR based diagnostic method to differentiate *K. pelamis* (skipjack tuna) has been developed in this study. The template DNA for PCR was prepared by the method devised by Nishiguchi *et al* (2002) with some modifications. Sequences of the DNA amplified by PCR were digested from restriction enzymes to differentiate two tuna species. Muscle tissues of *K. pelamis (n=10), T. obesus* (n= 10) and *T. albacares* (n= 10) were analyzed to evaluate this molecular assay. Developed and tested four STE buffers, which were modified its composition in order to obtain the maximum DNA quantity. The STE Buffer which contain 0.4 % SDS (pH 7.2) and 0.001 $moldm^{-3}$ EDTA (pH 8.0) resulted higher DNA quantity than other modifications. Therefore, it was used throughout the extractions. DNA isolation was optimized with tuna samples for the temperature and time of incubation. The incubation was performed at 55 °C and 65 °C for each temperature the incubation was performed at 17 hours (overnight) and one hour. The incubation performed at 65 °C for one hour was identified as the best incubation temperature for the DNA extraction.

*Stu*I restriction enzyme was used to digest the PCR products of *T. albacares.* It was failed to digest the PCR product of *T. albacares* due to a difference in the sequence available in the NCBI database form genomic and mitochondrial cytochrome b gene (figure 3). Therefore, an alternative restriction enzyme (*Sca*I) was used to overcome this condition.

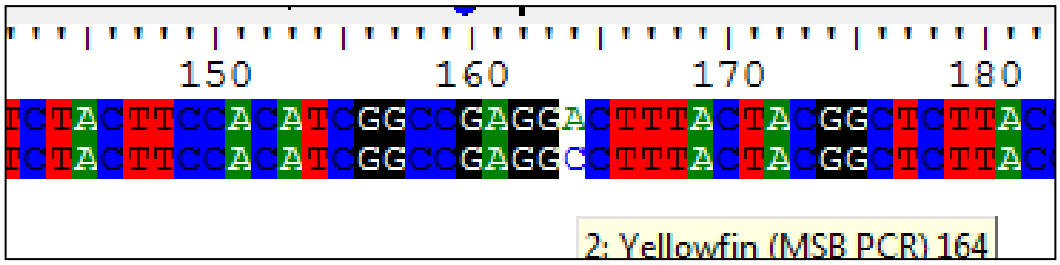



*Figure 3. Genomic and mitochondrial cytochrome b gene sequence of T. albacares*

## CONCLUSION

In conclusion, this study carries a reliable approach to identify and distinguish *K. pelamis* from the other tuna species commonly found in Sri Lanka.

## ACKNOWLEDGEMENT

As a degree holder; Bsc. in Aquatic Resources Technology from the Uva Wellassa University through which I learn and practice fisheries and biotechnology techniques.

## REFERENCES


Addgene.org, (2015). Addgene: Plasmid Cloning by Restriction Enzyme Digest (with Protocols). [Online] Available at: https://www.addgene.org/plasmid-protocols/subcloning/ [Accessed 11 Feb. 2015].

Aksoy, N. and Unlu, S. (2003). Increased Resolution of Macromolecules with Agarose Gel Electrophoresis Compared with Polyacrylamide Gel Electrophoresis. Macromol. Biosci., 3(9), pp.482-486.

Animalplace.net, (2015). Skipjack Tuna: Facts, Characteristics, Habitat and More « Animal Place. [online] Available at: http://www.animalplace.net/fishes/skipjack-tuna-facts-characteristics-habitat-and-more/ [Accessed 23 Jun. 2015].

Bertani, G. (1959). SENSITIVITIES OF DIFFERENT BACTERIOPHAGE SPECIES TO IONIZING RADIATIONS. (pp.387), Department of Medical Microbiology, University of Southern California School of Medicine.

Bio-rad.com, (2015). Protein Electrophoresis | Applications & Technologies | Bio-Rad. [online] Available at: http://www.bio-rad.com/en-lk/applications-technologies/protein-electrophoresis [Accessed 25 Jun. 2015].

Bio.davidson.edu, (2001). dideoxy Sequencing of DNA. [online] Available at: http://www.bio.davidson.edu/Bio111/seq.html [Accessed 14 Jul. 2015].

Chou, C.C., Lin, S.P., Lee, K.M., Hsu, C.T., Vickroy, T.W. and Zen, J.M. 2007. Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using copper nanoparticle plated electrodes. Journal of Chromatography B, 846(1):230-239

Dissanayake, D. (2005). Monitoring and assessment of the offshore fishery in Sri Lanka. UNU-FTP, [online] pp.9-11. Available at: http://www.unuftp.is/static/fellows/document/chamari05aprf.pdf [Accessed 29 Apr. 2015].

Fabrice, T. (2009). Molecular identification methods of fish species: reassessment and possible applications. Rev Fish Biol Fisheries 19: 265–293.

Harisha, S. (2007).Biotechnology procedures and experiments handbook. Hingham, Mass.: Infinity Science Press.

Hawaii-seafood.org, (2012). Hawaii Seafood - Yellowfin Tuna. [online] Available at: http://www.hawaii-seafood.org/yellowfin-tuna/ [Accessed 22 Jun. 2015].

Idtdna.com, (2015). The Polymerase Chain Reaction. [online] Available at: https://www.idtdna.com/pages/docs/educational-resources/the-polymerase-chain-reaction.pdf?sfvrsn=4 [Accessed 19 Jun. 2015].

Integrated DNA technologies, (2015). Breaking PCR: A Systematic Investigation of Intentional Violations of a Basic Polymerase Chain Reaction Amplification Protocol. [online] Available at: https://www.idtdna.com/pages/docs/technical-reports/breaking-pcr.pdf [Accessed 24 Jun. 2015].

Itano, D. (2005). A Handbook for the Identification of Yellowfin and Bigeye Tunas in Fresh Condition (v2). 2nd ed. [ebook] USA: University of Hawaii, JIMAR, pp.7-12. Available at: http://sih.ifremer.fr/content/download/6119/44603/file/BE_YF_ID_FRESH_v2.pdf [Accessed 30 Apr. 2015].

Lee, S. (2015). Modern Mass Spectrometry. [online] Princeton. Available at: http://www.princeton.edu/chemistry/macmillan/group-meetings/SL-mass%20spect.pdf [Accessed 25 Jun. 2015].

Lifetechnologies.com, (2015). Overview of Mass Spectrometry | Life Technologies. [online] Available at: https://www.lifetechnologies.com/lk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-mass-spectrometry.html [Accessed 26 Jun. 2015].

Lockley, A. and Bardsley, R. (2000). DNA-based methods for food authentication. Trends in Food Science & Technology, 11(2), pp.67-77.

Michelini, E., Cevenini, L., Mezzanotte, L., Simoni, P., Baraldini, M., De Laude, L. and Roda, A. (2007). One-Step Triplex-Polymerase Chain Reaction Assay for the Authentication of Yellowfin( Thunnusalbacares ), Bigeye ( Thunnusobesus ), and Skipjack ( Katsuwonuspelamis ) Tuna DNA from Fresh, Frozen, and Canned Tuna Samples. Journal of Agricultural and Food Chemistry, 55(19), pp.7638-7647.

Ncbi.nlm.nih.gov, (2015). Random Amplified Polymorphic DNA (RAPD). [online] Available at: http://www.ncbi.nlm.nih.gov/probe/docs/techrapd/ [Accessed 2 Jul. 2015].

Ncbi.nlm.nih.gov, (2015). Restriction Fragment Length Polymorphism (RFLP). [online] Available at: http://www.ncbi.nlm.nih.gov/probe/docs/techrflp/ [Accessed 14 Jul. 2015].

Obenrader, S. (2003). Sanger Method. [online] Bio.davidson.edu. Available at: http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Obenrader/sanger_method_page.htm [Accessed 14 Jul. 2015].

Oswald, N. (2010). The PCR Controls You Must Use. [Online] Bitesize Bio. Available at: http://bitesizebio.com/4074/the-pcr-controls-you-must-use/ [Accessed 02 Jan. 2015].

Russell, P. (2002). iGenetics. San Francisco: Pearson Education, pp.187-189.

Subasinghe, R. (2004). Efforts to increase Sri Lanka tuna production. [online] Available at: http://www.atuned.biz/public/viewarticle.asp?id=1219 [Accessed 16 Apr. 2015].

Sambrook, J., Fritsch,E.F., & Maniatis, T. (1989). The Basic Polymerase Chain Reaction. Molecular Cloning - A LABORATORY MANUAL (pp. 470-477). Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York

Tan, S. and Yiap, B. (2009). DNA, RNA, and Protein Extraction: The Past and The Present. Journal of Biomedicine and Biotechnology, 2009, pp.1-10.

Thaddeus, E. (1987). Restriction Fragment Length Polymorphism: Applications to Linkage Analysis, Journal of Growth, genetics and hormones. 3rd ed. pp.6-8.

Viñas, J. and Tudela, S. (2009). A Validated Methodology for Genetic Identification of Tuna Species (Genus Thunnus).PLoS ONE, 4(10), p.e7606.

Wageningen UR, (2015). Amplified Fragment Length Polymorphism (AFLP). [online] Available at: https://www.wageningenur.nl/en/show/Amplified-Fragment-Length-Polymorphism-AFLP.htm [Accessed 13 Jul. 2015].

Whfoods.com, (2015). Tuna. [online] Available at: http://www.whfoods.com/genpage.php?tname=foodspice&dbid=112#historyuse [Accessed 23 Jun. 2015].

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 27-35

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1106

# SCREENING OF SPRING WHEAT VARIETIES RESISTANT TO SOIL POLUTION WITH HEAVY METALS TO CREATE ECOLOGICALLY PURE PRODUCTION

R.A. Alybayeva, A.S. Alenova, V.I. Kruzhaeva and I.M. Salmenova

*Al-Farabi Kazakh National University, Department of UNESCO chair for Sustainable development, Almaty, Kazakhstan*

**Abstract**

The problem of obtaining safe foodstuff has remained relevant for a very long time. Long period of soil self-purification and the expensiveness of its artificial treatment force mankind to look for new ways to solve problems related to soil pollution with heavy metals. The most perspective direction in this field is the identification of plant facilities, characterized by minimal accumulation of heavy metals. The purpose of our research is to identify wheat germplasm, resistant to heavy metals (zinc, cadmium) primarily in Eastern Kazakhstan, and discover donors for selection for metal resistance and perspective wheat forms, intended to implement into production. The research subject is various genotypes of spring wheat from the collection of Eastern Kazakhstan Agricultural Scientific Research Institute. The experiments were held in condition of modeling environmental pollution on 10-day-old sprouts of different wheat genotypes, grown on nutrient mixture, containing $CaSO_4$ 0.1mm and Cd ions at a concentration of 40 mg / l (as $CdSO_4$ salt) or Zn at a concentration of 400 mg / l (a salt $ZnSO_4$). Since a direct negative impact of heavy metals on plants is displayed in inhibiting of their growth and development (root and sprout growth weakening), indicators of wheat genotypes growth processes were examined during the experiment and Wilkins coefficient was defined. The study revealed sensitive and metal resistant samples. Spring wheat varieties Zaulbinka and Altai turned out to be the most resistant to zinc translocation into the surface organs in the course of experiment among the studied genotypes, and Erythrospermum-606 is the most sensitive type of spring wheat. Spring wheat Altai has shown the biggest root resistance to the effect of zinc. Altai, Nargiz and Glubochanka can be considered the most resistant varieties of spring wheat to translocation of cadmium into the surface organs in terms of experiment, among the studied genotypes, and Erythrospermum-606 is the most sensitive variety of spring wheat. Spring wheat varieties: Nargiz, Ulbinka-25, Altai and Glubochanka have shown the biggest root resistance to the cadmium effect. Studies of membrane permeability for electrolytes in terms of environmental pollution by cadmium and zinc, have led to the conclusion that plant resistance can generally be caused by cell membranes' resistance to the action of the stressor. Membrane permeability for electrolytes in the most resistant wheat variety Altai changed less comparing to a more sensitive variety of spring wheat Erythrospermum-606 during the action of both cadmium and zinc. This fact proves that the overall resistance of plants may be due to stability of cell membrane to the action of the stressor.

**Keywords:** membrane permeability, heavy metals, wheat, growth parameters, accumulation of metals, sensitive and resistant genotypes

## BACKGROUND/ OBJECTIVES AND GOALS

In Kazakhstan, the development of a powerful industry was based on its rich natural resources. However, the industrial centers are the areas of highest contamination by heavy metals [1]. Sound environmental technologies are crucial to address heavy metal pollution. Development and use of plant varieties characterized by minimal accumulation of heavy metals can provide one such environmental solution.

Corresponding Author Email: raya_aa@mail.ru

Plants, grown in contaminated soils, exhibit significant interspecies differences in responses to pollution. The available literature data showed that a significant positive correlation between the concentration of heavy metals in the grain and genotypes, indicating the possibility of breeding varieties with a low potential for accumulation of heavy metals [2].

Characterization of heavy metal accumulation in wheat and identification of germplasm resistance to influence of heavy metals is an important step towards the creation and use of wheat varieties resistant to heavy metal pollutions in agricultural production systems. This requires the study of the gene pool of cultivated plants and the selection of donors that accumulate minimum amount of heavy metals and varieties that are promising for cultivation in areas contaminated with heavy metals

The aim of this study was to identify wheat germplasm resistant to heavy metals (zinc and cadmium), which are important to the Eastern Kazakhstan region and identification of donors for breeding and promising forms destined for agricultural production.

## METHODS

Studies were carried out on various genotypes of spring wheat from the collection of Eastern Kazakhstan Agricultural Scientific Research Institute. The following spring wheat genotypes were examined during the experiment: Altai, Ulbinka-25, Zaulbinka, Lyazzat, Glubochanka, Nargiz, Omskaya-18, Samal, Erythrospermum-606, Kutulukskaya.

Plant growth was held in terms of modeling environmental pollution on 10-day-old sprouts of different wheat genotypes, grown on nutrient mixture, which contains CaSO4 0.1mm and Cd ions at a concentration of 40 mg/l ($CdSO_4$ salt) or Zn at a concentration of 400 mg/l (as a $ZnSO_4$ salt). The plants were grown for 10 days in water culture with t-$22^0$C during the day and $18^0$C at night, with a 14-hour photoperiod, light intensity - 5 thousand lx, humidity - 65%.

The measurement of biometric indicators was carried out according to common methods. The plants were dissected to an over-ground part and roots. Roots and surface organs length were measured. In order to determine the plants' dry biomass, they were put in a drying cabinet and dried at t-$105^0$C till constant weight, cooled down to room temperature and weighed.

The tolerance index or Wilkins coefficient were calculated using the formula: $I_t = I_{me} / I_c$, where $I_{me}$ - growth of the roots on the solution of the tested metal, $I_c$ - growth of the roots on the solution without metal [3].

The testing of contrasting by stability spring wheat genotypes for the permeability of cell membranes was conducted in laboratory conditions. To research the permeability of wheat cell membranes, identified during experiments on study of growth parameters wheat varieties contrasting by resistance were used, resistant - Altai and sensitive – Erythrospermum-606.

Membrane permeability determination for electrolytes was conducted according to Dexter conduct metric method [4]. Modified Dexter method was used to determine the release of electrolytes from plants leaves tissues, exposed to the stress effect. A weighed sample of 500 mg leaves was immersed into 50 ml water with the known conductivity for 60 – 180 min. The exudate was drained out and electro conductivity was measured by conductivity meter at the end of the exposure. After the counting the exudate was poured into the same cup with the sample and was boiled up and its electro conductivity was re-measured. Calculation formula: % electrolytes elution = (a: b) x 100, where a - is the conductivity of dead tissues, b - is the conductivity of living specimens.

## RESULTS

Our study of cadmium and zinc effect on growth parameters of wheat sprouts in the laboratory conditions showed that the ions of both cadmium and zinc inhibit plant growth.

The research on wheat varieties from the Eastern Kazakhstan Agricultural Scientific Research Institute collection showed that by the growth of surface organs at a high concentration of cadmium, genotypes can be arranged as follows: Altai > Nargiz > Glubochanka > Ulbinka-25 > Samal > Omskaya-18 >

Kutulukskaya > Zaulbinka > Lyazzat > Erythrospermum-606 (Figure 1).

Regarding zinc, the results are the following: by the growth of surface organs at a high zinc concentration, genotypes can be arranged as follows: Zaulbinka > Altai > Ulbinka-25 > Lyazzat > Glubochanka > Kutulukskaya > Nargiz > Omskaya-18 > Samal > Erythrospermum-606 (Figure 2).

Based on the received data, we see that in both cases, by the growth of surface sprouts, Altai is the wheat variety least exposed to effect of zinc and cadmium ions, while surface sprouts of variety Erythrospermum-606 acutely react on the presence of heavy metal ions.

Samal, Omskaya-18, Kutulukskaya and Zaulbinka wheat varieties showed average resistance to the cadmium effect according to the growth parameters of surface organs. When introducing zinc to the cultivation medium, growth of surface organs of varieties Lyazzat, Glubochanka, Kutulukskaya, Nargiz, Omskaya-18 is inhibited at the average degree, comparing to other varieties, which is the indicator of average metal endurance.

By the root growth, at introducing cadmium to the nutrition medium, genotypes can be arranged the following way: Nargiz > Ulbinka-25 > Altai > Glubochanka > Omskaya-18 > Zaulbinka > Lyazzat > Kutulukskaya > Erythrospermum-606 > Samal (Figure 3).

Altai, Ulbinka-25 and Nargiz have the most resistant to cadmium root system. Root growth of varieties Nargiz, Ulbinka-25 and Altai is inhibited in much less degree comparing to other genotypes at a high cadmium concentration in cultivation medium. Varieties Glubochanka, Omskaya-18, Zaulbinka and Lyazzat have an average root resistance level. Plant roots of varieties Erythrospermum-606 and Samal turned to be the most non-resistant to the adverse cadmium effect (Figure 3). When studying the impact of zinc on growth parameters of varieties from the Eastern Kazakhstan Agricultural Scientific Research Institute collection, it was shown that by the root growth at a high zinc concentration genotypes can be arranged as follows: Altai > Ulbinka-25 > Zaulbinka > Lyazzat > Glubochanka > Nargiz > Omskaya-18 > Samal > Erythrospermum-606 > Kutulukskaya (Figure 4).

Varieties Altai, Ulbinka-25 and Zaulbinka have the most zinc resistant root system. Lyazzat, Glubochanka and Nargiz have an average roots resistance level. Altai, Ulbinka-25 and Zaulbinka varieties' root growth is inhibited less comparing to other genotypes that were introduced to zinc growth medium. Varieties Omskaya-18, Samal and Erythrospermum-606 are less resistant to the adverse effect of zinc. The most non-resistant to the adverse effect of zinc are plant roots of variety Kutulukskaya.

Varieties Nargiz, Ulbinka-25 and Altai have the highest, Glubochanka, Omskaya-18, Zaulbinka, Lyazzat Zaulbinka and Kutulukskaya have an average, and Erythrospermum-606 and Samal have the lowest Wilkins coefficient or index of tolerance at a high concentration of cadmium (Table 1). Based on the results of the study of root growth in polluted by cadmium ions medium and on index of tolerance varieties Nargiz, Ulbinka-25 and Altai can be identified as genotypes with the most resistant towards the adverse effect of cadmium root system.

Altai variety has the highest, varieties Ulbinka-25 and Zaulbinka have an average, and Kutulukskaya and Erythrospermum-606 varieties have the lowest Wilkins coefficient at a high zinc concentration (table 2). Based on the results of the study of root growth in polluted by zinc ions medium and on index of tolerance genotypes Altai and Ulbinka-25 can be identified as the most resistant toward the adverse effect of zinc root system.

During the research testing of spring wheat contrasting by resistance genotypes we tested on permeability of cell membranes. Membrane permeability change is one of the symptoms of plants reaction to the external influence. It is known, that the exit of electrolytes is the membrane permeability function, which in its turn describes the damage extent [5]. Therefore, genotypes resistant to adverse environmental factors must have different physicochemical membrane characteristics.

Study on the cadmium ions influence on cell membrane permeability of surface organs of resistant Altai and sensitive Erythrospermum-606 wheat genotypes, showed that the permeability of cell

membranes for electrolytes increase at a high cadmium (40 mg /l) concentration (table 3).

The leaf tissues cell membranes permeability of winter wheat variety Altai increased for 23% under the cadmium influence regarding control. Leaf cell membrane permeability of spring wheat variety Erythrospermum-606 increased for 75% under the cadmium influence (table 3).

It should be mentioned that cell membrane permeability for electrolytes of more resistant wheat variety Altai's changed less comparing to more sensitive genotype Erythrospermum-606.

Study on the influence of zinc ions on the cell membrane permeability of surface organs of resistant Altai and sensitive Erythrospermum-606 wheat genotypes, showed that cell membrane permeability for electrolytes increases at a high concentration of zinc (400 mg / l) (table 4). Cell membranes permeability of leaf tissues of spring wheat variety Altai increased for 13% under the zinc influence regarding control. Leaf cell membrane permeability of winter wheat variety Erythrospermum-606 increased by 45% under the zinc influence relatively to control (table 4).

It should be mentioned that cell membrane permeability for electrolytes of resistant wheat variety Altai changed less comparing to more sensitive Erythrospermum-606, both under the cadmium and under zinc effect. The reason for strong inhibition of spring wheat Erythrospermum's-606 growth processes, as well as the relative stability of Altai varieties to the heavy metals toxic effect can be cell membrane's different ability to resist the stress, particularly the strong effect of heavy metals.

Membrane permeability change under stressful conditions proves structural restructure of membranes, which defines plants' potentially possible mechanisms to resist adverse environmental factors [6]. As a result of change of cell membrane permeability under influence of various factors, the intensive output of electrolytes from cells is happening. Intensity of secretion of electrolytes depends on varietal features.

## CONCLUSION

As a result of a comprehensive study of zinc and cadmium effect on the growth of surface organs and sprout roots of different wheat genotypes, resistant and sensitive by growth parameters winter wheat genotypes were identified in the laboratory conditions.

Zaulbinka and Altai spring wheat varieties are the most resistant to zinc translocation into the surface organs in terms of the experiment, and Erythrospermum-606 is the most sensitive spring wheat variety. Altai spring wheat variety has shown the biggest root resistance towards the zinc effect.

Altai, Nargiz and Glubochanka spring wheat varieties can be considered the most resistant to cadmium translocation into the surface organs among the studied genotypes in terms of the experiment, and Erythrospermum-606 is the most sensitive spring wheat variety. Nargiz, Ulbinka-25, Altai and Glubochanka spring wheat varieties have shown the biggest root resistance towards the cadmium effect.

Altai spring wheat variety has demonstrated the biggest resistance to both zinc and cadmium translocation into the surface organs.

Membrane permeability of more resistant wheat variety was changing in less degree comparing to more sensitive wheat genotype, both under the cadmium and the zinc effect. This fact indicates that the overall resistance of plants may be due to their cell membrane resistance towards the stressor effect.

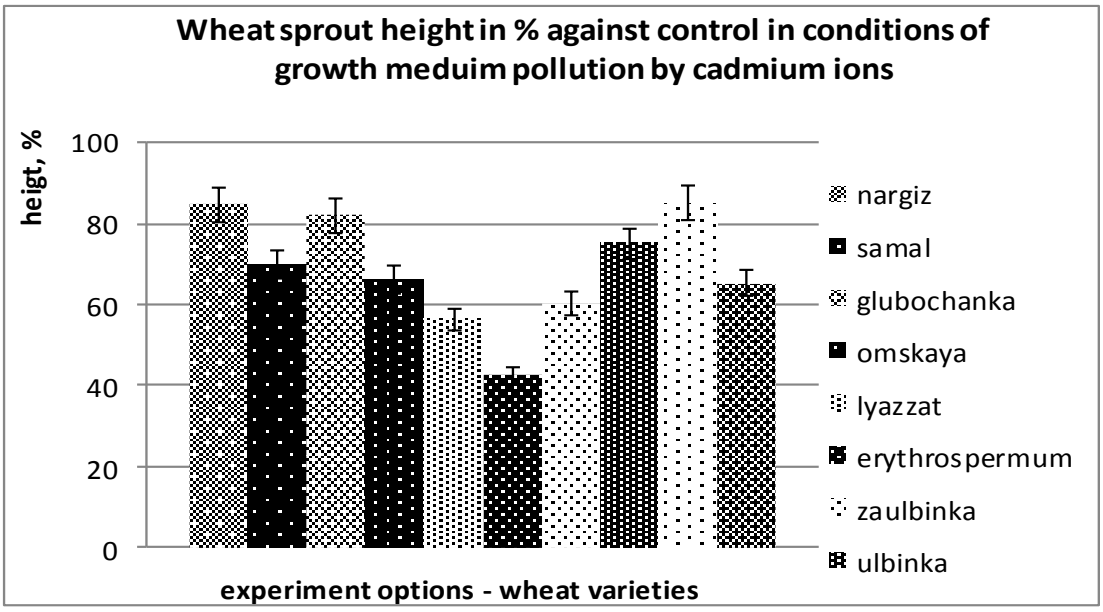



*Figure 1: Influence of cadmium ions presence in growth medium on sprout growth of different wheat genotypes*

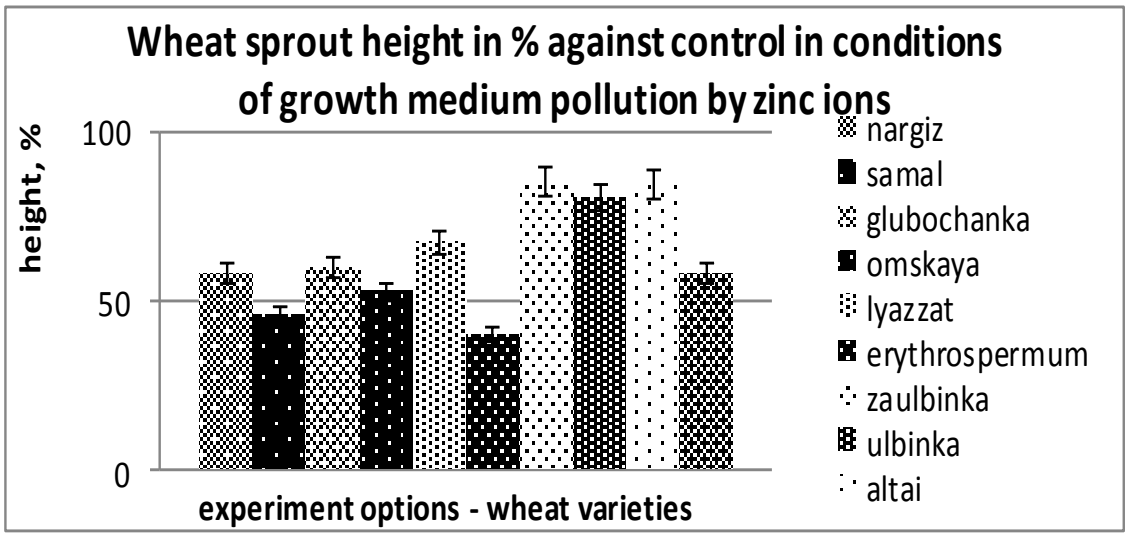



*Figure 2: Influence of zinc ions presence in growth medium on sprout growth of different wheat genotypes*

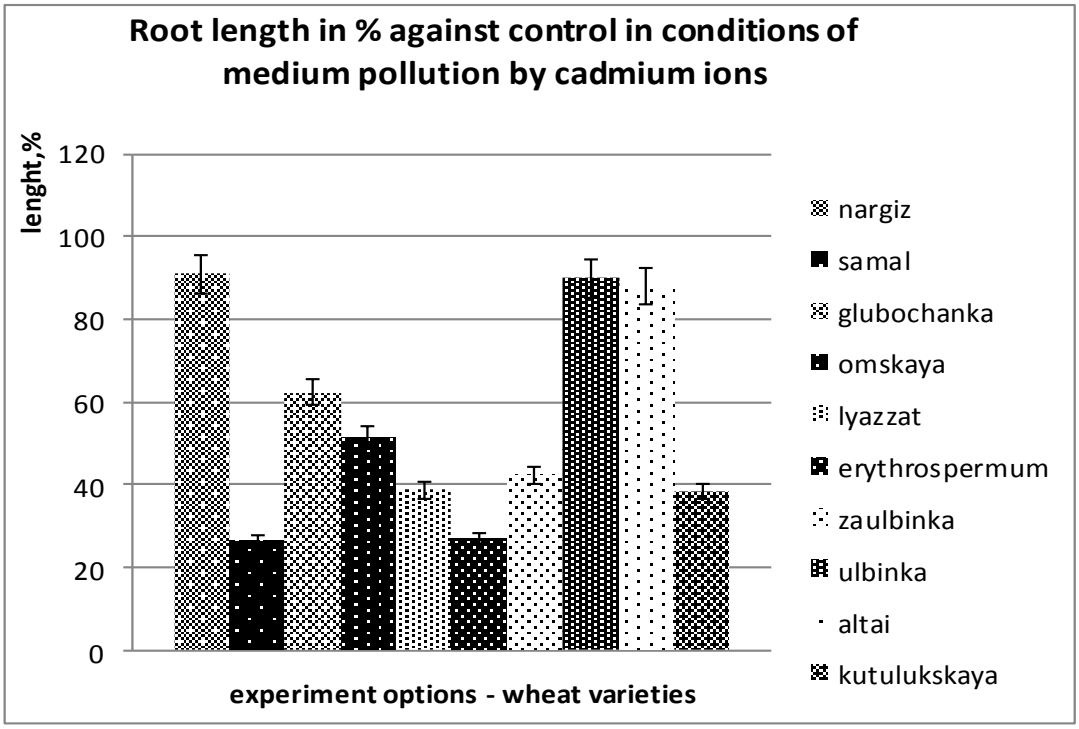



*Figure 3: Influence of cadmium ions presence in growth medium on root growth of different wheat genotypes*

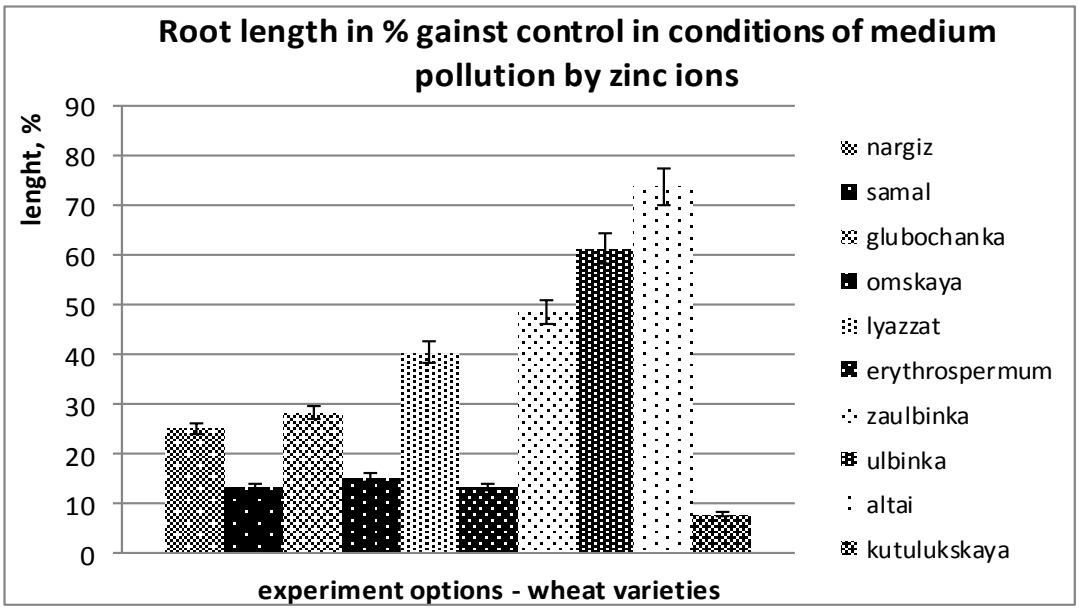



*Figure 4: Influence of zinc ions presence in growth medium on root growth of different wheat genotypes*

*Table 1: Wilkins Coefficient or tolerance index of wheat sprout roots in polluted by cadmium ions cultivation environment*

| Wheat varieties | Nar-giz | Samal | Glubochanka | Omskaya-18 | Lyaz-zat | Erythrospermum-606 | Zaul-binka | Ulbinka-25 | Altai | Kutulukskaya |
|---|---|---|---|---|---|---|---|---|---|---|
| Ime, | 12,08 | 8,36 | 15,17 | 12,63 | 10,33 | 4,6 | 6,9 | 8,8 | 11,15 | 9,6 |
| Ic | 13,29 | 31,46 | 24,36 | 24,60 | 26,70 | 16,9 | 16,3 | 9,8 | 12,7 | 25 |
| It | 0,91 | 0,27 | 0,62 | 0,51 | 0,39 | 0,27 | 0,42 | 0,90 | 0,88 | 0,38 |

*Table 2: Wilkins Coefficient or tolerance index of wheat sprout roots in polluted by zinc ions cultivation environment.*

| Wheat varieties | Nargiz | Samal | Glubochanka | Omskaya-18 | Lyazzat | Erythrospermum-606 | Zaul-binka | Ulbinka-25 | Altai | Kutulukskaya |
|---|---|---|---|---|---|---|---|---|---|---|
| $I_{me}$, | 3,32 | 4,17 | 6,87 | 3,72 | 10,79 | 2,2 | 7,9 | 6 | 9,4 | 1,9 |
| $I_c$ | 13,29 | 31,46 | 24,36 | 24,60 | 26,70 | 16,9 | 16,3 | 9,8 | 12,7 | 25 |
| $I_t$ | 0,25 | 0,13 | 0,28 | 0,15 | 0,40 | 0,13 | 0,48 | 0,61 | 0,74 | 0,08 |

*Table 3: Cadmium ions influence on the exit of electrolytes from leaf tissues of 10- days-old wheat sprouts.*

| Options | % the exit of electrolytes | % against control |
|---|---|---|
| Altai | | |
| Control | 2,50±0,04 | 100 |
| Cd 40 mg/l | 3,17±0,01 | 123 |
| Erythrospermum-606 | | |
| Control | 3,12±0,02 | 100 |
| Cd 40 mg/l | 5,52±0,01 | 175 |

*Table 4: Zinc ions influence on the exit of electrolytes from leaf tissues of 10- days-old wheat sprouts*

| Options | % the exit of electrolytes | % against control |
|---|---|---|
| Altai | | |
| Control | 2,50±0,04 | 100 |
| Zn 400 mg/l | 2,84±0,05 | 113 |
| Erythrospermum-606 | | |
| Control | 3,12±0,02 | 100 |
| Zn 400 mg/l | 4,63±0,015 | 145 |

## REFERENCES

Alybaeva R.A. (2007) Evaluation of the ecological status of soils in Ust-Kamenogorsk, Bulletin of the KNU, environmental Series 2 (21), 40-44.

Wu Feibo, Zhang Guoping (2002) Genotypic variation in kernel heavy metal concentrations in barley and affected by soil factors, Journal Plant Nutrition. 25 (6) 1163-1173.

Koval S.F. (1974) Investigation of the cell membranes properties and the resistance of plants by elution of electrolytes. News of the Sib. Branch of the Academy of Sciences of the USSR. Series of Biological Sciences, 15 (3), 161-167.

Dospekhov B.A. (1985) Methodology of field experience - Moscow: Agro-industrial Publishing.

Stevavic B., Sinzar I., Glisic O. (1998) Electrolyte leakage differences between poikilohydrous and homoiohydrous species of Gesneriaceae. Biologia Plantarum. 2 (40), 229-303.

Altergot V.F. (1976) Principles of physiological studies and problems of crop production in Siberia. In the book: Physiology of plant resistance to continental climate. Novosibirsk 4-14.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 36-48

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1107

# VARIATION IN TOTAL POLYPHENOL CONTENT, ANTIOXIDANT POTENTIAL, THEAFLAVIN AND THEARUBIGINS CONTENT OF BOPF GRADE SRI LANKAN BLACK TEAS (*Camellia sinensis* L.) OF DIFFERENT CLIMATIC ELEVATIONS AND BOPF GRADE COMMERCIALLY AVAILABLE BLACK TEAS IN SRI LANKA

[1*]R.S. Ratnayake and [2]K.K.D.S. Ranaweera

*[1]Department of Food Science & Technology, Faculty of Applied Sciences University of Sri Jayewardenepura , Gangodawila, Nugegoda, Sri Lanka*
*[2]Department of Food Science & Technology, Faculty of Applied Sciences University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka*

**Abstract**

Black tea is the most popular tea among three major types of tea namely green, oolong and black tea. Unblended Sri Lankan black teas are classified as high grown, medium grown and low grown based on their geographical origin. However some studies have been reported regarding the chemical parameters of black teas from different agro climatic elevations of Sri Lanka, such details like purity, cultivar and the particle size of teas have not been specified. And also there is no systematic screening has been reported for black tea commercialized in Sri Lankan market. So the main objective of this study was to analyze the total polyphenol content, antioxidant activity & total thearubigins & theaflavins content in unblended Sri Lankan black teas collected from factories representing different tea growing regions. And also to analyze the same biochemical parameters for the black teas that are commercialized in Sri Lankan market. During the month of April 2012, BOPF grade fermented black tea samples produced by TRI 2025 were collected from twelve tea plantations of different agro climatic elevations. All the teas were processed according to the orthodox-rotor vane method. Ten commercialized samples of different brands were also analyzed. Total phenolic content (ISO 14502-1 method), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, TF (flavognost method) and TR content were determined for each sample. Mid grown tea showed highest mean values in TPP content (15.9454 ± 2.5778 % GAE), TF content (22.2200 ± 3.9575 µmol/g) and TR content (14.6031 ± 1.8970 %). The antioxidant activity was well correlated with the total polyphenol content ($P < 0.05$) and thearubigins contents ($P < 0.05$). Lowest mean EC50 value (49.6791 ± 5.9475 µg/ml) was obtained by mid grown tea samples, indicating highest antioxidant activity among others. Rilagala plantation showed the maximum values in TPP content, antioxidant activity, TF and TR contents among others.

**Keywords:** Black tea, antioxidant activity, total polyphenolic content, theaflavin, thearubigin

## INTRODUCTION

Production of free radicals and reactive oxygen species (ROS) in cells and body tissues have been linked to aging process as well as leading to several human diseases such as diabetes, cirrhosis, cancer cardiovascular diseases and inflammatory disorders (Hertog *et al.,* 1993; Maxwell, 1995). In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates

Corresponding Author Email: *roger.ratnayake@gmail.com

polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents and pesticides (Halliwell, 1994). The antioxidants that scavenge free radicals have major role in the improvement of above diseased conditions.

Antioxidants protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, super oxide, peroxy radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage (Wilson, 1998).

Free radicals generated in the body can be removed by body's own natural antioxidants defenses such as glutathiones and catalases. However, endogenous antioxidant defenses are not completely efficient. Therefore, dietery antioxidants are required to lessen the overall effect of antioxidant stress due to excessive free radicals occurring in our system (Madsen & Bertelsen, 1995). Synthetic antioxidants such as Butyl hydroxyl anisole (BHA) or butyl hydroxyl toluene (BHT) are used in food industry to decelerate these processes. But, due to their unstable and highly volatile nature, they have been suspected to cause or promote negative health effects (Nabavi *et al.,* 2008). Therefore there is a trend to substitute them with naturally occurring antioxidants.

Plants are a potential source of natural antioxidants. Natural antioxidants can be phenolic compounds (Flavanoids, phenolic acids and tannins), nitrogen containing compounds (Alkaloids, chlorophyll, derivative amino acids, peptides and amino acids), carotenoids, tocopherols or ascorbic acid and its derivatives and these phytochemical antioxidants are secondary metabolites of plants (Velioglu *et al.,* 1998).

In searching for novel natural antioxidants, some plants have been extensively studied in the past few years for their antioxidant and free radical components (Pourmorad *et al.,* 2006). In this respect presence of flavonoids and other polyphenolic compounds have received the greatest attention (Sundarajan *et al.,* 2006).

*Camellia sinensis* is commonly known as Tea is one of the most widely consumed beverages in the world and well known to be rich in polyphenolic compound, which plays important roles as antioxidants (Worthy, 1991). The growing interest in the potential health benefits of tea have prompted numerous investigations on the chemical constituents of tea and their biological properties such as anticarcinogenic and antioxidant, antibacterial, anti-inflammatory activities (Gupta *et al.,* 2002). Although a number of mechanisms have been proposed for the beneficial effects of tea, the radical-scavenging and antioxidant properties of tea polyphenols are frequently cited as important contributors to these beneficial effects. Many reports have been studied extensively on the beneficial effects of tea on antioxidant activity and total phenolic content (Gadow *et al.,* 1997).

Many consumers use 'Broken Orange Pekoe Fannings' (BOPF) grade of black tea (a grade containing medium size leaf particles) due to its easy availability and pleasant flavour and taste. However, there is not a great deal of information on the phenolic content, antioxidant activity, theaflavin & thearubigins content of Sri Lankan BOPF grade black tea for a specific cultivar respect to agro climatic elevation. To date, no previous systematic studies have been reported on the phenolic compounds and antioxidant capacity of commercialized black tea from Sri Lankan market. This is worth examining since it is well recognized that pharmaco-theraputic potential of black tea infusion differs with many factors including country of origin, particle size (or grade of tea), brewing time and agro climatic elevation of sample (Modder and Amarakoon, 2002; Wijeratne, 2008).

The aim of this research was to analyze the total polyphenol content, antioxidant activity & total thearubigins & theaflavins content in Sri Lankan black tea originated from three major agro climatic elevations & black teas commercialized in Sri Lankan market.

Fermented black tea samples of BOPF grade tea produced by TRI 2025 cultivar in same season from four different factories in each elevation representing low-grown (below 600 m, average mean sea level; amsl), midgrown (between 600 and 1200 m, amsl) and high-grown (above 1200 m, amsl) was collected & analyzed for total polyphenol content, antioxidant activity & total thearubigins & theaflavins content.

And also, ten different black tea samples from different brands commercialized in Sri Lankan market was analyzed for same biochemical parameters.

**Main Objective**

To assess the Total polyphenol content, antioxidant activity, total theaflavins & thearubigin content of Sri Lankan black teas from different agro-climatic elevations & commercially available black teas from Sri Lankan market.

**Specific Objectives**

- To assess the variation in total polyphenol content, antioxidant activity, total theaflavins & thearubigin content of unblended Sri Lankan black teas & commercially available black teas (blended) of Sri Lanka.
- To identify the agro-climatic elevation that originates black tea with highest Total polyphenol content, antioxidant activity, total theaflavins & thearubigin content.
- To determine the correlation between total polyphenol content & antioxidant activity in above black teas.
- To assess the correlation between Theaflavins/Thearubigins & antioxidant activity in above black teas.
- To assess the variation in above parameters of each selected garden marks belonging to each agro-climatic region.

## LITERATURE REVIEW

Tea (*Camellia sinensis* (L.) O. Kuntze) is a member of Theaceae family. It is an evergreen, cross-pollinated tree or shrub that can grow to a height of 10-15 m in the wild, but is usually clipped to a height of 0.6-1.5 m in cultivation. The leaves are light green, short stalked, coriaceous, alternate, serrate margin, varying in length from 5-30 cm and about 4 cm width. Mature leaves are dark green coloured, smooth and leathery while young leaves are pubescent. Flowers are white fragrant, 2.5-4 cm in diameter, found in solitary or in clusters of two or four. Flowers bear numerous stamens with yellow anther and produce brownish red capsules. Fruit is a flattened, smooth, rounded, capsule with one to five chambers, mostly two or three (Mahmood *et al.,* 2010). Seed is composed of husk, seed capsule, cotyledon and embryo. The seed is rich in fat, starch, sugar with a small amount of saponin. The plant has a strong tap root.

Tea is propagated either from seeds or by cuttings. The tree or shrub is heavily branched with leaves preferentially picked as young leaves and leaf buds to produce tea. Older leaves are considered to be inferior in quality (Sharma *et al.,* 2007).

The two main types of processed tea are black tea and green tea. The difference between these two main types of tea is that black tea manufacture gives full rein to the oxidation of polyphenols by the polyphenoloxidase present in the flush, whilst in green tea manufacture the first operation is the heat destruction of polyphenoloxidase by steaming (Japan) or pan firing (china). It follows therefore, that the primary difference between black tea and green tea is that the former contains a mixture of the native unoxidized polyphenols, together with theaflavin and other oxidized polyphenols only. (Wicramasinghe, 1978)

Sri Lanka produces tea throughout the year and the growing areas are mainly concentrated in the central highlands and southern inland areas of the island. They are broadly grouped under these headings according to their elevations, with high growns ranging from 1200 m upwards, medium growns covering between 600 m to 1200 m. and low growns from sea level up to 600 m (SLTB, 2011)

Four main objectives of tea grading are separation of made tea particles into various shapes and sizes in conformity with trade requirements, size reduction of oversized particles so as to form more acceptable grades, cleaning of grades so as to present them in the most attractive and acceptable manner and preparation for marketing and transport of the produce (Samaraweera and Ziyad, 2008)

*Table 1: Major grades of tea produced in Sri Lanka*

| Main grade | Sub grade | Description |
|---|---|---|
| Very special (tips only) | Silver tips | Siler color dried buds |
| | Golden tips | Golden color dried buds |
| Tippy | FBOPF Sp | Similar in size to BOP 1 with a fair presence of tips |
| Flowery | FBOPF | Similar in size to BOP, BOPF but must contain tips |
| | FBOPF 1 | Larger than BOP, smaller than a FBOP with a show of tips |
| | FBOP | Smaller/shorter than BOP 1 with presence of tips, but larger than FBOPF1 |
| | FBOP 1 | Long, twisted, wiry leaf, fairly tippy. Longer than BOP1 |
| Pekoe | Pekoe 1 | Same as pekoe but smaller in size than pekoe |
| | Pekoe | Shotty, curly or semi-curly leaf of large size |
| Leafy | BOP 1 | Wiry and twisted, but shorter than OP 1 |
| | OP 1 | Long, wiry, well or partly twisted |
| | OP | Less wiry than OP1, but much more twisted than OPA |
| | OPA | Long bold leaf with fair twist |

Source: Tea Research Institute, 2008

**Chemicals in Tea**

Tea is composed of unique constituents among other plants. Caffeine is found only in a few other plants other than tea. Theanine, which is unique to tea, is a kind of amino acid constituting more than half the total amount of amino acids in tea. Major catechins in tea are also unique to tea. Vitamin C was found to be contained in tea after it was discovered in lemons. In 1827 caffeine was discovered in tea. At that time it was given the name theine, but when it was proven that the structure and properties of this substance were exactly the same as caffeine that was identified in coffee in 1820, the name theine was dropped (Yukihiko Hara *et al*., 2001).

In 1924, vitamin C was discovered in green tea by two Japanese scientists, M. Miura and M. Tsujimura. The astringency of tea, too, was investigated extensively by Tsujimura. In the years 1927 to 1935, Tsujimura isolated epicatechin, epicatechin gallate, and epigallocatechin. She purified them and determined their structural formulas. In 1950, with the new technique of column chromatography, the British scientist A. B. Bradfield succeeded in isolating epigallocatechin gallate and determined its structure by x-ray diffraction method. Tsujimura later identified her compound as being the same. Thus, the main four catechins in tea, which make up the major group of compounds in the soluble solids of tea, were identified in the early 1950s and Tsujimura, along with Bradfield, gained worldwide renowned for their pioneering work (Yukihiko Hara *et al*., 2001).

Later, around 1963–1965, Y. Takino *et al*., confirmed the benzotropolone structure of theaflavins. The chemistry of tea polyphenols in that of broader plant polyphenols was well reviewed by E. Haslam. Aroma components in tea were first researched more than 150 years ago by Mulder, who discovered essential oil in fresh tea leaves. In 1930s, Takei and. Yamamoto *et al*, were among the earliest scientists to contribute to the knowledge of tea aroma. Methods at that time were rather crude and tons of tea, not to mention time and patience, were necessary to isolate sufficient material for separation and identification of individual components. The work of these professors (Takei, who focused mainly on green tea, and Yamamoto, who focused on Taiwan black tea) became a vital basis for future research in the field. They identified more than 30 compounds from green and black teas. Today more than 600 aroma compounds have been identified. Theanine, γ-ethylamide of glutamic acid, was discovered in 1950 by Sakato. Theanine constitutes the ‘‘umami’’ or sweet taste in tea, particularly that of Gyokuro (the best quality green tea in Japan), and constitutes 2% of tea. The antagonistic action of theanine against the

stimulating action of caffeine in the nervous system and its vitalizing action on brain neurons are areas of interest that could be studied further (Yukihiko Hara *et al*., 2001).

The composition of *C. sinensis* plant varies with climate, season, agricultural practices, varieties of plant, age of leaf, types of leaf and processing methods (Balentine *et al.,* 1998; Yao *et al*., 2009).

The soluble part of tea consists of polyphenols (mainly catechins) to a large degree, amino acids (theanine), caffeine, minerals and vitamins. The catechin content is up to 30% of the dry weight, whereas, the content of caffeine is up to 5% of the dry weight (Balentine, 1997; Graham, 1992).

According to Mukhtar *et al.,* (2000) polyphenols in tea mainly include the following six groups of compounds: flavalonls, hydroxyl-4-flavanols, anthocyanins, flavones, flavonols and phenolic acids. Important and characteristic tea polyphenols are the flavanols of which catechins (flavan-3-ols) are predominant which contribute to the bitterness, astringency and sweet after taste of tea beverages (Hara *et al.,* 1995b). The major ones are: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), catechin (C) and gallocatechin (GC) (Hara *et al.,* 1995a; Liang *et al.,* 2003). Tea also contains favonols, mainly quercetin, kaempferol, myrecetin and their glycosides. In black tea, the enzyme-catalysed oxidation and polymerization of polyphenols during processing, commonly referred to as fermentation, leads to the formation of catechins and gallic acid complexes such as theaflavins and thearubigins, which are responsible for the characteristic dark brown colour in black tea (Balentine, 1997; Lee *et al.,* 2008). In addition, methylxanthines are present with 2 to 4% as caffeine and as a small amount of theophylline and of theobromine (Hara *et al.,* 1995a). Tea also contains many amino acids, but theanine, specific to the tea plant, is the most abundant, accounting for 50% of the total amino acids. Balentine (1997) reported that the amino acid degradation is involved in the biogenesis of the tea aroma. Chlorophyll, carotenoids, lipids and volatile compounds present in tea shoots play an important role in the development of the aroma (Hara *et al.,* 1995c).

Volatile fractions of tea leaves have been studied by Hara *et al.,* (1995d) and Shimoda *et al.,* (1995) in detail and more than 600 different molecules have been isolated. These include terpenoids and degradation products of amino acids, carotenoids and linoleic acid (Hara *et al.,* 1995a). Mineral constitutes about 4 to 9% of the inorganic matter of tea.

According to Hara *et al.,*(1995c) tea also provides useful amounts of minerals, manganese, potassium and fluoride ions to the diet, and also contains aluminum, iodine, selenium and nickel. This complex composition of tea leaves helps to understand the constituents of tea in particular those that may promote health.

**Antioxidant Properties of Tea Polyphenols**

Polyphenols are found in most plants, a diverse group of phenolic compounds (flavanols, flavonols, anthocyanins, phenolic acids, etc.) possess an ideal structural chemistry for free radical scavenging activity (Chanda & Dave, 2009). Antioxidative properties of polyphenols result from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (Shahidi & Wanasundara, 1992; Rice-Evans *et al.,* 1996). However, *C.sinensis* plant is unique in having huge amount of polyphenols up to 40% of the solids extracted in to the tea brew). Balentine (2001) reported that tea is one of the richest sources of flavanoids.

**Determination of Total Polyphenolic Content**

The amount of total phenol content can be determined by Folin-Ciocalteu reagent method or by Folin-Denis reagent method (Shahidi & Wanasundara, 1992). Generally this is a basic oxidation-reduction reaction. Mo (40) in Folin's reagent is reduced to Mo(40) with an electron donated by an antioxidant (Harbone & Williams,2000). Although the reducing capacity of a substance is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants.

Phenolic compounds react with Folin's reagent only under alkaline conditions and intensity of blue colour produced reflects the amount of phenolic compounds, which can be measured at 765 nm using

spectrophotometer (Conforti *et al*., 2006). However, the exact chemical nature of the Folin's reagent is not known and also the reagent is nonspecific to phenolic compounds as it can be reduced by many non phenolic compounds (vitamin C, Cu (I), etc.). Despite the undefined chemical nature of the Folin's reagent, this assay is simple, convenient and reproducible. As a result, a large number of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants.

Previously, Povichit *et al.,* (2010) investigated the total phenolic content of ethanolic extracts of fifteen medicinal plants by using Folin-Ciocalteu reagent and found that the leaves of *C. sinensis* plant contains highest phenolic content among the other studied plants. It is generally believed that plants which have more phenolic content show good antioxidant activity that is there is a direct correlation between total phenol content and antioxidant activity (Biglari *et al.,* 2008). Pawin and Sangsrichan (2009) found a positive and strong correlation of total phenolic content and antioxidant activity using ABTS and DPPH assays for Pickled Assam tea leaves (*C. sinensis* var. *assamica*), Chineese green tea, Oolong and black tea. It can be stated that phenolic content of the plant may be a good indicator of its antioxidant capacity.

**Evaluation of Antioxidant Activity**

A great number of *in vitro* methods have been developed to evaluate the potency of natural antioxidants either as pure compounds or as plant extracts (Robards *et al.,* 1999; Moure *et al.,* 2001). Antioxidant capacity assays may be broadly classified as hydrogen atom transfer and electron transfer based assays.

Oxygen radical antioxidant capacity (ORAC), total radical trapping antioxidant potential (TRAP) and β carotene bleaching test (BCBT) are some of the Hydrogen atom transfer reactions and trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay (DPPH), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide radical scavenging assay are based on electron transfer reactions (Chanda & Dave 2009).

Different antioxidant evaluating methods do not give the same absolute value but give similar relative rankings to the compound (Modder & Amarakoon, 2002c). Koleva *et al*., 2002 carried out a comparative study on three testing methods, DPPH, Static headspace gas chromatography and BCBT to screen out plant extracts for their antioxidant activity and found that the complex composition of the extracts and partition phenomena affected their activity in each assay. According to Salazar *et al.,* (2008) it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals.

A study by Molyneux (2004) reported that the molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl- β-picrylhydrazyl) is characterized as a stable free radical as they do not dimerise. The delocalization of the spare electron over the molecule also gives rise to the deep violet colour. When a solution of DPPH in ethanol is mixed with that of a substance that can donate a hydrogen atom, gives rise to the formation of reduced form of DPPH. This transformation results in colour change from violet to yellow and usually measured spectropheotometrically at 517 nm (Chanda & Dave, 2009).

**Theaflavins and Thearubigins Determination in Black Tea**

Approximately 76–78% of the tea produced and consumed worldwide is black tea (Cabrera et al., 2003). During the black tea manufacture, a natural enzyme catalyzes oxidation and condensation of green leaf tea catechins leading to the formation of theaflavins (TF) and thearubigins (TR) (Robertson, 1992).

The plain black teas are evaluated on the basis of their briskness, brightness, strength, body and total colour of liquors (Roberts & Smith, 1963). Black tea liquor brightness and colour are critical quality attributes used in tea trade to rank and price black teas (Biswas, Sarkar, & Biswas, 1973; McDowell, Feakes, & Gay, 1991). These attributes of black tea quality are mainly dictated by the levels of theaflavins and thearubigins (Robertson, 1992). Theaflavins are responsible for the astringency, brightness, colour and briskness of the black tea. Thearubigins contribute to the mouth feel (thickness)

and colour of the tea (Biswas, Biswas, & Sarkar, 1973).

Black tea quality is mainly influenced by total theaflavins (Wright, Mphangwe, Nyirenda, & Apostolides, 2002) or derived theaflavin digallate equivalents (Owuor & Obanda, 1997).

The formation of a single theaflavin requires a dihydroxy and trihydroxy flavan-3-ols. The ratio of dihydroxyflavan-3-ol to trihydroxy-3-ol in green leaf may thus have a major influence on the amount of theaflavins in black tea. The correct balance and amount of dihydroxyflavan-3-ol and trihydroxyflavan-3-ol are therefore necessary to ensure maximum formation of the theaflavins (Wright et al., 2002). The amount of the individual theaflavins are formed largely influenced by the amounts of the precursor catechins in green leaf, their redox potential and/or affinity for polyphenol oxidase and activity (Owuor & Obanda, 2007).

Fermentation is a critical stage in the manufacture of black tea during which oxidative condensation of catechins to TF and TR occurs. Catechins, together with their oxidation products are responsible for most of the sensory characteristics associated with black tea liquors (Biswas et al., 1973). Temperature and time are important factors in determining the extent of fermentation. Processing conditions which favour less degradation of simple theaflavins and the retention of higher epicatechin gallate (ECG) and epigallocatechin gallate (EGCg) levels produce more brisk tea liquors (Obanda, Owuor, & Mang'oka, 2001). Theaflavins and unoxidized catechins are thought to have considerable human health benefits (Apostolides & Weisberger, 1995). Therefore, tea clones or processing conditions with a high potential of achieving this, can enhance the health benefit potential of black tea and impact positively on liquor astringency.

The formation and contribution of TR to quality characteristics of black tea have also been reported but their structures remain speculative. In 1961, Roberts and Smith demonstrated TR could separate into two large groups, i.e. TRSI and TRSII, due to differences in chemical polarities. Roberts (1962) reported that TR was acidic brown pigments formed by the oxidative degradation of TF.

Traditional methods for the preparation and determination of polyphenols in fresh tea shoots or manufactured teas have been described by several researchers (Roberts, 1962; Roberts & Myers, 1958; Roberts & Smith, 1961). The most common methods are paper chromatography (Roberts, Cartwright, & Wood, 1956), column chromatography (Oshima & Nakabayashi, 1953; Whitehead & Temple, 1992) and colorimetric measurement (Muralidharan, 1997; Oshima & Nakabayashi, 1953; Roberts & Smith, 1961). All those methods are based on either the oxidation or reduction properties of tea polyphenols.

More recently developed analytical techniques are used to isolate, identify and determine individual polyphenolic compounds by HPLC (Harbowy & Balentine, 1997; Temple & Clifford, 1997; Yao & Nursten, 1997, 1998). However, colorimetric or spectrophotometric methods are still the most widely used, due to their simplicity for the determination of total phenolic compounds, TF and TR in tea (Harbowy & Balentine, 1997; Lakenbrink et al., 2000), particularly for tea industries.

The method developed for determining theaflavins and thearubigins depends on the fact that the theaflavins are almost quantitatively extracted from tea liquor by one extraction with either ethyl acetate or isobutyl methyl ketone. These solvents do not extract thearubigins of the S II type, but there is a partial extraction of the free-acid forms of the S I type thearubigins. Potassium and calcium salts are not extracted. The thearubigins extracted by ethyl acetate or isobutyl methyl ketone are soluble in aqueous sodium hydrogen carbonate, whereas the theaflavins are insoluble. Complete separation of theaflavins and thearubigins is therefore effected by shaking the ethyl acetate or isobutyl methyl ketone extract with aqueous sodium hydrogen carbonate.

Theaflavin and its gallate have well defined absorption maxima at 380 and 460 mμ. (Roberts, and Myers, 1959). Either of these wavelengths is suitable for direct spectrophotometry in the extract washed with sodium hydrogen carbonate, as no other substances are present that absorb at these wavelengths. The fall in optical density, which results from the washing with sodium hydrogen carbonate, affords a method of determining the extractable thearubigins.

Direct spectrophotometric determination of residual thearubigins in the aqueous layer after extraction with ethyl acetate or isobutyl methyl ketone is not possible, as a high proportion of the thearubigin molecules are present as anions, which are more deeply coloured than the free acids. Addition of excess aqueous oxalic acid reduces the colour intensity to that of the free acids, and spectrophotometry is possible after this acidification.

## METHODOLOGY

### Samples

Fresh tea samples of TRI 2025 were collected separately from each selected plantation during April 2012, and undergone to an orthodox rotor vane process. About 200 g of BOPF grade processed black tea sample from each factory on completion of processing were collected. The plantations were selected to represent the key tea producing regions in Sri Lanka. (See Table 1)

*Table 2: Names, Locations and sampling periods for the Sri Lankan Teas*

| Tea growing region | Plantation | Description of the location |
|---|---|---|
| High grown (>1200m) | Holyrood | Located in western slopes of the central hills of Dimbulla planting district, about 1340 m above sea level |
| | Somerset | Located in western slopes of the central hills, situated in the lower boundary of the misty village known as Nanuoya, about 1200 m above sea level |
| | Greatwestern | Situated in the foothills of the majestic Great Western range, about 1200 – 1800 m above sea level |
| | Mattakelle | Located in western slopes of the central hills, about 1400 m above sea level |
| Mid grown (1200-600m) | Craighead | Located in the middle slopes about 900 m -1100 above sea levels. There are no seasonal variations in harvesting patterns for mid grown teas |
| | Imboolpitiya | Located at an elevation about 800 m above sea levels. |
| | Dombagastalawa | Located at an elevation about above m600–900 m sea levels. |
| | Rilagala | Located at an elevation about 1000 m above sea levels. |
| Low grown (<600m) | Houpe | Located at an elevation of about 550 m above sea level. There are no seasonal variations in harvesting patterns for low grown teas |
| | Rilhena | Located at an elevation of about 500 m above sea level. |
| | Samawatte | Located at an elevation of about 450 m-500 m above sea level. |
| | Wellandara | Located at an elevation of about 500 m above sea level. |

Ten commercial bagged tea samples of different brands (A-J) were purchased from a Sri Lankan supermarket. When purchasing bagged tea products, they were selected with similar manufactured date.

For each commercial tea sample studied, three bags were sampled.

### Chemicals

Analytical Reagent Grade Absolute Methanol (Prolabo-UK), Analytical reagent grade sodium

carbonate anhydrous (Fisher Scientific, UK), Folin-ciocalteu phenol reagent (Prolabo-UK), Gallic acid (Sigma Aldrich Co. USA), Analytical Reagent Grade Absolute Ethanol (Prolabo-UK), DPPH (2,2-Diphenyl-1-picrylhydrazyl), IBMK (isobutyl methyl ketone), Flavognost Reagent (diphenylboric acid 2-aminoethyl ester) were purchased from Sigma Aldrich- USA.

**Extraction of Polyphenols**

The method described by the International Organization for Standardization (ISO) 14502-1 was used. Briefly, 0.200 ( 0.001 g of each sample was weighed in an extraction tube, and 5 mL of 70% methanol at 70 °C was added. The extract was mixed on a vortex for 10 min and heated at 70 °C. After cooling at room temperature, the extract was centrifuged at 3500 rpm for 10 min. The supernatant was decanted into a graduated tube. The extraction step was repeated twice. Both extracts were pooled and the volume adjusted to 10 mL with cold 70% methanol. One millilitre of the extract was diluted with water to 100 mL.

**Estimation of the Total polyphenol content by Folin-Ciocalteu assay**

The total polyphenol content (TPC) was determined by spectrophotometry, using Gallic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. Briefly, 1.0 mL of the diluted sample extract was transferred in duplicate to separate tubes containing 5.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The TPC was expressed as Gallic acid equivalents (GAE) in g/100 g material. The concentration of polyphenols in samples was derived from a standard curve of Gallic acid ranging from 10 to 50 $\mu$g/mL (Pearson's correlation coefficient: $r^2$ =0.9910).

**Determination of the Free Radical Scavenging Activity by DPPH Free-Radical Scavenging Assay**

Scavenging activities of the extracts on the stable free radical DPPH were assayed using the modified Blois' method, in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample.

The diluted working solutions of the test extracts were prepared (1.00, 5.00, 10.00, 20.00, 30.00 µg/ml). 0.04% w/v of DPPH was prepared in absolute ethanol and 2 ml of this solution was mixed with 2 ml of sample solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using UV-mini Spectrophotometer. Ethanol (2 ml) with DPPH solution (0.04%, 2 ml) was used as blank. The optical density was recorded and % inhibition was calculated using the formula given below:

$$I\ \% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\ \%$$

$A_{control}$ = Absorbance of the control

$A_{sample}$ = Absorbance of the sample / ascorbic acid solution

**Total TF content analysis (Flavognost Method)**

Total TF were determined by the Flavognost method (Hilton, 1973). A tea infusion was made with 375 ml of boiling water, added from an overhead boiler into a tared flask, and 9 g of tea. The flask was shaken for 10 min, the infusion filtered rough cotton wool, and allowed to cool to room temperature, and then 10 ml were pipetted into 10 ml of isobutylmethylketone (4-methylpentan-2-one, IBMK). The mixture was shaken for 10 min and allowed to stand until the layers separated. Two millilitres of the upper layer were pipetted into a test tube, followed by 4 ml ethanol and 2 ml Flavognost reagent (2 g diphenylboric acid-2-aminoethyl ester dissolved in 100 ml ethanol). The contents were mixed and colour allowed 15 min to develop. The absorbance (A) at 625 nm was read against an IBMK/ethanol (1:1 v/v) blank.

$$\text{Theaflavin content}(\mu\text{mol/g}) = A_{625nm} \times 47.9 \times 100/DM$$

**Determination of Total Thearubigins**

Fifty milliliters of the cool, well-shaken and filtered standard tea infusion from TF analysis were mixed with 50 ml isobutyl methyl ketone (IBMK) and gently shaken to avoid formation of an emulsion. The layers were allowed to separate and 4-ml portion of the IBMK layer was taken and made to 25 ml with methanol in a volumetric flask (Solution A). Two milliliter portions of the aqueous layer were diluted to 10 ml with distilled water and then to 25 ml with methanol (Solution B). Twenty-five milliliters of the remaining initial IBMK layer were taken in a separate flask and mixed with 25 ml of 2.5% aqueous sodium hydrogen carbonate. The mixture was vigorously shaken before the layers were allowed to separate and the aqueous layer discarded. A 4-ml portion of the washed IBMK layer was made to 25 ml with methanol (Solution C). Two milliliters of a saturated oxalic acid aqueous solution and 6 ml of water were added to a 2-ml portion of the aqueous layer left from the first extraction with IBMK, and diluted to 25 ml with methanol (Solution D).

The absorbencies $A_A$, $A_C$, $A_D$ of solutions A, C and D at 380 nm were obtained using a spectrophotometer (UV- mini, Shimadzu) with distilled water as the blank. Each black tea sample was extracted in triplicate for the determination of the Total Thearubigins content.

$$\%\ \text{Thearubigin Content (Total)} = \left(\frac{375 \times 0.02 \times 6.25 \times [2A_D + A_A - A_C]}{0.733 \times 9 \times DM/100}\right)$$

**DATA ANALYSIS**

All determinations were carried out in three triplicate and data were subjected to analysis of variance. Analysis of variance was performed using the ANOVA procedure. The data were normally distributed at the 95% confidence level and Anderson-Darling test was used for the determination of normality of the data. Two normality assumptions were made for the residuals at the 95% confidence level according to the p value of the test parameters. *P* values less than 0.05 were considered statistically significant. Significant differences between means were determined by Tukey test. *P* values less than 0.05 were considered statistically significant. Two-Sample-T Test was carried out to analyze the significance of the tested parameters between black tea samples from different agro climatic elevations and commercial black tea samples. All statistical analyses were performed using the MINITAB14 software.

**RESULTS & DISCUSSION**

According to the results, mid grown tea had showed highest mean values in TPP content (15.9454 ± 2.5778 % GAE), TF content (22.2200 ± 3.9575 µmol/g) and TR content (14.6031 ± 1.8970 %). And also the lowest mean $EC_{50}$ value (49.6791 ± 5.9475 µg/ml) was obtained by mid grown tea samples, which indicated the highest antioxidant activity by scavenging DPPH free radicals. For TPP content & $EC_{50}$ value, there is no significance difference showed between means of mid grown and low grown teas.

The results of antioxidant activity were much similar to the trend of total polyphenolic content in different black tea samples and showed a positive linear correlation between total polyphenolic content and $EC_{50}$ values (P = 0.018). There was no correlation was found between Theaflavins content and antioxidant activity. But a positive linear correlation between antioxidant activity and TR content (P = 0.037) was found.

Black tea samples from Rilagala plantation of mid grown region was found to be the finest with respect to TPP content (18.6595 ± 0.6977 % GAE), antioxidant activity ($EC_{50}$ = 42.2706 µg/ml) and TR content (16.9016 ± 0.1261 %); followed by

Imboolpitiya plantation of mid grown region and Samawatte plantation of low grown region.

From two sample T-test results, it is found that there was significant difference between the TPP content ($P < 0.05$), TF content and TR content ($P < 0.05$) of black tea samples from different agro climatic elevations and commercial black teas.

## CONCLUSION

The study demonstrates considerable variation among both unblended and commercialized Sri Lankan black tea samples for TPP, antioxidant properties as determined by DPPH, TF and TR contents. It clearly shows that there is considerable potential for using selected combinations of plantations and season to select high antioxidant teas.

The results of antioxidant activity were much similar to the trend of total polyphenolic content in different black tea samples and showed a positive linear. A positive linear correlation between antioxidant activity and TR content was also found. There was also evidence for an interaction effect for TPP with DPPH among plantations. Various reasons may be behind these differences such as climate and soil fertility. In terms of selecting high antioxidant teas, attention needs to be paid to plantation and the growing region.

This study indicates that the bioactive molecules present in black tea produced by *C. sinensis* can be used as a valuable antioxidant source. Further studies to elucidate the role of respective polyphenols in tea responsible for antioxidant activity are very promising.

## ACKNOWLEDGEMENT

This research was supported financially by Ceylon Tea Services PLC, Peliyagoda, Sri Lanka. Authors wish to thank Ms. Harshini Rajakulendran & Mr. Indika Palagasingha of Ceylon Tea Services PLC for their excellent assistance in this study.

## REFERENCES

Akio Kobayashi, Hisako Sato, Royoko Arikawa and Tei Yamanishi (1965), Flavour of Black tea, Journal of Agr. Biol. Chem , Vol.29,No.10, pp.902-907.

Alain Chaintreau (2000), Simultaneous distillation and extraction from birth to Maturity review, Journal of Flavour and Fragrance, vol. 16: 136–148

Anthony J. Wright and Michael J. fishwick (1978), Lipid Degradation During manufacture of black tea, Journal of Phytochemistry, Vol.18,pp, 1511-1513.

Biswas A.K., Biswas A.K., and Sarkar A.R, (1973), "Statistical Evaluation of the Biochemical Constituents and their Effects on Colour, Brightness and Strength of Black Teas", Journal of the Science of Food and Agriculture, Volume 24, Issue 12, pp. 1457–1477, December 1973

Chi‐Tang Ho, Jen‐Kun Lin, Fereidoon Shahidi (1944), Tea and tea products, chemistry and health-promoting properties, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

Davies K. J. Oxidative stress: the paradox of aerobic life Biochem. Soc. Symp. 61, 31, 1995

De Silva, Rasangika *Sri Lankawe te wagawe arambaya, Samantha publishers, 1st ed,* 2005, 27-30

Frenkel K., Chrzan K. Hydrogen peroxide formation and DNA base modification by tumor promoter-activated polymorphonuclear leukocytes Carcinogenesis 8, 455, 1987

Gupta, S., Saha, B., & Giri AK (2002). Comparative antimutagenic and anticlastogenic effects of green tea and black tea: a review. *Mutat. Res,* **512**(1), 37-65.

Gadow, V.A. Joubert, E., & Hansmann, C.F. (1997). Comparison of the antioxidant activity of rooibos with green, oolong, and black tea. *Food Chem*, **60**(1), 73-77.

Ghiselli A., Serafini M., Natella F., Scaccini C. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Rad. Biol. Med. 29, 1106, 2000

Halliwell B, Gutteridge J. M. C., Cross C. E. Free radicals, antioxidants and human disease: where are we now? J. Lab. Clin. Med. 119, 598, 1992

Halliwel, B. (1994). Free radicals, Antioxidants, and human disease: Curiosity, Cause, or Consequence? *Lancet,* **344**, 721-724.

Harbowy ME, Balentine DA (1997). Tea chemistry. Critical Rev. Food Sci. Nutrition. Volume 16, pg. 415-480

Hertog, M.G. Feeskens, E.J., Hollman, P.C., Katan, M.B., & Kromhout, D. (1993). Dietery antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet,* **342**, 1007-1011.

History of tea, http://en.wikipedia.org/wiki/History_of_tea_in_China, viewed 14th December 2012.

Jose A. B. Baptista, Joaquim F. da P Tavares and Rita C. B. Carvalho (1998), Comparison of catechins and aromas among different green teas using HPLC/SPME-GC, Journal of Food Research International, Vol. 31, No. 10, pp. 729±736.

Kinsella J. E., Frankel E., German B., Kanner J. Possible mechanism for the protective role of antioxidants in wine and plant foods Food Technol. 47, 85, 1993

Lakenbrink, C., Lapczynski, S., Maiwald, B., Engelhardt, U.H., 2000. Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. Journal of Agricultural and Food Chemistry 48, pg. 2848–2852

Leopold Jirovetz, Gerhard Buchbauer, Martin Benoit Ngassoum, Margit Geissler (2002), Aroma compound analysis of *Piper nigrum* and *Piper guineense* essential oils from Cameroon using solid-phase microextraction–gas chromatography, solid-phase microextraction–gas chromatography– mass spectrometry and olfactometry, Journal of Chromatography A, vol. 976 (2002) 265–275.

Liu, Y., Guo, Y., and Zheng, Z. (2003), Investigations on the variation of main chemical substances and the formation of white tea during manufacturing. J. Fujian Tea.

Li-Fei Wang a, Joo-Yeon Lee b, Jin-Oh Chung a, Joo-Hyun Baik a, Sung So a, Seung-Kook Park b(2006), Discrimination of teas with different degrees of fermentation by SPME–GC analysis of the characteristic volatile flavour compounds, Journal of Food Chemistry, vol. 109 (2008) 196–206.

Madsen, H.L., & bertelsen, G. (1995). Spices as antioxidants. *Trends Food Sci. Technol*, **6,** 271-277

McDowell, I., Feakes, J., Gay, C., 1991, Phenolic composition of black tea liquors as a means of predicting price and country of origin, Journal of the Science of Food and Agriculture, Volume 55, Issue 4, pp. 627–641

Modder, W.W.D., & Amarakoon, A.M.T. (2002a). The chemicals in tea shoots and their health effects, In *Tea & Health* (pp13). The Tea Research Institute in Sri Lanka.

Modder, W.W.D., & Amarakoon, A.M.T. (2002b). Tea & cancer, In *Tea & Health* (pp103-104). The Tea Research Institute in Sri Lanka.

Modder, W.W.D., & Amarakoon, A.M.T. (2002c). Antioxidant Activity, In Tea & Health (pp 61-64). The Tea Research Institute in Sri Lanka.

Muralidharan, D. (1997). Spectrophotometric analysis of catechins and condensed tannins using Ehrlichs reagent. Journal of the Society of Leather Technologists and Chemists, Volume 81, pg. 231–233

Nabavi, S.M., Ebrahimzadeh, M.A., Nabavi, S.F., & Jafari, M. (2008). Free radical scavenging activity and antioxidant capacity of *Eryngium caucasicum Trautv* and *Froripia subpinnata. Pharmacologyonline,* **3,** 19-25

N. Togari, A. Kobayashi' & T. Aishima (1995), Pattern recognition applied to gas chromatographic profiles of volatile components in three tea categories, Journal of Food Research International, Vol. 28, No. 5, pp. 495-502

Ninomiya M., Unten L., Kim M. Chemical and physicochemical properties of green tea polyphenols; in Chemistry and applications of green tea, ed. Yamamoto T., Juneja L. R., Chu D. C., Mujo K., CRC Press, New York pp 23-35, 1997

Obanda, M. and Owuor, P.O., 1992. Effect of wither and plucking on the biochemical and chemical parameters of selected Kenyan tea. Discov.Innov. 6, pp. 190–197.

Obanda, M., Owuor, P. O., & Taylor, S. J. (1997). Flavanol composition and caffeine content of green leaf as quality potential indicators of Kenyan black teas. Journal of the Science and Food Agriculture, Volume 74, pp. 209–215.

Obanda, M., Owuor, P.O. And Mang'oka, R. (2001), Changes in the chemical and sensory quality parameters of black tea due to variations of fermentation time and temperature, Food Chem, Volume 75, pg. 395–404

Oshima, Y., & Nakabayashi, T. (1953a). Studies on catechins and pigments in tea leaves. Part 5. Paper chromatography of tea flavones. Journal of Agricultural Chemistry, Japan, Volume 27, pg. 754–756.

Oshima, Y., & Nakabayashi, T. (1953b). Studies on catechins and pigments in tea leaves. Part 6. Isolation of tea flavones by column chromatography. Journal of Agricultural Chemistry, Japan, Volume 27, pg. 756–759.

Oshima, Y., & Nakabayashi, T. (1953c). Colorimetric estimation of gallic acid and flavonoid pigments in tea leaves. Journal of Agricultural Chemistry, Japan, Volume 26, pg. 377–381

Ostrowska J., Stankiewicz A., Skrzydlewska E. Antioxidative properties of green tea. Bromatol. Toxicol. Chem. 2, 131, 2001

Pourmorad, F., Hosseeinimehr, S.J. & Shahabimajd, N (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *Afr. J. Biotechnol,* **5,** 1142-1145

Renu Rawat, Ashu Gulati, G.D. Kiran Babu, Ruchi Acharya, Vijay K. Kaul, Bikram Singh (2006), Characterization of volatile components of Kangra orthodox black tea by gas chromatography-mass spectrometry, Journal of Food Chemistry, vol.105 (2007) 229–235.

R. L. Wickremasinghe, Emily L. Wick and Tei Yamanishi (1972),Gas chromatographic –mass spectroscopic analysis of "Flavory" and "non flavor" Ceylon Black tea aroma concentrates prepared by two different methods, Journal of Chromatography, vol. 79 (1973) 75-80

R.L. Wickremasinghe (1978), Monograph on Tea Production in Sri Lanka, Tea Research institute of Sri Lanka.

Robertson, A. (1992). The chemistry and biochemistry of black tea production – the non-volatiles. In: K.C. Willson (Ed.) & M.N. Clifford, Tea: Cultivation to consumption, (pg. 574-580). Kluwer Acad. Publ., Dordrecht, The Netherlands.

Roberts, E. A. H., (1956), The leuco-anthocyanins of unprocessed tea-leaf, Journal of the Science of Food and Agriculture, Volume 7, Issue 4, pages 253–257.

Roberts, E. A. H., Cartwright, R. A., & Oldschool, M. (1957), The phenolic substances of manufactured tea, Journal of the Science and Food Agriculture, Volume 8, pg. 72–80.

Roberts, E. A. H., and R. F. Smith (1961), Spectrophotometric measurements of theaflavins and thearubigins in black tea liquors in assessments of quality in teas, Analyst, Volume 86, Issue 1019, pg. 94-98

Robert R. Selvendran, John Reynolds and Terence Galliard (1977), Production of Volatiles By Degradation of Lipids During Manufacture Of Black Tea, Journal of Phytochemistry, Vol.17,pp,233

Roy Teranishi and Robert A. Flath (1981), Flavour Research (Resent advances), Western regional Research center, Agriculture Research Service, California

Ryoyasu Saijo (1967), Volatile Flavour of Black Tea, Journal of Agr. Biol. Chem., Vol.31, No.11, p.1265-1269.

Schlesier K., Harwat M., Bohm V., Bitsch R. Assessment of antioxidant activity by using different in vitro methods Free Radic. Res. 36, 177, 2002

Spiro Michael and William E. Price, Determination of theaflavins in tea solution using the flavognost complexation method, ***Analyst***, 1986,**111**, 331-33

Sundarajan, R., Haja, N., Venkatesan, K., Mukherjee, K., Saha, B., Bandyopadhyay, A., & Mukherjee, P. (2006). *Cytisus scoparius* link – Anatural antioxidant. BMC Complement. *Altern*. Med, **6,** 8-14.

Tei Yamanishi, Alio kobayashi, Hisako Sato, Akiko Ohmura and Hideko Nakamura (1965), Flavory of black tea, Journal of Agr. Biol. Chem, Vol.29,No.11, p.1016-1020.

Tei Yamanishi, Michiko Nose and Yoichi Nakatani (1970), Studies on the flavour of green tea, Journal of Agr. Biol. Chem, Vol. 34, No.4, p.599-608.

Tei yamanishi, Akio Kobayashi, Atsuko Uchida and Yoko Kawashima (1996), Studies on the flavour of green tea, Journal of Agr. Biol. Chem, Vol 30, No.11, p. 1102-1105.

Temple, C. M., & Clifford, M. N. 1997. The stability of theaflavins during HPLC analysis of a decaffeinated aqueous tea extract. Journal of Agricultural and Food Chemistry, 74, pp. 536–540.

The Chemistry of Tea, http://nobleharbor.com/tea/teachemistry.html, viewed 13th November 2012.

Velioglu, Y.S., Mazza., G, Gao, L., & Oomah, B.D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem*, **46**(24), 4113-4117.

Whitehead, D. L., and Temple, C. M., 1992. Rapid method for measuring thearubigins and theaflavins in black tea using C18 absorbent cartridges. Journal of the Science and Food Agriculture, 58, pp. 149–152

Wright, L.P., Mphangwe, N.I.K., Nyirenda, H.E. and Apostolides, Z., 2002. Analysis of the theaflavin composition in black tea (Camellia sinensis) for predicting the quality of tea produced in Central and Southern Africa, Journal of the Science of Food and Agriculture, 82, pp. 517-525.

Wilson, R.L. (1998). Free radicals and tissue damage, mechanistic evidence from radiation studies. In" Biochemical Mechanisms of Liver Injury, (pp.12), Academic Press, New York.

Worthy, W. (1991). Fruit, vegetables, green tea may cut cancer risk. *Chem. Eng*, **69**(37), 27-29

Yang Zhao, Pei Chen, Longze Lin, J.M. Harnly, Liangli (Lucy) Yu, Zhangwan Li (2010),Tentative identification, quantitation, and principal component analysis of green pu-erh, green, and white teas using UPLC/DAD/MS,Journal of Food Chemistry, vol. 126 (2011) 1269–1277

Yao, L. H., & Nursten, H. E. (1997). The chemical oxidation of individual (-)-epicatechin gallate and (-)-epigallocatechin gallate. Journal of Food and Fermentation and Industry, Volume 23, pg. 21–25.

Yao, L. H., & Nursten, H. E. (1998). Reversed-phase HPLC with photodiode array detection of the oxidation products from (-)- ECG, (-)-EGCG and black tea liquor. Journal of Food and Fermentation and Industry, Volume 24, pg. 11–16.

Yuerong Liang*, Jianliang Lu, Lingyun Zhang, Shan Wu, Ying Wu (2002), Estimation of black tea quality by analysis of chemical composition and colour difference of tea infusions, Journal of Food Chemistry, vol. 80 (2003) 283–290.

Yukihiko Hara(2001), Green tea, health benefits and applications, Tokyo Food Techno Co., Lt, Tokyo, Japan.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 49-53

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1108

# REAL TIME PCR ASSAY FOR THE DIFFERENTIATION OF MYCOBACTERIAL SPECIES IN BRONCHIAL WASHINGS

[1]T.P. Keerthirathne, [1*]D.N. Magana-Arachchi, [2]S.D.S.S. Sooriyapathirana and [3]R.M.D. Madegedara

*[1]National Institute of Fundamental Studies, Hantana road, Kandy, Sri Lanaka*
*[2]Department of Molecular Biology and Biotechnology, University of Peradeniya, Sri Lanka*
*[3]Respiratory Disease Treatment Unit & Teaching Hospital, Kandy, Sri Lanka*

**Abstract**

Pulmonary infections caused by Nontuberculous Mycobacteria (NTM) species has to be carefully interpreted due to their ubiquitous nature. NTM infections are more common than before in non-immunosuppressed hosts. Real-time PCR designed for *Mycobacterium* species, allows precise identification through melting point analysis. This study was designed for identification of *Mycobacterium* species present in bronchial washings. Ethical clearance was obtained from the Post-Graduate Institute of Science, University of Peradeniya. Bronchial washings (n=150) were collected from patients, suspected of having pulmonary diseases, attending the General Hospital Kandy. The samples were processed according to modified Petroff's method and inoculated onto Löwenstein–Jensen medium. Culture positives were subjected to Ziehl–Neelsen (ZN) staining, DNA were extracted from AFB isolates using the standard CTAB (N Cetyl-N, N, N-trimethyl ammonium bromide) method. SYBR green mediated real-time PCR assay was conducted to identify rapid and slow growers in two parallel reactions. Primers specific for *Mycobacterium* genus, *Mycobacterium tuberculosis* complex (MTC), *M. avium* complex (MAC), *M. chelonae- M. abscessus* group (MCAG) and *M. fortuitum* group (MFG) were used. Among the 26 AFB isolates 25 were found to be belonging to the *Mycobacterium* genus. Two MTC isolates and three MAC isolates were confirmed; following reaction I. Reaction II confirmed the presence of *Mycobacterium* genus and the presence of MCAG for two isolates. Application of SYBR green mediated real time PCR assay in clinical microbiology could improve the diagnostics due to the increased specificity. Moreover, it is a tool that can be used for the rapid detection of pathogenic NTM species.

**Keywords:** NTM, SYBR green, real-time PCR, AFB

## INTRODUCTION

The genus Mycobacterium includes non-tuberculous mycobacteria (NTM) apart from Mycobacterium tuberculosis (MTB) [1]. NTM are diverse, ubiquitous organisms that can found in the environment, including in water and soil. NTM organisms are well known for its infrequent infection on both immunocompetent and immunocompromised individuals [2]. Accurate detection and identification of NTM is important to the species level because patients with NTM infections show clinical signs that are similar to those of patients with tuberculosis (TB), causing clinical misleading during therapeutic actions [3]. Nevertheless, the presence of NTM in the patients with TB can have a considerable influence on clinical management because incorrect diagnosis of pulmonary TB due to the presence of NTM can lead to unsuitable and unnecessary treatment of patients with NTM infections [4] which could lead to the emergence of drug resistant Mycobacterium strains. Thus, it's important to rapidly distinguish NTM from Mycobacterium tuberculosis complex (MTC) to administer appropriate treatment.

Different methods are used for the identification of NTM in different regions of the world. Even though, most laboratories use conventional methods, improved molecular methods have been reported as having the ability to differentiate and identify more

Corresponding Author Email: *cellbiology@ifs.ac.lk

NTM species rapidly [5]. Espy M. J. *et al* (2006) states that "Real-time PCR provide equivalent sensitivity and specificity as conventional PCR combined with Southern blot analysis" [6]. Moreover, when compared with conventional PCR methods, as the nucleic acid amplification and detection steps are performed in the same closed system the possibility for contamination by the release of the amplicon is neglectful. Speedy detection is also an added advantage in the use of real-time PCR technique. The objective of the study was to identify the Mycobacterium species present in bronchial washings using SYBR Green mediated real time PCR.

## METHODOLOGY

Ethical clearance was obtained from the Postgraduate Institute of Science, University of Peradeniya, Sri Lanka. Bronchial washings (n=150) from the patients suspected of having pulmonary infections, were obtained from the General Hospital Kandy, Sri Lanka. The samples were liquefied and decontaminated with 4% NaOH according to the modified Petroff's method and was inoculated on to Löwenstein–Jensen medium (L-J medium), LJ media containing Thiophene – 2 carboxylic acid hydrazide (TCH) and p-nitrobenzioc acid (PNB) and incubated at 37 $^0$C and at 28 $^0$C respectively. Inoculated PNB containing cultured media were incubated under light and dark conditions. The inoculated culture tubes were incubated for 8 to 12 weeks. When the growth was detected, Ziehl–Neelsen stain (ZN stain) was carried out in order to confirm the presence of acid fast organisms. DNA was extracted from confirmed acid fast bacilli (AFB) positive isolates according to the standard CTAB (N-Cetyl-N, N, N-trimethyl ammonium bromide) method [7].

Primers targeting the internal transcribed spacers (ITs) of MTC and MCAG (*M. chelonae-M. abscessus* group), the 16S rRNA genes of MAC (*M. avium* complex), MFG (*M. fortuitum* group) and *Mycobacterium* genus were used for the real time multiplex, PCR assay. Amplification was monitored by the measurement of the SYBR Green fluorescence. Subsequent to the cycling process melting curves were generated by inclining the temperature from 60 $^0$C to 95 $^0$C at 0.2 $^0$C/s [8].

The real time multiplex, PCR assay was conducted in two separate reactions where primers specific for MTC and MAC were in reaction I and primers specific for MCAG and MFG were in reaction II which helped in identifying slow and rapid growers respectively (Table 01). Primers targeting AFB genus were included in both the reactions. Each reaction was carried out in a 25 µl volume which contained 2.0 µl of 25 mM $MgCl_2$, 0.25 µl of 5 u / µl Taq polymerase, 5.0 µl of 5X PCR Buffer, 2.5 µl of 1mM dNTP mix and 1.0 µl 0f each primer (10µm). The PCR amplification process was initiated by ramping the temperature at 95 $^0$C for 5 minutes followed by 40 cycles of the amplification process (95 $^0$C for 15s, 60 $^0$C for 30s and 72 $^0$C for 30s).

*Table 1: Primer sequences which were used for the study*

| Organism | Target region | Primer sequence | Reaction |
|---|---|---|---|
| MAC | 16S | **F:**CCTCAAGACGCATGTCTTC<br>**F:**GACCTTTAGRCGCATGTCTTT<br>**R:** ACCTACCGTCAATCCGAGAA | I |
| MTC | ITS | **F:** GCGAGAGCCGGGTGCATG<br>**R:** AACAGTGTGTTGGTGGCCAA | I |
| *Mycobacterium* genus | 16S | **F:** CCGCAAGRCTAAAACTCAAA<br>**R:** TGCACACAGGCCACAAGGGA | I / II |
| MCAG | 16S | **F:** TAAGGAGCACCATTTCCCAG<br>**R:** CGACGTTTTGCCGACTAACC | II |

| | | | |
|---|---|---|---|
| MFG | ITS | **F:** CCACGCGCTTCATGGTGT<br>**F:** CCGCGCTCTTCATGGGGT<br>**F:** ACCACGCAATTTCATGGTGT<br>**R:**ACTTGCGCTTCGTCCCTAT | II |

## RESULTS

Following the sample processing and culture, 55 samples yielded positive cultures. However, only 26 were AFB positive. The SYBR green mediated real time multiplex PCR assay confirmed the presence of *Mycobacterium* genus in 25 AFB positive isolates. Reaction I of the real time multiplex PCR assay confirmed the presence of AFB genus (n=25) and two MTC (n=2) isolates (Figure 01) and three MAC (n=3) isolates. Reaction II also confirmed the presence of genus *Mycobacterium* in addition to the presence of MCAG (Figure 02) in two samples (n=2) (Table 02).

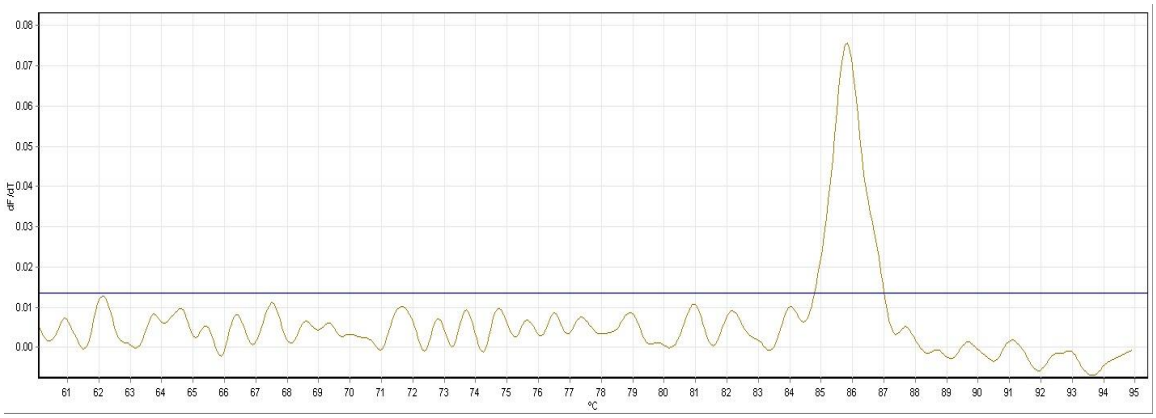



*Figure 01: Melting curve generated following the SYBR Green mediated real-time PCR reaction I indication the presence of MTC (Tm= 85.8 0C)*

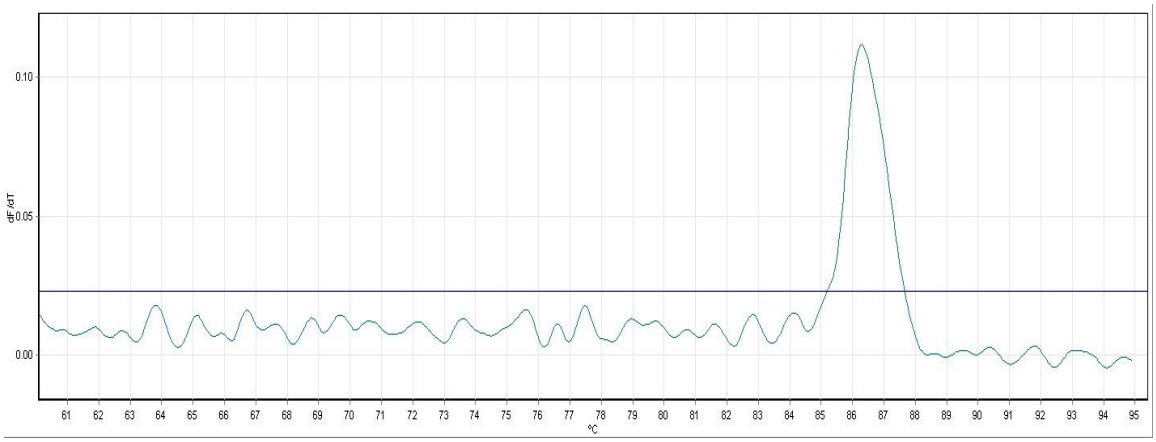



*Figure 02: Melting curve generated following the SYBR Green mediated real-time PCR reaction II indication the presence of MCAG (Tm= 86.3 0C)*

*Table 02: Number of Mycobacterium isolates identified during the study*

| Organism | Reaction | Number of Isolates identified |
|---|---|---|
| *Mycobacterium* genus | I / II | 25 |
| MTC | I | 2 |
| MAC | I | 3 |
| MCAG | II | 2 |

## DISCUSSION

NTM infections are an increasing public health problem in many countries in the world. Limited laboratory facilities and poverty, which may in some cases, lead to unhygienic life style, are significant challenges for infection control approaches in most of the developing countries. Therefore, understanding the trends and true prevalence of NTM is important in controlling the NTM s well as MTB infections.

The early classification of Mycobacteria was based on growth rate, pigmentation, and clinical significance [9]. Mycobacteria can be broadly classified as rapid growers and slow growers [10] Fast growers commonly have two identical copies of the 16S rRNA gene, whereas slow growers are thought to have only one [11]. The isolates identified throughout this study there were 23 Rapid growers while there were only two (n=2) slow growers.

Throughout this study a considerable proportion of the isolates were identified as NTM, which were identified as slender pink rods upon ZN staining. There are incidences reported, where *M. celatum* isolates were isolated from bronchial washings [12] which is a potential human pathogen [13]. The isolate which was identified upon 16S rRNA sequencing, produced smooth, convex, non-pigmented colonies on L-J medium. Even though this species is identified as slow growing the *M. celatum* isolate identified during this study was found to be rapidly growing which formed colonies on L-J medium in less than 7 days. Increase in incidence of MAC was reported in early 1980's simultaneously with the beginning of the AIDS widespread [14]. During this study three isolates were identified as MAC which was appeared as smooth, opaque colonies. It is believed that the antimicrobial resistance of the MAC is because of the lack of drug penetration due to the complex cell wall structure [15]. Three isolates were also identified as MCAG following reaction II of real time PCR which were also rapidly growing.

In conclusion, Real time PCR assay is a method of excellent sensitivity and specificity. Moreover, the low contamination risk has made real-time PCR technology appealing to be used in the clinical microbiology for diagnostics.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge the National Research Council, Sri Lanka, grant number 11-059 for providing the equipment, Real-Time PCR machine. The corresponding author for the paper, D. N. Magana-Arachchi can be contacted via email at cellbio@ifs.ac.lk .

## REFERENCES

1. Tortoli, E., Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev, 2003. 16(2): p. 319-54.

2. Ley, S., et al., Non-tuberculous mycobacteria: baseline data from three sites in Papua New Guinea, 2010-2012. Western Pac Surveill Response J, 2015. 6(4): p. 24-9.

3. Hong, Y.J., et al., Usefulness of three-channel multiplex real-time PCR and melting curve analysis for simultaneous detection and identification of the Mycobacterium tuberculosis complex and nontuberculous mycobacteria. Journal of clinical microbiology, 2011. 49(11): p. 3963-3966.

4. Maiga, M., et al., Failure to recognize nontuberculous mycobacteria leads to misdiagnosis of chronic pulmonary tuberculosis. PLoS One, 2012. 7(5): p. e36902.

5. Dvorska, L., et al., Strategies for differentiation, identification and typing of medically important species of mycobacteria by molecular methods. VETERINARNI MEDICINA-PRAHA-, 2001. 46(11/12): p. 309-328.

6. Espy, M., et al., Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clinical microbiology reviews, 2006. 19(1): p. 165-256.

7. Somerville, W., et al., Extraction of Mycobacterium tuberculosis DNA: a question of containment. J Clin Microbiol, 2005. 43(6): p. 2996-7.

8. Richardson, E.T., D. Samson, and N. Banaei, Rapid Identification of Mycobacterium tuberculosis and nontuberculous mycobacteria by multiplex, real-time PCR. J Clin Microbiol, 2009. 47(5): p. 1497-502.

9. Runyon, E.H., Anonymous mycobacteria in pulmonary disease. The Medical clinics of North America, 1959. 43(1): p. 273-290.

10. García-Agudo, L. and P. García-Martos, Clinical significance and antimicrobial susceptibility of rapidly

growing mycobacteria. Science against microbial pathogens: communicating current research and technological advances, 2011. 77.

11. Reischl, U., et al., 16S rRNA sequence diversity in Mycobacterium celatum strains caused by presence of two different copies of 16S rRNA gene. Journal of clinical microbiology, 1998. 36(6): p. 1761-1764.

12. Couto, I., et al., Identification of nontuberculous mycobacteria in clinical samples using molecular methods: a 3-year study. Clinical Microbiology and Infection, 2010. 16(8): p. 1161-1164.

13. Roxo, E., Philip Suffys, Adalgiza da Silva Rocha and Diogo dos Santos Netto (Fun.

14. Cespedes, M.S. and J.A. Aberg, Mycobacterium avium Complex (MAC).

15. Inderlied, C., C. Kemper, and L. Bermudez, The Mycobacterium avium complex. Clinical microbiology reviews, 1993. 6(3): p. 266.

Proceedings of 1st International Conference on Bioscience and Biotechnology, Vol. 1, 2016, pp. 54-67

ISSN: 2513–2695 online
DOI: 10.17501/biotech.2016.1109

# INVESTIGATION OF METHYL TETRAHYDRO-FOLATE REDUCTASE C677T POLYMORPHISM IN A SAMPLE OF PATIENTS IN A HOSPITAL BASED DIAGNOSTIC SETTING IN SRI LANKA

Varnakulasingham, H[1*] and Manamperi, A[2]

[1]*University of Wolverhampton*
[2]*University of Kelaniya*

**Abstract**

The present study was conducted to identify the prevalence of MTHFR gene mutation in a group of Sri Lankan patients seeking routine diagnostic testing for the C677T mutation through PCR followed by RFLP diagnostic methods. Human MTHFR gene codes for the production of MTHFR enzyme in humans which plays major role in homocysteine and folate metabolism in humans. The mutation in MTHFR gene at nucleotide position 677 has two possibilities: C (cytosine, which occurs in the wild type gene) or T (thymine, which occurs in the mutated gene).In C677T variant, modification from C to T gives rise to the replacement of alanine by valine residue in protein at 222 aa position. Eight serum samples were collected from the MTHFR mutation suspected patients. Genomic DNA extraction was done and extracted DNA was subjected to PCR amplification. PCR products were subjected to RFLP assay with overnight digestion. The substitution of "C" to "T" at 677 position of MTHFR gene creates a restriction site for the enzyme Hind111. RFLP products were subjected to gel electrophoresis and further analysis. The results obtained shows that, of the 8 samples tested, 2 had 677CT genotype, 1 had 677TT genotype whereas the remaining 5 had 677CC wild type. Homozygous 677TT genotype leads to lower MTHFR activity and hyperhomocysteinaemia, which can cause defects in DNA repair. However, as this is a small sample of the population, it is recommended to carry out studies with a large sample volume to ascertain the prevalence of this mutation in the community.

**Keywords:** Methylene tetrahydrofolate reductase (MTHFR) gene mutation (C677T variant), Genotype, Hyperhomocysteinaemia, DNA repair, Restriction fragment length polymorphisms (RFLP), Polymerase chain reaction (PCR), Hind111.

## INTRODUCTION

Methylene tetrahydrofolate reductase (MTHFR) is one of the most important enzymes in human body. Human MTHFR gene codes for the production of methylene tetrahydrofolate reductase (MTHFR) enzyme in humans. The 5, 10-methylene tetrahydrofolate reductase (MTHFR) enzyme catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate and a co substrate for homocysteine remethylation to methionine (Curtin et al., 2004). Folate is known as Vitamin B9 and it is required for the, Synthesis of DNA, RNA and SAMe, Single carbon metabolism or methylation, Amino acid metabolism (for neurotransmitter, serotonin, dopamine and norepinephrine production and detoxification), Formation and maturation of RBC (red blood cells), WBC (white blood cells) and platelet production and Essential for detoxification of homocysteine. For all these functions to happen with in the body, it needs to convert folate to active 5-MTHF (5-methyltetrahydrofolate) with the help of co-factors (Leclerc, Sibani and Rozen, 2000).

The MTHFR gene is located on chromosome 1 at P arm p36.3 in humans. There are several DNA sequence variants are associated with this gene. In 2012 a report state that over 40 point mutations of

Corresponding Author Email: hesika1993@gmail.com

this gene have been identified (Crider et al., 2012).There are two commonly recognized polymorphic variants in the gene encoding for this enzyme: They are C677T, and A1286C variant. Both are missense changes which can cause decrease enzyme activity. Reduced enzyme activity of MTHFR is a genetic risk factor for hyperhomocysteinemia, especially in the presence of low serum folate levels. Elevated Homocysteine level in blood is associated with various diseases including cardiovascular diseases, stroke, neural tube defects, Spina bifida, High blood pressure, Glaucoma, certain types of cancer. (Eg. colorectal cancer, breast cancer, lung cancer etc.) and type 2 diabetes mellitus. Mild to moderate hyperhomocysteinemia has been identified as a risk factor for several diseases like cancers and cardiovascular diseases (Curtin et al., 2004).

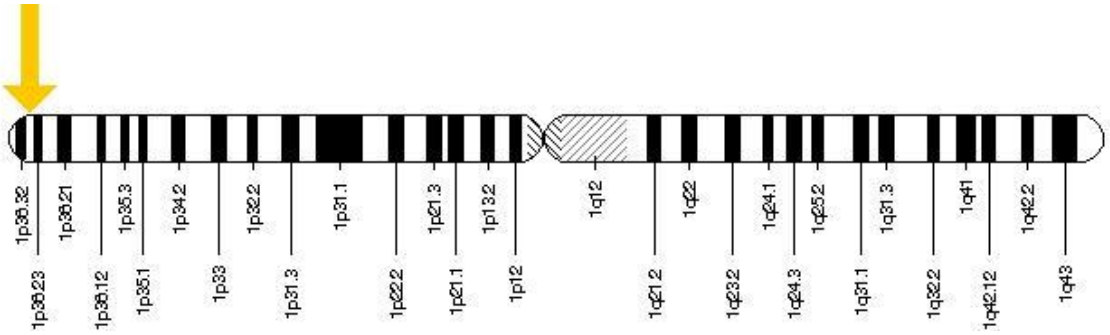



*Figure 01 - The MTHFR gene is located on chromosome 1 (Albers et al., 2005).*

According to this research mutation in the 677 base pair position of the MTHFR gene has been investigated. The mutation in MTHFR gene at nucleotide position 677 has two possibilities: C (cytosine, which occurs in the wild type gene) or T (thymine, which occurs in the mutated gene).In the C677T variant the nucleotide position at 677 is modified from C to T and this gives rise to the replacement of alanine by valine residue in the protein at 222 amino acid position (Kirke, 2004). Individuals with two copies of 677CC or 677TT have wild type or mutated genes respectively in both homologous chromosomes. Some individuals are heterozygous for this mutation where one homolog carries C residue and the other homolog carries T residue (Wang et al., 2012). Homozygous TT genotype is considered as harmful because it is associated with high concentration of total plasma Homocysteine level and lower MTHFR enzyme activity of 30%, which can cause defects in DNA repair system in the enzyme. On the other hand, 677CT genotype has MTHFR enzyme activity of about 65% when compared to wild type (Curtin et al., 2004).

**Biochemical Mechanisms of Folate and Homocysteine Cycles**

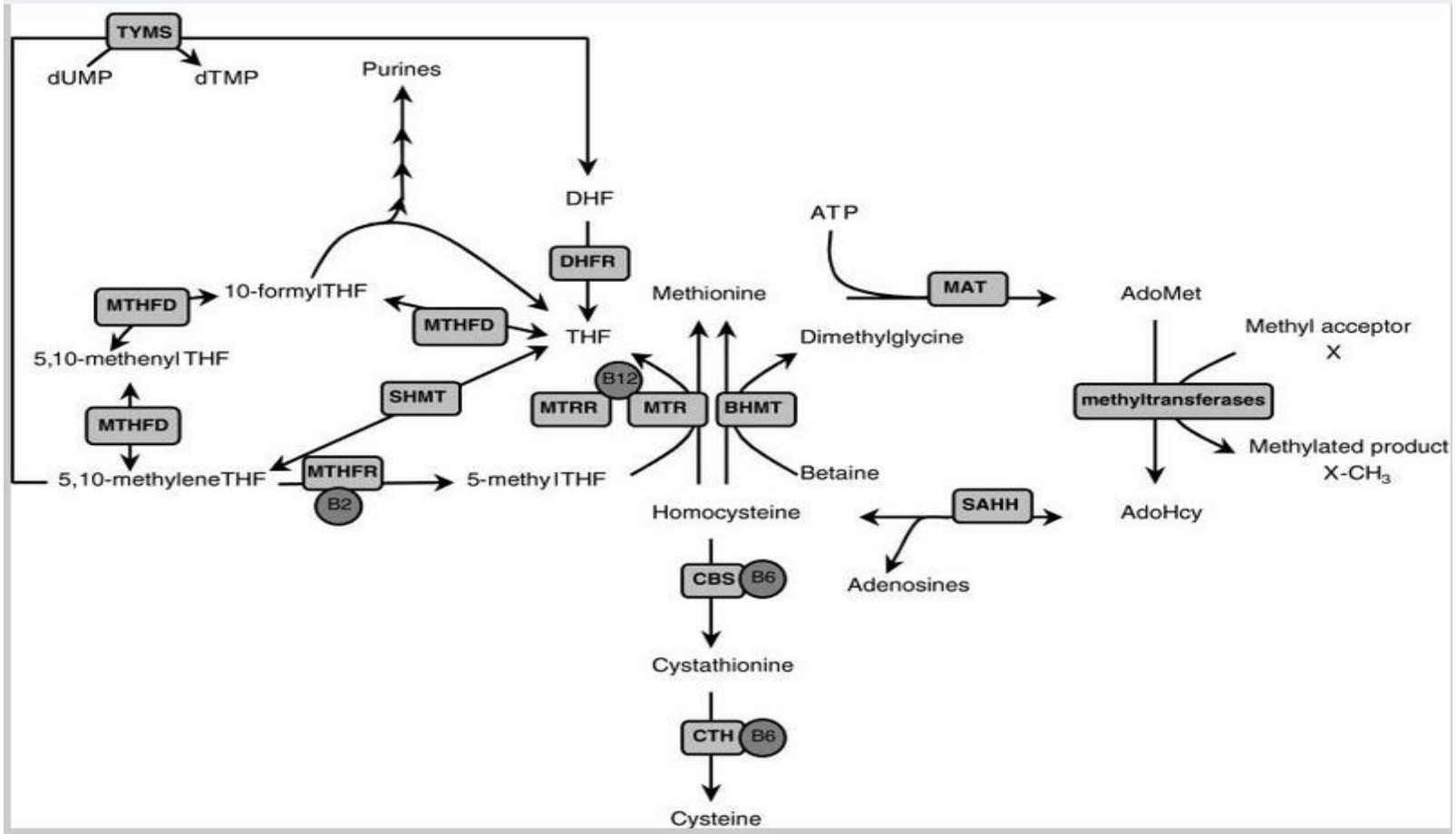



*Figure – 02 Schematic representation of the folate cycles and homocysteine metabolism (Blom and Smulders, 2011).*

**Folate cycle**

5-methylTHF functions as a methyl donor for homocysteine remethylation. The resulting THF can directly be converted into 5, 10-methyleneTHF by the action of serine hydroxymethyltransferase (SHMT). SHMT is a vitamin B6-dependent enzyme that uses serine as a one-carbon donor. Conversion of THF into 5, 10-methyleneTHF, via 10-formylTHF and 5, 10-methenylTHF, is catalyzed by the trifunctional enzyme methylenetetrahydrofolate dehydrogenase (MTHFD) that has formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase activities (Blom et al., 2009). 5, 10-methylenetetrahydrofolate can be used as a cofactor for the conversion of deoxyuridine monophosphate (dUMP) into deoxythimidine monophosphate (dTMP). This reaction is catalyzed by the enzyme thymidylate synthase (TYMS) and produces dihydrofolate (DHF), which can induces reduction back to THF by the action of dihydrofolate reductase (DHFR). In addition to being a cosubstrate for dTMP synthesis, 5, 10-methyleneTHF can also be reduced to 5-methylTHF by the riboflavin (vitamin B2)-dependent enzyme methylenetetrahydrofolate reductase (MTHFR). MTHFR enzyme has a great importance in the regulation of 5-methylTHF level for homocysteine remethylation (Forges et al., 2007).

**Homocysteine metabolism**

Homocysteine is a sulphur containg amino acid that is formed by the demethylation of the essential amino acid methionine through S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). Methionine adenosyltransferase (MAT) catalyse the formation of s-adenosylmethionine (AdoMet), which acts as the methyl donor in many methylation reactions such as methylation of DNA, RNA, hormones and lipids (Forges *et al*., 2007).The transmethylation of S-adenosylmethionine (AdoMet) by methyltransferase (MT) form S-adenosylhomocysteine (AdoHcy) which is an inhibitor of many methyltransferases. Hydrolysis of S-adenocsylhomocystein (SAH) by S-adenosylhomocystein hydrolase (SAHH) forms adenosine and homocysteine. The resulting homocysteine can be further metabolized in two pathways: transsulpuration pathway or remethilation pathway. In trassulpurination pathway, homocysteine is irreversibly degraded to cystathionine and cysteine. In remethilation pathway, it is remethylated to methionine (Blom *et al*., 2009).

**Remethylation**

Homocysteine can be re-methylated to methionine by two different pathways.

***Methionine synthase pathway***

Homocysteine (Hcy) is remethylated into methionine (Met) by MTR with 5-methylTHF as a methyl donor and cobalamin (B12) as a co-enzyme. 5-MethylTHF is produced by the FAD-dependent enzyme 5, 10-Methylene tetrahydrofolate reductase (Leclerc, Sibani and Rozen, 2000). 5, 10-MethyleneTHF is also a one-carbon donor in the synthesis of thymidylate and after conversion into 5,10-methenyltetrahydrofolate (MethenylTHF) and further into 10-formyltetrahydrofolate (FormylTHF), in the synthesis of purines. After the release of one-carbon all of these substituted folates are converted to THF which is finally recycled into MethyleneTHF during the conversion of serine to glycine by the enzyme serine hydroxymethyltransferase (SHMT). Met is further transformed into SAM. After release of the methyl group, SAM is converted into SAHH (S-adenosylhomocysteine hydrolase), which in turn hydrolyzed into Hcy (Crider et al., 2012).

***Betaine homocysteine methyltransferase (BHMT) pathway***

The alternative pathway by which Hcy is remethylated into Met takes place in the liver and uses betaine as a methyl donor; this reaction is catalyzed by the enzyme betaine homocysteine methyltransferase (BHMT) (Forges *et al*., 2007).

According to this mechanism 5, 10-methylene tetrahydrofolate reductase (MTHFR) enzyme catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. During the absence of MTHFR due to C677T mutation 5-Methyl tetrahydro folate not produced. Along with that remethylation pathway also stopped because 5-Methyl tetrahydro folate acts as a factor in remethylation pathway. Therefore blood homocysteine level get elevated and cause hyperhomocysteneamia in the patients with this mutation.

**Factots That Can Cause Hyperhomocystenaemia**

The cause of hyperhomocysteinaemia is multifactorial. In addition to genetic factors, a number of environmental factors also involve in elevated homocysteine concentrations. They are

***Age and sex***

Increasing age and male gender are strongly associated with increased homocysteine concentrations (Dedoussis *et al*., 2005). The gender difference of homocysteine concentration is attributed to many factors.

***Lifestyle factors***

Coffee consumption is independently associated with increased homocysteine concentration while association with smoking is dose dependent (Chrysohoou et al., 2004). The association between alcohol consumption and homocysteine concentration appears to be J shaped in both sexes. Therefore low intake of alcohol (1-2 units) is associated with lower homocysteine concentration resulting protective effect on cardiovascular mortality (Crider et al., 2012). High body mass index (BMI) too is associated with lower homocysteine levels (Blom and Smulders, 2011).

***Other factors***

Several drugs, such as antiepileptic drugs (Phenobarbitol, Valproate, Phenytoin etc), Methotrexate or lipid-lowering drugs (Cholestyramine, Fibric acid derivatives), Diuretics, Nitrous oxide, Estrogen-containing oral contraceptives, Metformin, Niacin, Theophylline and Sulfasalazine also can be increase plasma homocysteine level (Karabacak et al., 2014).

***Dietary factors***

Plasma homocysteine levels are regulated by several factors. Among the nutritional factors, deficiencies of vitamins B6, B12, and folate are associated with elevated plasma homocysteine concentrations. Supplementation with folic acid alone and in combination with vitamins B6 and B12 reduces the plasma homocysteine concentration (Yasui *et al*., 2000). The meta-analysis of the size of reduction of homocysteine concentration achieved by different doses of folic acid with or without vitamin B 12 and B6 in clinical trials. Both their first and second analyses concluded that addition of vitamin B12 to Folic acid reduced 25% of blood homocysteine level, while no significant effect was observed with the addition of vitamine B6 (Blom and Smulders, 2011).

Even though the relative importance of the environmental factors is not known, they exert their effects on homocysteine metabolism which is primarily determined by genetic composition of the individual. The genetic factors are more prominent in the young than in the old age group because cumulative effects of the environmental factors take time to modify the phenotype of the individual (Micheal *et al*., 2009).

**Genetics of Homocysteine**

A large number of studies had been conducted over the last several decades implicating genetic aetiology of homocysteine. One comprehensive analysis of studies conducted up to 2004 has identified 135 genes are related to homocysteine metabolism (Sharma *et al*., 2006).These genes are classified in to two groups; Genes that are involved in modulating homocysteine levels and Genes that are modulated by elevated homocysteine levels. Defects of these genes, primarily due to single nucleotide polymorphism (SNP) alter the homocysteine metabolism, often resulting elevated homocysteine levels. They include not only the genes that code for the enzymes involved in homocysteine metabolism, but also genes that code for the enzymes involved in metabolism of cofactors (vitamin B6 and vitamin B12) or co substrates which are needed for homocysteine homeostasis (Kluijtmans *et al*., 2003).

Homocysteine modulate these genes either by altering their methylation status or by unknown mechanisms. Defects of these genes cause variable diseases. Most of these gene products are enzymes involved in homocysteine metabolism. Rare mutations of these genes cause severe hyperhomocysteinaemia with characteristic clinical features. Even though any types of mutations are possible in these genes theoretically, single and repeat nucleotide polymorphisms are the commonest genetic variants reported by the researchers (Brulhart *et al*., 1997).

**Clinical Signs and Symptoms**

There are no symptoms at all in MTHFR gene mutations, but they can cause severe irreversible health conditions. Environmental poisoning can increase when not enough Homocysteine gets converted into Glutathione. Glutathione is most powerful antioxidant and responsible for detoxifying the body from the environmental heavy metals. Some symptoms of this can be: nausea, diarrhoea, abdominal pain, liver and kidney dysfunction, hypertension, tachycardia, pulmonary fibrosis, asthma, immune problems, hair loss and rashes (Ueland et al., 2001).

Central Nervous System disorders can be caused by homocysteine not getting converted into SAMe. SAMe is responsible for Serotonin, Dopamine, and Norepinephrine (neurotransmitters responsible for mood and motivation) production. Cardiovascular problems often occur when Homocysteine levels in the body are too high. Heart attack, Stroke, Blood clots, Peripheral neuropathy, Anemia even Miscarriages and Congenital birth defects can be related to this issue among others. These problems are typically more related to the C677T gene mutation (Wilcken, 2003).

**Diagnosis**

In blood circulation 80-90% of homocysteine molecules are protein bounded, 10-20% of the total homocysteine is present as homocysteine-cysteine mixed disulfide and homocystine dimer and less than 1% remaining is present as a free reduced form (Brustolin, Giugliani and Félix, 2010). Blood homocysteine level can be measured through a routine blood test. Before the collection of blood sample fasting is probably not necessary, as short-term dietary factors will not affect the test results (van der Griend *et al*., 1998).

Methionine-load test is the other one used to measure the homocysteine level. Homocysteine level is measured before and after the intake of 100 mg/kg of methionine dissolved in orange juice (Varga et al., 2005). This test is commonly used to diagnose abnormal homocysteine metabolism in people who have a high risk for cardiovascular disease with normal baseline homocysteine levels (Suliman, 2001). There are fairly variable classifications for what can be considered as an elevated homocysteine level. Typically, homocysteine level less than 13 μmol/L is considered as normal level and level between 13 μmol/L and 60 μmol/L is moderately elevated, and a value greater than 60 μmol/L to 100 μmol/L is strictly elevated (Varga *et al*., 2005). These are serum level diagnosis. The above tests are based on looking at biochemical markers to evaluate serum homocystein level. However, to analyse the genetic polymorphism of the MTHFR gene flanking C677T mutation, and several molecular based tests can be employed.

Several techniques have been developed in different countries for the immediate detection of this mutation with high throughput and in narrowed period of time. Those techniques are including PCR, Fluorescence scanning, MS-PCR, Allele specific PCR, and microarray technology. These techniques are fast and strong but in other hand they are highly expensive. For cost effective and easy screening of large number of samples PCR-RFLP has also been developed (Koksal *et al*., 2006). Restriction fragment length polymorphism (RFLP) analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. RFLP can be used to trace inheritance patterns, identify specific mutations, and for other molecular genetic techniques (Samtani *et al*., 2013). Even though the southern blotting technique is accurate, PCR PFLP is used in this project. In disease diagnostic purposes it is important to provide the results with in a particular time therefore PCR RFLP is more preferable than the southern blotting (Samtani *et al*., 2013).

**Treatment**

Usually, those who with C677T mutations have decreased ability to produce methylfolate, increased cardiovascular risk, increased risk of blood clots, increased pain and inflammation and increased chemical sensitivity. Therapeutic measures are used to increasing the body's inherent ability to restore function and to reduce these adverse effects (Crider *et al*., 2012).Basic goal of this treatment is to lower the level of homocysteine to normal. Treatment may consist of giving supplements of folic acid, vitamin B-12 and vitamin B-6. It also may include anticoagulant medications such as aspirin, clopidogrel, heparin, low-molecular weight heparin, or warfarin, to prevent the formation of blood clots.

Patients with the severe form of hyperhomocysteinemia are often treated with high doses of vitamin B-6 or betaine, and the amount of methionine consumed in the diet may be restricted (Yasui *et al*., 2000).

Now a day MTHFR mutations can be easily diagnosed in the laboratories. Person who have one or more of the gene mutations can supplement with methyl-folate and methyl B12 (active forms of these B vitamins). They can be supplement with liposomal or acetyl-glutathione. Glutathione drugs are poorly absorbed so either the liposomal form or a precursor, called as n-acetylcysteine (NAC) can be used (Ueland *et al*., 2001). There are other medicines also, that contain methyl-folate: Deplin, MetanX, CerefolinNAC are a few. Methyl B12 can also be given as shots, nasal sprays, and sublingually. The intramuscular shots are the most effective method (Varga *et al*., 2005).

**Prevalence**

Prevalence studies of MTHFR C677T polymorphism in Sri Lankan populations are unexpectedly sparse even though several researchers proving that hyperhomecysteinaemia were significantly associated with ischaemic heart disease in Sri Lankans. Particular study reported that, a lower prevalence for T allele (4.5%) frequency after studying 67 Sri Lankans. This percentage is an unexpected finding since other Asian populations have higher values (Mendis et al., 2002). Another wide-ranging study involving 80 Sinhalese, 80 Tamils and 80 Moors reported prevalence figures of 13%, 9% and 9% respectively for each ethnic group. Still these values can be considered as comparatively low and this study reveals that the highest prevalence of MTHFR polymorphism is among Sri Lankan Sinhalese compared to Tamils and Moors (Dissanayake et al., 2009). Similar study on Tamils in southern India also reported lower prevalence of (10.4%) (Angeline et at., 2004).

Genetic polymorphism is a population reliant on phenomenon. Researchers have reported varying frequency of MTHFR C677 T polymorphism in every population they tested. The T allele frequency is higher in European and Asian populations compared to Africans and Americans while the least prevalence was reported in Africa. They reached the conclusion after studying 881 unrelated individuals from 16 worldwide populations. In fact they attributed the higher prevalence of myocardial infarction and neural tube defects in Europeans than in Africans to the variation of the T allele frequency in the two populations (Schneider et al., 1998).Their findings were complemented by analysing the polymorphism in over 7000 new-borns from 16 different areas in the world. They concluded that the variation of polymorphism depend not only on racial or ethnic background, but also the geographical location of the study population too (Wilcken et al., 2003).

**OBJECTIVES**

**General objective-**

To identify the prevalence of methylenetetrahydrofolatereductase (MTHFR) gene mutation in a group of Sri Lankan patients and seeking routine diagnostic testing for the C677T mutation through the PCR based diagnostic methods

**Specific objective -**

To identify the prevalence of mutation in sample population.

To establish PCR for MTHFR gene flanking C677T mutation.

To establish Restriction fragment length polymorphisms (RFLP).

**METHODOLOGY**

**Extraction of genomic DNA from human blood**

Eight samples were used and DNA was extracted using ceygen column based extraction kit (Ceygen biotech, Colombo) according to manufacturer's instructions.

In a 1.5 ml micro centrifuge tube 200 µl of Binding Buffer (BB) and 40 µl of Protenase K were added to 200 µl of blood. Mixture was vortexed and incubated at $56^0$C for 10 minutes. Tube was centrifuged to remove any drops from the inside of the lid and 100 µl of Isopropanol was added. Then the mixture was vortexed. Mixture was transferred to a GenospinG™ column in a collection tube and Centrifuge at 8000rpm for 1 minute. Flow through liquid was

discarded from the collection tube. 500 µl of Inhibition Removal Buffer (IRB) was added to the column and centrifuged at 8000 rpm for 1 minute. Then flow through liquid was discarded. 500µl of Wash Buffer (WB) was added to the column, centrifuged at 8000 rpm for 1 minute and then flow through liquid was discarded. This step was done twice. Empty column was centrifuged at 13000 rpm for 10 seconds. The GenoSpin G™ was placed in microcenrtifuge tubes and 50 µl of Elution Buffer (EB) was added. It was kept at room temperature for 5 minutes and then centrifuged at 8000 rpm for 1 minute. Then the GenoSpin G™ column was discarded and the extracted DNA was kept at -20°C until further analysis.

**Polymerase chain reaction (PCR) amplification procedure of extracted DNA**

*Table – 01: PCR reagents*

| Reagents | Volumes (µl) | Volumes (µl) x10 |
|---|---|---|
| PCR water | 7.5 | 75 |
| Ceygen master mix | 10.0 | 100 |
| MTHFR 1primer (10µM) | 1.0 | 10 |
| MTHFR 2primer (10µM) | 1.0 | 10 |
| Tag polymerase 5U/µl | 0.5 | 05 |
| Template DNA | 5.0 | - |
| Total | 25.0 | 200 |

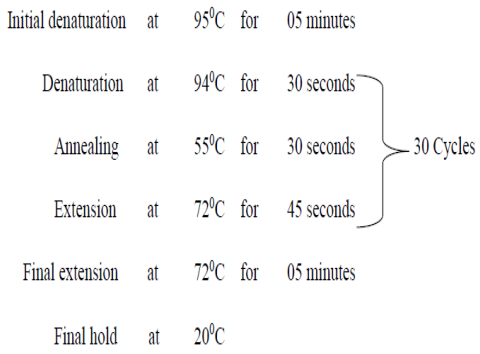
200 µl of PCR mix was prepared with reagents mentioned in the table 01. It was aliquot into 10 PCR tubes to prepare positive control, negative control and 8 samples. Each PCR tube contained 20 µl of PCR master mix and then 5 µl of DNA template and PCR water or known positive DNA in test samples, negative control and positive control respectively.

MTHFR C677T amplification was performed with qualitative polymerase chain reaction. Extracted DNA was amplified by PCR using primer pair MT-1 and MT-2. The sequences of the primers are shown below. These specific primers flank the polymorphism spanning a 223 base pair region of the MTHFR gene.

*Table – 02 MT-1 and MT-2 Primer sequences (Leclerc, Sibani and Rozen, 2000).*

| Primer | Sequence |
|---|---|
| MT- 1 | 5'-TTGAGGCTGACCTGAAGCACTTG -3' |
| MT- 2 | 5'-AGGACGGTGCGGTGAGAGTG-3' |

***Cyclic parameters for MTHFR PCR programme***




**RFLP assay for PCR products**

Before the establishment of RFLP PCR products were run under the gel electrophoresis to confirm the presence of PCR bands.140 µl of restriction digestion mix was prepared with reagents mentioned in the table 03. For restriction digestion, each tube contained 14µl of the restriction digestion mixture and 1 µl of known positive PCR product and the negative control vial contained 1 µl of water. Samples were kept for overnight incubation at 37°C and then kept for 20 minutes in 80°C to inactivate Hind 111 enzyme.

*Table- 03: Restriction digestion of PCR product*

| Reagents | Volumes (µl) | Volumes (µl) x10 |
|---|---|---|
| PCR water | 7.5 | 75 |
| Buffer 2 | 1.5 | 15 |
| Hind 111 enzyme | 5.0 | 50 |
| PCR product | 1.0 | - |
| Total | 15.0 | 140 |

**Preparation of agarose gel and gel electrophoresis**

70ml of TBE buffer was measured and taken in to the conical flask. 1.2g of Agarose powder was taken and mixed with it. Conical flask was covered with aluminum foil and heated in a microwave oven for 2minutes and 20 seconds until all the solids were completely melted. Then 4.5 µl of ethidium bromide was added to the solution and then it was poured into a gel casting tray with a comb for the preparation of wells and it was allowed to solidify about 40-50 minutes at room temperature. After the gel was solidified comb was removed and then the gel was placed in the electrophoresis chamber and covered with TBE buffer.

**Detection and analysis**

10 µl of positive control and sample products were added with 2 µl of loading dye separately; mixed well and loaded into the gel. Positive control, samples and one uncut PCR product were loaded. 500 ng of molecular weight marker was loaded in to the first lane of the gel. Gel was run under 100- 120mA electric field for 30- 45 minutes. Finally migrated DNA bands were visualized under UV trans illuminator.

**RESULTS**

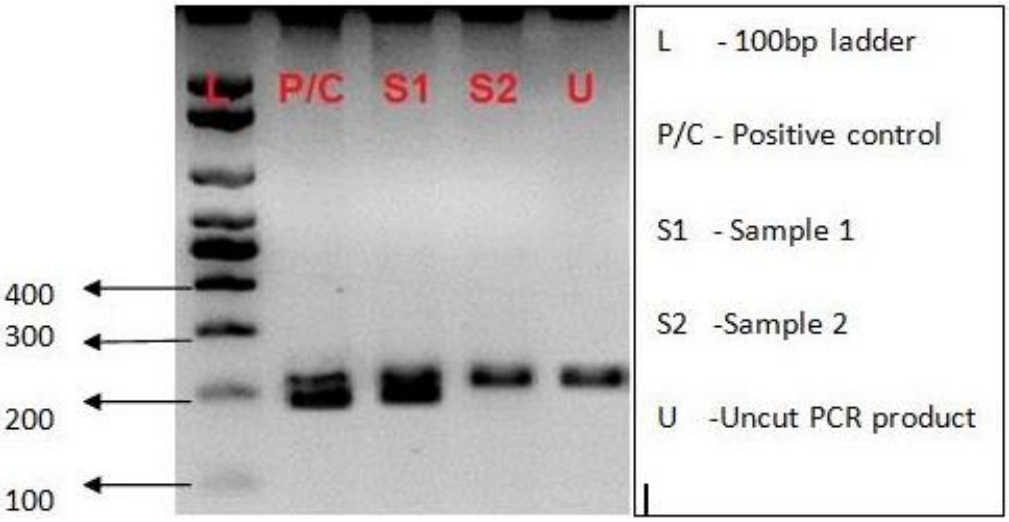



*Figure 03- Gel picture of sample 1 and sample 2 MTHFR genotypes*

Sample1 is heterozygous (CT) genotype

Sample 2 is homozygous (CC) genotype

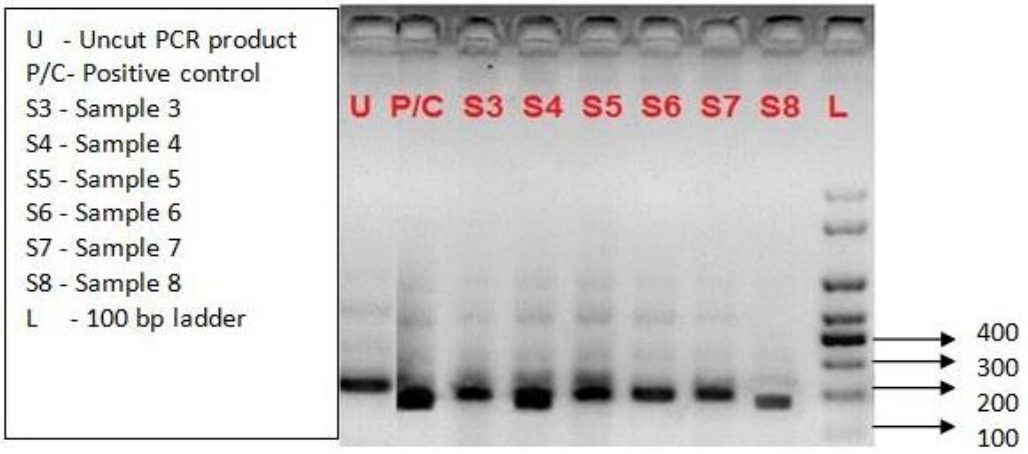



*Figure 04- Gel picture of MTHFR genotypes from sample 3 to sample 8*

Sample 4 is the heterozygous (CT) genotype

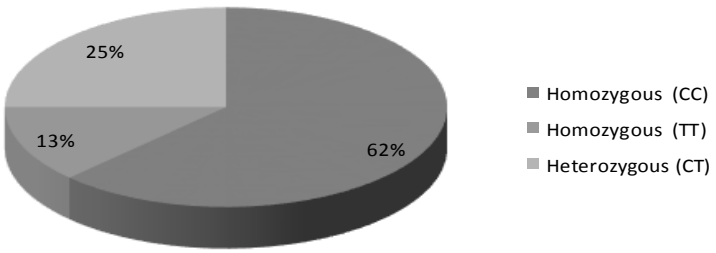
Sample 8 is the homozygous (TT) genotype

Other samples are homozygous (CC) genotype

*Table 04: Interpretation of MTHFR C677T polymorphism*

| Genotype | Number of bands expected | Number of bands visible |
|---|---|---|
| **Homozygous (CC)** | 1 (223 bp) | 1 (223 bp) |
| **Homozygous (TT)** | 2 (25 bp and 198 bp) | 1 (198 bp) |
| **Heterozygous (CT)** | 3 (25 bp,198 bp and 223 bp) | 2 (198 bp and 223 bp) |

*Chart 01: Percentage of MTHFR genotypes in Sri Lankans*



The substitution of "C" to "T" at 677 position of MTHFR gene creates a restriction site for the enzyme Hind111. Restriction digestion of the 677TT amplicon results two fragments of 198bp and 25bp while the 677CC amplicon does not undergo digestion. As a result the homozygous 677CC genotype (wild type) would give rise to one band of 223bp in the electrophoresed gel while the homozygous 677TT genotype would produce 2 bands of 25bp and 198bp in length. Also the heterozygous 677CT genotype would give rise to 3 bands of 25bp, 198bp and 223bp in length.

## DISCUSSION

Among the 8 samples tested 2 were had heterozygous 677CT genotype, one had homozygous 677TT genotype and the other 5 had 677CC wild type. Homozygous TT genotype is considered as harmful because it is associated with high concentration of total plasma Homocysteine level and lower MTHFR enzyme activity of 30%. On the other hand, 677CT genotype has MTHFR enzyme activity of about 65% when compared to wild type (Curtin et.al, 2004). The substitution of “T” for “C” at 677 position of MTHFR gene creates a restriction site for the enzyme Hind111 (Koksal et al., 2007). During electrophoresis of the PCR products 25bp fragment migrates out of the gel due to the small fragment size. Therefore, it is not visible in the gel. But that did not hinder the genotype differentisingle bands of two homozygous genotypes were of different sizes which can be easily separated using the marker DNA (Samtani et al., 2013).

The primer melting temperature can indicate DNA-DNA hybrid stability and it is important for the optimization of PCR annealing temperature (Ta). Really high Ta values can cause insufficient primer-template hybridization and produce less PCR products; in turn low annealing temperatures can cause a high number of primer mis pairings and formation of non-specific bands. The GC content is related to the primer melting temperature and plays a role in the annealing step of PCR. A previous study reported that a GC content of 45-60% results in more specific hybridization. Maintain the melting temperature above 50°C is important to avoid the formation of non-specific bands (Loo, Griffiths and Gan, 2012). Considering the fact that MTHFR C677T polymorphism is the commonest cause for hyperhomocysteinaemia, the heterogeneity of the results of the individual studies could be due to the variation of the population prevalence of the T allele. Although a lot of gaps exist in T allele distribution data in our country, it is widely expected for the prevalence to be varied significantly among different ethnic groups even in the same locality. Vitamin status especially B 12 and Folic acid influence the homocysteine level and in turn the heterogeneity of the study results. Other genetic and environmental factors too can contribute for the variation of the results of the different studies, but the relative contribution of them to the final outcome is difficult to assess (Leclerc, Sibani and Rozen, 2000).

In similar studies, it seems that conclusion of each individual study should be interpreted in the context of ethnic origin, geographical background, vitamin status of the sample population and the prevalence of the T allele in the particular ethnic groups. Prevalence of MTHFR C677T mutation differs in various populations and decreasing from East to West. According to the most comprehensive study up to date, T allele frequency is 34-44% in China, 25-46% in Europe, 21-41% in America and 28% in Australia. The highest frequency was reported in Mexico (57%) while the lowest frequency was reported among American blacks (12.6%) (Wilcken et al., 2003). Similar study reported that allele frequency in Africa (6.6%) was the lowest and comparable to Sri Lanka (4.5%) (Cronin et al., 2005). The observed frequency of MTHFR C677T in the presently studied population (even though ethnic groups not included) is higher than that reported Cronin on 2005 but it is comparable to the frequencies reported by Dissanayake on 2009. Although this study was conducted with in the small sample population and the extension of the research work to include larger sample population with ethnic groups in Sri Lanka was limited by the time, research period and financial constraints.

Therapeutic interventions if any would be in the form of vitamin (Folic acid and vitamin B12 mainly) supplementation for a patient with high homocysteine level due to the presence of the polymorphism, since it has been established fact that folic acid has a significant homocysteine lowering effect which is increased by the addition of vitamin B 12 (Yasui et

al., 2000). High intake of folate, which is plentiful in vegetables and fruits, has been related with reduced risk of several cancers (Leclerc, Sibani and Rozen, 2000). Folate deficiency was suggested to increase the risk of cancer through impaired DNA repair and disruption of DNA methylation that may lead to proto-oncogene activation (Varga et al., 2005). Preventive measures such as food fortification with folic acid and vitamin B 12 can be started if the vitamin status of the population is lower or the prevalence of T allele is higher in the target population. FDA approved programme for folic acid fortification of cereal and grain products was initiated in USA in 1998 and post fortification analysis of its effects on homocysteine level is still underway (Leclerc, Sibani and Rozen, 2000).

In summary, related studies has shown that, even though lower frequency of MTHFR C677T polymorphism is present in Sri Lankans the frequency is increasing over the year. But the present study has many limitations. Limited numbers of participants, failure to include Sri Lankan ethnic groups and also sample details are blinded. The financial and time constrains were the main reasons for the above mentioned limitations. Future studies with larger sample population, looking at different ethnic groups in different parts of the country, study of homocysteine level and nutritional status of the population together with MTHFR genotype will give better and accurate frequency of MTHFR C677T polymorphism in Sri Lanka.

## CONCLUSION

The results obtained from this study shows that, of the 8 samples tested, 2 had 677CT genotype, 1 had 677TT genotype whereas the remaining 5 had 677CC wild type. In the last decade several studies had been conducted to discover the direct or indirect influence of increased levels of homocysteine in several diseases. These studies confirmed that several polymorphisms in genes which are related to folate and homocysteine methionine pathway result hyperhomocysteinemia and suggesting that these variants may play a major role in many multifactorial disorders which are high prevalence in the world wide population. Folic acid supplements with vitamin B12 are better and cost effective way to decrease hyperhomocysteinemia. However, as this is a small sample of the population, it is recommended to carry out studies with a large sample volume to ascertain the prevalence of this mutation in the community. RFLP and PCR based diagnostic methods are useful for the identification of these mutations.

## ACKNOWLEDGEMENT

I would like to express my gratitude and appreciation to all those whogave me the possibility to complete this report. A special thanks goes tomy project supervisor, Prof. Aresha Manamperi, University of kelaniya who gave propersuggestions, encouragement and helpful in areas where the guidancewas much necessary. I wish to thank my lecturers, tutors, facilitators (Durdans hospital molecular diagnostic laboratory) family and friends who gave me a great support to finish my research project successfully.

## REFERENCES

Angeline, T., Thiruvarutselvi, G., Isabel, W., Aruna, R. M., Devi, R. and Jeyaraj, N. (2009) MTHFR (Ala 222 Val) polymorphism and AMI in patients with type II diabetes mellitus., Indian journal of clinical biochemistry : IJCB, 24(2), pp. 137–41

Barbosa, P. R., Stabler, S. P., Machado, A. L. K., Braga, R. C., Hirata, R. D. C., Hirata, M. H., Sampaio-Neto, L. F., Allen, R. H. and Guerra-Shinohara, E. M. (2008) Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women., European journal of clinical nutrition, Macmillan Publishers Limited, 62(8), pp. 1010–21

Blom, H. J. (2009) Folic acid, methylation and neural tube closure in humans., Birth defects research. Part A, Clinical and molecular teratology, 85(4), pp. 295–302

Blom, H. J. and Smulders, Y. (2011) Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects., Journal of inherited metabolic disease, 34(1), pp. 75–81

Brulhart, M. C., Dussoix, P., Ruiz, J., Passa, P., Froguel, P. and James, R. W. (1997) The (Ala-Val) mutation of methylenetetrahydrofolate reductase as a genetic risk factor for vascular disease in non-insulin-dependent diabetic patients., American journal of human genetics, 60(1), pp. 228–9

Brustolin, S., Giugliani, R. and Félix, T. M. (2010) Genetics of homocysteine metabolism and associated disorders., Brazilian journal of medical and biological research = Revista brasileira de pesquisas médicas e biológicas / Sociedade Brasileira de Biofísica ... [et al.], 43(1), pp. 1–7

Crider, K. S., Yang, T. P., Berry, R. J. and Bailey, L. B. (2012) Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role., Advances in nutrition (Bethesda, Md.), 3(1), pp. 21–38

Cronin, S., Furie, K. L. and Kelly, P. J. (2005) Dose-related association of MTHFR 677T allele with risk of ischemic stroke: evidence from a cumulative meta-analysis., Stroke; a journal of cerebral circulation, 36(7), pp. 1581–7

Curtin, K. (2004) MTHFR C677T and A1298C Polymorphisms: Diet, Estrogen, and Risk of Colon Cancer, Cancer Epidemiology Biomarkers & Prevention, 13(2), pp. 285–292

Deeparani, T., Pillai, M. R. and Elavazhagan, T. (n.d.) Detection of MTHFR C677T and A1298C Gene Polymorphism in Congenital Heart Disease, [online] Available from: http://www.researchgate.net/publication/239557433_Detection_of_MTHFR_C677T_and_A1298C_Gene_Polymorphism_in_Congenital_Heart_Disease (Accessed 12 May 2015).

Dissanayake, V. H. W., Weerasekera, L. ., Gammulla, C. G. and Jayasekara, R. W. (n.d.) Prevalence of genetic thrombophilic polymorphisms in the Sri Lankan population - implications for association study design and clinical genetic testing services, [online] Available from: http://archive.cmb.ac.lk/research/handle/70130/1956 (Accessed 13 May 2015).

Ford, E. S. (2002) Homocyst(e)ine and cardiovascular disease: a systematic review of the evidence with special emphasis on case-control studies and nested case-control studies, International Journal of Epidemiology, 31(1), pp. 59–70

Forges, T., Monnier-Barbarino, P., Alberto, J. M., Guéant-Rodriguez, R. M., Daval, J. L. and Guéant, J. L. (2007) Impact of folate and homocysteine metabolism on human reproductive health., Human reproduction update, 13(3), pp. 225–38

Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A. and van den Heuvel, L. P. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase., Nature genetics, 10(1), pp. 111–3

Gallagher, P. M., Meleady, R., Shields, D. C., Tan, K. S., McMaster, D., Rozen, R., Evans, A., Graham, I. M. and Whitehead, A. S. (1996) Homocysteine and Risk of Premature Coronary Heart Disease: Evidence for a Common Gene Mutation, Circulation, 94(9), pp. 2154–2158

Gilbody, S., Lewis, S. and Lightfoot, T. (2007) Methylenetetrahydrofolate reductase (MTHFR) genetic polymorphisms and psychiatric disorders: a HuGE review., American journal of epidemiology, 165(1), pp. 1–13

Graham, I., Refsum, H., Rosenberg, I. H., Ueland, P. M. and Shuman, J. M. (eds.) (1997) Homocysteine Metabolism: From Basic Science to Clinical Medicine, Developments in Cardiovascular Medicine, Boston, MA, Springer US, [online] Available from: http://www.springerlink.com/index/10.1007/978-1-4615-5771-5 (Accessed 13 May 2015).

Husemoen, L. L. N., Linneberg, A., Fenger, M., Thuesen, B. H. and Jørgensen, T. (2009) Changes in lifestyle, biological risk factors and total homocysteine in relation to MTHFR C677T genotype: a 5-year follow-up study., European journal of clinical nutrition, Macmillan Publishers Limited, 63(10), pp. 1233–40

Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J. and Rozen, R. (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations., Circulation, 93(1), pp. 7–9

Kalkan, G., Yigit, S., Karakuş, N., Ateş, O., Bozkurt, N., Ozdemir, A. and Pancar, G. Ş. (2013) Methylenetetrahydrofolate reductase C677T mutation in patients with alopecia areata in Turkish population., Gene, 530(1), pp. 109–12

Karabacak, E., Aydin, E., Ozcan, O., Dogan, B., Gultepe, M., Cosar, A. and Muftuoglu, T. (2014) Methylenetetrahydrofolate reductase (MTHFR) 677C>T gene polymorphism as a possible factor for reducing clinical severity of psoriasis., International journal of clinical and experimental medicine, 7(3), pp. 697–702

Kirke, P. N. (2004) Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study, BMJ, 328(7455), pp. 1535–1536

Kluijtmans, L. A. J., Young, I. S., Boreham, C. A., Murray, L., McMaster, D., McNulty, H., Strain, J. J., McPartlin, J., Scott, J. M. and Whitehead, A. S. (2003) Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults., Blood, 101(7), pp. 2483–8

Koksal, V., Baris, I. and Etlik, O. (2007) Primer-engineered multiplex PCR-RFLP for detection of MTHFR C677T, prothrombin G20210A and factor V Leiden mutations., Experimental and molecular pathology, 83(1), pp. 1–3

Kruman, I. I., Kumaravel, T. S., Lohani, A., Pedersen, W. A., Cutler, R. G., Kruman, Y., Haughey, N., Lee, J., Evans, M. and Mattson, M. P. (2002) Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease., The Journal of neuroscience : the official journal of the Society for Neuroscience, 22(5), pp. 1752–62

Leclerc, D., Sibani, S. and Rozen, R. (2000) Molecular Biology of Methylenetetrahydrofolate Reductase (MTHFR) and Overview of Mutations/Polymorphisms, Landes Bioscience, [online] Available from: http://www.ncbi.nlm.nih.gov/books/NBK6561/ (Accessed 11 May 2015).

Liang, Y., Li, Y., Li, Z., Liu, Z., Zhang, Z., Chang, S. and Wu, J. (2012) Mechanism of folate deficiency-induced apoptosis in mouse embryonic stem cells: Cell cycle arrest/apoptosis in G1/G0 mediated by microRNA-302a and tumor suppressor gene Lats2., The international journal of biochemistry & cell biology, 44(11), pp. 1750–60

Loo, K. W., Griffiths, L. R. and Gan, S. H. (2012a) A novel multiplex PCR-RFLP method for simultaneous detection of the MTHFR 677 C > T, eNOS +894 G > T and - eNOS -786 T > C variants among Malaysian Malays., BMC medical genetics, 13(1), p. 34

Mansoori, N., Tripathi, M., Luthra, K., Alam, R., Lakshmy, R., Sharma, S., Arulselvi, S., Parveen, S. and Mukhopadhyay, A. K. (2012) MTHFR (677 and 1298) and IL-6-174 G/C genes in pathogenesis of Alzheimer's and vascular dementia and their epistatic interaction., Neurobiology of aging, Elsevier, 33(5), pp. 1003.

Mendis, S., SBP, A. and Kenji, T. (1997) Association between hyperhomocysteinemia and ischemic heart disease

in Sri Lankans, International Journal of Cardiology, Elsevier, 62(3), pp. 221–225

Micheal, S., Qamar, R., Akhtar, F., Khan, M. I., Khan, W. A. and Ahmed, A. (2009) MTHFR gene C677T and A1298C polymorphisms and homocysteine levels in primary open angle and primary closed angle glaucoma, Molecular Vision, [online] Available from: http://www.molvis.org/molvis/v15/a244/ (Accessed 4 May 2015).

Samtani, R., Kaur, L., Saraswathy, K. N. and Ghosh, P. K. (2013) Title: Simultaneous detection of FVL and MTHFR (C677T) mutations using PCR RFLP method, [online] Available from: http://www.researchgate.net/publication/236165857_Title_Simultaneous_detection_of_FVL_and_MTHFR_(C677T)_mutations_using_PCR_RFLP_method (Accessed 4 May 2015).

Schmitz, C., Lindpaintner, K., Verhoef, P., Gaziano, J. M. and Buring, J. (1996) Genetic Polymorphism of Methylenetetrahydrofolate Reductase and Myocardial Infarction: A Case-Control Study, Circulation, 94(8), pp. 1812–1814

Schneider, J. A., Rees, D. C., Liu, Y. T. and Clegg, J. B. (1998) Worldwide distribution of a common methylenetetrahydrofolate reductase mutation., American journal of human genetics, 62(5), pp. 1258–60

Spotila, L. D. (2003) Age Dependence of the Influence of Methylenetetrahydrofolate Reductase Genotype on Plasma Homocysteine Level, American Journal of Epidemiology, 158(9), pp. 871–877

Suliman, M. E. (2001) Effects of methionine loading on plasma and erythrocyte sulphur amino acids and sulph-hydryls before and after co-factor supplementation in haemodialysis patients, Nephrology Dialysis Transplantation, 16(1), pp. 102–110

Trovato, F. M., Catalano, D., Ragusa, A., Martines, G. F., Pirri, C., Buccheri, M. A., Di Nora, C. and Trovato, G. M. (2015) Relationship of MTHFR gene polymorphisms with renal and cardiac disease., World journal of nephrology, 4(1), pp. 127–37

Ueland, P. M., Hustad, S., Schneede, J., Refsum, H. and Vollset, S. E. (2001) Biological and clinical implications of the MTHFR C677T polymorphism, Trends in Pharmacological Sciences, 22(4), pp. 195–201

Van der Griend, R., Haas, F. J., Duran, M., Biesma, D. H., Meuwissen, O. J. and Banga, J. D. (1998) Methionine loading test is necessary for detection of hyperhomocysteinemia., The Journal of laboratory and clinical medicine, 132(1), pp. 67–72

Varga, E. A., Sturm, A. C., Misita, C. P. and Moll, S. (2005) Cardiology patient pages. Homocysteine and MTHFR mutations: relation to thrombosis and coronary artery disease., Circulation, 111(19), pp. e289–93

Weisberg, I. (2001) The 1298A→C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine, Atherosclerosis, 156(5), pp. 409–415

Wilcken, B. (2003) Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide, Journal of Medical Genetics, 40(8), pp. 619–625

Yasui, K., Kowa, H., Nakaso, K., Takeshima, T. and Nakashima, K. (2000) Plasma homocysteine and MTHFR C677T genotype in levodopa-treated patients with PD, Neurology, 55(3), pp. 437–440

Zhang, M.-J., Li, J.-C., Yin, Y.-W., Li, B.-H., Liu, Y., Liao, S.-Q., Gao, C.-Y. and Zhang, L.-L. (2014) Association of MTHFR C677T polymorphism and risk of cerebrovascular disease in Chinese population: an updated meta-analysis., Journal of neurology, 261(5), pp. 925–35