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

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

DNA characterization and polymorphism of *KISS1* gene in Egyptian small ruminant breeds

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Genetic information especially of the Quantitative Trait Loci (QTL) which affect different performance traits is considered one of the most effective tools in the breeding programs of livestock. Several genes were reported as candidate genes that effect litter size performance and one of these genes is the *KISS1* which is considered as a regulator of puberty onset. The polymorphisms of *KISS1* gene have some relationships with high prolific and sexual precocity. The objective of this study was the detection of the restriction fragment length polymorphism (RFLP) and single nucleotide polymorphisms (SNPs) of *KISS1* gene in six major Egyptian small ruminant breeds. The primers used in this study flanked a 377 bp fragment from intron 1 of *KISS1* gene in sheep and goat. These PCR amplified fragments were digested with *Xmn*I endonuclease. According to the presence or absence of the restriction site (GAANN^NNTTC) at position 121^122, we genotyped the 122 tested animals as AT (54.92%) and TT (45.08) with the absence of AA genotype. The overall frequencies of alleles A and T were 27.46 and 72.54%, respectively. The sequence analysis of purified PCR products representing these two detected genotypes declared the presence of a SNP (T→A) at position 125 in the amplified fragment which is responsible for the elimination of the restriction site and consequently the presence of two different alleles T and A. The nucleotide sequences of sheep *KISS1* alleles T and A as well as goat *KISS1* alleles T and A were submitted to GenBank database and have accession numbers: KP835797, KP835798, KP835799 and KP835800, respectively. It is concluded that small ruminant breeds have high frequency of *KISS1* allele T which was associated with greater litter size. We recommend to increase this allele in Egyptian small ruminant breeds and also to select the animals which possess TT genotypes of *KISS1* gene and enter them in breeding programs of Egyptian small ruminants to increase their fecundity traits.

Key words: Sheep, goat, *KISS1*, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), single nucleotide polymorphisms (SNPs).

INTRODUCTION

Genetic information especially of the QTL which affect different performance traits is considered one of the most effective tools in the breeding programs of livestock. Several genes were reported as candidate genes that

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Abbreviations: RFLP, Restriction fragment length polymorphism; **LH,** luteinizing hormone; **FSH,** follicle-stimulating hormone; **PCR,** polymerase chain reaction.

effect on litter size performance and many studies were carried out to identify these genes and their relation with litler size and prolific status in different farm animals including small ruminants. Some of these genes are G protein-coupled receptor 54 (*GPR54*) gene (Cao et al., 2011), cocaine-amphetamine-regulated transcript (*CART*) gene (Wang et al., 2011), kit ligand (*KITLG*) gene (An et al., 2012) and bone morphogenetic protein receptor IB (*BMPR-IB*) gene (Chu et al., 2010). The *KISS1* gene encodes a family of neuropeptides called kisspeptins, which activate G protein-coupled receptor-54 and play a role in the neuroendocrine regulation of GnRH secretion (Smith et al., 2005). *KISS1* neurons in the hypothalamus has a critical role in reproductive maturation and function including brain-level sex differentiation, puberty onset and the neuroendocrine regulation of gonadotropin secretion and ovulation (Caraty et al., 2010). Also, kisspeptins are reported as regulators for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in different mammalian species (Gottsch et al., 2004; Dhillo et al., 2005).

According to the importance of *KISS1* as a regulator of puberty onset, there is a hypothesis that the polymorphisms of *KISS1* gene have some relationships with high prolific and sexual precocity. One novel nonsynonymous single nucleotide polymorphism (G/T) substituting one amino acid in kisspeptin (P/T) has been found to be statistically related to central precocious puberty in human (Luan et al., 2007). These findings indicate that the *KISS1* gene could be an excellent candidate gene for reproductive traits in humans and livestock. In view of the above considerations, the objective of this study was to detect the genetic polymorphism of *KISS1* gene in six major Egyptian small ruminant breeds; Barki, Rahmani and Ossimi sheep breeds as well as Barki, Baladi and Zaraibi goat breeds. Also, this work aimed to identify the single nucleotide polymorphisms in different *KISS1* genotypes which were detected in native small ruminant breeds.

MATERIALS AND METHODS

Blood samples and genomic DNA extraction

The whole blood samples were collected from 122 animals belonging to six native major small ruminant breeds; 32 from sheep Barki, 18 from sheep Rahmani, 24 from sheep Ossimi, 14 from goat Baladi, 16 from goat Barki and 18 from goat Zaraibi. Genomic DNA was extracted from the whole blood according to the method described by Miller et al. (1988) with minor modifications. Briefly, blood samples were mixed with cold 2x sucrose-triton and centrifuged at 5000 rpm for 15 min at 4°C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37°C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1x TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer and then diluted to the working concentration of 50 ng/µl which is suitable for polymerase chain reaction.

Polymerase chain reaction (PCR)

A PCR amplification reaction was performed using specific primer that was designed on the basis of DNA sequence of the *KISS1* gene (Accession: D00476) (An et al., 2013a): F: CCC GCT GTA ACT AGA GAA AG; R: CAT CCA GGG TGA GTG ATA CT

A PCR cocktail consisted of 1.0 µM of forward and reverse primer, 0.2 mM dNTPs, 10x of PCR reaction buffer and 1.25 units of Taq polymerase (Fermentas) was used. The cocktail was added into PCR tubes with 100 ng of sheep or goat DNA. The reaction was run at 94°C for 5 min, 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide to test the amplification success.

Restriction fragment length polymorphism (RFLP)

The PCR products were digested using restriction enzyme; *Xmn*I (Fermentas). 10 µl of PCR product was digested with 1 µl of FastDigest restriction enzyme for 15 min at 37°C. The restriction fragments were subjected to electrophoresis in 2% agarose ethidium bromide gel in 1x TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in FX Molecular Imager apparatus (BIO-RAD).

Sequencing analysis and single nucleotide polymorphism

The PCR products -representatives for each detected genotype of *KISS1* gene in different sheep and goat breeds were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite to identify each single nucleotide substitution between different detected genotypes. Results of endouclease restriction were carried out using FastPCR. The nucleotide sequence of each genotype for Egyptian sheep and goat *GH* gene was submitted to GenBank (NCBI, BankIt).

RESULTS AND DISCUSSION

Kisspeptins, the product of the *KISS1* gene, play an essential role in the regulation of reproductive functions, acting primarily at the hypothalamic level of the gonadotropic axis (An et al., 2013a). Kisspeptins have been identified as the natural ligands for an orphan G-protein-coupled receptor, GPR54, and it is becoming clear that the hypothalamic KISS1/GPR54 system plays a crucial permissive role in controlling the onset of puberty by regulating the release of gonadotrophin-releasing hormone (GnRH) from hypothalamic neurons (Messager et al., 2005; d'Anglemont de Tassigny et al., 2007; Smith et al., 2007). In a view of the importance of *KISS1* as a regulator of puberty onset, there is a hypothesis that the polymorphisms of *KISS1* have some relationships with high prolifi in small ruminant (Cao et al., 2010). So far, there have been some studies of the *KISS1* gene as a candidate gene for reproductive traits in animals, which revealed that the *KISS1* gene plays an important role in animal reproduction (Tomikawa et al., 2010). We aimed in this study to detect RFLP and SNP polymorphisms of KISS1 gene in three Egyptian sheep breeds; Barki, Rahmani and Ossimi as well as three Egyptian goat

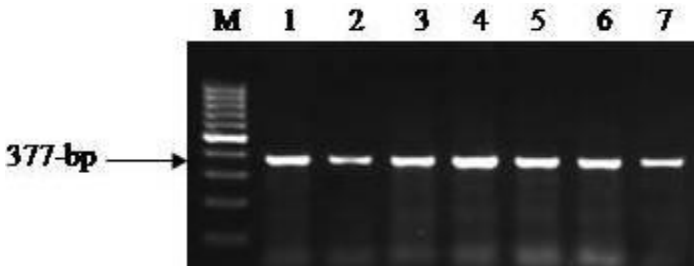

Figure 1. Ethidium bromide-stained gel of PCR products representing amplification of *KISS1 g*ene in Egyptian sheep and goat animals. Lane M: 100 bp ladder marker. Lanes 1 to 7, 377 bp PCR products amplified from sheep and goat DNA.

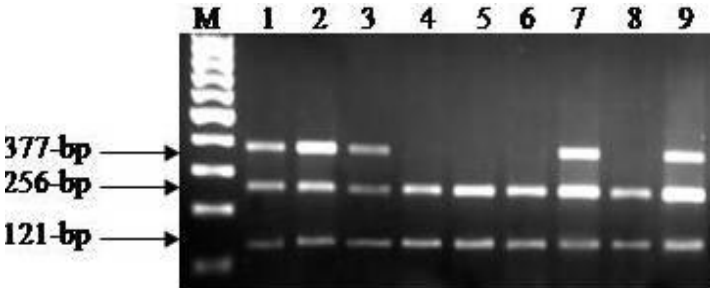

Figure 2. The electrophoretic pattern obtained after digestion of PCR amplified fragment of *KISS1* gene from sheep and goat DNA with *Xmn*I restriction enzyme. Lane M: 100 bp ladder marker. Lanes 1 to 3, 7 and 9, AT heterozygous genotype with three digested fragments at 377, 256 and 121 bp. Lanes 4 to 6 and 8, TT homozygous genotype with two digested fragments at 256 and 121 bp.

CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAAATTTTAAAAAGATAT
TCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAACT^TCTTCTCTCCTGGGATCGGGTGCTCTT
TCTGGGTAAGGGAGGATCCCCCGGAGAATAACAATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCT
TCTTGGCAAAATCTTTTAGGTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTG
TAGCTGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGTATCACTACCC
CTGGATG

Figure 3. Endonuclease restriction of amplified fragment from sheep and goat *KISS1* using FastPCR GAACT^TCTTC; the restriction site in red.

breeds; Barki, Baladi, and Zaraibi. The primers used in this study flanked a 377 bp fragment from intron 1 of *KISS1* gene in sheep and goat. The amplified fragments obtained from all tested sheep and goat animals were at 377 bp (Figure 1).

These PCR amplified fragments (377 bp) were digested with *Xmn*I endonuclease. Depending on the presence or absence of the restriction site (GAANN^NNTTC) (N = A or T or C or G) at position 121^122, we can easily differentiate between 3 different genotypes: AA with undigested one fragment at 377 bp, TT with two digested fragments at 256 and 121 bp and AT with three digested fragments at 377, 256 and 121 bp. The results showed the presence of two genotypes; AT and TT with the absence of AA genotype in 122 tested animals for this gene (Figures 2 and 3). The frequencies of AT and TT genotypes were 62.5 and 37.5% in sheep Barki animals (32 animals), 55.56 and 44.44% in sheep Rahmani animals (18 animals) and 45.83 and 54.17% in sheep Ossimi animals (24 animals), respectively, with the total frequencies of 55.41 and 44.59% for AT and TT genotypes, respectively, in 74 tested sheep animals for this gene. In tested goat animals, the frequencies of AT and TT genotypes were 57.14 and 42.86% for Baladi (14

Table 1. The genotype and allele frequencies of *KISS1* gene in Egyptian sheep and goat breeds.

Animal	Breed	Number of animals	Genotype frequencies		Allele frequencies	
			AT%	TT%	A%	T%
Sheep	Barki	32	62.50	37.50	31.25	68.75
	Rahmani	18	55.56	44.44	27.78	72.22
	Ossmi	24	45.83	54.17	22.92	77.08
	Total	74	55.41	44.59	27.70	72.30
Goat	Baladi	14	57.14	42.86	28.57	71.43
	Barki	16	62.50	37.50	31.25	68.75
	Zaraibi	18	44.44	55.56	22.22	77.78
	Total	48	54.17	45.83	27.08	72.91
	Overall	122	54.92	45.08	27.46	72.54

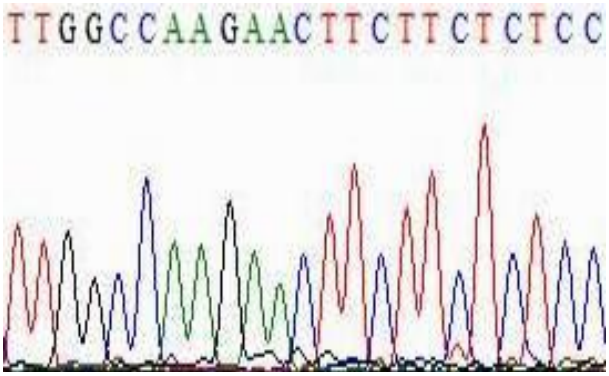

Figure 4. Genotype T/T.

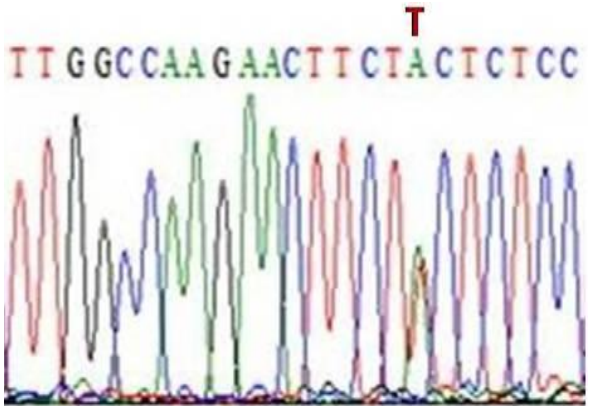

Figure 5. Genotype T/A.

animals), 62.5 and 37.5% for Barki (16 animals) and 44.44 and 55.56% for Zaraibi (18 animals), respectively, with total frequencies of 54.17 and 45.83% for AT and TT genotypes, respectively, in 48 tested goat animals for this gene. The overall frequencies for genotypes AT and TT as well as alleles A and T in all 122 tested animals were 54.92, 45.08, 27.46 and 72.54%, respectively (Table 1). The nucleotide sequences of two different genotypes T/T (Figure 4) and T/A (Figure 5) which were detected in this study declared the presence of one SNP substitution (T→A) at position 125 in the amplified fragments of sheep and goat *KISS1* gene (Figure 6) which is responsible for the elimination of the restriction site GAACT^TCTTC and consequently the appearance of two different alleles T and A. The nucleotide sequences of sheep *KISS1* alleles T and A as well as goat *KISS1* alleles T and A were submitted in GenBank database and have accession numbers: KP835797, KP835798, KP835799 and KP835800, respectively.

An et al. (2013a and b) detected polymorphisms of the goat *KISS1* gene in three Chinese goat breeds using PCR-RFLP and DNA sequencing methods. Two novel SNPs were identified in the intron 1 of the *KISS1* gene. The 2124T>A and 2270C>T SNPs were significantly associated with litter size where the combined alleles of T in both loci with greater litter size than the combined alleles of A and C. The frequencies of alleles T and A in first locus were 0.60 and 0.40, 0.59 and 0.41 and 0.55 and 0.45 in the three tested goat breeds. Whereas, the frequencies of alleles T and C in the second locus were 0.60 and 0.40, 0.59 and 0.41 and 0.64 and 0.36 in these tested goat breeds. On the other hand, Cao et al. (2010) used three pairs of primers to clone the goat *KISS1* and scan polymorphisms and four pairs to detect polymorphisms in sexual precocious and sexual late-maturing goat breeds. The genotype distribution did not show obvious difference between sexual precocious and sexual late-maturing goat breeds and no consistency within the sexual late-maturing breeds. This study preliminarily indicated an association between allele C in *KISS1* gene and high litter size in Jining Grey goats. Chu et al. (2012) analyzed SNPs in exon 1 of *KISS1* gene in high fecundity sheep breeds (small Tail Han and Hu breeds) and low fecundity sheep breeds (Dorset, Texel and Corriedale breeds) by PCR-SSCP. Polymorphisms in

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Allele T: 1   CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAA  60
Allele A: 1   CCCGCTGTAACTAGAGAAAGCCCATGTGCAGAAGGCCCTGTGCTGCCAAAACTAAAATAA  60
              ************************************************************

Allele T: 61  ATTTTAAAAAGATATTCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAAC  120
Allele A: 61  ATTTTAAAAAGATATTCCAGTGCAAAGAGATCATGGACGCGGGTGTCCTTGGCCAAGAAC  120
              ************************************************************

Allele T: 121 TTCTTCTCTCCTGGGATCGGGTGCTCTTTCTGGGTAAGGGAGGATCCCCCGGAGAATAAC  180
Allele A: 121 TTCTACTCTCCTGGGATCGGGTGCTCTTTCTGGGTAAGGGAGGATCCCCCGGAGAATAAC  180
              **** ********************************************************

Allele T: 181 AATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCTTCTTGGCAAAATCTTTTAG  240
Allele A: 181 AATGTCATCACCGCCCGGGGGGACGCTGAGCTCTTGGCTCTTCTTGGCAAAATCTTTTAG  240
              ************************************************************

Allele T: 241 GTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTGTAGC  300
Allele A: 241 GTGATGCTAAAAACAGGCATGCCTAAGTAGCGATCCACTTGCTGGGATGATGGCTGTAGC  300
              ************************************************************

Allele T: 301 TGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGT  360
Allele A: 301 TGGAAAAGAGGTCATCGTCCCTCCCATCTCTGCCAGGCCCAGGGCCCTCCTAGGAAGAGT  360
              ************************************************************

Allele T: 361 ATCACTACCCCTGGATG 377
Allele A: 361 ATCACTACCCCTGGATG 377
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Figure 6. Nucleotide sequences and alignment between *KISS1* alleles T and A T/A substitution at position 125.

exon 1 of *KISS1* gene were detected in prolific Small Tail Han sheep (AA, AB and BB genotypes) and in Hu sheep (AA and CC genotypes) and on the other hand, no polymorphism was found in low fecundity sheep breeds (only AA genotype). These results preliminarily indicated that the *KISS*1 gene may have some associations with prolificacy in sheep.

Our result matches with the previous results obtained by An et al. (2013a and b), where they studied the genetic polymorphism of *KISS1* gene in three Chinese goat breeds and recorded the association of T→A substitution with the litter size. They reported that frequency of allele T ranged from 0.55 to 0.60 and frequency of allele A ranged from 0.40 to 0.45 in the three tested Chinese goat breeds. These frequencies very close to the frequencies of T and A alleles in our animals. The allele T was reported to be associated with greater litter size, so we recommend to increase this allele in Egyptian small ruminant breeds and also to select the animals possess TT genotypes of *KISS1* gene and enter them in breeding programs of Egyptian small ruminants to increase their fecundity traits

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Genetic analysis of eight x-chromosomal short tandem repeat loci in Iraqi population using the Mentype® Argus X-UL PCR amplification kit

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X-Chromosome short tandem repeat (STR) typing can complement existing DNA profiling protocols and can also offer useful information in cases of complex kinship analysis. This is the first population study of 8 X-linked STRs in Iraq. The purpose of this work was to provide a basic data of allele and haplotype frequency for x-linked markers and methods that are commonly used to analyze microsatellites, and the subsequent possibilities of using these specifities especially in forensic genetics. FTA® Technology (FTA™ paper DNA extraction) was utilized to extract DNA. Amplification was performed using the Mentype® Argus X-UL PCR amplification kit. Products were detected using ABI PRISMR 3100 Genetic Analyzer (Applied Biosystems). Forensic efficiency parameters showed that DXS7132, DXS7423, DXS8378, HPRTB, DXS10074, DXS10101, DXS10134 and DXS10135 are suitable for forensic application in Iraq. All the analyzed markers were in Hardy-Weinberg equilibrium (HWE); therefore Hardy-Weinberg laws could be applied for match probability calculation.

Key words: Allele frequency, haplotype frequency, Iraq, short tandem repeat (STR), X-chromosome.

INTRODUCTION

X-Chromosomal short tandem repeats (X-STRs) are particularly helpful in paternity testing and kinship analyses, such as father-daughter, mother-son and grandmother-granddaughter kinship testing, or the kinship testing of putative sisters (Excoffier and Lischer, 2010). The Argus X-8 kit enables simultaneous amplification of eight STR loci located on human chromosome X, that is DXS7132, DXS7423, DXS8378, DXS10074, DXS10101, DXS10134, DXS10135, HPRTB and the locus of Amelogenin (Gomes et al., 2009) (Figure 1). Autosomal markers are helpful in solving most of the forensic tasks in DNA analysis. However, some of them need the implementation of STRs on the sex chromosomes (gonosomes) (Diegoli and Coble, 2011; Mohammed and Imad, 2013). Gonosomal STR markers are helpful in the investigation of relationships among individuals of different generations, especially when key persons of the pedigree are missing. Furthermore, the use of gonosomal STRs in the analysis of DNA traces in forensic purposes is strongly rising (Becker et al., 2008; Muhanned et al., 2015).

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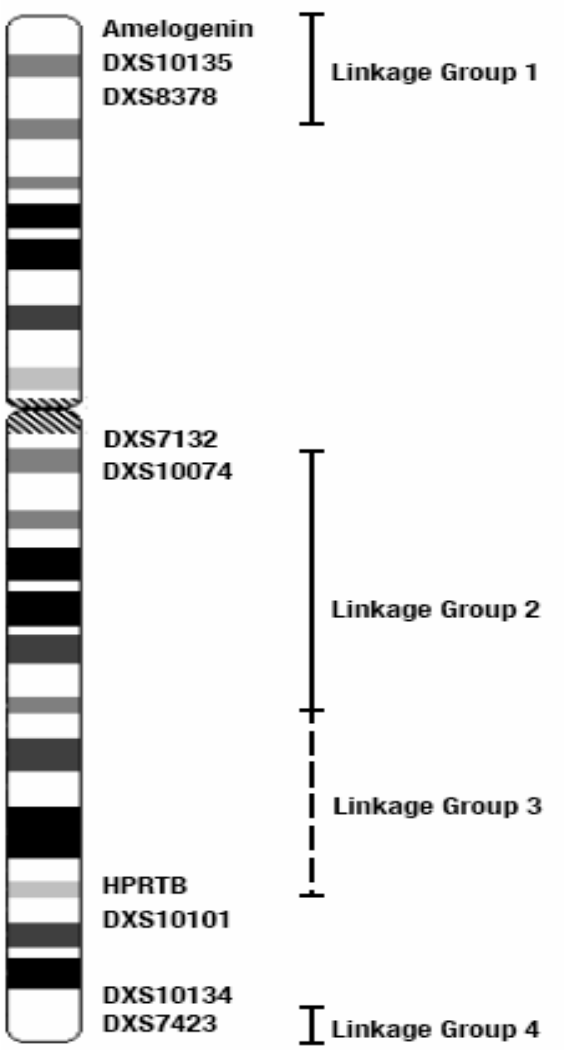

Figure 1. The ideogram of the Mentype® Argus X-8 markers on X-chromosome (www.biotype.de).

Chromosome X short tandem repeats (X-STRs) analysis has recently attracted attention of the forensic community because of its usefulness in complex kinship testing. It is worth-while including X-STRs with autosomal markers for the cases when father/daughter relationships are to be tested. Analysis of ChrX short tandem repeat markers (STRs) can successfully embrace the answer that unravels the challenge presented in particular cases of kinship analysis, when the offspring is female (Szibor et al., 2003). Since fathers transmit the same X chromosome to all their daughters, they are particularly useful in deficiency paternity cases when the child is a female, in maternity testing, and in paternity cases involving blood relatives (Desmarais et al., 1998; Tun et al., 1999; Zarrabeitia et al., 2000; Szibor et al., 2003; Jia et al., 2004; Lee et al., 2004; Imad et al., 2014a,b). Forensic X-STR markers, as per size of amplicons, are just like autosomal and Y chromosomal STRs (Shin et al., 2005; Gomes et al., 2007; Hill et al., 2008; Diegoli and Coble, 2011).

The major advantage of X-chromosomal (ChrX) STRs arises in deficiency paternity cases, that is, when a putative father is not available and DNA from paternal relatives has to be analyzed instead (Szibor, et al., 2000; Imad et al., 2014c). Female individuals fathered by the same man share their paternal ChrX. Males inherit their only ChrX from their mother. Hence, in cases in which the putative grandmother is available for genotyping, the possible ChrX alleles of the putative father can be determined (Ellegren, 2000; Szibor et al., 2003). ChrX marker typing is highly effective in mother-son kinship and in father-daughter testing. However, linkage and possible linkage disequilibrium between the ChrX markers used have to be taken into consideration (Szibor et al., 2006).

In the present study, we investigated the polymorphisms and haplotypes of the Argus X-8 loci in Iraq and evaluated their efficiency in forensic practice.

MATERIALS AND METHODS

Preparation of blood stain samples

Biological samples of blood were collected from 120 healthy unrelated males randomly selected from population living in the middle and south population from Iraq. Total DNA from blood cells was extracted using the Chelex® DNA extraction kit and FTA™ paper DNA extraction. This special paper is useful in storing collected DNA blood samples because it contains a matrix which protects the sample from nuclease degradation and bacterial growth.

DNA amplification for X- chromosomal STR

All samples were amplified using the Mentype® Argus X-8 STRs add 1.2 mm punches from FTA® storage cards containing whole blood into the appropriate wells of the reaction plate. The preferred protocol for use with the GeneAmpR PCR System 9700 thermal cycler is provided below. The estimated total cycle time is 1.5 h, 94°C for 4 min, then: 94°C for 30 s, 58°C for 120 s, 72°C for 75 s, for 30 cycles, then: 68°C for 60 min.

PCR amplicon analysis (capillary electrophoresis)

Capillary electrophoresis is a method in which DNA is separated by size in order to be analyzed. The fragments were separated in POP-4™ polymer using the ABI PRISM® 3130xl Genetic Analyzer instrument for capillary electrophoresis and data collection. The analysis software program used for this study is Applied Biosystems GeneMapper® ID version 3.2, which has precise base sizing capabilities and designates appropriate allele calls. To set up for capillary electrophoresis, a master mix is prepared containing HiDi Formamide and GeneScan™ 500 LIZ (Figure 2). 1 µl of amplified STR product, controls and allelic ladder are added to the appropriate wells. 9 µl of master mix is added to all the reactions, giving a total 10 µl reaction volume. The mixture was denatured at 95°C for 5 min and then immediately snap-cooled on ice for 3 min until the amplicons were loaded on the ABI 3130xl. Analysis of the data was done using GeneScan® 3.7 (Applied Biosystems)

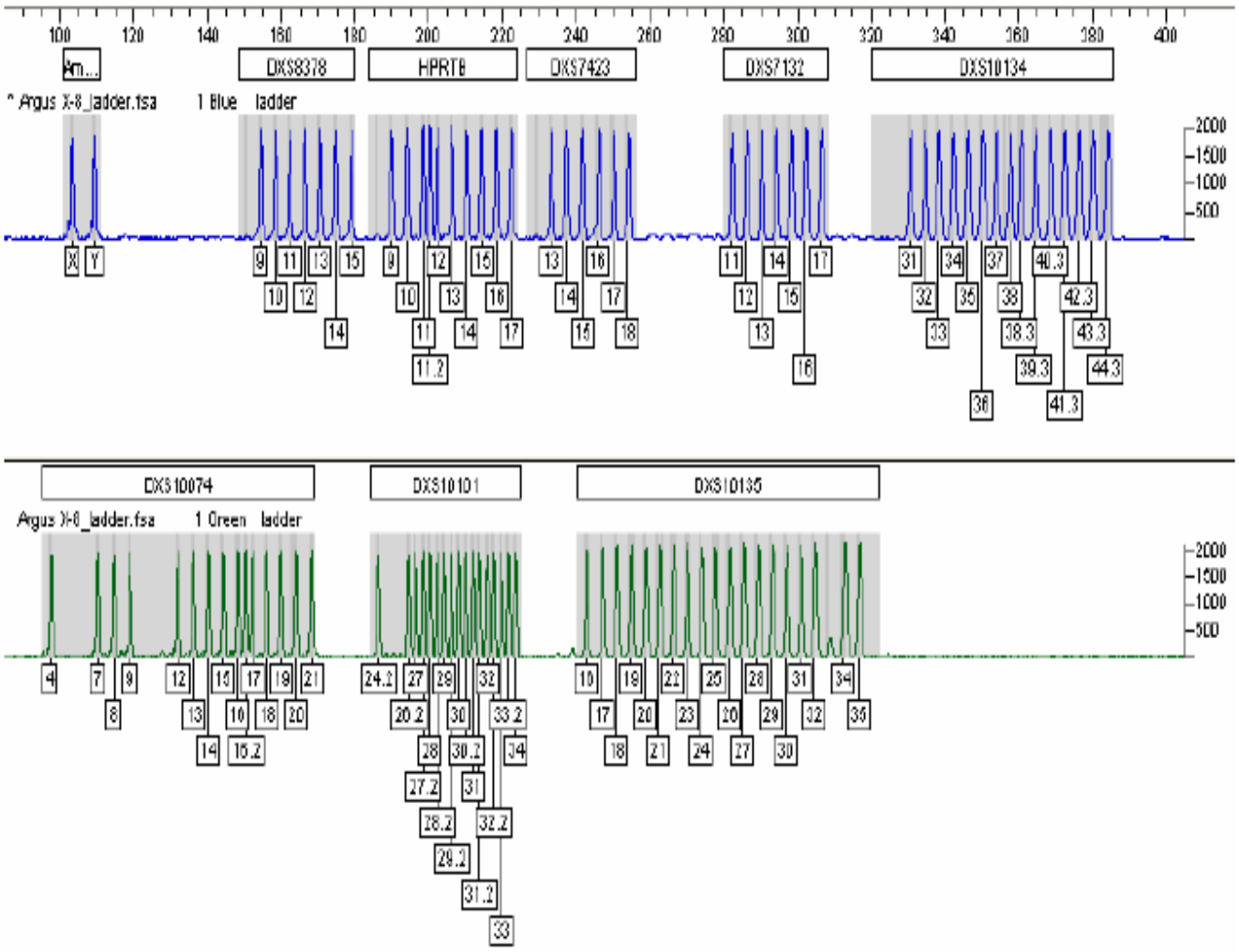

Figure 2. Electropherogram of the allelic ladder Mentype® Argus X-8 analysed on an ABI PRISM® 310 genetic analyzer.

software. Amplicons were then converted to allele numbers using the Genotyper® 3.7 (Applied Biosystems).

Statistical analysis

Once frequencies are obtained, specific statistical tests are conducted on the data to evaluate whether the database will be useful when applied to human identity testing. Allele frequencies for each locus were calculated for males collectively by hand. Observed heterozygosities (HET), polymorphism information content (PIC), power of exclusion (PE) and power of discrimination (PD) were calculated with PowerStats v12 software (http://www.promega.com). Hardy-Weinberg equilibrium (HWE) was calculated by an exact test with Arlequin v3.5 software (Excoffier and Lischer, 2010). Power of discrimination in males (PD) wascalculated with chromosome X web version.

RESULTS AND DISCUSSION

X-Linked markers in Mentype® Argus X-8 PCR amplification kit proved to be highly polymorphic with a high power of discrimination. Allele frequencies for each of the eight short tandem repeat loci in the Iraqi population sample are shown in Table 1. In the locus HPRTB, (allele 13) the highest allele frequencies were found. Haplotype frequencies of four linkage groups were counted in 120 men. The linkage groups 1, 2, 3 and 4 revealed 60, 90, 65 and 88 haplotypes, respectively (Tables 2 and 3). The most frequent haplotypes were 12-12, 12-15 and 17-15 for Linkage Group 1; 11-34 and 13-27 for Linkage Group 2; 31-17 and 38-13 for Linkage Group 3; and 20-13 for Linkage Group 4. DXS10135 was the most polymorphic locus (with 25 alleles, PIC = 0.931), whereas the lowest values were observed for DXS7423 and DXS8378 (both with 5 alleles, PIC 0.506 and 0.589 respectively) (Figure 3). Power of exclusion (PE) ranged from 0.308 to 0.781 in male samples (Figure 4). Power of discrimination (PD) ranged from 0.599 to 0.952 in male samples (Figure 5). In a complex kinship testing, X-STR genotyping can supplement the analysis of autosomal, mitochondrial and Y-chromosomal markers. In the last few years, the need

Table 1. Allele frequencies at eight X-STR loci of 120 unrelated males from Iraq.

Allele	HPRTB	DXS10101	DXS7132	DXS10074	DXS10134	DXS7423	DXS10135	DXS8378
7	0.03032	-	-	0.04007	-	-	-	-
8	-	-	-	0.09916	-	-	-	-
9	-	-	-	0.00810	-	-	-	0.02091
10	0.02083	-	-	0.00275	-	-	-	0.29810
11	0.20090	-	0.00262	0.00300	-	-	-	**0.37100**
12	0.00282	-	0.10970	0.02610	-	-	-	0.19229
13	**0.43620**	-	**0.40010**	0.00991	-	0.08971	-	0.04007
14	0.29410	-	0.28113	0.05190	-	**0.40980**	-	-
15	-	-	0.10002	**0.30160**	-	0.30151	-	-
15.3	-	-	-	-	-	-	0.00459	-
16	0.06910	-	0.03960	0.19500	-	0.21500	0.00265	-
17	-	-	0.00251	0.26600	-	0.02390	0.01184	-
18	-	-	-	0.03957	-	-	0.04251	-
19	-	-	-	0.10061	-	-	0.06256	-
19.1	-	-	-	-	-	-	0.00265	-
20	-	-	-	0.00640	-	-	0.04243	-
20.1	-	-	-	-	-	-	0.03151	-
21	-	-	-	-	-	-	0.05944	-
21.1	-	-	-	-	-	-	0.02073	-
22	-	-	-	-	-	-	0.05697	-
22.1	-	-	-	-	-	-	0.00610	-
23	-	-	-	-	-	-	0.08400	-
23.1	-	-	-	-	-	-	0.01301	-
24	-	-	-	-	-	-	0.08204	-
25	-	-	-	-	-	-	0.08902	-
25.1	-	-	-	-	-	-	0.00825	-
25.2	-	0.02192	-	-	-	-	-	-
26	-	-	-	-	-	-	**0.09320**	-
26.2	-	0.00298	-	-	-	-	-	-
27	-	0.00310	-	-	-	-	0.08989	-
28	-	0.06210	-	-	-	-	0.04100	-
28.2	-	0.01292	-	-	-	-	-	-
29	-	0.10330	-	-	-	-	0.04812	-
29.2	-	0.05972	-	-	-	-	-	-
30	-	0.11922	-	-	0.00401	-	0.02799	-
30.2	-	0.03077	-	-	0.00299	-	-	-
31	-	**0.22011**	-	-	0.01270	-	0.03092	-
32	-	0.07921	-	-	0.00412	-	0.00698	-
32.2	-	0.20930	-	-	-	-	-	-
33	-	0.06033	-	-	0.06009	-	-	-
33.2	-	0.03991	-	-	-	-	-	-
34	-	0.05001	-	-	0.13299	-	0.00419	-
34.2	-	0.00960	-	-	-	-	-	-
35	-	-	-	-	0.20390	-	-	-
35.2	-	-	-	-	**0.36022**	-	-	-
36	-	-	-	-	0.20716	-	-	-
37	-	-	-	-	0.00215	-	-	-
37.1	-	-	-	-	0.00801	-	-	-
37.2	-	-	-	-	0.00719	-	-	-
37.3	-	-	-	-	0.05911	-	-	-
38	-	-	-	-	0.01996	-	-	-

Table 1. Contd

38.3	-	-	-	-	0.02113	-	-	-
39	-	-	-	-	0.04093	-	-	-
39.3	-	-	-	-	0.00416	-	-	-
40	-	-	-	-	0.01968	-	-	-
41	-	-	-	-	0.00296	-	-	-
41.3	-	-	-	-	0.01930	-	-	-
42.3	-	-	-	-	0.02617	-	-	-
43.3	-	-	-	-	0.00805	-	-	-

Table 2. Haplotype frequencies for eight X-STR loci in linkage groups 1 and 2 in Iraq.

Linkage group 1				Linkage group 2			
Haplotype		Number	Frequency	Haplotype		Number	Frequency
DXS7132 - DXS10074				HPRTB - DXS10101			
11	11	1	0.0083	7	29.2	1	0.0083
11	12	2	0.0166	7	30	1	0.0083
11	13	2	0.0166	7	30.2	1	0.0083
11	14	1	0.0083	7	31	2	0.0166
11	15	1	0.0083	7	32	1	0.0083
12	7	1	0.0083	7	32.2	2	0.0166
12	8	4	0.0333	7	33	2	0.0166
12	9	1	0.0083	7	33.2	1	0.0083
12	10	1	0.0083	7	34	1	0.0083
12	11	1	0.0083	7	34.2	1	0.0083
12	12	10	0.0833	10	25.2	1	0.0083
12	13	1	0.0083	10	26.2	1	0.0083
12	14	1	0.0083	10	27	1	0.0083
12	15	6	0.0500	10	28	1	0.0083
12	15.3	1	0.0083	10	28.2	1	0.0083
13	10	1	0.0083	10	29.2	1	0.0083
13	11	1	0.0083	10	30	2	0.0166
13	12	1	0.0083	10	30.2	1	0.0083
13	13	5	0.0416	10	31	1	0.0083
13	14	1	0.0083	10	32	1	0.0083
13	15	1	0.0083	10	32.2	1	0.0083
13	15.3	1	0.0083	10	33	2	0.0166
14	7	1	0.0083	10	33.2	1	0.0083
14	8	2	0.0166	10	34	1	0.0083
14	9	3	0.0250	10	34.2	1	0.0083
14	10	1	0.0083	11	28	1	0.0083
14	11	1	0.0083	11	28.2	1	0.0083
14	12	1	0.0083	11	29.2	1	0.0083
14	13	4	0.0333	11	30	1	0.0083
14	14	1	0.0083	11	30.2	1	0.0083
14	15	1	0.0083	11	31	1	0.0083
14	15.3	2	0.0166	11	32	1	0.0083
15	7	2	0.0166	11	32.2	1	0.0083
15	8	2	0.0166	11	33	1	0.0083
15	9	1	0.0083	11	33.2	1	0.0083
15	10	1	0.0083	11	34	6	0.0500

Table 2. Contd

15	11	1	0.0083	11	34.2	1	0.0083
15	12	5	0.0416	12	25.2	1	0.0083
15	13	1	0.0083	12	26.2	1	0.0083
15	14	2	0.0166	12	27	1	0.0083
15	15	1	0.0083	12	28	1	0.0083
15	15.3	3	0.0250	12	28.2	1	0.0083
16	9	1	0.0083	12	29.2	1	0.0083
16	10	1	0.0083	12	30	1	0.0083
16	11	1	0.0083	12	30.2	1	0.0083
16	12	3	0.0250	12	31	1	0.0083
16	13	1	0.0083	12	32	1	0.0083
16	14	6	0.0500	12	32.2	1	0.0083
16	15	1	0.0083	12	33	1	0.0083
16	15.3	5	0.0416	12	33.2	1	0.0083
17	7	1	0.0083	12	34	4	0.0333
17	8	1	0.0083	12	34.2	1	0.0083
17	9	4	0.0333	13	25.2	3	0.0250
17	10	2	0.0166	13	26.2	1	0.0083
17	11	2	0.0166	13	27	6	0.0500
17	12	1	0.0083	13	28	1	0.0083
17	13	1	0.0083	13	28.2	1	0.0083
17	14	1	0.0083	13	29.2	1	0.0083
17	15	6	0.0500	13	30	1	0.0083
17	15.3	1	0.0083	13	30.2	1	0.0083
-	-	-	-	13	31	4	0.0333
-	-	-	-	13	32	2	0.0166
-	-	-	-	13	32.2	1	0.0083
-	-	-	-	13	33	1	0.0083
-	-	-	-	13	33.2	1	0.0083
-	-	-	-	13	34	1	0.0083
-	-	-	-	13	34.2	1	0.0083
-	-	-	-	14	27	1	0.0083
-	-	-	-	14	28	1	0.0083
-	-	-	-	14	28.2	1	0.0083
-	-	-	-	14	29.2	1	0.0083
-	-	-	-	14	30	1	0.0083
-	-	-	-	14	30.2	1	0.0083
-	-	-	-	14	31	1	0.0083
-	-	-	-	14	32	1	0.0083
-	-	-	-	14	32.2	1	0.0083
-	-	-	-	14	33	1	0.0083
-	-	-	-	14	33.2	1	0.0083
-	-	-	-	16	25.2	1	0.0083
-	-	-	-	16	26.2	1	0.0083
-	-	-	-	16	27	5	0.0416
-	-	-	-	16	28	2	0.0166
-	-	-	-	16	28.2	1	0.0083
-	-	-	-	16	29.2	1	0.0083
-	-	-	-	16	30	1	0.0083
-	-	-	-	16	30.2	1	0.0083
-	-	-	-	16	31	1	0.0083
-	-	-	-	16	32	1	0.0083

Table 2. Contd

-	-	-	-	16	32.2	1	0.0083
-	-	-	-	16	33	1	0.0083

Table 3. Haplotype frequencies for eight X-STR loci in linkage groups 3 and 4 in Iraq.

Linkage group 3				Linkage group 4			
Haplotype		Number	Frequency	Haplotype		Number	Frequency
DXS10134 - DXS7423				DXS10135 - DXS8378			
30	15	1	0.0083	15.3	11	1	0.0083
30	16	6	0.0500	15.3	12	1	0.0083
30	17	1	0.0083	15.3	13	1	0.0083
30.2	13	1	0.0083	16	10	2	0.0166
30.2	16	1	0.0083	16	12	2	0.0166
31	13	1	0.0083	16	13	1	0.0083
31	14	1	0.0083	17	9	1	0.0083
31	15	1	0.0083	17	10	1	0.0083
31	16	1	0.0083	17	11	1	0.0083
31	17	10	0.0833	17	12	2	0.0166
32	14	1	0.0083	17	13	1	0.0083
32	15	1	0.0083	18	11	1	0.0083
32	16	1	0.0083	18	12	1	0.0083
32	17	1	0.0083	18	13	1	0.0083
33	15	1	0.0083	19	9	1	0.0083
33	16	1	0.0083	19	12	1	0.0083
33	17	1	0.0083	19	13	1	0.0083
34	14	2	0.0166	19.1	9	1	0.0083
34	17	2	0.0166	19.1	10	4	0.0333
35	13	2	0.0166	19.1	12	1	0.0083
35	16	2	0.0166	19.1	13	5	0.0416
35	17	1	0.0083	20	9	1	0.0083
35.2	13	1	0.0083	20	10	1	0.0083
35.2	14	1	0.0083	20	11	1	0.0083
35.2	15	1	0.0083	20	12	1	0.0083
35.2	16	2	0.0166	20	13	9	0.0750
35.2	17	2	0.0166	20.1	11	1	0.0083
35.2	13	1	0.0083	20.1	12	1	0.0083
35.2	14	1	0.0083	20.1	13	1	0.0083
36	15	1	0.0083	21	9	1	0.0083
36	16	3	0.0250	21	10	1	0.0083
37	14	1	0.0083	21	11	1	0.0083
37	15	1	0.0083	21	12	1	0.0083
37	16	1	0.0083	21	13	1	0.0083
37.1	13	4	0.0333	21.1	10	1	0.0083
37.1	14	5	0.0416	21.1	11	1	0.0083
37.1	17	1	0.0083	21.1	12	1	0.0083
37.2	13	1	0.0083	22	9	1	0.0083
37.2	14	1	0.0083	22	10	1	0.0083
37.2	15	2	0.0166	22	11	1	0.0083
37.2	17	1	0.0083	22	12	1	0.0083
37.3	16	2	0.0166	22	13	1	0.0083

Table 3. Contd

37.3	17	1	0.0083	23	10	1	0.0083
38	13	10	0.0833	23	11	1	0.0083
38	15	1	0.0083	23	12	1	0.0083
38	16	1	0.0083	23.1	11	1	0.0083
38	17	1	0.0083	23.1	12	1	0.0083
38.3	14	1	0.0083	23.1	13	1	0.0083
38.3	16	1	0.0083	24	10	7	0.0583
39	13	1	0.0083	24	11	3	0.0250
39	17	1	0.0083	24	12	2	0.0166
39.3	15	1	0.0083	24	13	2	0.0166
39.3	16	4	0.0333	25	9	2	0.0166
39.3	17	1	0.0083	25	10	1	0.0083
40	17	1	0.0083	25	11	1	0.0083
41	13	1	0.0083	25	12	1	0.0083
41	16	1	0.0083	25	13	1	0.0083
41.3	15	1	0.0083	25.1	10	1	0.0083
41.3	16	1	0.0083	25.1	11	1	0.0083
41.3	17	1	0.0083	25.1	12	1	0.0083
42.3	13	1	0.0083	26	9	1	0.0083
42.3	14	1	0.0083	26	10	1	0.0083
42.3	17	1	0.0083	26	11	1	0.0083
43.3	14	1	0.0083	27	11	1	0.0083
42.3	16	1	0.0083	27	12	1	0.0083
-	-	-	-	27	13	1	0.0083
-	-	-	-	28	9	1	0.0083
-	-	-	-	28	10	1	0.0083
-	-	-	-	28	11	1	0.0083
-	-	-	-	28	12	1	0.0083
-	-	-	-	28	13	3	0.0250
-	-	-	-	29	10	1	0.0083
-	-	-	-	29	11	1	0.0083
-	-	-	-	29	12	1	0.0083
-	-	-	-	30	9	1	0.0083
-	-	-	-	30	10	1	0.0083
-	-	-	-	30	11	1	0.0083
-	-	-	-	30	12	1	0.0083
-	-	-	-	30	13	1	0.0083
-	-	-	-	31	10	1	0.0083
-	-	-	-	31	11	1	0.0083
-	-	-	-	31	12	1	0.0083
-	-	-	-	32	10	1	0.0083
-	-	-	-	32	11	1	0.0083
-	-	-	-	32	12	1	0.0083
-	-	-	-	32	13	1	0.0083
-	-	-	-	33.2	11	1	0.0083
-	-	-	-	33.2	12	1	0.0083

for commercially available and validated X-STR kits has increased due to a growing number of complex kinship cases. Intensive studies of the X chromosome discover a lot of closely linked X-STR markers, which can be included

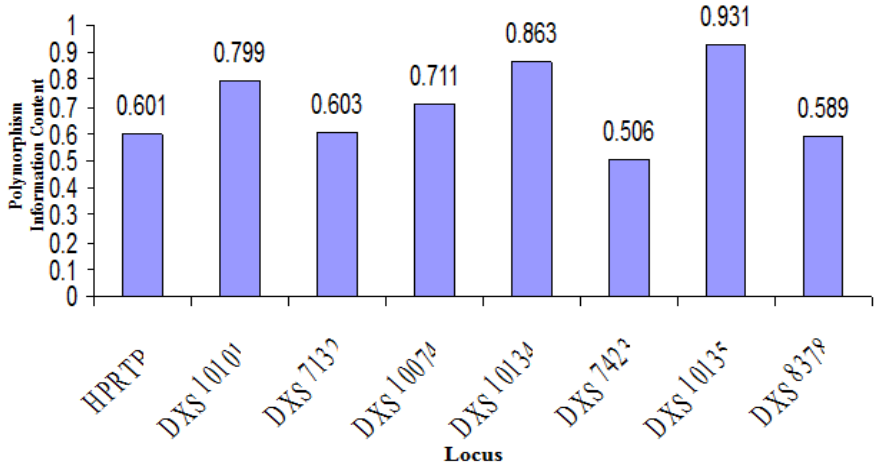

Figure 3. Forensic efficiency parameters: Polymorphism information content (PIC).

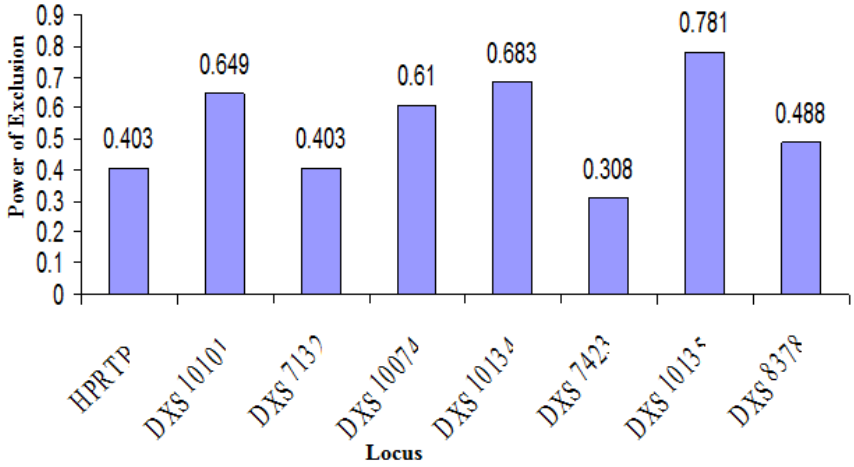

Figure 4. Forensic efficiency parameters: Power of exclusion (PE).

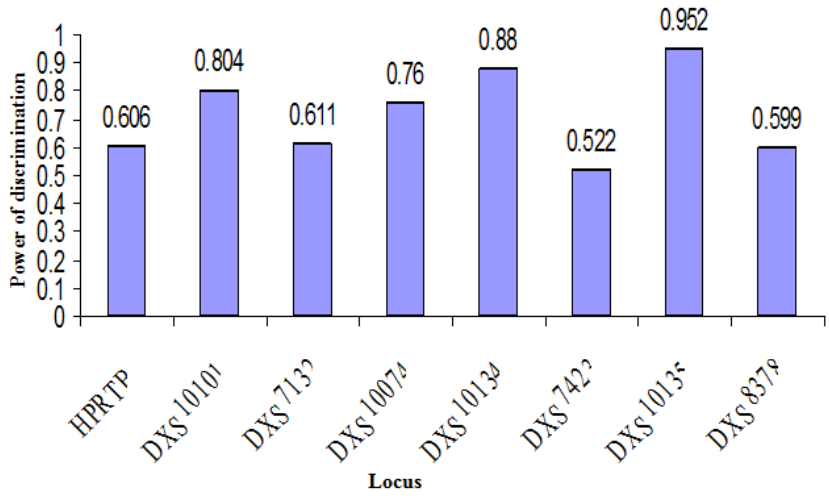

Figure 5. Forensic efficiency parameters: Power of discrimination (PD).

in the commercially available kits (Hering et al., 2006; Edelmann et al., 2008; Edelmann et al., 2009; Hundertmark et al., 2008; Ferreira et al., 2010).

Recent forensic casework, population genetics and anthropological studies have used the relatively new commercially available Investigator Argus X-12 kit. Investigator Argus X-12 presents an improvement as compared to Mentype® Argus X-8 in the sense of increased discriminatory power due to four linkage groups with three markers per group (Amelogenin; DXS10148, DXS10135, DXS8378; DXS7132, DXS10079, DXS10074; DXS10103, HPRTB, DXS10101; DXS10146, DXS10134, DXS7423) (Qiagen. Investigator Argus X-12 PCR handbook. Hilden: Qiagen; 2010). Further studies are planned to get an overview of the X-STR variability in all Croatian regions, and there are plans for inclusion of 12 X-STR loci in the database.

Conclusion

This study is the first of its kind in Iraq, where there are no prior study on the assessment of x-chromosome STRs. X-linked markers in Mentype® Argus X-8 PCR amplification kit proved to be highly polymorphic with a high power of discrimination. Our results suggest that all eight X-STRs described here can efficiently be used in parentage analysis and provide a powerful tool in forensic case work, in particular, to identify the female DNA profile in mixture analysis.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Citrus tristeza virus: An increasing trend in the virus occurrence and distribution in citrus fruits of Northwest, Pakistan

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Citrus tristeza closterivirus (CTV) is one of the most damaging fruit viruses playing havoc in citrus orchards around the world. Here, we report, an ELISA-based indexing of citrus trees over a period of eight years (2002 to 2010) in Northwest Pakistan, revealing that the incidence of CTV is increasing mainly with the distribution of infected rootstocks, putting citrus industry at the verge of complete annihilation. The surveys revealed that the average incidence of CTV in 10 major citrus growing districts in the Northwest of Pakistan has steadily increased from 24% (in 2002) to 31, 35, 39 and 44 in 2004, 2006, 2008, 2010, respectively. Maximum per cent increase of CTV was in citrus orchards in district Haripur, that is, 27.50% and followed by 26% increase of the virus incidence in district Swat during 2002 to 2010. The incidence of the virus was correlated with use of sour orange as root stock with rough lemon. In orchards, where rough lemon was used as root stock; the incidence of CTV was comparatively low. A comparative study of the virus incidence conducted during 2006 to 2012 in sweet orange trees grafted on sour orange and rough lemon in Northwest indicated an average per cent incidence 43 and 37, respectively. This is the first comprehensive study on citrus fruits to determine an increasing trend of CTV in Northwest of Pakistan where citrus industry has been a major source of income for local farmers. An extensive rescue plan needs to be placed to avoid complete destruction of this export-based industry.

Key words: Citrus, Tristeza, citrus tristeza closterivirus (CTV), ELISA, incidence, Pakistan.

INTRODUCTION

Citrus fruits represent approximately 40% of all fruit crops growing in Pakistan and mainly concentrated in Punjab and Khyber Pakhtunkhwa (KP) Province (Catara, 1987; Catara et al., 1988). The range of cultivated citrus

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Abbreviations: CTV, Citrus tristeza virus; **DAS-ELISA,** double antibody sandwich-enzyme-linked immunosorbent assay; **KP,** Khyber Pakhtunkhwa; **PARC,** Pakistan agricultural research council.

cultivars in Pakistan is limited. Kinnow is a major citrus cultivar in Punjab, whereas sweet orange (blood-red) is mainly grown in KP. Many cases of citrus decline have been reported from Punjab and KP, Pakistan in 1970s and citrus tristeza was considered as the major cause of the decline (Bove, 1995). In a preliminary survey conducted by a group of Italian and Pakistani experts in 1988 with the co-operation of Ministry of Foreign Affairs and Pakistan Agricultural Research Council (PARC), citrus was reported to be infected by a number of virus and virus-like diseases in KP and Punjab, regions Pakistan and only CTV was confirmed by enzyme-linked immuno-sorbent assay (ELISA) and electron microscopy (Catara et al., 1988). After a period of about 15 to 20 years, the situation had been deteriorated to the extent that most of the citrus orchards are about to collapse in KP (Arif et al., 2005a) as well as in Punjab Provinces (Iftikhar et al., 2009) of Pakistan. Almost 100% of citrus trees in most parts KP are infected with one or more virus and virus-like diseases (Arif et al., 2005a) that resulted in high economic losses. The major virus and virus-like diseases of citrus trees reported in Pakistan are tristeza, infectious variegation, exocortis, cachexia-xyloporosis, greening and stubborn (Catara, 1987; Catara et al., 1988; Bove, 1995; Arif et al., 2005a; Iftikhar et al., 2009). Most of these diseases are wide spread in new plantations due to un-certified infected bud wood being used as scion. As a result, the citrus industry that could earn foreign exchange in billion is on decline and is almost collapsed. New plantation would also be un-successful until pathogen-free bud wood is provided to the citrus growers.

Tristeza caused by citrus tristeza virus (CTV) is the most economically destructive disease of citrus fruits world-wide. The virus is restricted to phloem tissues of the infected plants. It occurs in most citrus producing areas of the world, especially in the areas where CTV-sensitive sour orange (*Citrus aurantium* L.) is used as rootstock (Bar-Joseph et al., 1989; Rocha-Pena et al., 1995). CTV was originated in the Orient from where it was spread world-wide through infected bud-wood and plants in the quest for new citrus varieties (Bar-Joseph et al., 1979; Roistacher et al., 1991; Roistacher and Moreno, 1991; Rocha-Pena et al., 1995). Several epidemics of CTV have been reported from Argentina and Brazil during the 1930s, over 30 million citrus trees were killed (Bar-Joseph et al., 1989), in Spain during 1960s and in Venezuela during 1980s, about 10.0 and 6.6 million trees were killed, respectively (Cambra et al., 1988; Rocha-Pena et al., 1995).

The virus particles are flexuous, approximately 2000 × 11 nm in size (Gonsalves et al., 1978; Bar-Joseph and Lee, 1989), having single stranded positive-sense RNA of about 20 Kb (Gonsalves et al., 1978; Bar-Joseph et al., 1985; Bar-Joseph and Lee, 1989) and encapsidated by a coat protein (Bar-Joseph and Lee, 1989; Sekiya et al., 1991). Molecular characterization of the CTV isolates in various countries has been established (Biswas, 2010; Al-Sadi et al., 2012; Davino et al., 2013). CTV is transmitted by several aphid species in a semi-persistent manner (Raccah et al., 1989; Yokomi et al., 1994). However, each aphid species vary in transmission efficiency. The most efficient aphid vector is *Toxopetra citricida* (Roistacher and Bar-Joseph, 1987; Rocha-Pena et al., 1995) where *Aphis gossypii* Glover is also an efficient vector of severe strains of CTV in many areas around the world (Roistacher and Bar-Joseph, 1987; Yokomi and Garnsey, 1987). *Aphis spiraecola* Patch (Yokomi and Garnsey, 1987), *Toxoptera aurantii* (Norman and Grant, 1958), *Aphis craccivora* Koch and *Dactynotus jacae* (Roistacher and Bar-Joseph, 1987; Bar-Joseph and Lee, 1989) have also been reported as vectors of CTV. *A. gossypii* and *A. spiraecola* are the major vectors of CTV in Pakistan (Catara, 1987).

In Pakistan, occurrence of CTV has long been suspected in citrus fruits but virus identity was confirmed by using ELISA and electron microscope from samples collected from various parts of KP and the Punjab provinces (Catara et al., 1988; Catara et al., 1991; Anwar and Mirza, 1992). In Northwest (KP province) of Pakistan, comprehensive surveys were conducted to report virus and virus diseases of citrus fruits and CTV was emerged as major pathogen (Arif et al., 2005a). The occurrence and distribution of CTV was also reported from selected orchards of KP and the Punjab provinces by Iftikhar et al. (2009). In this paper, we report an increasing trend of CTV in citrus fruits in Northwest of the country including KP province, based on more than eight years of comprehensive survey study, requiring a rescue plan to avoid complete annihilation of citrus industry.

MATERIALS AND METHODS

Field surveys: sampling and indexing of mother citrus trees for CTV

Field surveys were conducted from March to September 2002 to 2010 in 10 major citrus growing districts (Peshawar, Nowshera, Charsadda, Mardan, Swabi, Haripur, Malakand, Swat, Dir, D. I. Khan) of the KP province, Pakistan. Two citrus orchards were selected in each district and orchards were selected where 100 to 200 citrus trees were present at 10 to 20 years of age. During surveys, trees were examined diagonally as described by Hughes and Gottwald (1998) or by selecting 10 × 10 m, 20 × 20 m area at two-three sites in an orchard (Arif et al., 2005a). In some cases where total numbers of trees were in hundreds, then a scheme of random assessment was made (Arif et al., 2005a). The total number of trees in selected sites or in some cases total numbers of trees in an orchard were counted, assessed and trees showing symptoms of the virus were recorded. Trees showing characteristic symptoms of CTV were tagged for future reference. For serological indexing for CTV, leaf samples were collected from infected and healthy trees from all 20 orchards in 10 districts surveyed. Samples were collected in plastic bags, kept in ice bags and transported to the laboratory for serological detection. The specimen of insects associated with citrus plantation were collected in Petri dishes containing moistened blotting paper and kept for identification. Detailed information on insecticides sprays, source of nursery plants and rootstocks, cultivars and symptoms of other biotic effects

were also recorded. Percent incidence of CTV was determined in each orchard/site or district using ELISA based surveys as: (% CTV incidence = total trees infected/total trees tested × 100). Mean values were calculated for each district surveyed.

Serological studies

DAS-ELISA was used for the detection of CTV (Clark and Adams, 1977). The tests were performed in polystyrene micro-plates containing 96 wells (NUNC, Immunoplate II, Thermal Scientific, Waltham, MA, USA). ELISA-plates were coated with 100 µl aliquots of CTV-specific antibody (Agdia, USA) with coating buffer, pH 9.6. Plates were kept at room temperature in a humid box for 3 to 4 h. Leaf samples were extracted by crushing through pestle and mortar in extraction buffer, pH 7.4. Leaf tissues were extracted in extraction buffer at 1:10 ratio (w/v), and 100 µl of prepared sample was dispensed in each well after washing. After first incubation was completed the plates were washed with 1 × PBST buffer, pH 7.4. Positive control wells were filled with using same amount of sap from known CTV-infected and healthy citrus plants as negative control. ELISA-plates were incubated inside a humid box for 2 h at room temperature or overnight in refrigerator (4°C). After washing, 100 µl of enzyme conjugate (Agdia) was dispensed in each well of the plate and incubated in a humid box for 2 h at room temperature. The plates were washed four times with 1 × PBST. 100 µl of OPD solution [(100 ml of OPD solution, pH 5.0 was prepared by dissolving hydrogen peroxide (30%) 0.4 ml, citric acid (anhydrous) 5.1 g, sodium phosphate, dibasic (anhydrous) 7.33 g in 900 ml of distilled water and volume was adjusted to 1000 ml by adding more distilled water)] was added per well and the plates were incubated 1 h in humid box at room temperature or overnight (that is, 16 h) at 4°C. The reaction was stopped by adding 50 µl 3 M sulphuric acid to each well. The reaction was assessed visually or measured at A_{405} nm using Titertek Multiskan, (Model MC-Photometer) (Flow Laboratories, Covina, CA, USA). Inc.). The samples were considered to be positive when the $A_{405\ nm}$ values exceeded the mean of the virus-free samples by at least a factor of three.

RESULTS

CTV was present in all major citrus growing areas of the Northwest of Pakistan (KP province) surveyed (Table 1). The characteristic symptoms of CTV were commonly observed on sweet oranges grafted on sour orange. The infected trees exhibited vein clearing in leaves and developed phloem necrosis and inner face tiny projections (Figure 1). The affected wood produced tiny projections going in to small holes in the inner face of the bark. Vein clearing and stem pitting were also observed on sweet orange trees sour orange rootstocks. ELISA-based indexing of citrus trees revealed that the incidence of CTV is increasing, with the distribution of infected rootstocks from 2002 to 2010 (Table 1). The average percent incidence of CTV in the major citrus growing districts of the Northwest (KP province) was recorded as: 24% (2002), 31% (2004), 39% (2006), 41% (2008) and 44 % (2010), respectively (Table 1). In KP province, the minimum and maximum range of CTV was 16 to 31% during 2002, 25 to 38% during 2004, 27 to 42% during 2006, 35 to 47 during 2008 and 40 to 48% during 2010, respectively. Therefore, the range of increase of CTV during 2002 to 2010 in KP province was from 16 to 48% (Table 1). Sweet orange was the major commercially grown citrus species in the province, therefore, ELISA-based indexing was done mainly in the orchards. The incidence of CTV was at maximum range (44%) and widely spread in 10 districts surveyed. In KP province, in most cases, where the incidence of the virus was high, sweet orange was grafted on sour orange root stock. In a few orchards at District Haripur, rough lemon was used as root stock; and thus, the incidence of CTV was comparatively low (Table 2). A comparative study of the virus incidence conducted during 2006 to 2012 in sweet orange trees grafted on sour orange and rough lemon in KP province indicated an average per cent incidence of CTV was 43 and 37, respectively (Table 2).

Maximum per cent increase of CTV was reported in citrus orchards in Haripur and followed by virus incidence in district Swat. In 2002, the virus incidence at Haripur was only 16.50%, while at Swat district was 20%, respectively. The results reported in Table 3 shows an increasing trend of the virus in 10 major citrus growing districts of KP province during 2002 to 2010. Figures 2 and 3 show the increasing trend of CTV in each districts/division and KP province as a whole, Pakistan, during 2002 to 2010. Further details of CTV increase in citrus fruits in major district/divisions of KP province, Pakistan such as Peshawar, Mardan, Malakand, Hazara D. I. Khan, and as a whole in KP, are given as Table 3. The identity of the virus was further confirmed through graft inoculation (by taking buds from selected infected trees) on different citrus species (*C. aurantium*, *Citrus lemon* cv. Eureka and *Citrus sinensis*). The graft inoculated citrus species mainly exhibited vein clearing and chlorosis symptoms. A comparison of CTV symptoms on naturally infected citrus species in field and reactions of the plants artificially inoculated in screen house are given in Table 4. Citrus species (*C. limon*) cv. Eureka was reported better indicator plant for the detection of CTV due to the production of vein clearing and chlorosis symptoms comparatively less time period after graft inoculation.

DISCUSSION

The results confirmed the wide spread infection of CTV in all citrus growing areas of the Northwest of Pakistan. Citrus tristeza along with other graft-transmitted diseases has been reported in Northwest, Pakistan (Arif et al., 2005a). Citrus tristeza virus, exocortis viroid and greening posed a real threat to citrus cultivation, not only in Pakistan but also in most citrus producing areas of the world (Catara et al., 1988; Bar-Joseph et al., 1989; Lee et al., 1994; Rocha-Pena et al., 1995). In Northwest of Pakistan, CTV was reported in a few locations with low intensity during 1987-1988 (Catara, 1987; Catara et al., 1988), however, greening was reported to be potential threat to citrus in this area (Coehran, 1976; Catara et al.,

Table 1. Percent incidence of Citrus tristeza virus in major citrus growing areas of Northwest of Pakistan during 2002 to 2010.

Division	District	Location	Year-2002		Year-2004		Year-2006		Year-2008		Year-2010	
			Tested/ Infected	% Incidence	Tested/ Infected	% Incidence	Tested/ Infected	% Incidence	Tested/ Infected	% Incidence	Tested/ Infected	% Incidence
Peshawar	Peshawar	Malakandher	45/10	22	40/12	30	30/10	33	20/8	40	35/15	45
		ARI Tarnab	55/14	25	52/20	38	40/16	40	45/19	42	40/16	40
	Charsadda	Tangi, Abazo	80/20	25	60/18	30	38/13	34	16/7	44	38/17	44
		Charsadda	70/16	23	50/14	28	42/14	33	25/9	36	42/18	43
	Nowshera	Manki Sharif	110/27	25	90/24	27	54/18	33	24/10	42	54/24	44
		Akora Khattak	90/24	27	85/23	27	23/7	30	15/7	47	23/11	48
Mardan	Mardan	Bakshali	194/60	31	100/34	34	54/20	37	24/11	46	56/26	46
		Palo Deri	190/54	28	102/33	32	44/14	32	34/15	44	44/18	40
	Swabi	Marghuz	180/61	34	110/40	36	30/11	37	24/10	42	30/13	43
		Dook	160/33	21	102/29	28	30/12	40	25/11	44	30/14	46
Hazara	Haripur	Haripur-1	162/27	17	56/16	29	50/18	36	30/12	40	50/22	44
		Haripur-2	145/23	16	40/12	30	25/9	36	40/14	35	25/11	44
Malakand	Malakand	Dargai, Dobandai	80/25	31	60/18	30	42/16	38	40/16	40	42/18	43
		Dargai, Kal Dera	80/17	21	44/14	32	45/12	27	42/17	40	45/20	44
	Swat	Barikot	80/16	20	28/8	29	30/8	27	50/21	42	30/14	46
		Fazalabad	100/20	20	50/16	32	24/10	42	30/11	37	24/11	46
	Dir	Timergara	62/14	23	40/10	25	20/8	40	26/10	38	20/9	45
		Khungi Payan	58/12	21	56/18	32	18/5	28	15/7	46	18/8	44
D. I. Khan	D. I. Khan	D. I. Khan-1	36/11	31	30/8	27	20/7	35	24/10	42	24/11	46
		D. I. Khan-2	44/9	20	18/6	33	25/9	36	30/12	40	25/12	48
Average			2021/493	24	1213/373	31	576/223	39	579/237	41	695/308	44

[I]Results based on DAS-ELISA detection of citrus tristeza virus from leaf samples collected from fields.

1988). During the course of about 15 years of these investigations, comprehensive and systematic surveys and indexing of mother plants revealed that the average incidence of CTV has increased from 24 to 44% and is almost prevalent in all areas surveyed. This rapid and consistent increase in incidence and distribution of CTV shows the potential threat of CTV to citrus fruits in Northwest of Pakistan. The common practice of

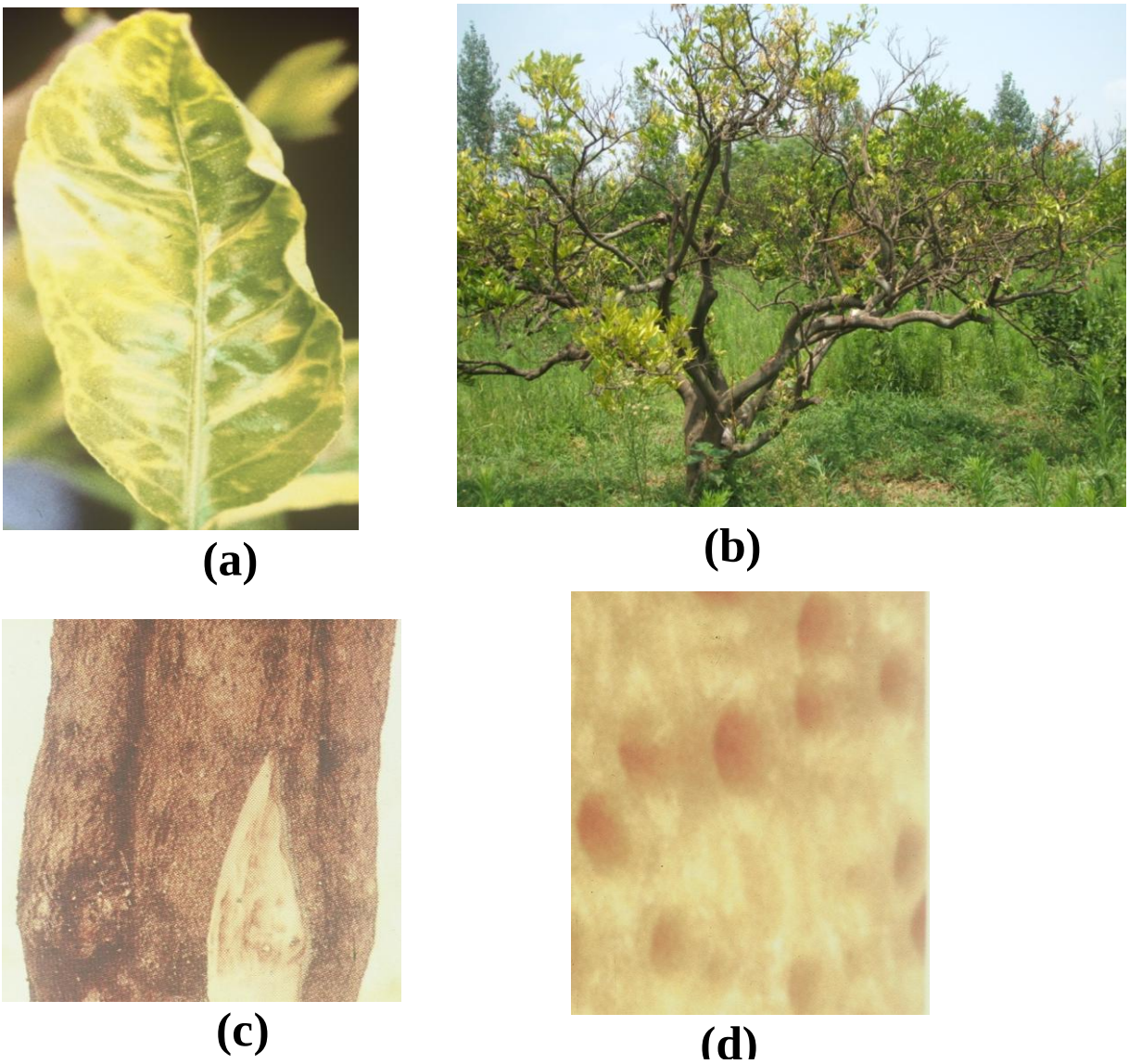


Figure 1. Field infection of citrus tristeza virus-sweet orange trees grafted on sour orange root stock. **(a)** Leaf showing vein clearing and chlorosis. **(b)** Decline and die-back. **(c)** Phloem necrosis. **(d)** Inner face tiny projections.

Table 2. Comparison of citrus tristeza virus incidence in sweet orange trees grafted on sour orange and rough lemon root stock in Northwest of Pakistan.

Year	Sweet orange[1] trees grafted on :			
	Sour orange [2]		Rough lemon[3]	
	Number of samples (tested/ infected)	% incidence	Number of samples (tested/ infected)	% incidence
2006	120/48	40	120/42	35
2008	130/53	41	130/44	34
2010	135/60	44	135/50	37
2012	140/66	47	140/56	40
Average % incidence		43		37

[1]Sweet orange (*Citrus sinensis* L. Osbeck), [2]Sour orange (*Citrus aurantium* L.). [3]Rough lemon (*Citrus jambhiri* Lush).

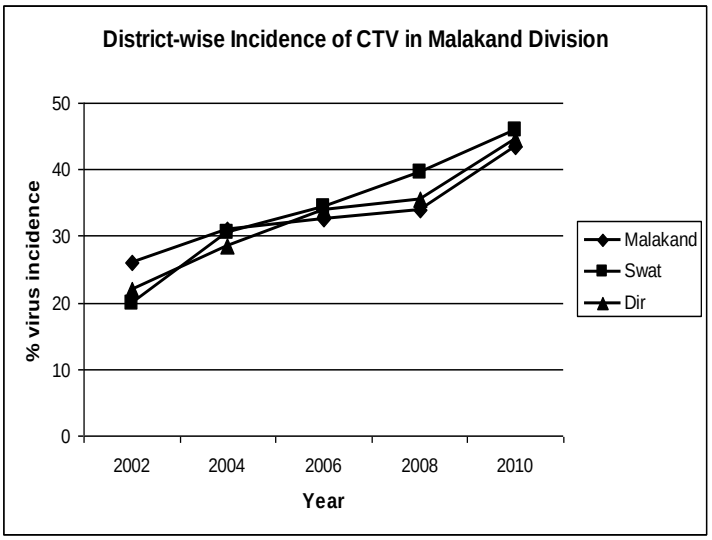
Table 3. Percent increase of Citrus tristeza virus in Northwest of Pakistan during 2002 to 2010.

Division	District	Average % incidence	% increase			
		Year-2002	Year-2004	Year-2006	Year-2008	Year-2010
Peshawar	Peshawar	23.50	+10.50 (34.00)[1]	+13.00 (36.50)	+17.50 (41.00)	+19.00 (42.50)
	Charsadda	24.00	+5.00 (29.00)	+9.50 (33.50)	+16.00 (40.00)	+19.50 (43.50)
	Nowshera	26.00	+1.00 (27.00)	+5.50 (31.50)	+18.50 (44.50)	+20.00 (46.00)
Mardan	Mardan	29.50	+3.50 (33.00)	+5.00 (34.50)	+15.50 (45.00)	+13.50 (43.00)
	Swabi	27.50	+4.50 (32.00)	+11.00 (38.50)	+15.50 (43.00)	+17.00 (44.50)
Hazara	Haripur	16.50	+13.00 (29.50)	+19.50 (36.00)	+21.00 (37.50)	+27.50 (44.00)
Malakand	Malakand	26.00	+5.00 (31.00)	+6.50 (32.50)	+14.00 (40.00)	+17.50 (43.50)
	Swat	20.00	+10.50 (30.50)	+14.50 (34.50)	+19.50 (39.50)	+26.00 (46.00)
	Dir	22.00	+6.50 (28.50)	+12.00 (34.00)	+20.00 (42.00)	+22.50 (44.50)
D. I. Khan	D. I. Khan	25.50	+4.50 (30.00)	+10.00 (35.50)	+15.50 (41.00)	+21.50 (47.00)

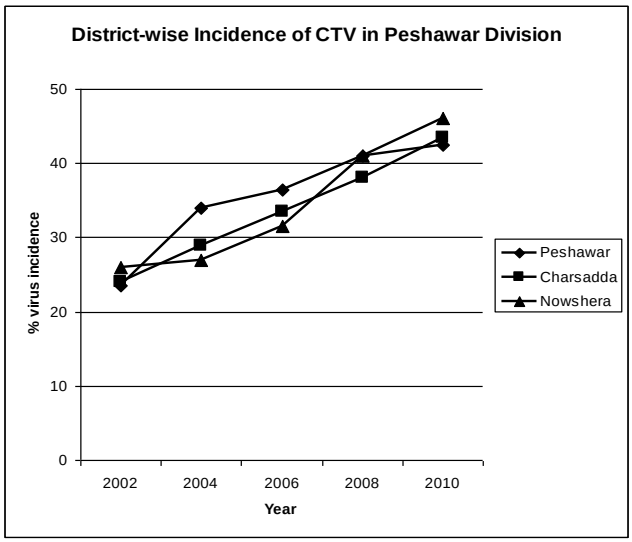
[1]Average per cent incidence of CTV in each district/ year.

grafting of sweet orange on sour orange root stock could be one of the main reasons for its wide spread occurrence and distribution. Sour orange has been one of the most popular root stocks world-wide (Grosser et al., 2004) and in Northwest of the country, citrus growers mainly used it as root stock for the production of high quality sweet orange. Sour orange is known to be susceptible to various graft-transmitted pathogens including CTV (Grosser et al., 2004). The use of infected bud scion could also be the cause of its rapid spread in the target areas. This practice facilitates the growth both virus-host relationship and result in citrus decline in almost 10 to 12 years depending on host genotype, virus strains and suitable environmental conditions. These infected trees also served as reservoir hosts for viruses and its vectors. Another reason for its wide spread occurrence of the virus in Northwest of Pakistan is the abundance of aphid vectors. CTV is transmitted by many aphid species (Catara, 1987; Roistacher and Bar-Joseph, 1987). Although, the most important aphid vector, *T. citricida* (Kirdaldy) has not been reported in major citrus growing districts of Northwest of the country including KP province (Arif et al., 2005a) and the Punjab province, Pakistan (Catara, 1987; Iftikhar et al., 2009). However, the virus is also efficiently transmitted by *A. gossypii* and *A. citricola* in Northwest, Pakistan (Arif, unpublished; Catara, 1987). The efficiency of *A. gossiypii* as vector of CTV has been well documented (Roistacher, 1984; Roistacher and Bar-Joseph, 1987) Small scale transmission studies has also indicated that a single *A. gossypii* can efficiently transmits CTV from sour orange to sweet orange and back from sweet orange to sour orange (Arif et al., 2005a). In this study, *A. gossiypii* was found in abundance during March to April on succulent citrus shoots, together with a number of other insect species but their role in transmission of virus and virus-like pathogens have not been determined. Based on the wide spread and quick decline syndrome, citrus greening has been previously reported as potential threat (Catara et al., 1988). No doubt, greening is playing pivotal role in the deterioration of citrus industry in Pakistan and other parts of the world. Citrus decline seems to be a complicated disease syndrome and involvement of CTV, *Citrus exocortis viroid* (CEVd) and *Spiroplasma citri,* the other pathogens could also be a possibility (Arif et al., 2005a). Detailed studies are required for isolation and characterization of the pathogens associated with the quick decline syndrome of citrus in Pakistan.

The results of this study indicate that CTV is quite prevalent in Northwest of Pakistan and its incidence is continuously increasing with time. Consistent and serious efforts are required to minimize the effect of the virus on citrus trees and for the successful production of citrus in Northwest and elsewhere in the country. It is essential that quarantine regulations should be strictly enforced to prevent introduction of CTV through exchange of and movement of infected nursery plants/ bud-wood from other parts of the country into the KP province and outside Pakistan. Awareness of citrus growers, farming community, researchers, extensionists on CTV problem and its prevention, is required. Eradication and immediate disposal of source of inoculum by removing old citrus trees and use of sour orange, *C. aurantium* for ornamental purposes should be discouraged. Rootstocks other than sour orange should be searched and tested for future plantation. Experience of rough lemon as root stock in many parts of Pakistan and elsewhere in world, is encouraging to prolong the life of CTV infected trees. Use of thermotherapy (Arif et al., 2005b) and cross protection (Abbas et al., 2005) techniques could be used to eliminate the virus in infected bud-wood and trees, respectively. Infestation of insects regardless of CTV vector, that is, *A. gossypii, Aphis citricolla,* on citrus nurseries and trees should carefully be monitored and controlled through insecticidal sprays. More precisely, an integrated approach could be adopted by using



(a)



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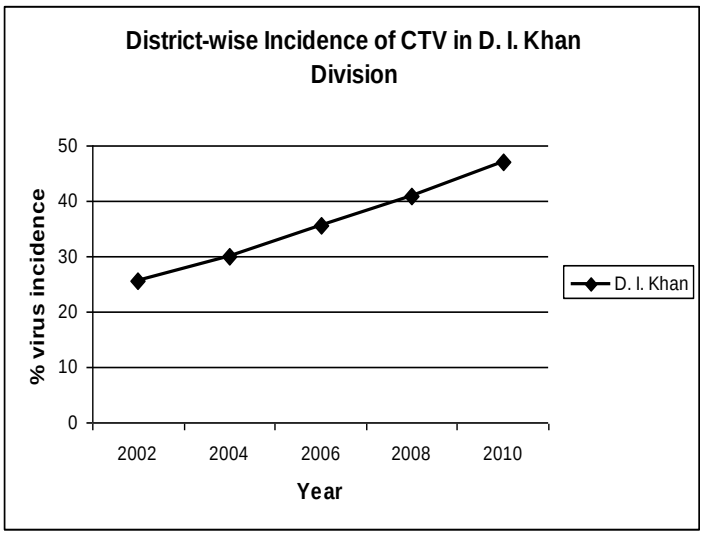

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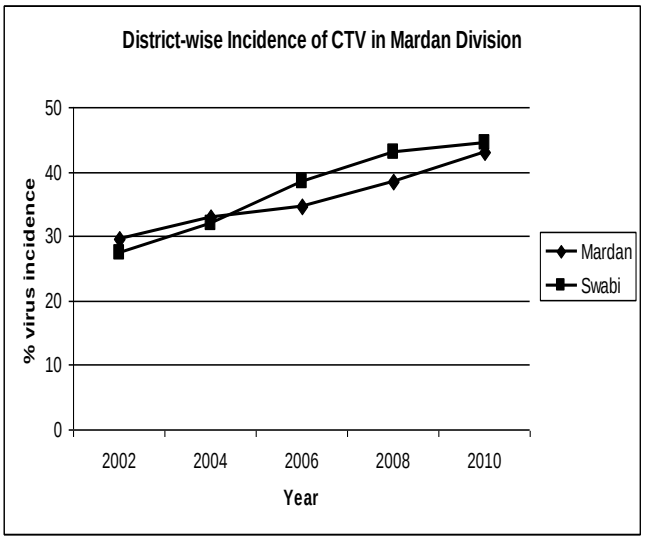

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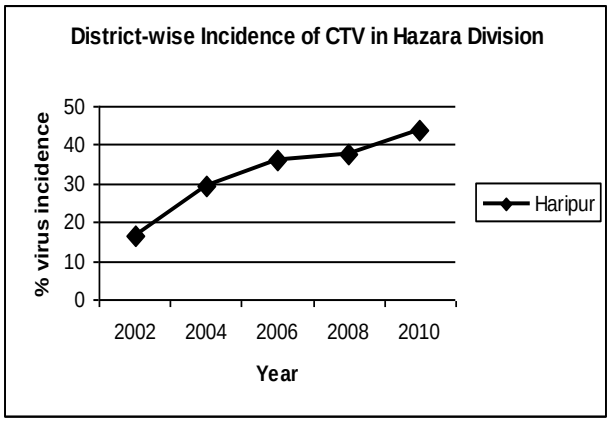

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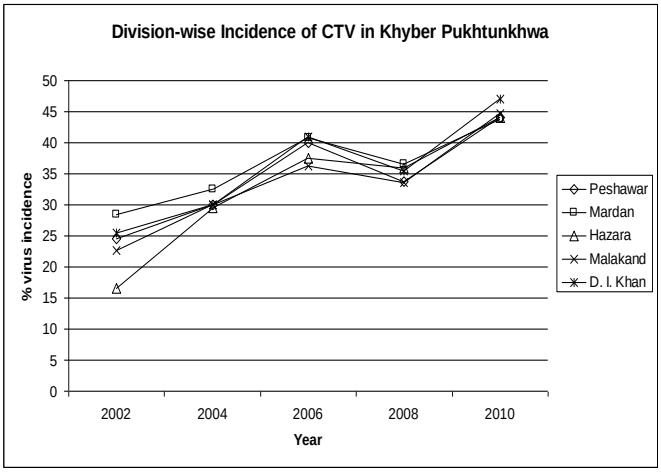

(f)

Figure 2. Curves showing increasing trend of Citrus tristeza virus in five major divisions and in Khyber Pakhtunkhwa Province. **(a)** Malakand. **(b)** Peshawar. **(c)** D. I. Khan. **(e)** Mardan. **(e)** Hazara divisions. **(f)** Overall in Khyber Pakhtunkhwa province.

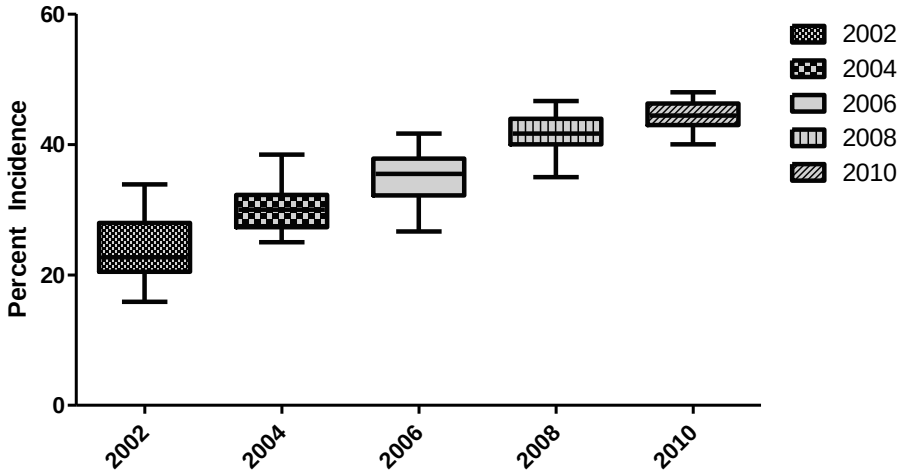

Figure 3. Box whisker plot showing percent incidence of citrus tristiza virus in Northwest of Pakistan during year 2002-2010.

Table 4. Reaction of citrus species against Citrus tristeza virus-prevalent isolates.

Pathogen	Naturally infected trees		Graft-inoculated plants	
	Host	**Symptoms**	**Host**	**Symptoms**
Citrus tristeza virus (CTV)	Sweet orange[1]	Vein clearing	*Citrus aurantium* (L.) [2]	Vein clearing and chlorosis
	Sweet orange grafted on sour orange	Phloem necrosis, inner face tiny projections	*C. limon*[3] (L.) Barm. cv. Eureka	Vein clearing and chlorosis
	Sweet orange / Mexican lime[4] / grapefruit[5]	Vein clearing and stem pitting	*C. sinensis* (L.) Osbeck	Vein clearing

[1]Sweet orange (*Citrus sinensis* (L.) Osbeck*);* [2]Sour orange (*Citrus aurantium* L.); [3]Eureka lemon *(Citrus limon* (L.) Barm.; [4]Mexican lime *Citrus aurantifolia* (Christm.) Swingle; [5]Grape fruit (*Citrus paradise* Macf.).

pathogen-free bud-wood, avoidance sources of infection, proper and timely use of insecticides, implementation of quarantine regulations and the use of resistant/ tolerant root stocks, for successful citrus production in Pakistan.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDEMENTS

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Full Length Research Paper

Estimation of optimal size of plots for experiments with radiometer in beans

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An experimental error can lead to rework and, consequently, to the loss of financial and human resources. One way to reduce this problem is the estimation of the optimum size of experimental plot to carry out the treatments. The objective of this study was to estimate the optimal size of plots for reflectance measurements in beans by the modified maximum curvature method and the maximum distance method. Reflectance readings were made on bean plants with the aid of the GreenSeeker® equipment, obtaining basic units of 0.45 m² in an area of lines 6 and 8 m in length, performing 46 combinations of experimental area. X_0 was determined using the modified maximum curvature and the maximum distance method. To increase the R^2, the calculations have been redone using 20 combinations of experimental area. By adopting the bigest obtained area, it was concluded that the optimum size of an experimental plot for works with reflectance in beans is 5.40 m² and the combination that presents the best distribution is 2 lines totalling 6 m long.

Key words: Reflectance, experimental error, modified maximum curvature, maximum distance.

INTRODUCTION

The spectral response of vegetation usually shows that plants absorb more solar energy in the visible region and the bands used for determining the vegetation indices (VI) are in the red and near infrared region (Monteiro et al., 2012).The GreenSeeker® is an instrument that provides the normalized difference vegetation index (NDVI) via reflectance measurements, the interpretation of which can provide information in a rapid and targeted way on nutritional conditions, physiological state, stress and potential crop yields, even in cloudy days, which prevent the acquisition from satellites (Malenovský et al., 2009; Gutiérrez-Soto et al., 2011; Martin et al., 2012; Ali et al., 2015). The reflectance, percentage of light reflected by the culture, can also detect variations in leaf area of plants attacked by diseases, serving as a parameter to estimate damage to production and determine the economic

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Abbreviations: MMC, Modified maximum curvature; **MMC,** maximum curvature method; **MD,** maximum distance.

damage threshold (Hikishima et al., 2010). For experiments with beans, the size of the portions differ according to the purpose of the study. To check the efficiency of the severity assessment of angular leaf spot in common bean based in healthy and diseased areas of the leaf, Parrella et al. (2013) adopted portions 8 m² (4 m long and 2 m wide). Doblinski et al. (2010) adopted a 2 m² area in the study of diffuse pollution of swine wastewater on the beans. To estimate the productivity of grains and wheat plant height using reflectance measurements Xavier et al. (2006) adopted 3.6 m² plots (3 m long by 1.2 m wide). In order to quantify the damage and the relationship between severity, reflectance and productivity in the pathosystem of Asian soybean rust, Hikishima et al. (2010) adopted as experimental unit an area of 6.75 m², or 3 lines of 5 m in length.

The economy of human and financial resources, without losing experimental precision, is considered an important factor in the design of experiments. To plan the tests and assess the magnitude of the experimental accuracy is important to determine the level of credibility of the results obtained in the research (Storck, 2011; Storck et al, 2011). The establishment of optimum plot size, in any culture, is one of the ways to increase the experimental precision and maximize the information obtained in an experiment (Silva et al., 2012), and is a recognized way to reduce experimental error, while there are several methods for its estimation based on different principles (Lorentz et al., 2012).The experimental error, which is the existing variance between experimental units that received the same treatment, is estimated by applying repetition, which is one of the principles of the trial and to avoid it is necessary to know the characteristics of the experimental area and the grown culture (Oliveira et al., 2005). Works with the right size of plots allow optimal use of resources, while also allowing the researcher greater control and management of their experiment, when performed in a smaller area (Lackey and Stein, 2014).For determining the optimum plot size through the method of maximum modified curvature and the maximum distance method a blank experiment is necessary, with the culture of interest and then the experimental area is subdivided into smaller portions, called basic units from which the data is collected independently while identifying the relative position. After the taking of the data, plots of different sizes and shapes are simulated through the sum of contiguous plots (Lorentz et al., 2012).

The objective of this study was to estimate the optimal size of plots for reflectance measurements in beans by the modified maximum curvature method and the Lorentz et al. (2012) called maximum distance.

MATERIALS AND METHODS

The experiment was conducted in a growing area of the State University of Londrina (UEL), in Londrina-PR, in the dry season of 2013. The cultivated beans were the IPR Andorinha (registration No. 30617, Ministry of Agriculture, Livestock and Supply), seeded with 0.45 m spacing between rows and 11 plants per meter.With the aid of the GreenSeeker® equipment were collected reflectance values in six rows wide by 23 m long at intervals of each meter thereby obtaining 138 readings. The basic unit for this study was set at 0.45 m², obtained through the minor form: 0.45 m × 1 m (Table 1). The optimum plot size was estimated using initially the method of the modified maximum curvature proposed by Lessman; Atkins (1963) apud Meier and Lessman (1971). In this method, the measure of variability given by coefficient of variation (CV_x) and the portion size with X basic units is clarified by $CVx=aX^{-b}$, where aand b are the parameters to be estimated. The optimum plot size was estimated by the expression:

$$X_0 = exp\left\{\left[\frac{1}{2b+2}\right]\log\left[\frac{(ab)^2(2b+1)}{b+2}\right]\right\}$$

Where, X_0 is the value of the abscissa at the point of maximum curvature, which corresponds to the optimum plot size (Meier and Lessman, 1971).

The method of maximum distance was then calculated, where its resolution of the geometry formed by a y_c curve, described by $CV_x=aX^{-b}$, and a secant line to this curve, y_r. We look for the point of the curve y_c that is at the largest distance from the line y_r, since the line segment along this distance is perpendicular to the line y_r (Lorentz et al., 2012).The solution method presented by Lorentz et al. (2012) proposes to express the line perpendicular to the line y_r as an aid to find the point sought of the y_c curve. So, this line perpendicular to the line y_r will be called the y_p, expressed by $y_p=ex+f$. The angular coefficient c and the linear coefficient d, both from the line y_r, are fixed and can be obtained from two y_r points common to the y_r curve.The common point between y_c e $y_r$which is more to the left, given by (x_{cri}, y_{cri}), and the common point more to the right, given by (x_{crf}, y_{crf}), then *c* and *d* are expressed, respectively, by $c = \frac{y_{crf}-y_{cri}}{x_{crf}-x_{cri}}$ and $d = y_{cri} - cx_{cri}$ or $d = y_{crf} - cx_{crf}$, and these expressions for *d* are obtained by isolating the equation for y_R,having been replaced in this, the point (x_{cri}, y_{ri}), or the point (x_{crf}, y_{crf}). The angular coefficient e of the line y_p is also fixed and may be obtained by using the condition that the y_R and y_P lines are perpendicular to each other. In this way, $e = \frac{-1}{c}$.The determination of the linear coefficient *f* of the line y_P is part of the interactive method proposed by Lorentz et al. (2012) and that has as its solution: $x_{rpj} = \frac{f-d}{c-e}$.The distance between the points (x_{cj}, x_{cj}) and (x_{rpj}, x_{rpj}), this distance is on line y_{pj}, which is perpendicular to y_r, and is given by

$$d_{cr} = \sqrt{(yc_j - y_{rpj})^2 + (xc_j - x_{rpj})^2}$$

RESULTS AND DISCUSSION

By the method of the modified maximum curvature (MMC) the estimates of *a* and *b* were 2.0012 and 0.058, respectively (Figure 1), thus, the optimum plot size was the minimum measure, that is, 0.45 m² because X_0 = 0.365. The result is justified by the low coefficient of variation, with 2.423 maximum and 1.401 minimum (Table 2). In order to obtain a more representative R², we limited following

Table 1. Size (X), shape and total number of plots for the determination of the optimal size in studies using reflectance.

Simulation	Size (WxL)	Form (WxL)	Area(m²)	Number of plots
1	1	0.45 m × 1 m	0.45	138
2	2	0.45 m × 2 m	0.90	66
3	2	0.90 m × 1 m	0.90	69
4	3	0.45 m × 3 m	1.35	42
5	3	1.35 m × 1 m	1.35	46
6	4	0.45 m × 4 m	1.80	30
7	4	0.90 m × 2 m	1.80	33
8	4	1.80 m × 1 m	1.80	23
9	5	0.45 m × 5 m	2.25	24
10	5	2.25 m × 1 m	2.25	23
11	6	0.45 m × 6 m	2.70	18
12	6	0.90 m × 3 m	2.70	20
13	6	2.70 m × 1 m	2.70	22
14	6	1.35 m × 2 m	3.15	21
15	7	0.45 m × 7 m	3.25	18
16	8	0.45 m × 8 m	3.60	12
17	8	0.90 m × 4 m	3.60	15
18	8	1.80 m × 2 m	3.60	11
19	9	1.35 m × 3 m	4.05	14
20	10	0.90 m × 5 m	4.50	12
21	10	2.25 m × 2 m	4.50	11
22	12	1.35 m × 4 m	5.40	10
23	12	0.90 m × 6 m	5.40	9
24	12	1.80 m × 3 m	5.40	7
25	12	2.70 m × 2 m	5.40	11
26	15	1.35 m × 5 m	6.75	8
27	15	2.25 m × 3 m	6.75	7
28	16	1.80 m × 4 m	7.20	5
29	18	1,35 m × 6 m	8.10	6
30	18	2.70 m × 3 m	8.10	7
31	20	1.80 m × 5 m	9.00	4
32	20	2,25 m × 4 m	9.00	5
33	21	1.35 m × 7 m	9.45	6
34	24	1.35 m × 8 m	10.80	4
35	24	1.80 m × 6 m	10.80	3
36	24	2.70 m × 4 m	10.80	5
37	25	2.25 m × 5 m	11.25	4
38	28	1.80 m × 7 m	12.60	3
39	30	2.25 m × 6 m	13.50	3
40	30	2.70 m × 5 m	13.50	4
41	32	1.80 m × 8 m	14.40	2
42	35	2.25 m × 7 m	15.75	3
43	36	2.70 m × 6 m	16.20	3
44	40	2.25 m × 8 m	18.00	2
45	42	2.70 m × 7 m	18.90	3
46	48	2.70 m × 8 m	21.60	2

* L = length, W = Width.

simulations to the number 20 and calculations were redone and we obtained the same value of 0.45 m² through the modified maximum curvature method (MMC), because X_0 = 0.587. In the method of maximum distance (MD), in

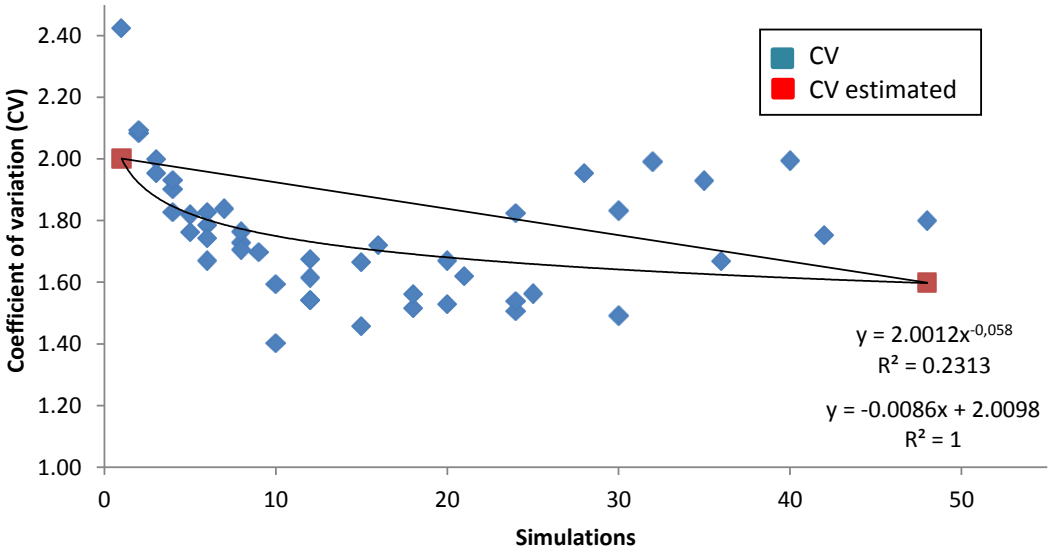

Figure 1. Regression of observed CV and estimated CV data for the variables *a*, *b*, *c* and *d* for 46 simulations.

Table 2. Standard deviation, mean and variation coefficient of reflectance data.

Simulation	Standard deviation	Average	Coefficient of variation (%)
1	20.652	852.242	2.423
2	17.750	852.242	2.082
3	17.834	852.242	2.092
4	17.032	851.992	1.999
5	16.658	852.242	1.954
6	16.189	851.000	1.902
7	15.562	852.242	1.826
8	16.440	851.431	1.930
9	14.987	851.000	1.761
10	15.482	851.200	1.818
11	15.527	849.713	1.827
12	15.206	851.992	1.784
13	14.226	852.242	1.669
14	14.850	852.242	1.742
15	15.663	851.992	1.838
16	14.656	847.770	1.728
17	15.010	851.366	1.763
18	14.519	851.431	1.705
19	14.494	854.158	1.696
20	13.558	851.138	1.593
21	11.966	853.750	1.401
22	13.735	851.000	1.613
23	13.131	852.242	1.540
24	14.264	851.261	1.675
25	13.131	852.242	1.540
26	12.403	851.000	1.457
27	14.168	850.942	1.665
28	14.633	850.225	1.721
29	12.869	849.713	1.514

Table 2. Contd.

Simulation	Standard deviation	Average	Coefficient of variation (%)
30	13.305	851.992	1.561
31	13.006	850.225	1.529
32	14.190	849.940	1.669
33	13.796	851.992	1.619
34	12.769	847.770	1.506
35	15.486	849.208	1.823
36	13.100	851.000	1.539
37	13.281	849.940	1.562
38	16.623	851.261	1.952
39	15.543	848.744	1.831
40	12.687	851.000	1.490
41	16.860	846.859	1.990
42	16.414	850.942	1.928
43	14.178	849.713	1.668
44	16.882	846.587	1.994
45	14.922	851.992	1.751
46	15.261	847.770	1.800

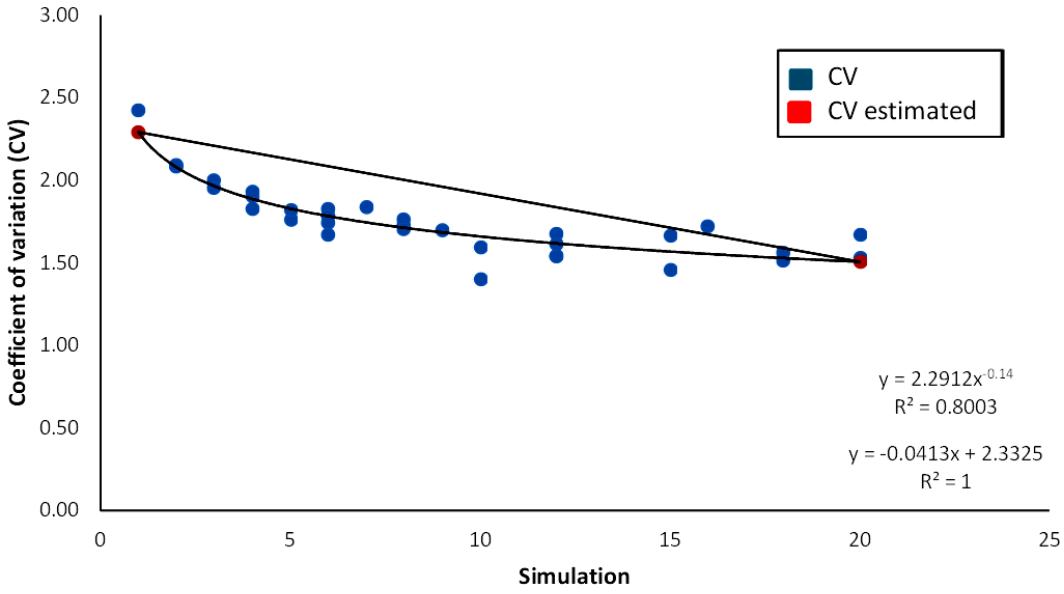

Figure 2. Regression of observed CV and estimated CV data to obtain the variables *a*, *b*, *c* and *d* for 20 simulations.

addition to the values of *a* and *b*, were obtained the values of *c* and *d* by linear regression of the estimated CV (*c* = - 0.0086 and *d* = 2.0098, Figure 1) and the value of *e* was obtained by *e* = - 1/C (*e* = 116.28). The value of the optimum plot size was 5.40 m². In order to obtain a more representative R^2, we limited following simulations to the number 20 and calculations were redone. Thus, were obtained the following values: *a* = 2.2912; *b* = - 0.14; *c* = - 0.0413; *d* = 2.3325 (Figure 2) and *e* = 24.21, resulting in an optimum plot size of 2.70 m² by the maximum distance method (MD). In order to confirm the result obtained with the coefficient of variation, calculations were redone using the variance (V), obtaining the following values: *a* = 381.53; b = 0.2810; *c* = - 11.427; *d* = 392.96 (Figure 3) and *e* = 0.0875, resulting in the same optimal plot size, 5.40 m². Monteiro et al. (2012) adopted plots that were 3.20 m long and 2.20 m wide (7.02 m²) for studies with radiometer in beans, a value higher than estimated by the method of

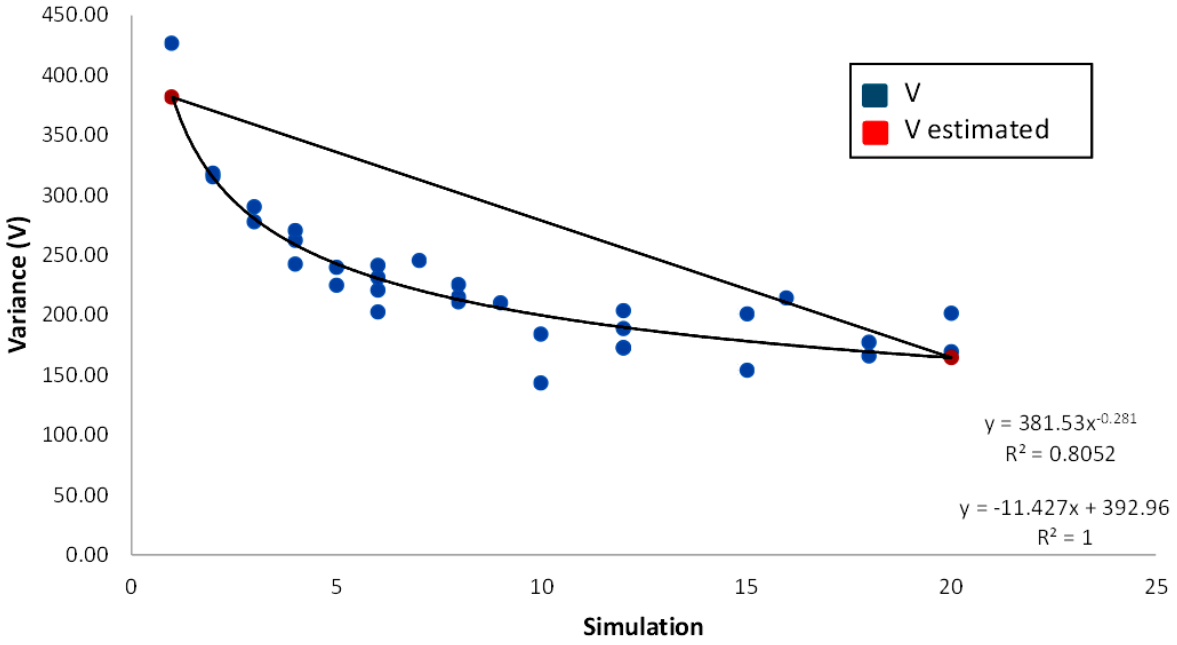

Figure 3. Regression of observed V and estimated V data to obtain the variables *a*, *b*, *c* and *d* for 20 simulations.

maximum distance (5.40 m²).

Conclusion

With optimum size values of plots obtained by the MMC for 46 and 20 combinations equal to 0.45 m² for MD with 20 simulations equal to 2.70 m² and MD for 46 simulations, and for variance equal to 5.40 m², it could be concluded that the highest value is the most suitable for works in beans with application of radiometer. Adopting as a criterion the lowest CV, the optimal size area is 5,40 m² with combination 2x6 that is two lines (0.90 m) wide and 6 m long.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effect of some phytohormones on growth characteristics of *Chlorella sorokiniana* IAM-C212 under photoautotrophic conditions

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The effects of some phytohormones [indole acetic acid (IAA), indole butyric acid (IBA), gibelleric acid (GA_3), and kinetin] on cell dry weight, cell number, cell size, protein and chlorophyll contents of *Chlorella sorokiniana* IAM-C212 were investigated under photoautotrophic conditions. Treatment with IAA (15 mg/l) and IBA (15 mg/l) resulted in significantly higher dry cell weight and cell number than the control ($P < 0.05$) but there were no significant effects of GA_3 and kinetin on cell growth as expressed by dry cell concentration (g/l) and cell population (cells/ml). Treatment with IAA at a concentration of 10 or 15 mg/l gave the highest cell dry concentration of 4.68 g/l after eight days of cultivation, which is more than 9 times higher than the value obtained in the control culture (without phytohormone). The optimum concentration of each of the phytohormones for *C. sorokiniana* cell enlargement was 20 mg/l. At this concentration, the average cell sizes were 81.07, 78.67, 78.07, 66.90 and 68.1 µm for GA_3, kinetin, IAA, IBA and control, respectively. Addition of 15 mg/l of IAA or GA_3 to the culture resulted in significantly higher extractable chlorophyll contents than the control ($P < 0.05$) but the effects of IBA and kinetin were not significant ($P > 0.05$). The protein contents of the cells cultivated with 20 or 10 mg/l of GA_3, 15 mg/l of kinetin, and 15 mg/l of IBA or IAA were 46.64, 45.83 and 45.81%, respectively. In the control experiment, the protein content was 43.38% after eight days of cultivation, showing that treatment with these phytohormones had no significant effect on the protein contents of the cells ($P > 0.05$). Combination of IBA and GA_3 exhibited synergistic effect on growth and productivity of *C. sorokiniana* but there was no synergistic effect when IAA was combined with either GA_3 or kinetin.

Key words: Phytohormones, *Chlorella sorokiniana*, cell growth, cell size, protein content, chlorophyll content.

INTRODUCTION

Microalgae biomass has numerous applications which include its use as a nutritional supplement for humans and animals to improve immune response and fertility, sources of biofuels, cosmetic supplement, natural food

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Abbreviations: IAA, Indole acetic acid; **IBA,** indole butyric acid; **GA_3,** gibelleric acid.

colourants, sources of high-value functional ingredients such as polyunsaturated fatty acids, ω-3 fatty acids and pigments (Griffiths and Harrison, 2009; Heydarizadeh et al., 2013; Salama et al., 2014). Many of these applications have commercial values but their production costs are still high due to low growth rates of many microalgae species. Many of these applications of microalgae demand the use of monocultures and controlled cultivation systems. These requirements have led to increased emphasis on development of cultivation strategies for efficient and cost-effective production of algae biomass. A lot of work has been done on photobioreactor design and optimization for efficient cultivation of microalgae but the reported productivities are still low due to some technical problems with light supply and distribution inside such reactors (Ogbonna and Tanaka, 2000; Oliver et al., 2013; Park et al., 2013). Furthermore, efforts to develop strains with high growth rates and productivity through genetic engineering approach have not yielded the desired results (Potvin and Zhang, 2010; Brennan et al., 2012).

Phytohormones have been used to improve productivities in higher plants since the 1930s (Piotrowska et al., 2008). Auxins (Indoleacetic acid, Phenylacetic acid, Indolebutyric acid and Naphthalene acetic acid) are a class of phytohormones that primarily increase growth in plants (Woodward and Bartel, 2005; Salama et al., 2014). Indole acetic acid (IAA) is a natural auxin and one of its primary effects is activation of the Plasmalemmal H^+ - ATPase involved in the process of growth by elongation (Tarakhovaskaya et al, 2007). Auxins accomplish these tasks by increasing cell wall plasticity, increasing water intake, altering respiratory patterns and altering nucleic acid metabolism (Woodward and Bartel, 2005). Cytokinins (trans-zeatin and kinetin) are plant growth substances which play a role in senescence and chloroplast development, primarily by promoting cell division (Tarakhovskaya et al., 2007). Endogenous Cytokinin-like activity has been documented in various microalgae (Stirk et al., 2002; Filomena et al., 2013). Effects of cytokinins have been determined in higher plants by exogenous addition of cytokinins. Most of the data on the effects of exogenous cytokinins on algal growth and development are on the members of the division Rhodophyta. Cytokinins (alone or in combination with auxins) were shown to accelerate red algal growth in the culture and in some cases, facilitate callus formation (Yokoya et al., 1999; Bajguz and Piotrowska-Niczyporuk, 2013). Thus, cytokinins, as cell-division promoting substances, may induce a faster growth rate in algae cells as they do in higher plant species. This fact, along with the detection of cytokinin-like activity in algae cells, is encouraging and highlights the potential for these substances to promote bioactive molecules and biofuel productions from algae. Gibberellins are diterpenoid acids that affect many areas of plant growth. They promote stem elongation, fruit generation and seed germination (Nakajima et al., 2006).

Application of Gibberellins caused cells to increase in size (Gonai et al., 2004). Little evidence for endogenous gibberellins activity has been observed in green algae. Although increased growth in response to gibberellins has been documented in some algae (Jennings, 1968; Joseph and Chennubhotla, 1999), there is scarce evidence for its actions beyond those in higher plants. Microalgae share some physiological similarities with higher plants. Although contemporary research on phytohormone physiological actions remain almost completely focused on the higher plants, there are few studies devoted to auxins and other classes of phytohormones in green algae such as *Chlorella* and *Scenedesmus*, and in Cyanobacteria such as *Spirulina* (*Arthrospira*) species (Czerpak et al., 1994; 1999). Studies with *Chlorella* species showed that the use of phytohormones have considerable stimulating effects on algal growth and productivity (Czerpak et al., 1994; 1999). Although, all the phytohormones used in their studies had considerable stimulating effect on *Chlorella pyrenoidosa*, it is imperative to determine whether such effect is common to all species of *Chlorella* and to determine their effects on other growth characteristics. To the best of our knowledge, this is the first report on the effects of these various phytohormones on the growth characteristics of *Chlorella sorokiniana* IAM-C212. The specific objectives of this study are: (i) to determine the optimal concentrations of the selected phytohormones for cell biomass (dry cell weight, cell number and cell size) as well as the chlorophyll and protein contents of the cells, and (ii) to evaluate synergistic effects of combinations of the selected phytohormones on the growth characteristics of *C. sorokiniana* IAM-C212.

Microalgae growth involves chlorophyll development, cell division and cell enlargement. Thus, representatives of classes of phytohormones that have been reported to stimulate these processes in higher plants, namely auxins (cell enlargement), cytokinines (chloroplast development and cell division), and gibberellins (cell elongation and enlargement) were selected for this study.

MATERIALS AND METHODS

Strain of microalgae and sub-cultures

Axenic strain of *C. sorokiniana* IAM-C212 used in this study was obtained from the Culture Collection Centre, University of Tokyo, Japan and maintained in basal growth medium as modified by Ogbonna et al. (1997). The basal growth medium was composed of (g/l): urea, 1.2; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.02; sodium citrate, 0.05; Fe-solution, 0.16 ml; and A_5 solution, 0.8 ml. The Fe-solution was composed of 25 g of $FeSO_4.7H_2O$ and 33.5 g EDTA per liter of distilled water. A_5 solution was composed of (g/l): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.22; $CuSO_4.5H_2O$, and MoO_4, 0.015. The pH of the culture medium was adjusted to

6.5 ± 0.2 before inoculation. The culture was maintained in a temperature controlled growth chamber at 25 ± 2°C illuminated with six 12 watts energy saving bulbs fixed on two parallel wooden boxes each placed at a distance of 10 cm from the conical flasks containing the culture medium. The mean light intensity on the surface of the flasks was 90±5 µmol m^{-2} s^{-1}. The stock was sub-cultured every month.

Selection of the phytohormones

The four phyto-hormones used in this study were indoleacetic acid (IAA), indolebutyric acid (IBA), gibberellic acid (GA_3) and kinetin. The IAA, IBA, GA_3 and Kinetin were purchased from Wako Pure Chemical Industrial Ltd, Tokyo, Japan.

Phytohormone stock solutions

Twenty milligrams of each of the phytohormones was first dissolved in the appropriate solvent (GA_3 in 5.0 ml of de-ionized water, IAA and IBA in 0.5 ml of 95% ethanol, and kinetin in 0.1 N hydrochloric acid) and then made up to 200 ml with de-ionized water to obtain 100 mg/l which served as the stock solution. Desired concentrations: 5, 10, 15, and 20 mg/l were prepared from the stock solution.

Synergistic study

The effect of combined phytohormones on the growth characteristics of *C. sorokiniana* IAM-C212 was studied using the following phytohormone combinations: IBA combined with GA_3; and IAA combined with GA_3.

Cultivation method

Five hundred milliliter (500 ml) Erlenmeyer flasks containing 300 ml of the basal growth medium supplemented with various concentrations of phytohormones or combinations were inoculated with 15 ml of a 4 day old culture of *C. sorokiniana* containing 5.00 × 10^8 cells/ml and incubated statically but mixed manually three times daily for 8 days in a growth chamber illuminated by six-12 watts energy-saving bulbs fixed on two parallel rectangular wooden boxes. The mean light intensity on the surface of the flasks was 90 ± 5 µmol m^{-2} s^{-1} while the temperature was controlled at 30 ±2°C. A 10 ml of the culture broth was aseptically drawn on 48 hourly bases for assay.

Analyses

The dry cell weights were determined using triplicate samples of the growth culture solution. A 10 ml of algal culture was filtered through a pre-weighed Whatman No 1 filter paper after centrifuging at 3000 rpm for 15 min. The filter paper was washed with 5 ml of 0.1 N HCl to remove the precipitated salts and dried overnight at 80°C in an oven. Dried filter paper with biomass was cooled in a desicator and weighted again to estimate the final dry weight of the algae (Ogbonna et al., 1997). The cell number was measured on 48 hourly bases by counting the cell number using light microscope and Neubaur counting chamber. The cell size was measured using Moticam Images Plus 2.0 digital camera (Motic China Group Ltd) connected to a microscope and computer system. For determination of chlorophyll contents, a 10 ml of algal culture broth was centrifuged at 3000 rpm for 20 min, and the chlorophyll was extracted from the algae pellet using 4 ml of methanol (95%). The amount of chlorophyll extracted in the methanol was determined spectrophotometrically according to the method described by Ogbonna et al. (1997), using the following equation:

Chlorophyll (µg/ml^{-1}) = 25.5 (A650-A 750) + 4.0 (A 665 - A 750).

Here, A650, A665 and A750 are absorbance at 650, 665 and 750 nm, respectively. The values were converted to mg/g by dividing by the respective cell concentration in the sample.

The protein content was determined using 0.2 g of dry algal sample to estimate the nitrogen content of the biomass. The nitrogen contents were multiplied with the nitrogen - to - protein conversion factor of 6.25. The nitrogen content was measured according to AOAC method of analysis (2010). A 0.2 g weight of the dry biomass was added into a clean and dry digestion flask (Kjedhal flask). Selenium powder (0.05 g), Copper sulphate (0.5 g) and Sodium sulphate (2 g) were added. This was followed by the addition of 20 ml of concentrated H_2SO_4. The solution was swirled until it darkened and then heated in a fume cabinet until it became clear. The digested sample was diluted to 100 ml with distilled water and 5 ml taken for distillation. A 10 ml of 50% sodium hydroxide was added to 5 ml of the sample in a Markham apparatus and the solution allowed to distil over 10 ml of boric acid mixed indicator until the indicator turned light green. A 50 ml volume of the distillate was titrated against 25 ml of 0.01 N HCl until the first pink appearance occured. The percentage nitrogen was calculated.

Statistical analysis

All the data were subjected to one way analysis of variance (ANOVA). Where, there were significant effects, least significant difference (LSD) was used to separate the means. The results were expressed as mean ± standard error of the mean.

RESULTS AND DISCUSSION

Effect of phytohormones on dry cell weight of *C. sorokiniana*

Treatment with IAA, IBA and kinetin resulted in significantly higher biomass concentration than the control ($P < 0.05$) while treatment with GA_3 resulted in lower biomass concentration. IAA at a concentration of 10 mg/l gave the highest cell concentration of 4.685 g/l and productivity of 0.586 g/l/day as shown in Figures 1 and 2. This compares with the control (without phytohormone) which had an average biomass concentration of 0.489 g/l. Using Least Significant Difference, the effectiveness of the phytohormones in increasing the dry cell concentration of *C. sorokiniana* can be ranked as IAA (4.685 g/l) > IBA (1.664 g/l) > Kinetin (0.621 g/l) > GA_3 (0.471 g/l). The optimum concentration of each phytohormone was between 10 and 15 mg/l and the effectiveness of the phytohormone concentrations was ranked as (10 = 15 mg/l) > 20 > 5 mg/l. As shown in Figure 1, the effect of IAA was not significant in the first 3 days and the average cell concentration was 0.157 g/l compared with the 0.101 g/l

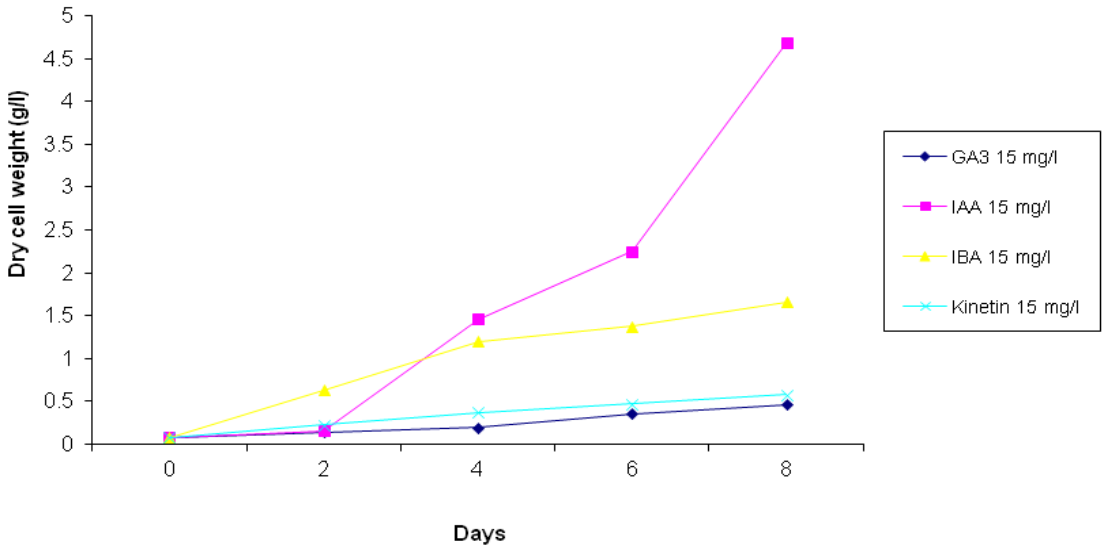

Figure 1. Effect of phytohormones on dry cell weight of *Chlorella sorokiniana* IAM C212.

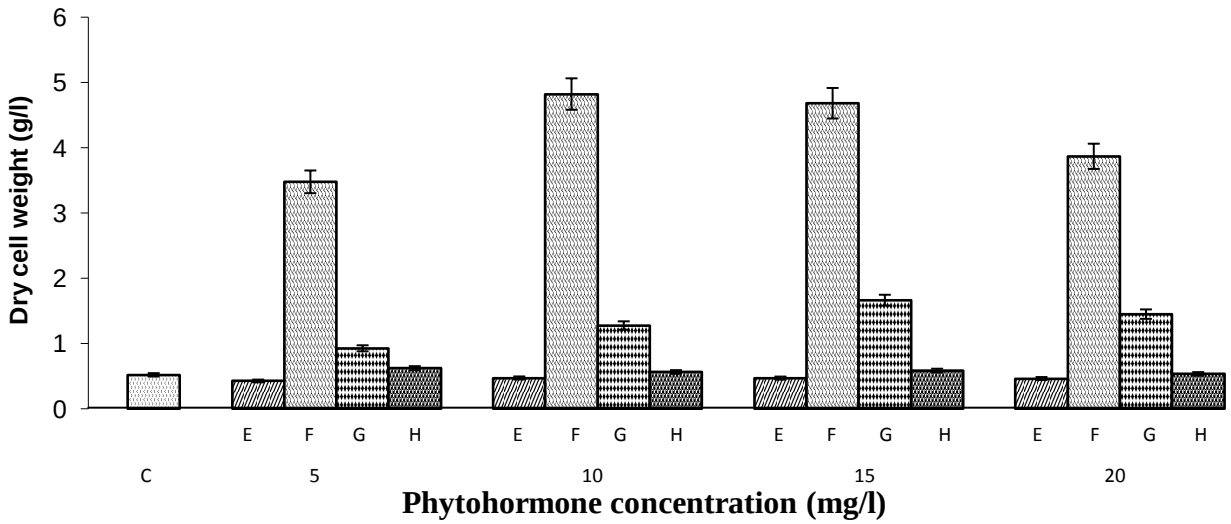

Figure 2. Effect of phytohormones on dry cell weight of *Chlorella sorokiniana* IAM C212. Symbols: C, Control; E, GA_3; F, IAA; G, IBA; H, kinetin.

obtained in the control culture during that period. In contrast, IBA increased the biomass productivity in the first 3 days, but maintained a gradual and steady increase thereafter, resulting in an 8 day average of 1.664 g/l compared with the 0.489 g/l obtained in the control experiment. These differences may be explained on the basis of nutrient transport into the cells. The algal cells tend to have a residual negative charge. At neutral or alkaline pH, organic acids such as IAA and IBA do not enter the cells due to their ionic forms (Dibb-Fuller and Morris, 1992). At acidic pH values, these compounds are non-ionized and can enter the negatively charged cells. IAA having lower molecular weight than IBA diffuses faster across the cell membrane of the algae cells. The cell will try to maintain its internal pH by neutralizing or expelling the protons but this will slow down the growth as it diverts energy from growth-related functions (Rayle and Cleland, 1992). This brings about increased lag phase. In other words, the length of the lag phase may reflect the time necessary for the algae cells to bring the external environment within their optimum growth range. Hunt et al. (2010) experimenting with Naphthaleneacetic

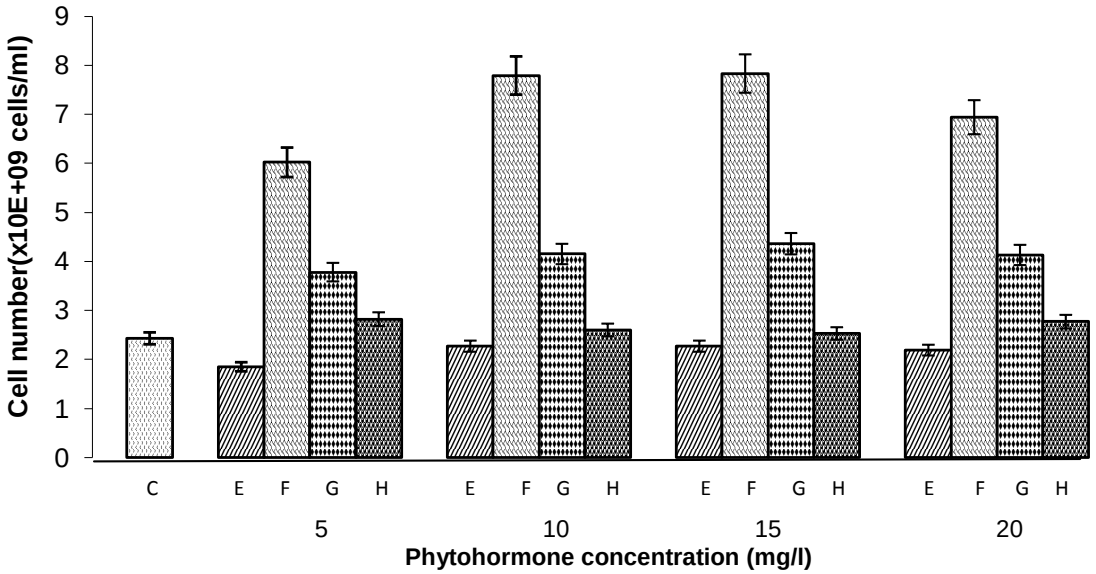

Figure 3. Effect of phytohormones on cell number of *Chlorella sorokiniana* IAM C212. Symbols: C, Control; E, GA_3; F, IAA; G, IBA; H, Kinetin.

acid (NAA) over a 10 day static culture observed that NAA did not enhance the growth of *C. sorokiniana* in the first 5 days of the cultivation but significantly enhanced growth and productivity of the algae cells thereafter. They opined that it could be as a result of longer lag and prolonged exponential phases of algae cells exposed to NAA. Since IAA and NAA belong to the auxin family, IAA may have elicited similar response from *C. sorokiniana* IAM-C212 in this experiment. Unlike NAA, it took a shorter period of time (3 days) for the algae cells to get acclimatized to IAA in this experiment.

Effect of phytohormones on *C. sorokiniana* cell number

The result of the effect of different concentrations of phytohormones on cell number of *C. sorokiniana* after 8 days of cultivation is shown in Figure 3. There were also significant effects of the phytohormones on cell number ($P < 0.05$). Amongst the phytohormones, IAA at a concentration of 15 mg/l gave the highest cell number with an average value of 7.83×10^9 cells/ml. This compares with the control (without phytohormone) which had an average value of 2.43×10^9 cells/ml. The effectiveness of the phytohormones on the cell number of *C. sorokiniana* can be ranked as IAA (7.83×10^9 cells/ml) > IBA (4.36×10^9) > Control (2.43×10^9 cells/ml) > Kinetin (2.27×10^9 cells/ml) > GA3 (2.19×10^9 cells/ml) and the effectiveness of the various concentrations was ranked as (15 =10 mg/l) > (20 = 5 mg/l). Contrary to its effects in higher plants (initiation of cell division), kinetin did not bring about an appreciable increase in cell number of the algae cells. Cytokinin signal transduction pathway begins with binding to a two-component receptor system, involving the cytokinin receptor, CR2 (Inoue et al., 2001). Along these pathways, regulatory proteins play a critical role in increasing and decreasing the cytokinin signal. The increased growth from cytokinins is a product of the activation of these regulators of the cell division cycle and differentiation (Sheen, 2001; Rióu-Khamlichi et al*.,* 1999). These regulatory proteins may be lacking or inactive in *C. sorokiniana* IAM C212.

Effect of phytohormones on cell size of *C. sorokiniana*

The result of the effect of different concentrations of phytohormones on cell size of *C. sorokiniana* after 8 days of cultivation is shown in Figure 4. The optimum concentration of phytohormones for *C. sorokiniana* cell enlargement was 20 mg/l for each of the phytohormones. At this concentration, the average values of the cell sizes were 81.07, 78.67, 78.07 and 66.90 µm for GA_3, Kinetin, IAA, and IBA, respectively. This compares with the control (without phytohormone) which had an average value of 68.43 µm. Treatment with GA_3, Kinetin and IAA significantly increased the cell size ($P < 0.05$) while there was no significant difference between the average sizes of the cells treated with IBA and those of the control. The effectiveness of the phytohormones in increasing the size

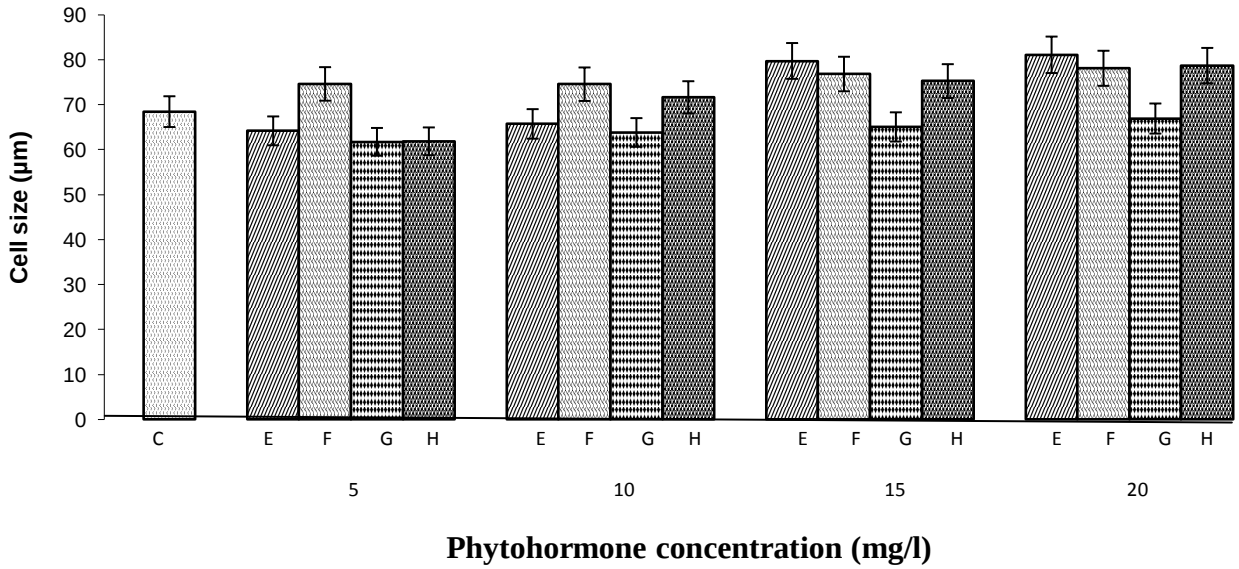

Figure 4. Effect of phytohormones on cell size of *Chlorella sorokiniana* IAM C212. Symbols: C, Control; E, GA_3; F, IAA; G, IBA; H, kinetin.

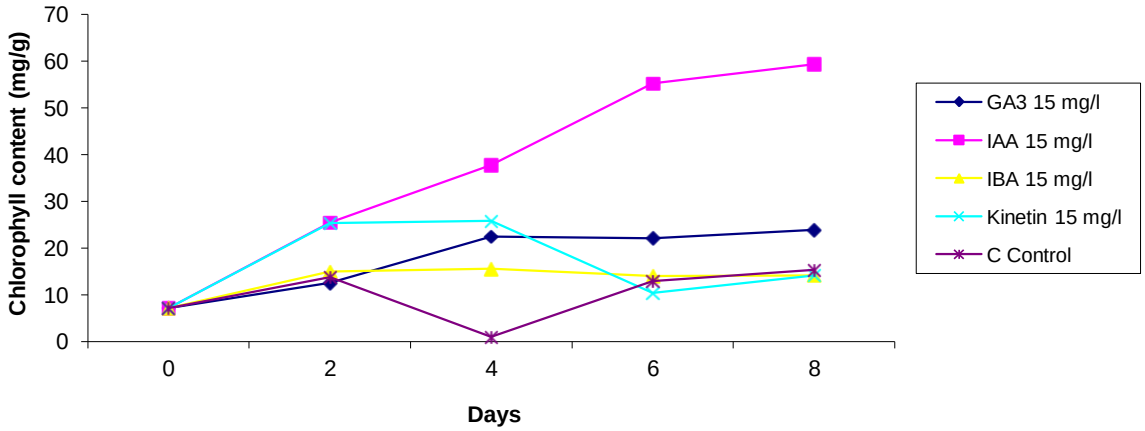

Figure 5. Effect of phytohormones on *Chlorella sorokiniana* IAM C212.

of the cells can be ranked as GA_3 > (Kinetin = IAA) >IBA. The best performing phytohormone was GA_3 at 20 mg/l concentration. This is in line with the report of Gonai et al. (2004). The harvesting of unicellular microalgae biomass is a challenging phase of the algae biomass production process (Wang et al., 2008; Brennan et al., 2012) and accounts for 20 to 30% of the total costs of production according to Gudin and Therpenier (1986). Small sizes of some algae cells make the recovery of biomass difficult (Chen et al., 2011). The discovery of phytohormones and the optimum concentrations that lead to increases in cell size of *C. sorokiniana* is very significant as it will lead to reduced cost in downstream processing of the microalgae biomass. Unfortunately, there was a negative correlation between the cell size of *C. sorokiniana* IAM C212 and biomass productivity in this study. GA_3 was the least effective in increasing biomass productivity but the most effective in increasing the cell size.

Effect of phytohormones on chlorophyll contents of *C. sorokiniana* IAM C212

The effects of the phytohormones on chlorophyll contents of *C. sorokiniana* followed the same trend as the dry weight with IAA at a concentration of 15 mg/l giving the highest value of 50.94 mg/g-cell biomass (Figures 5 and 6). This compares with the control (without

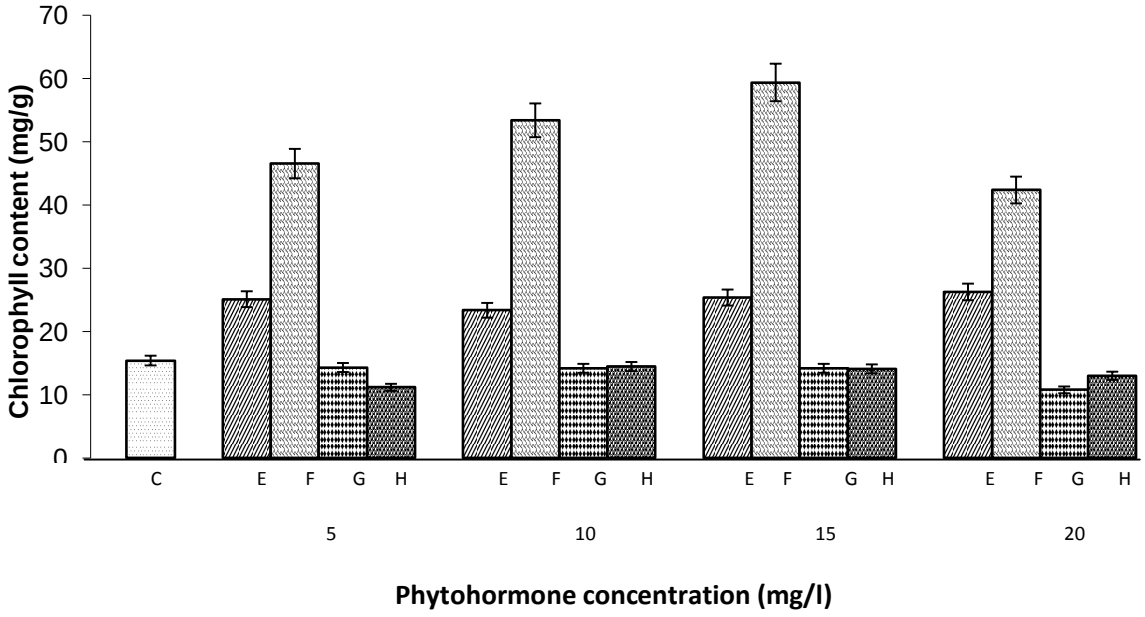

Figure 6. Effect of phytohormones on chlorophyll content of *Chlorella sorokiniana* IAM C212. Symbols: C, Control; E, GA_3; F, IAA; G, IBA; H, kinetin.

phytohormone) which had an average value of 15.40 mg/g. This represents more than 3-fold increase in chlorophyll contents over the control. However, it is only IAA and GA_3 that resulted in significant increase in chlorophyll contents of the cells. The effectiveness of the phytohormones in increasing the chlorophyll contents of the microalgae can be ranked as IAA (50.94 mg/g) > GA_3 (25.37 mg/g) > {Control (15.40 mg/g), IBA (14.30 mg/g), Kinetin (14.10 mg/mg)}. The effectiveness of the various concentrations of these phytohormones in increasing the chlorophyll contents of *Chlorella* can be ranked as 15 mg/l > (10 = 20 mg/l) > 5 mg/l. Surprisingly, GA_3 which was the least effective in enhancing biomass productivity was more effective than IBA in increasing the chlorophyll content. Presently, chlorophyll content of algae biomass has significant effect on their market values. Chlorophyll is an essential compound in many everyday products. It is used not only as an additive in pharmaceutical and cosmetic products but also as a natural food colouring agent. Chronic ulcer is a significant health problem in the society, with lengthy periods required for its treatment (Hosikian et al., 2010). Chlorophyll's ability to increase the rate of healing is a breakthrough for ulcer sufferers. Because of this property, chlorophyll is used not only in the treatment of ulcers and oral sepsis but also in proctology. Fruit and vegetable consumption has been associated with decreasing the risks of cancer. Phytochemicals present in these foods, particularly chlorophyll and its derivatives, have been suggested to play a key role in cancer prevention due to their antioxidant and antimutagenic activities (Lanfer-Marquez et al., 2005; Ferruzzi and Blakeslee, 2007). The discovery of a phytohormone that simultaneously leads to higher biomass production and high chlorophyll content is a useful contribution to advancing biotechnological applications of microalgae in cosmetic, pharmaceutical and food industries.

Effect of phytohormones on protein content of *C. sorokiniana*

The effect of phytohormones on protein content of *C. sorokiniana* is shown in Figure 7. The effects of the phytohormones on protein contents of the cells were not significant ($P > 0.05$). Treatment of *Chlorella* with 10 or 20 mg/l of GA_3, 15 mg/l of kinetin, and 15 mg/l of IBA or IAA gave protein contents of 46.64, 45.83 and 45.81%, respectively. These compare with the control which had 43.38% protein after eight days of cultivation. Although not statistically significant, GA_3 enhanced the protein content of *C. sorokiniana* despite its ineffectiveness in stimulating growth and biomass productivity. The results of this study imply that there is a positive correlation between the cell size of the green alga and their protein content. Salama et al. (2014) reported that the protein content of the microalga *Scenedesmus obliquus* was decreased at high concentration of IAA (10^{-5} to 10^{-6} M). This is in line with our finding in this research using *C sorokiniana* IAM C212. There is a negative correlation between cell growth rate and protein content as the fast growing cells tends to accumulate carbohydrates more

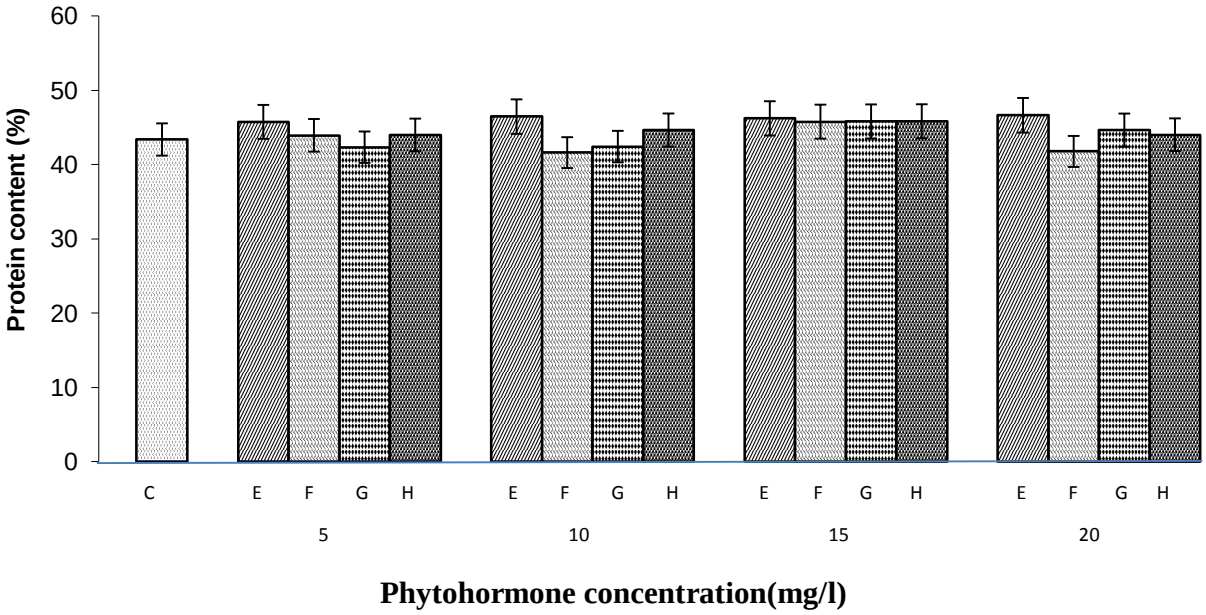

Figure 7. Effect of phytohormones on protein content of *Chlorella sorokiniana* IAM C212. Symbols: C, Control; E, GA_3; F, IAA; G, IBA; H, kinetin.

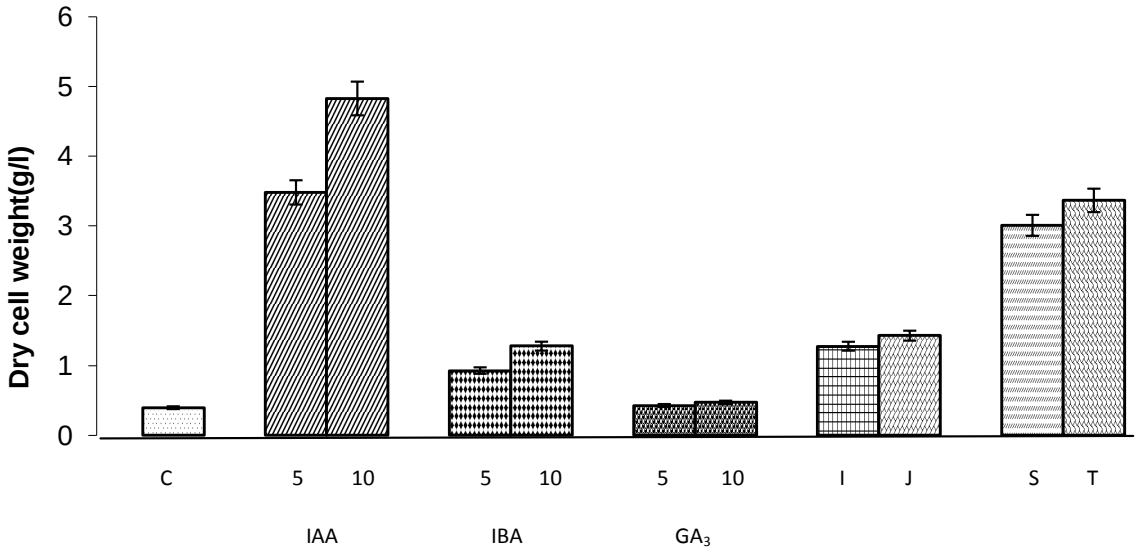

Figure 8. Effect of combined phytohormone on dry cell weight of *Chlorella sorokiniana* IAM C212. Symbols: I, IAA (10 mg/l) + GA (5 mg/l); J, IAA (12.5 mg/l) + GA (2.5 mg/l); S, IBA (10 mg/l) + GA (5 mg/l); T, IBA (12.5 mg/l) + GA (2.5 mg/l).

and slow growing cells tend to accumulate protein more (Markou and Nerantzis, 2013).

Effect of combinations of phytohormones on the dry cell weight of *C. sorokiniana*

The effect of combinations of phytohormones on the dry cell weight of *C. sorokiniana* is shown in Figure 8. The combination of phytohormones IBA (12.5 mg/l) + GA (2.5 mg/l) exhibited the highest effect with an average dry cell weight of 3.36 g/l after 8 days of cultivation. This compares with the control (without phytohormones) which recorded 0.394 g/l dry cell weight after 8 days of cultivation. Individually, neither IBA (1.66 g/l) nor GA_3 (0.47 g/l) at concentration of 15 mg/l did better than their

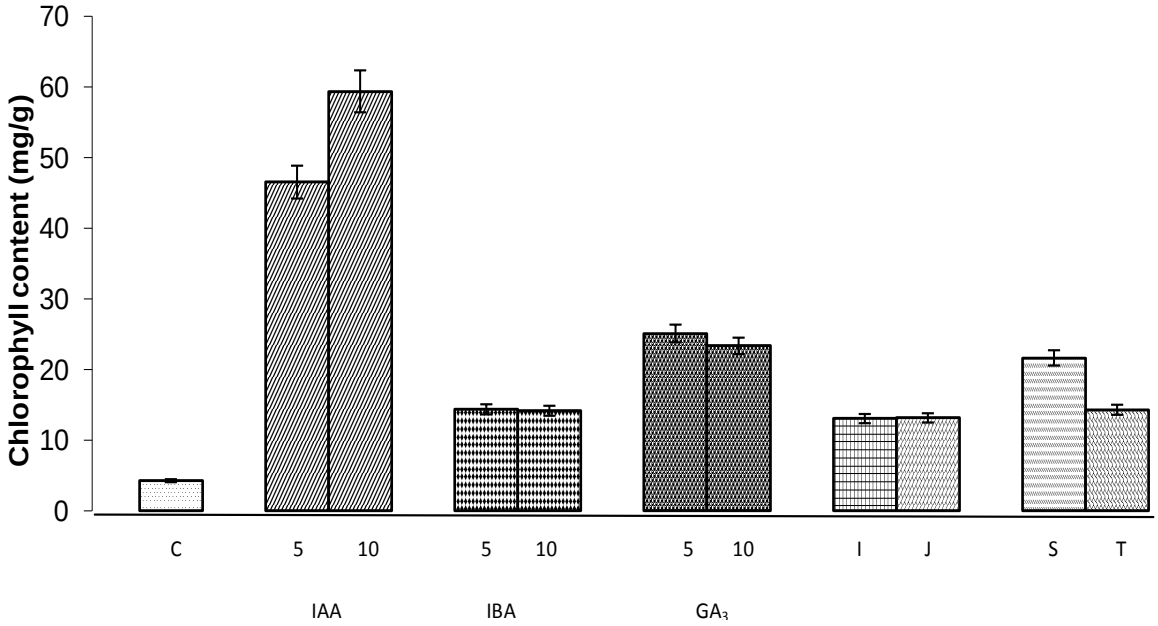

Figure 9. Effect of combined phytohormones on chlorophyll contents of *Chlorella sorokiniana* IAM C212. Symbols: I, IAA (10 mg/l) + GA (5 mg/l); J, IAA (12.5 mg/l) + GA (2.5 mg/l); S, IBA (10 mg/l) + GA (5 mg/l); T, IBA (12.5 mg/l) + GA (2.5 mg/l).

combination in enhancing cell concentration. There is a clear synergistic effect of combining IBA with GA_3 on cell dry weight as the resulting cell concentration (3.36 g/l) is 1.57 times higher than the sum of the cell concentrations obtained separately with the two phytohormones (1.66 + 0.47 = 2.13 g/l). IAA in all combinations with GA_3 did not exhibit any synergistic effect since the cell concentration obtained with IAA alone was higher than the values obtained with a combination of IAA (12.5 mg/l) and GA_3 (2.5 mg/l) or a combination of IAA (10 mg/l) and GA_3 (5 mg/l). As shown in Figure 9, the combination of IBA (12.5 mg/l) and GA_3 (2.5 mg/l); and IBA (10 mg/l) and GA_3 (5 mg/l), exhibited synergistic effect on *C. sorokiniana* in terms of biomass production and chlorophyll contents. Hunt et al. (2010) combined NAA (5 mg/l) and GA_3 (10 mg/l) and recorded about 38% increase in biomass productivity. To the best of our knowledge, no combination of GA_3 with IBA, a less performing member of auxin family, has been reported before now. In this study, we combined IBA and GA_3 and recorded over 7-fold increase in biomass concentration compared with GA_3 alone, and 1.96 fold increases over IBA alone. Furthermore, the combination of IBA and GA_3 simultaneously led to higher biomass and chlorophyll content, which is attractive for cosmetic and pharmaceutical industries. Hunt et al. (2010) reported that NAA in all combinations with GA_3, IBA, and zeatin showed only marginal increase in average productivity over the control.

Conclusion

Phytohormones can be used to increase the growth (dry cell concentration and cell number), cell size, protein and chlorophyll contents of *C. sorokiniana* IAM C212. However, the choice of the phytohormone and the concentration depend on the specific objective. Auxins (IAA and IBA) had pronounced effects on both dry cell concentration and cell numbers but only IAA had significant effect on the cell size. On the other hand, chlorophyll contents were more affected by IAA and GA_3. IAA and GA_3 have been reported to enhance cell elongation in higher plants and in this study, they also resulted in significant increase in the size of *C. sorokiniana*. Although kinetin is known to enhance cell division in higher plants, there was no significant effect of addition of kinetin to the medium on cell number of *C. sorokiniana.* Our results also show that there were synergistic effects of IBA and GA_3 on cell growth and chlorophyll contents of the cells.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Fatty acid profile and bioactivity from *Annona hypoglauca* seeds oil

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Plants from *Annona* (Annonaceae) genus are present in tropical regions, where they have economic and medicinal potential. Information on the fatty acids profile and bioactivity from seed oil of *Annona* species are incipient. The objective of this work was to investigate *Annona hypoglauca* seeds oil in terms of its yield, composition and biological activity (acetylcholinesterase enzyme inhibition, bactericidal and fungicidal activity). Fatty acids profiles were determined by Gas Chromatography equipped with Flame Ionization Detector. Oil yield reached about 15% and the major constituents detected were ω-9 oleic acid (42.65%) and ω-6 linoleic acid (29.63%). *A. hypoglauca* oil was potent for acetylcholinesterase inhibition (79.55%), and presented high and selective bioactivity against *Candida albicans*.

Key words: *Annona hypoglauca*, ω-9 oleic acid, ω-6 linoleic acid, acetylcholinesterase, *Candida albicans*.

INTRODUCTION

The Annonaceae family consists of about 112 genera and 2,440 species (Couvreur et al., 2011), occurring in tropical regions. Brazil has 33 native genera with about 250 species (Souza and Lorenzi, 2008). Species from *Annona* genus present various activities such as anthelmintic (Ferreira et al., 2013), antioxidant (Julián-

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Abbreviations: BHI, Brain Heart Infusion; **GC-FID,** gas chromatography using flame ionization detector; **AD,** Alzheimer's Disease.

Loaeza et al., 2011), antidiabetic (Florence et al., 2014), anticancer (Wang et al., 2014), antiacetylcholinesterase (Tsai and Lee, 2010), antimicrobial (Jamkhande et al., 2014), among others. Activity has been reported for various parts of the plant such as roots (Jamkhande et al., 2014), stem bark (Dutra et al., 2014), leaves (Matsumoto et al., 2014), fruit pulp (Clerici and Carvalho-Silva, 2011) and seeds (Ribeiro et al., 2014). A huge diversity of substances associated to a range of medicinal and nutritional (Pareek et al., 2011) benefits has also been described.

Annona hypoglauca species has in extracts of its wood antitumor activity against breast adenocarcinoma has been described for *A. hypoglauca* (Suffredini et al., 2007). Other screenings have shown cytotoxic activity against tumor cell lines such as prostate, lung, colon, central nervous system, leukemia, among others (Rinaldi, 2007). Activity of *A. hypoglauca* against the bacterium *Streptococcus mutans* has also been reported (Barnabé et al., 2014). The information on biological activities of *A. hypoglauca* is incipient. This work was addressed to study *A. hypoglauca* seeds oil in terms of its yield, composition and biological activities, which included examination on whether acetylcholinesterase was inhibited by the oil and its possible bactericidal and fungicidal actions.

MATERIALS AND METHODS

Source and processing of *A. hypoglauca* seeds

The plant was identified by Ricardo de Oliveira Perdiz (Studies Center of Amazonian Biodiversity, CENBAM, Brazil) and deposited in UFRR Herbarium (UFRR 3654). The fruits were obtained in Mucajaí city, Roraima, taken to the Environmental Chemistry Laboratory (Research Center, Post-Graduate Course in Science and Technology – NPPGCT-UFRR) and washed. Seeds were removed, washed, and dried at room temperature and then were placed in a drying oven at 40°C with air circulation. Seeds were ground and sieved on a 20 to 40 mesh fabric to obtain a homogeneous granulation (Jorge and Luzia, 2012).

A. hypoglauca oil seeds processing

The oil was obtained by extraction from hexane solvent in a Soxhlet apparatus for 6 h. The solvent was evaporated on rotaevaporator and the oil (10.0328 g) was properly packaged in an amber vial under nitrogen atmosphere and stored in a freezer (Jorge and Luzia, 2012).

Hydrolysis and methylation of lipids

An aliquot (10 mg) of *A. hypoglauca* oil was transferred to a 2 mL cryotube, which contained 100 µL of a mixture made of ethanol (95%) and KOH 1 mol/L (5%). After vortexing for 10 s, esters in the oil were hydrolyzed in a microwave oven (Panasonic Piccolo) at 80 W (power 2) for 5 min. After cooling and neutralization with 400 µL of hydrochloric acid 20%, 20 mg NaCl and 600 µL of ethyl acetate were added. Afterwards, free fatty acids were obtained by using an adapted protocol of the one reported by Christie (adapted from Christie, 1989). Thus, after vortexing for 10 s and rest for 5 min, aliquots (300 µL) of the ethyl acetate layer was taken, placed in microcentrifuge tubes and dried by evaporation. Free fatty acids were methylated using 100 µL of BF_3/methanol (14%) and the reaction mixture was heated for 10 min in a water-bath at 60°C. After dilution with 400 µL methanol, fatty acid methyl esters were analyzed by Gas Chromatography.

Oil analysis by GC-FID

Free fatty acids were resolved by Gas Chromatography using HP7820A (Agilent) system equipped with flame ionization detector. An Innowax column (HP) 15 m × 0.25 mm × 0.20 µm was used and the following temperature gradient: 100°C min and 0.7°C/min up to 240°C; injector (1/30 split) to 250 and 260°C detector. Hydrogen was used as carrier gas (3 mL/min) and injection volume was 1 µL. The data acquisition program used was EZChrom Elite Compact (Agilent). The peaks were identified using FAME Mix C14-C22, CRM18917 Supelco fatty acid methyl esters standard.

Biological screening

AChE inhibition assay

Aliquots of a working solution (25 µL) (sample in DMSO 10 mg/mL) were added to microplate wells and positive and negative controls were also prepared. To the first five wells of a column (positive control) 25 µL of an eserine solution prepared at 10 mg/mL (31 mM; 2.7 mM in the whole reaction mixture 275 µL) in Tris/HCl at pH 8.0) was added. Then, 25 µL of acetylthiocholine iodide (ATChI, Sigma A5751) 15 mM; the reaction mixture, 125 µL of 5',5-dithio-bis (2-nitrobenzoate) (DTNB, Sigma D8130) (3 mM) and 50 µL of Tris/HCl (50 mM, pH 8) containing 0.1% (m/v) bovine serum albumin was added to each well. Absorbance was measured at 405 nm every 1 min for 8 times. Then 25 µL (0.226 U/mL) of Electric eel AChE (type VI-S) provided by Sigma (C3389-500UN) in Tris/HCl was added to each well. Absorbance was measured at 405 nm by 10 times (Frank and Gupta, 2005; Ellman et al., 1961).

Filamentous fungi assay

Filamentous fungi used in this test were *Aspergillus flavus* (CCT 4952) and *Fusarium proliferatum* (CML 3287). DMSO was used for sample preparation and the concentration of sample in the assay was 250 mg/mL. Sabouraud broth was used for fungal growth. A spore suspension at a concentration of 5×10^{-5} spores/mL was used after spores counting on a Neubauer chamber. The sample incubation time was 48 h after which absorbance was read at 490 nm on a microtitre plate reader. Data were processed using the Outlier method, Grubbs test with 95% significance level. The percentage of inhibition was calculated by using the formula.

% inhibition = 100-AC-AC × 100AH-AM

AC = absorbance of the sample; AC = absorbance of control sample; AH = absorbance of microorganisms control and AM = absorbance culture medium control.

Antibacterial and antifungal assay

Escherichia coli (ATCC 25922), Salmonella *tiphymurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus sanguinis (*ATCC 49456*)* and *Candida albicans* (ATCC 18804) were used in the assay following the procedures for Minimum Inhibitory

Table 1. Composition and quantification of fatty acids in *A. hypoglauca* seeds oil (percentage in the mixture of fatty acids).

Fatty acids	Retention time (min)	Amount (%)			
		A. hypoglauca	Olive (Carvajal-Zarrabal et al., 2014)	Corn (Carvajal-Zarrabal et al., 2014)	Canola (Carvajal-Zarrabal et al., 2014)
Lauric acid (C12:0)	1.56	0.09	0	0	0
Myristic acid (C14:0)	2.86	0.13	0	0	0
Palmitic acid (C16:0)	4.74	16.35	11.32	7.19	7.50
Palmitoleic acid (C16:1)	4.90	0.55	0.11	0.02	0.20
Stearic acid (C18:0)	6.87	6.65	4.34	4.51	3.30
Oleic acid (ω-9) (C18:1)	7.07	42.65	74.12	32.08	32.0
Linoleic acid (ω-6) (C18:2)	7.56	29.63	7.64	54.26	37.0
α-Linolenic acid (ω-3) (C18:3)	8.20	0.65	0.61	0.10	7.70
Eicosanoic acid (C20:0)	8.98	0.50	0	0	0
Behenic acid (C22:0)	11.04	0.11	0	0	0

Concentration (MIC) described below. Concentrations assayed were 500, 250, 125, 62.5, 31.25, 15.6, and 3.9 µg/mL (Zacchino and Gupta, 2007). Samples were weighed and dissolved in DMSO to 50 mg/mL. Forty µL of this solution was added to a flask containing 960 µL of BHI (Brain Heart Infusion) broth (working solution). A pre-inoculum was prepared in which the bacteria and the yeast, stored under refrigeration, were transferred with a platinum loop to test tubes containing 3 mL of freshly made BHI broth. The tubes were incubated at 37°C for 18 h. Then, the pre-inoculum (500 µL) was transferred to tubes containing 4.5 mL of sterile distilled water. The tubes were homogenized and the concentration adjusted to 0.5 of McFarland turbidity standard (10^8 CFU/mL), thereby obtaining the inocula used in the bioassays. Assays were performed in 96-microwell plates in duplicate. One hundred µL of BHI broth was added to each well. In the first well 100 µL of working solution was also added. The solution was homogenized and 100 µL transferred to the next well and so on until the last well, from where 100 µL was discarded. Then, 100 µL of microorganism inocula was added to wells. Eight different concentrations of each sample were tested. A positive control devoid of the working solution allowed us to examine microorganism growth. A negative control, which lacked the inoculum permitted us to discount the color coming from the working solution. A control plate containing 100 µL of BHI culture medium and 100 µL of sterile distilled water were added to the experiment as a control of BHI broth sterility. Microorganism growth was measured in ELISA plate reader (492 nm) immediately after ending the experiment (0 h). They were incubated at 37°C and read again after 24 h of experiments, ending the test.

RESULTS AND DISCUSSION

Fatty acids profile and quantification by gas chromatography using flame ionization detector (GC-FID)

Extraction of *A. hypoglauca* seeds provided 15.05% yield in oil. This oil was subjected to hydrolysis and methylation before being analyzed by GC-FID. Table 1 shows the identified fatty acids (%) from seed oil of *A. hypoglauca*. The relative abundance of fatty acids in seeds of other oil-making plants is also shown. The ω-9 in *A. hypoglauca* is higher than the corn oil and canola and ω-6 higher than the olive oil and ω-3 was similar to the concentration in olive oil and superior to the corn oil, canola was superior. These unsaturated fatty acids are important to human health, because they act on reducing blood lipid, cholesterol, but the human body does not produce such acid (Lopez-Huertas, 2010; Simopoulos, 2006).

Bioactivity of *A. hypoglauca*

Inhibition of acetylcholinesterase

But fatty acids can go beyond reducing blood lipids and reduce cardiovascular problems. The fatty acids present in vegetable oils or fish fats may have anti-inflammatory activities (Calder, 2005; Calder, 1998; Zhao et al., 2005), mainly against neuroinflammation. The neuroinflammation can lead to brain disorders such as Alzheimer's disease (AD) and Parkinson's disease, but there is a relationship between the use of polyunsaturated fatty acids present in oils or fats at low risk of developing these disorders (Bazinet and Layé, 2014). Thus, seek for new therapeutic agents from natural products such as extracts, essential oils and fixed oils with potential for inhibition of acetylcholinesterase (AChE) to treat people with AD is a modern challenge (Mukherjee et al., 2007). In this way, in testing the capacity of AChE inhibition, *A. hypoglauca* seed oil showed to be able to inhibit 79.55% of AChE activity. According to Vinutha et al. (2007) inhibitions values higher than 50% indicate potent inhibition; agents providing values below 30% are considered weak inhibitors and those leading to 30 to 50% inhibition are considered moderate inhibitors. The best known and prevailing neurodegenerative diseases are Parkinson and Alzheimer (AD). Symptoms of the latter include a regression of various physiological functions, causing difficulties in language, memory, emotional

Table 2. Bioactivity of oil from *A. hypoglauca* seeds against fungi and bacteria.

% Oil inhibition against yeast			
Concentration (µg mL^{-1})	***C. albicans***	**Miconazole (%)**	**Nystatin (%)**
500	91.08	92.33	93.30
250	90.61	91.05	90.77
125	91.01	90.71	90.14
62.5	90.02	90.55	90.18
31.25	91.09	91.15	90.92
15.625	94.57	91.61	91.49
9.375	91.45	91.52	91.26
3.90625	91.27	91.28	91.42

% Oil Inhibition against filamentous fungi		
Concentration (µg mL^{-1})	***A. flavus***	***F. proliferatum***
250	11.69%	3.69%

% Oil inhibition against bacteria gram(-)				
Concentration (µg mL^{-1})	***E. coli***	**Ampicillin (%)**	***S.* Typhimurium**	**Ampicillin (%)**
500	30.867	100.0	77.637	100.0
250	21.169	100.0	7.234	100.0
125	17.737	100.0	24.939	100.0
62.5	15.499	100.0	59.829	100.0
31.25	16.792	96.270	57.241	100.0
15.625	18.781	95.375	17.277	100.0
9.375	22.910	94.529	56.930	100.0
3.90625	26.789	79.559	63.763	100.0

% Oil inhibition against bacteria gram(+)				
Concentration (µg mL^{-1})	***S. aureus***	**Ampicillin (%)**	***S. sanguinis***	**Ampicillin (%)**
500	24.902	100.0	17.658	100.0
250	28.937	100.0	14.117	3.223
125	23.917	100.0	19.110	2678
62.5	20.177	100.0	14.389	0.000
31.25	12.106	100.0	7.490	0.000
15.625	8.268	86.713	18.021	0.000
9.375	13.780	34.744	20.835	0.000
3.90625	5.807	14.862	25.465	0.000

or personality behavior, and cognitive abilities (Singh et al., 2013). Since the number of people afflicted of AD increases exponentially, it is estimated that in 2050 about 115 million people might be affected by AD around the world (WHO, 2012).

Bioassays with filamentous fungi

A. hypoglauca oil showed low inhibition of fungi viability (*A. flavus*, 11.69%; and *F. proliferatum*, 3.69%, Table 2). These unsatisfactory results discarded the use of the oil as a means to control these filamentous fungi. *A. flavus* and *F. proliferatum* are phytopathogenic fungi that generate large economic damage. For *A. flavus*, for example, various types of grains contaminated with the mycotoxin aflatoxin lead to asperlogilosis in humans with severe effects on the respiratory tract (Hedayati et al., 2007). In turn, *F. proliferatum* can produce rot in some crops, such as corn on the cob; more recently, it has been reported that the fungus causes rot of soybean root (Chang et al., 2015).

Bioassay with *C. albicans*

When testing the capacity of *A. hypoglauca* seeds oil against *C. albicans* outstanding results were obtained

(Table 2). In some concentrations, oil from seeds of *A. hypoglauca* was superior to the inhibition promoted by the standard utilized. It is worth noting that inhibition caused by the oil was greater than 94% in the concentration of 15.625 mg/L, while at the same concentration miconazole and nystatin only reached 92% inhibition (Table 2). *C. albicans* is an opportunistic pathogen host in humans. The patient may even need to be hospitalized because of *C. albicans* infection called candidemia. People with low immunity, the elderly, patients with cancer, diabetes, surgery, among, others, have increased risk of fungal infections (Giolo and Svidzinski, 2010).

Bioassays with bacteria

The antibacterial activity test using the *A. hypoglauca* seed oil against *E. coli*, S. *tiphymurium*, *S. aureus*, *S. sanguinis* and *C. albicans* showed notable inhibition, except for *E. coli* which was poorly inhibited: 31% with 500 mg L^{-1} oil and 27% with 3.90625 mg L^{-1}. The antibacterial activity against *S. typhimurium* was greater. Using 500 mg L^{-1} of seed oil the inhibition extent reached 78% and at the lowest concentration tested (3.90625 mg L^{-1}) the inhibition level was rather high (64%). *E. coli* and *S. typhimurium* are Gram(-) bacteria that may cause diarrhea, intense fever and even death. They are transmitted principally by fecal-oral contamination, very common in countries without basic sanitation, as well as the improper handling of food (Moura et al., 2012; Butler, 2011). The activity of the oil against *S. aureus* was not satisfactory, since less than 30% inhibition was observed at all concentrations used. Similarly, *S. sanguinis* inhibition was below 40% at all concentrations. The low inhibition of *A. hypoglauca* seed oil towards some bacterial strains and high inhibition against *C. albicans* is indicative of a selective action mechanism, which deserves further investigation. *S. aureus* and *S. sanguinis* are Gram(+) bacteria which also cause grave health problems for humans, for instance skin infections and pneumonia (*S. aureus*), periodontal disease and severe endocarditis (*S. sanguinis*) (Evans et al., 2014; Sung et al., 2008). The WHO (World Health Organization) (2014) pointed out that there is global concern about the indiscriminate use of antibiotics, because this makes it that fungi and bacteria develop resistance to current drugs. Thus, it is necessary to look for new drugs that meet this need.

Conclusion

The oil from *A. hypoglauca* seed can be considered as an alternative source of vegetable oil. It can be used as raw material in the pharmaceutical and food industries, as it is constituted by essential fatty acids, where ω-9 and ω-6 unsaturated fatty acids occur in high proportion. Of note was the potent inhibition that the oil exerted on AChE, about 80% of the activity was suppressed. Although the bioactivity against filamentous fungi (*A. flavus* and *F. proliferatum*) was low, it was excellent for inhibiting the yeast *C. albicans*, exceeding 90%. At some concentrations, the activity of the oil was superior to that displayed by clinically used standards. The inhibition level did not reach 50% against *E. coli* and was above 77% for S. *tiphymurium*. The inhibition of Gram-positive bacteria, *S. aureus* and *S. sanguinis* was unsatisfactory. The excellent bioactivity of the oil against *C. albicans* may be related to selectivity and this is the most outstanding action detected for this oil.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Simulated inhibitory effects of typical byproducts of biomass pretreatment process on the viability of *Saccharomyces cerevisiae* and bioethanol production yield

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The abundance of second generation feedstock reinforces the consideration of biofuel over fossil fuel, as bioethanol can be produced from lignocellulosic materials. However, the pretreatment required for oxidation of lignocellulose into hexose often results in the production of inhibitors likely to impede the activity of *Saccharomyces cerevisiae* during bioethanol production. This study aimed to investigate the comparative inhibitory effects of acetic acid and vanillin on the viability of *S. cerevisiae* and the production yield of bioethanol. Different concentrations of inhibitors were spiked in the fermentation broth then the production of bioethanol monitored overtime and correlated with cell viability. The results showed that the inhibition of *S. cerevisiae* by vanillin is more potent compared to acetic acid; however the reduction of bioethanol yield after 12 h was more pronounced with acetic acid (42.8% reduction) than with vanillin (33.3% reduction) which was ascribed to the simultaneous production of weak acids during the fermentation process. The viability test has shown that in the presence of lower concentrations of inhibitors, *S. cerevisiae* can adapt for the first 12 h of fermentation and then may improve ethanol production yield overtime. At lower concentrations (2 g/l vanillin and 4 g/l acetic acid) the effect of inhibitors on the viability of *S. cerevisiae* and ethanol productivity does not last and can be overcome by the adaptation of the yeast. However, the presence of higher concentrations (4 g/l vanillin and 6 g/l acetic acid) results to nearly total inhibition of bioethanol production and the remediation of such effect may therefore require a detoxification process.

Key words: Bioethanol production, *Saccharomyces cerevisiae*, inhibition, acetic acid, vanillin, cell viability.

INTRODUCTION

Globally bioethanol technology is rapidly expanding due to progressive depletion of non-renewable fuel reserves and the potential for carbon neutral processes to contribute in the reduction of emission rate of polluting

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Abbreviations: ACE, Associated chemical enterprises; **CFUs,** colony forming units; **OD,** optical density; **HPLC,** high performance liquid chromatograph.

gasses to the atmosphere. Bioethanol production is also sustainable, reasonably cost effective, and easy to add into fuel distribution systems (Tomas-Pejoet al., 2008). Currently, first and second generation feed stocks are available for the production of bioethanol. First generation feedstock includes food crops and is therefore likely to negatively impact on the bio diverse regions which are destroyed to avail land required to grow crops. The main disadvantage of this approach is the increased cost of food as crops are used to produce bioethanol (Naik et al., 2010). Second generation feedstock mainly consists of lignocellulosic materials which are widely abundant, comprising about 50% of the biomass on earth and are available as industrial, agricultural, forestry and municipal residues (Almeida et al., 2007). Lignocellulosic materials for ethanol production have been classified into six groups by Sanchez and Cardona (2008). Herbaceous biomass, crop residues, cellulose wastes, softwood, hardwood and municipal solid wastes. These materials are inexpensive and abundant as they consist of the non-eatable parts of plants. Currently, the production of second generation bioethanol is an expensive process which does not make it a viable commercial setup, as the process of the conversion of lignocellulosic materials into bioethanol is not yet optimized. However, this approach does not affect the food crops,therefore minimizing the overall impacted cost of the second generation biofuel compared to the first generation biofuel (Naik et al., 2010).

The challenge is that second generation feedstock has a very complex structure as they are made of hemicellulose, lignin and cellulose. The production of bioethanol from this feedstock therefore requires a preliminary step of pre-treatment to release digestible sugar monomers; the problem with the pre-treatment is the formation of inhibitors which inhibit the growth of fermenting organisms. Some of these inhibitors generally found in the hydrolysates include aromatic compounds (that is, phenolics), furans (furfurals and 5-hydroxymethylfurfural), weak acids (acetic, levulinic and formic acids), raw material extractives (acidic resins, tannic, and terpene acids), and heavy metals (iron, chromium, nickel and copper) (Chandel et al., 2011). The formation of these components can lead to the inhibition of the growth of microorganisms by affecting the rate of the sugar uptake with simultaneous decay in the product formation (Palmqvist and Hahn-Hagerdal, 2000). The effect of such inhibitors on the production of biofuel has been intensively studied; several authors (Cao et al., 2010; Veeravalli et al., 2013; Liu et al., 2015) have reported the inhibition of hydrogen production as well as a shift in microbial community caused by furan derivatives present in the hydrolysate. The inhibition of the fermentation process by inhibitors in the lignocellulosic hydrolysates has also been alluded to (Delgenes et al. 1996; Bellido et al. 2011; Huang et al. 2011). Using the hydrolysate derived from wheat straw pretreatment with steam explosion for ethanol fermentation by *Pichiastipitis*, Bellido et al. (2011) observed a considerable reduction of the ethanol productivity. On the other hand, Huang et al. (2011) observed that weak acids such as acetic acid and formic acid were more potent inhibitors of yeast during bioethanol production as compared to phenols and aldehyde.

To overcome the effects of inhibitors from lignocellulosic hydrolysates on the fermentation process, physical, chemical and biological detoxification methods are often considered (Klinke et al., 2004). However, consideration of a detoxification step in the fermentation process may increase the cost as well as the production time. Although, *Saccharomyces cerevisiae* can tolerate the presence of inhibitors for a short while, this is often done at the cost of an extended lag phase and reduces ethanol productivity (Palmqvist et al., 1999; Larsson et al., 2000; Almeida et al., 2007; Landaeta et al., 2013). There is therefore a need to further investigate the behaviour of *S. cerevisiae* in the presence of inhibitors from lignocellulosic hydrolysates as well as the impact on bioethanol production yield.The inhibition may therefore lead to ineffective use of lignocellulosic biomass and insufficient yield for the commercialization of the process. Identifying the effects that the inhibitors, specifically acetic acid and vanillin (phenol), have on the growth of *S.cerevisiae* will then be correlated with the reduction in bioethanol yield.

METHODOLOGY

Chemicals

Acetic acid (95.5%) and vanillin (>99%) which act as the main inhibitors during bioethanol production from lignocellulosic biomass where purchased from Associated Chemical Enterprises (ACE) and MERCK, respectively. Chemical ingredients for the preparation of growth media included peptone, yeast extract which were purchased from SIGMA-ALDRICH, while Glucose and agar powder were obtained from ACE. Other common chemicals used included ethanol (99.9%) (SIGMA-ALDRICH) and sodium hydroxide (>98%)(ROCHELLE CHEMICALS).

Preparation of media

The growth supporting broth medium for yeast was prepared using Yeast extract, Peptone and Dextrose (YPD). YPD broth medium contained 10, 20 and 10 $g.L^{-1}$ of yeast extract, peptone and dextrose in de-ionized water. Agar medium contained 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ dextrose and 15 $g.L^{-1}$ agar in de-ionized water. The pH was adjusted to 6.5 using 0.1 M NaOH. Sterilization of the broth and agar media where done at 121°C for 20 min.

Batch fermentation

Batch fermentation was carried out in 250 ml Erlenmeyer flask, mainly using glucose as substrate for the yeast *S. cereviseae*. The inoculum was prepared by adding 0.005 g of dry *S. cerevisiae* cells

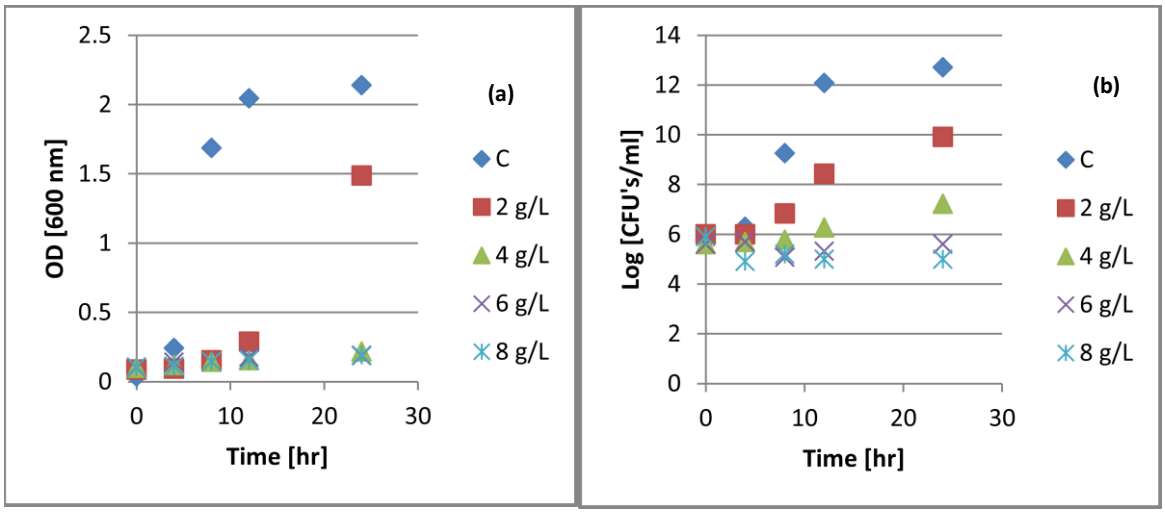

Figure 1. Inhibition of *S. cerevisiae* growth in presence of various concentrations of vanillin: (a) expression of growth by absorbance; (b) expression of growth by colonies count.

to one litre of sterilized broth and incubated overnight at 30°C in a shaking incubator (120 rpm). The culture was inoculated in 20% glucose solution contained in 100 mL GL 45 laboratory glass bottles with blue PP screw caps and pouring rings then incubated at 30°C for 48 h.

Determination of minimum inhibitory concentration

Yeast grown aerobically for 24 h in YPD broth was inoculated in broth spiked with different concentrations of acetic acid and vanillin (2, 4, 6 and 8 gper liter of broth). All experiments were conducted in Erlenmeyer flasks containing 50 mL broth, pH 6, 120 rpm shaking speed and incubated at 30°C. Samples were analyzed at set time intervals (3, 6, 8, 12 and 24 h) to determine the minimum inhibitory concentration.

Determination of the effect of inhibitors on bioethanol yield

An aliquot of 4 ml of yeast culture was added to glucose (46 mL, 20 $g.L^{-1}$) in 100 mL GL 45 laboratory glass bottles with blue PP screw caps and pouring rings. Adequate volume of acetic acid and vanillin was added to the glucose mixtures to make a final concentration of 4 or 6 $g.L^{-1}$ and 2 or 4 $g.L^{-1}$, respectively. Samples were analysed at set time intervals over a period of 48 h.

Quantification and viability of yeast cells

The growth of *S. cerevisiae* in the fermentation broth in the absence and presence of inhibitors was quantified through measurement of the absorbance. The total *S. cerevisiae* cells were measured at a wavelength of 600 nm using a spectrophotometer (Shimadzu). This measurement of the optical density (OD) gave an indication of the total cells (alive, injured or dead) present. The amount of viable yeast cells was determined using culture method. The culture was serially diluted with sterilized de-ionized water. Diluted cells were plated on agar medium (30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4$, $7H_2O$, 20 g/L agar) in Petri dishes then incubated at 30°C for 48 h. The number of colonies counted and the average of duplicate plates was expressed as colony forming units (CFUs).

Analytical method

The fermentation liquor was filtered through a 0.2 µmmicro pore syringe filter and the ethanol was quantified in the filtrate using a high performance liquid chromatograph (HPLC). An Agilent 1200 HPLC fitted with a refractive index detector was used with an isocratic mobile phase of 0.005 M H_2SO_4.

RESULTS

Vanillin and acetic acid belong to the groups of phenolic compounds and weak acid respectively; they are generated during pre-treatment and hydrolysis of second generation feedstock used for the production of bioethanol. Vanillin is a phenolic compound derived from lignin breakdown and acetic acid is a derivative from hemicellulose breakdown during pre-treatment. Although, there are a large variety of phenols and acids formed during pre-treatment, vanillin and acetic acid were chosen in this study as they occur in the largest quantities. Few studies have been previously carried out to determine the inhibitory effect of these compounds; the particularity of this study is to correlate the inhibitory effect to the viability of the yeast and also to delineate the factors contributing to the decrease of ethanol yield in the presence of inhibitors.

Effect of inhibitors concentration on the growth of *S. cerevisiae* over time

Effect of vanillin

Figure 1a and b show that the inhibition effect of vanillin increased with the concentration and exposure time. The minimum inhibitory concentration (MIC) could be estimated as 2 g/l. When the inhibitor was present at

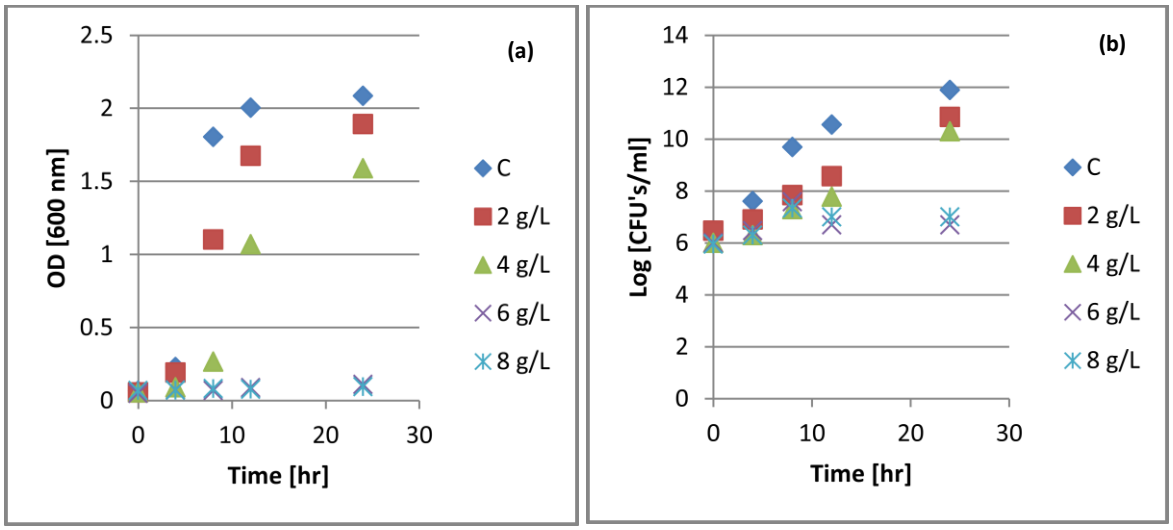

Figure 2. Inhibition of *S. cerevisiae* growth in presence of various concentrations of acetic acid: (a) expression of growth by absorbance; (b) expression of growth by colonies count.

concentrations of 2, 4 or 6 g/l, there was a similar trend between the OD measurement and colony count; however a dissimilarity was observed after 8 h incubation and in the presence of 8 g/l vanillin, as the cell count indicated no growth while the OD value of 0.2 was recorded; this implies that the cells were no longer viable after 8 h incubation in the presence of 8 g/l vanillin.

Effect of acetic acid

Data plotted in Figures 2a and b clearly indicate the inhibition of *S. cerevisiae* in the presence of acetic acid; it was observed that the inhibition effect also increased with the concentration and time. The MIC was found to be 2 g/l with only little effect on the growth of the yeast. There was no perfect correlation between the adsorbance and the cell counts as shown by the behaviour of the yeast at 6 and 8 g/l of acetic acid. This implies that at those concentrations, although the cells multiply in the first 8 h, metabolic rearrangement may also take place resulting in the decrease of the yeast's biomass (Yousef and Uneja, 2002). Exposing yeast to various environmental stress conditions, Tibayrenc et al. (2010) also found that there was an increase of population of significantly smaller cells size.Comparing the effects of the two inhibitors, it can be observed that in general vanillin has a pronounced inhibitory effect than acetic acid; for the same MIC (2 g/l), vanillin caused more reduction of growth than acetic acid; and at 8 g/l, vanillin had a lethal effect while acetic acid only had a static effect. It has been reported (Klinke et al., 2003; Almeida et al., 2007) that phenolic compounds are stronger inhibitors than acids because of their aldehyde and ketone groups. It is suggested that phenolic compounds act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices; while the inhibitory effect of acetic acids has been ascribed to uncoupling and intracellular anion accumulation (Russel, 1992).

Bioethanol yield influenced by MIC level of inhibitors

The minimum inhibitory concentrations of 2 g/l of vanillin and 4 g/l of acetic acid were chosen to determine their effect on the production of ethanol by *S. cerevisiae*. It is important to use relatively low concentrations to mimic the level produced following pretreatment of biomass.

Inhibitory effect of vanillin

The impact of vanillin on the production of ethanol in the first 36 h was quite obvious as shown in Figure 3, the constant reduction of bioethanol production compared to the control not exposed to the vanillin; however after 36 h, the yeast seem to recover and perform better in the presence of vanillin resulting in higher production of ethanol; this could be explained by the cell count as an increase was recorded while the OD remained lower than the control values, implying that the cells may have lost weight but remained more active after longer exposure to vanillin. The simultaneous production of weak acids during ethanol production may have also played a role in the stabilization of ethanol production rate after 36 h, as discussed later. Figure 4a and b both express the growth of *S. cerevisiae* during bioethanol production, the results clearly show that OD values could not be strictly corroborated to the number of cells, as the trend of the

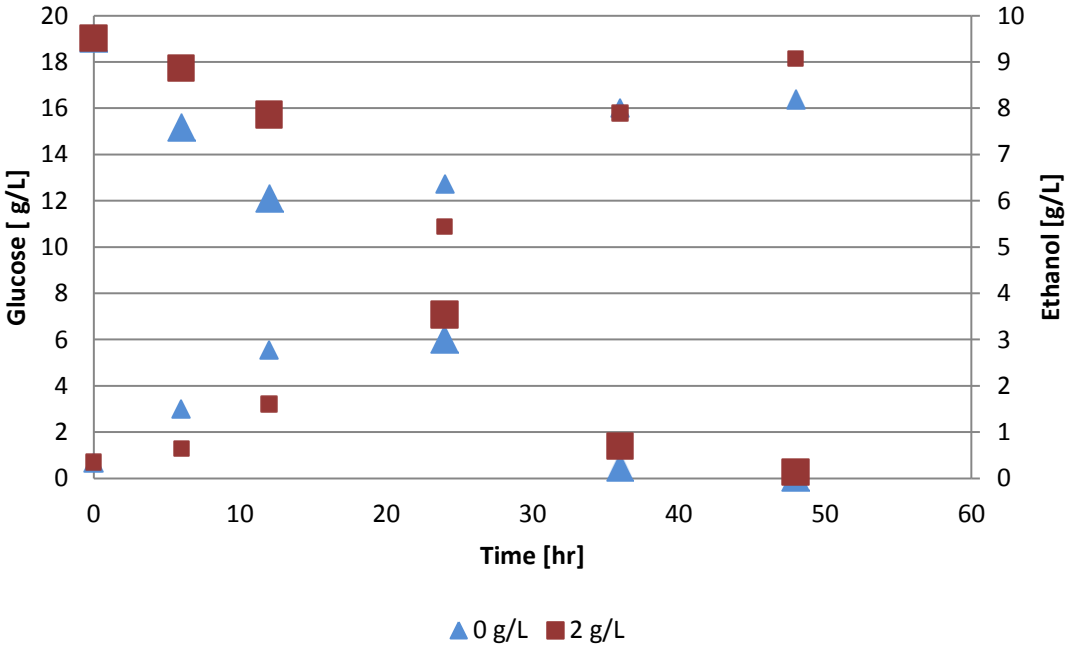

Figure 3. Glucose consumption and ethanol production in the presence of 2 g vanillin. Large symbols (glucose), small symbols (ethanol).

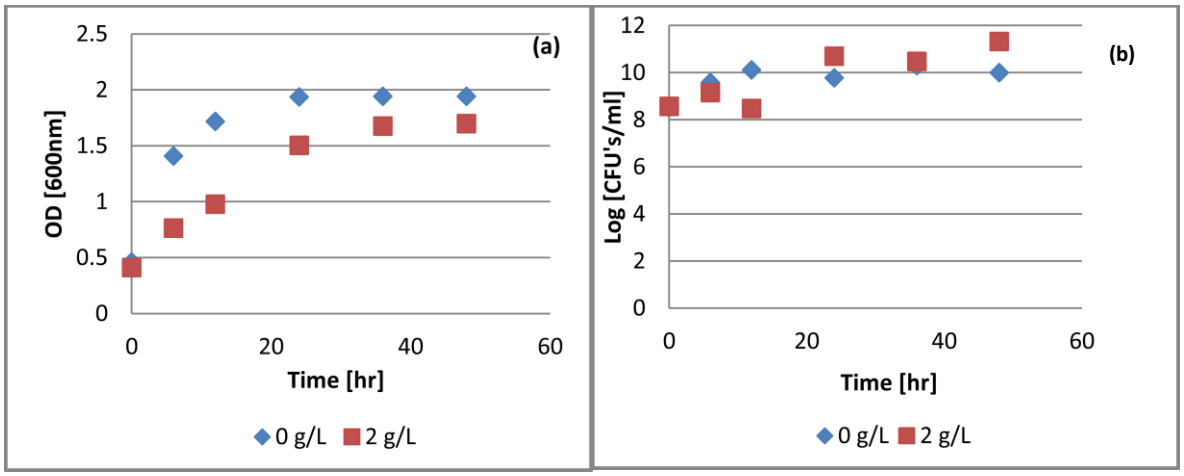

Figure 4. Growth expression of *S. cerevisiae* during fermentation and in the presence of vanillin (2 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

OD plots do not express clearly the rapid multiplication of cells in the presence of the inhibitor after 10 h; the cells probably lose weight during adaptation to the presence of the inhibitor, but continue to grow rapidly compared to the control.

Inhibitory effect of acetic acid

Figure 5 shows that there was a decrease of glucose concentration as the ethanol was formed, clearly indicating that ethanol production results from the use of glucose by *S. cerevisiae*; however, the rate of glucose breakdown was slow at the beginning and therefore lower production of ethanol for the first 12 h in the presence of acetic acid; the trend changed after 12 h as more ethanol was produced in the flask containing the acetic acid. This could have merely been ascribed to the adaptation of *S. cerevisiae*, but the patterns of OD values and cell count (Figure 6a and b) do not confirm this, further discussion

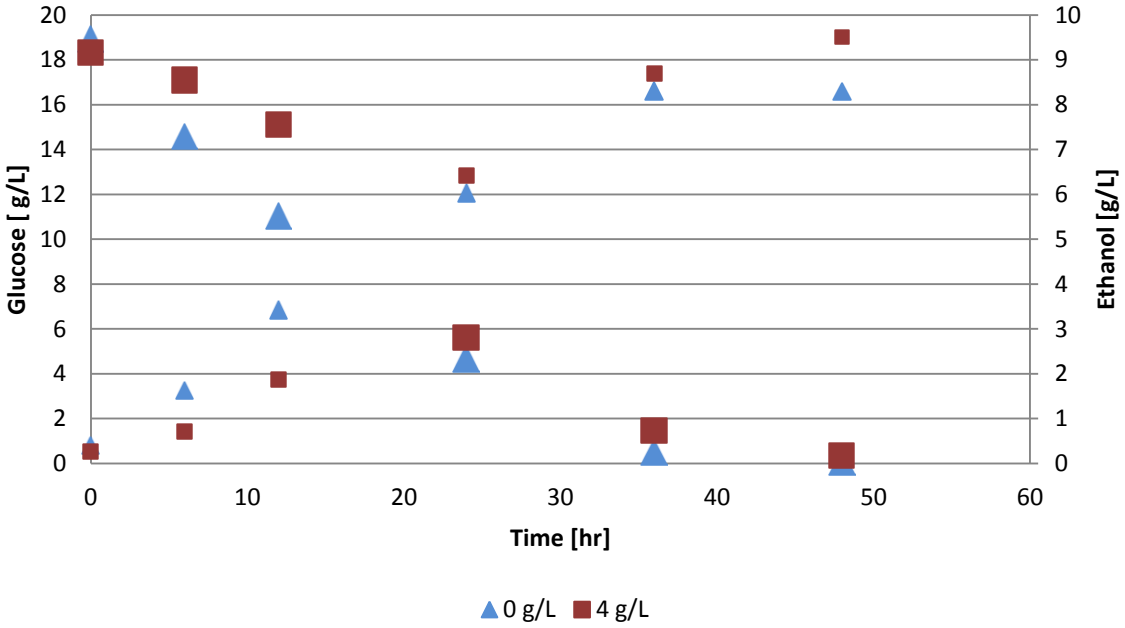

Figure 5. Glucose consumption and ethanol production in the presence of 4 g acetic acid: Large symbols (glucose), small symbols (ethanol).

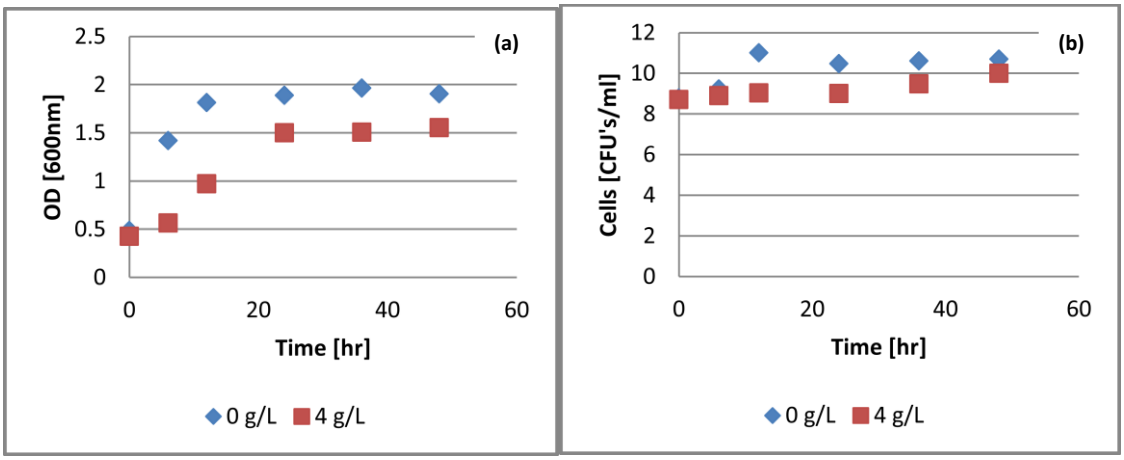

Figure 6. Growth expression of *S. cerevisiae* during fermentation and in the presence of acetic acid (4 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

will be done in the following sections. The plots of optical density and cell count in Figure 6a and b indicate an extended lag phase and more sluggish exponential growth phase in the presence of the inhibitor. However, after 48 h there was as much cells in the control sample as in the sample with the inhibitor, implying that the yeast adapted overtime.

Bioethanol yield influenced by higher concentrations of inhibitors

The inhibitory effects at relatively higher concentration of vanillin (4 g/l) and acetic acid (6 g/L) on the growth of *S. cerevisiae* was observed in Figures 1 and 2, respectively. A significant effect on the bioethanol production yield could therefore be expected at higher concentrations of inhibitors.

Inhibitory effect of vanillin

Figures 7 and 8 clearly indicate the effects of higher concentrations of vanillin on the ethanol production yield and the viability of *S. cerevisiae.*For the total duration of the fermentation process the ethanol yield in the

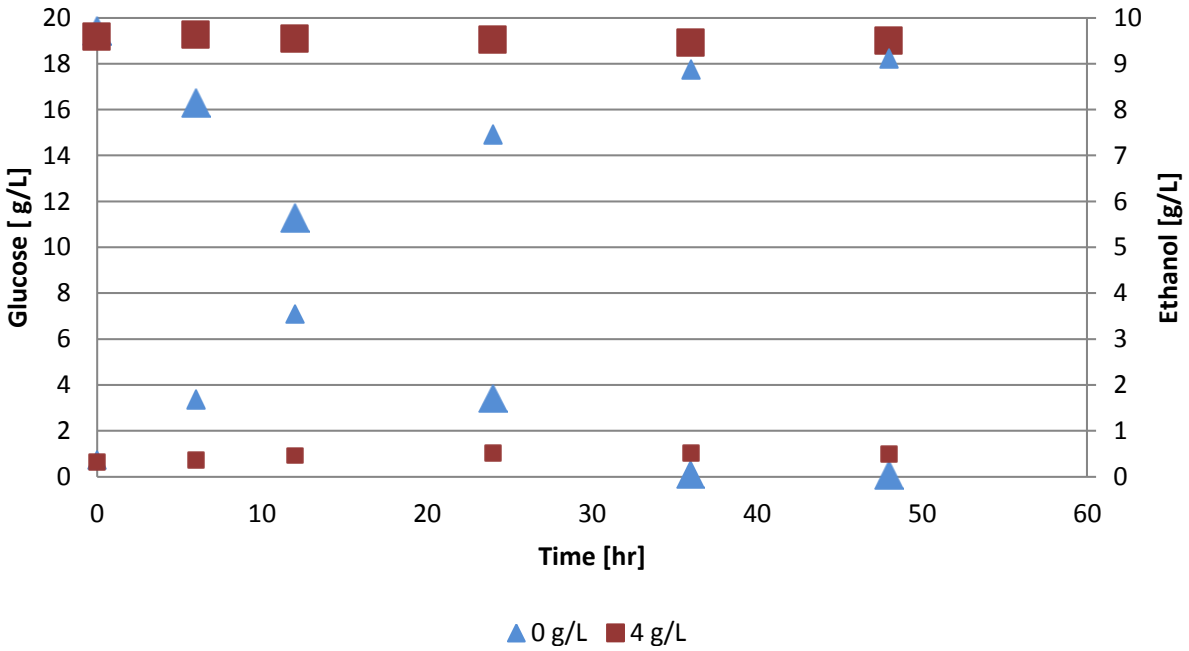

Figure 7. Glucose consumption and ethanol production in the presence of 4 g/L vanillin: large symbols (glucose), small symbols (ethanol).

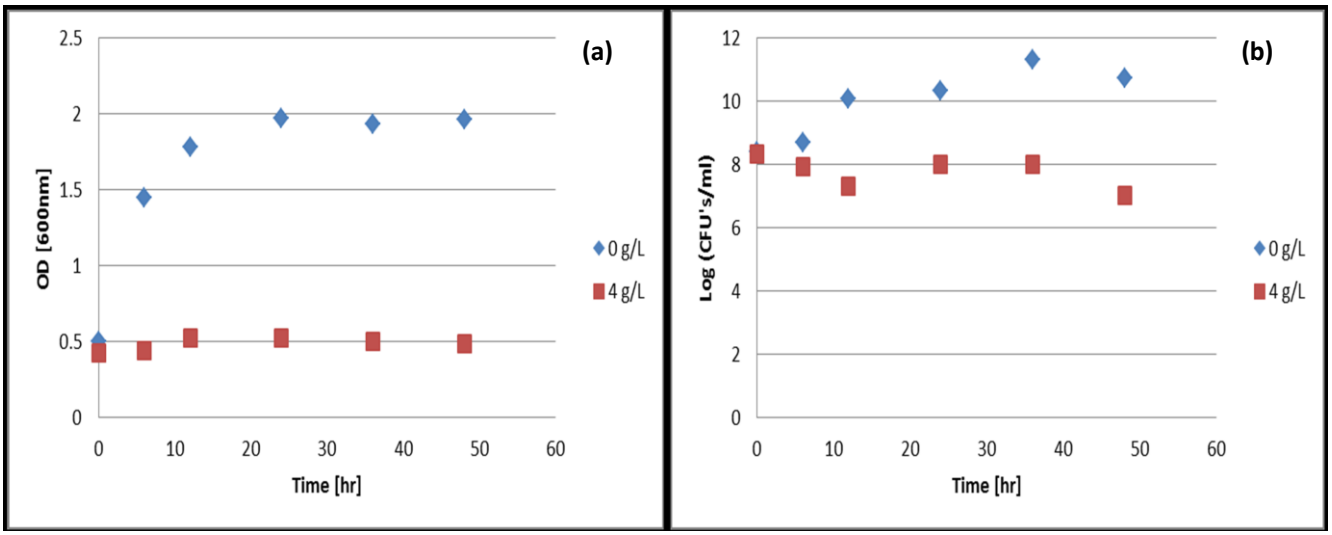

Figure 8. Growth expression of *S. cerevisiae* during fermentation and in the presence of vanillin (4 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

presence of 4 g/L vanillin (Figure 7) remained constant throughout the 48 h. Glucose concentration also remained constant at about 20 g/L in the presence of the inhibitor. When comparing these results to that of the effect of the MIC of vanillin (2 g/L) it can be observed that the final ethanol concentration decreases from 9 g/L in the presence of lower (2 g/L) of inhibitor to 0.5 g/L at higher (4 g/L) concentration of the inhibitor, respectively. Thus, the fermenting organism is very sensitive to the slight increase of the concentration of vanillin, the two fold increase led to almost 95% reduction of the bio-ethanol yield, showing the impact of inhibitor when using pre-treatment and hydrolysis methods that produce more than two gram per litre of vanillin from second generation feedstock. Figure 8 shows a total inhibition of cells growth as expressed by the absorbance (a) and the viability test

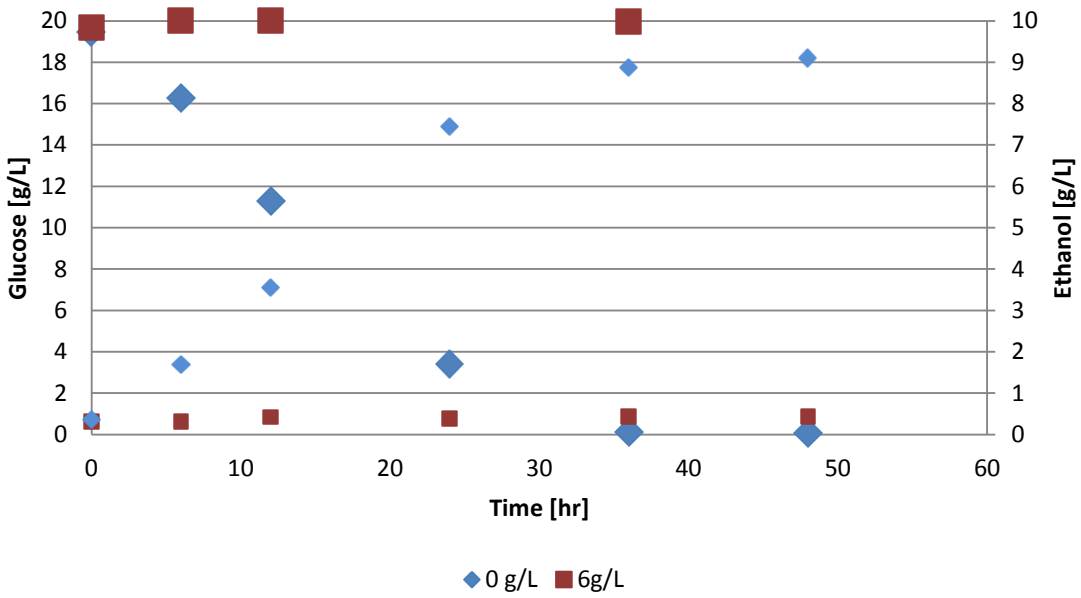

Figure 9. Glucose consumption and ethanol production in the presence of 6g/L acetic acid: Large symbols (glucose), small symbols (ethanol).

expressed by CFU values (b) confirming the inhibitory effect of 4 g/L vanillin. According to the colonies counts there is attempt by the cells to adapt to the presence of the inhibitor in the interval time between 25 to 35 h; the inhibition effect is however persistent because of the cumulative effects of other inhibitors such as lactic and acetic acids produced during fermentation.

Inhibitory effect of acetic acid

During the 48 h of fermentation, the ethanol yield in the presence of 6 g/L acetic acid (Figure 9) remained constant. Glucose concentration also remained constant in the presence of the inhibitor. It is quite evident that increasing the concentration of acetic acid from 4 to 6 g/L has resulted to a more pronounced inhibitory effect on the yeast, preventing adequate organization of the metabolic activities required for the fermentation of glucose to bioethanol; hence the concentration of glucose remaining constant throughout the 48 h. The inhibitory effect of 6 g/L of acetic acid on *S. cerevisiae* growth could be observed in Figure 10a and b, as the OD values did not increase during the 48 h of incubation; this implies that there was no growth as the cells were exposed to the inhibitor, but the cells grow well in the absence of inhibitor. The effect related to increased concentration of acetic acid could be noted when comparing the OD values at 4 and 6 g/L of the inhibitor. The count of colonies, provide information about the viability of the cells; it is observed in Figure 10b that the cells number decreases overtime indicating a microbicidal effect of 6 g/L of acetic acid; this effect is more pronounced than with 4 g/L acetic acid. This therefore explains the drastic drop of 95% of bioethanol yield.

Formation of week acids during bioethanol production

In this study the formation of weak acids during the fermentation of glucose was monitored to determine their contribution in the inhibition of *S. cereviseae* and subsequently the effect on the yield of bioethanol. It was observed that the amount of weak acids formed varied with the initial concentration of the inhibitors in the fermentation broth.

In the presence of MIC level of inhibitors

Figure 11a and b below show that there was formation of acetic and lactic acids during the degradation of glucose and formation of ethanol by *S. cerevisiae*; it can however be seen that in the presence of the inhibitor (vanillin) the production of weak acids is lowered. The accumulation of these weak acids has contributed to significantly reduce after 12 h, the performance of the yeast not previously exposed to inhibitors (Figure 3).The formation of weak acids including lactic and acetic acids was observed during the production of ethanol in the absence and presence of acetic acid (4 g/L) (Figure 12a and b). However, in the presence of acetic acid the inhibition effect led to the reduction of the amount of lactic acid formed while the increase of the amount of acetic acid was likely due to the combination with the residual

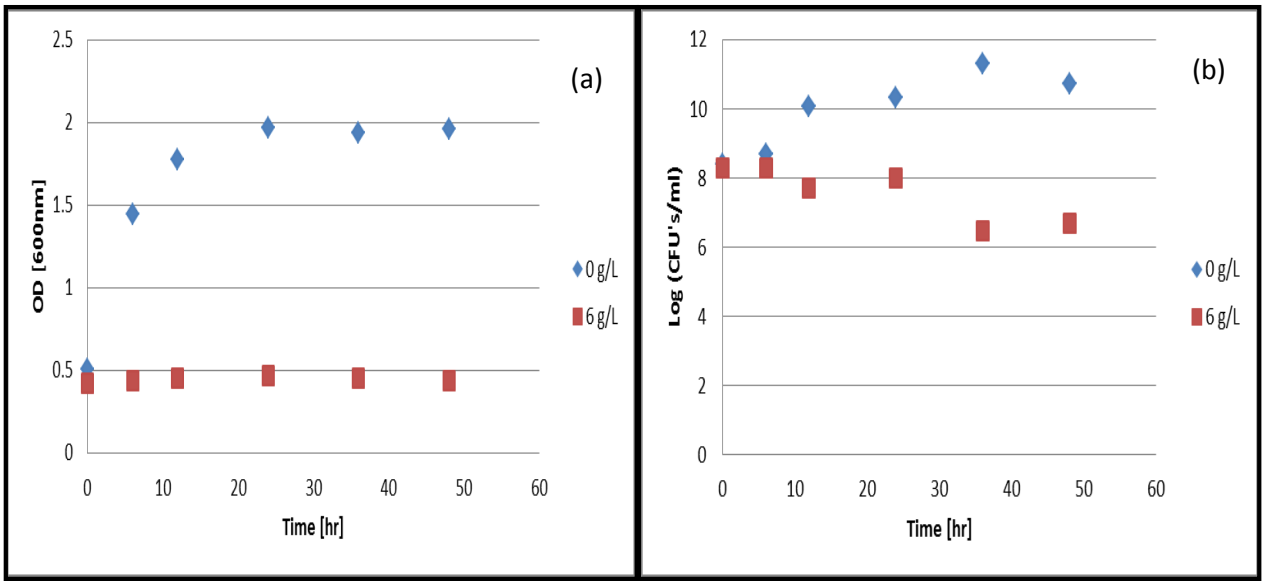

Figure 10. Growth expression of *S. cerevisiae* during fermentation and in the presence of acetic acid (6 g/L): (a) expression of growth by absorbance; (b) expression of growth by colonies count.

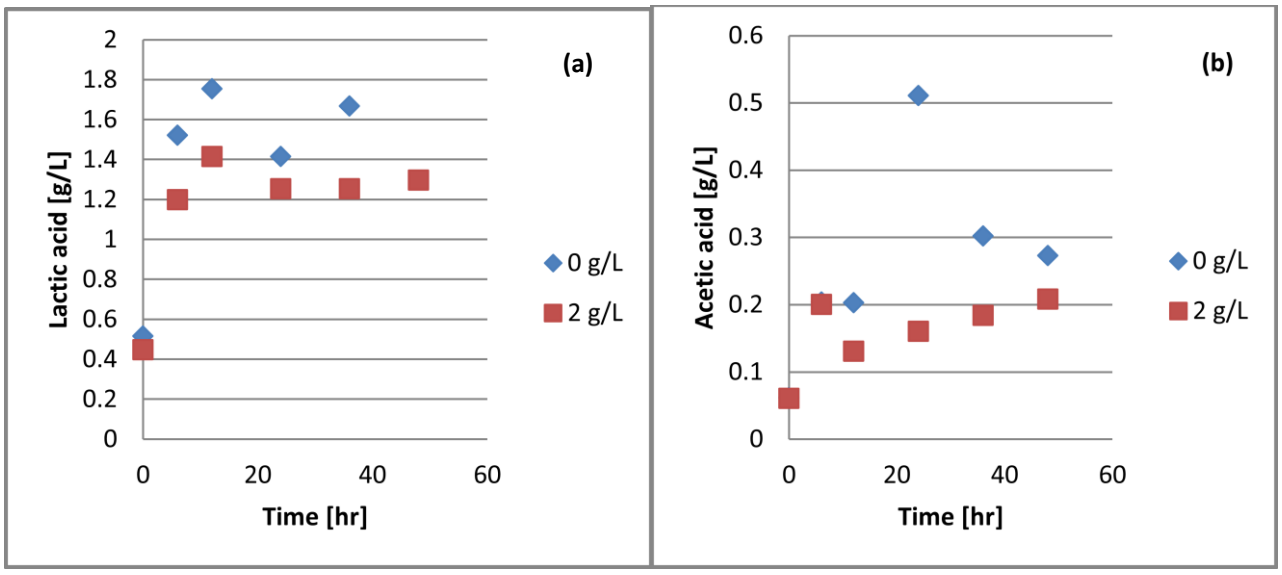

Figure 11. Formation of acetic acid and lactic acid during fermentation and in presence of vanillin (2 g/L): (a) Lactic acid formation, (b) acetic acid formation.

acid.The formation of weak acids in the control samples after 24 h probably led to the inhibition of *S. cerevisiae*, this explains why the performance of the yeast exposed to inhibitors from the first hour was better than the control after 24 h.

In the presence of higher concentration of inhibitors

Figure 13a and b show the formation of lactic acid and acetic acid during fermentation, the amounts of these acids is relatively low compared to that obtained during fermentation in the presence of lower (2 g/L) concentration of vanillin. Acetic acid final concentration was halved from 0.2 to 0.1g/L, certainly as a result of reduced cell activity. The amount of cells is directly related to the amount of acetic acid and lactic acid produced. In Figure 14a and b lactic and acetic acids formation is reduced drastically in the presence of 6 g/L acetic acid; this translates to the significant reduction of activity.

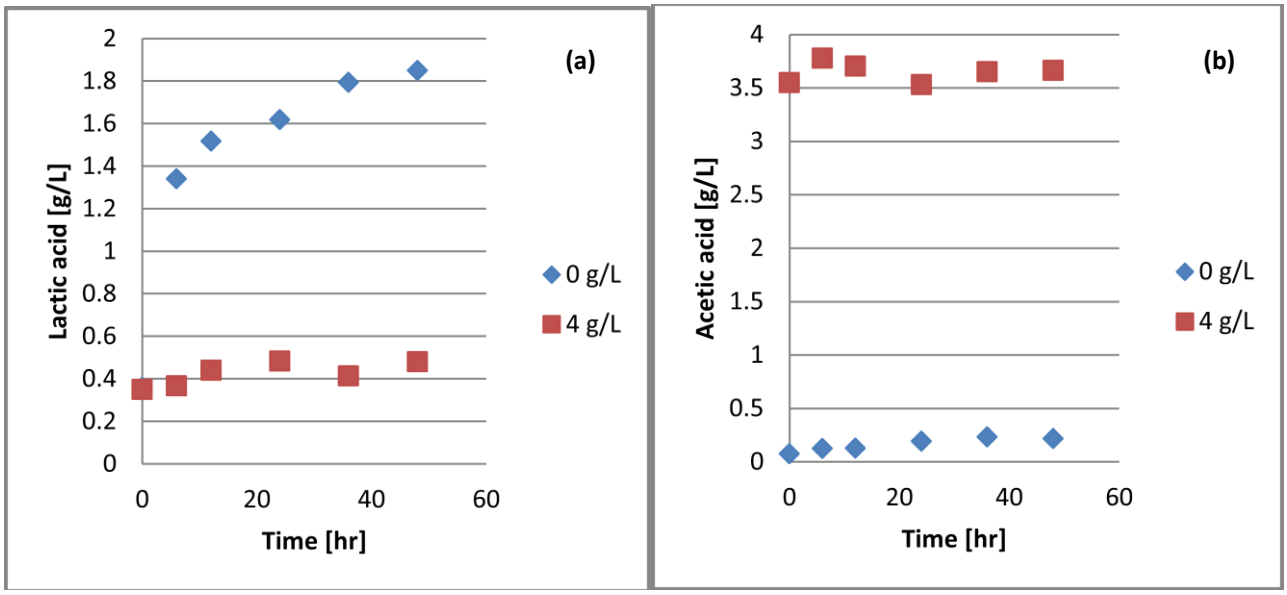

Figure 12. Formation of acetic acid and lactic acid during fermentation and in presence of acetic acid: (a) lactic acid formation (b) Acetic acid formation.

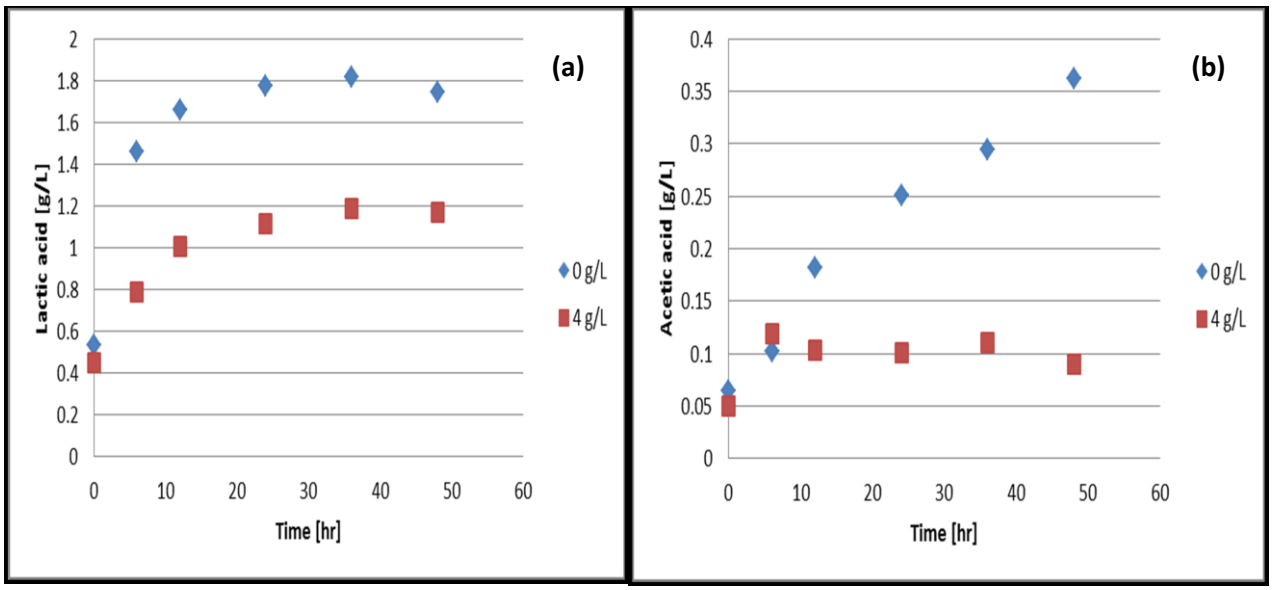

Figure 13. Formation of acetic acid and lactic acid during fermentation and in the presence of vanillin (4 g/L): (a) Lactic acid formation, (b) Acetic acid formation.

Therefore, the inhibition during the 48 h period results predominantly from the activity of the acetic acid introduced at the beginning of the fermentation.

DISCUSSION

By exposing the yeast to lower and higher concentrations of inhibitors it was possible to better understand its fermentability behaviour; the inhibition of yeast at lower concentration of inhibitors brought about two scenarios. A deceleration phase was observed during the adaptation of yeast in the first 12 h, resulting in lower consumption rate of glucose and lower ethanol productivity.

The ethanol productivity value dropped from around 0.26 g/L h in the control sample to about 0.121 and 0.137 g/L h in the presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively, representing approximately 50%

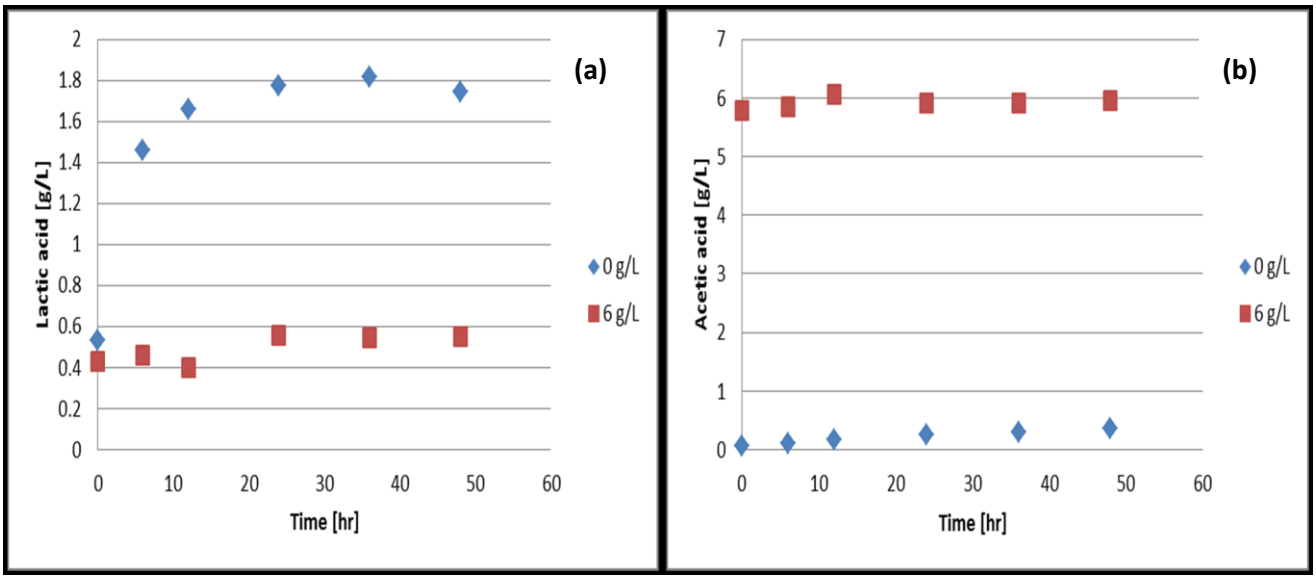

Figure 14. Formation of acetic acid and lactic acid during fermentation and in the presence of acetic acid (6 g/L): (a) lactic acid formation, (b) acetic acid formation.

reduction. In the second phase the yeast had adapted and the cells were very active, judging by the higher productivity values 0.213 and 0.236 g/L h in presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively; these values were equal or higher than the control value of 0.219 g/L h. It is however important to mention that the acetic acid and lactic acid formed during fermentation in the control sample, were much likely to inhibit the non-adapted yeast.

The recorded changes in bioethanol productivity in the presence of inhibitors were not always correlated with the OD values, but reflected the growth pattern expressed as cell plate count or viability which translates into the ability of cells to grow and replicate. After consumption of almost all the glucose, it was found that at 48 h the inhibitory effects on the yeast's growth did not affect the bioethanol yield, but rather increased the yield from 0.412 g/g in the control sample to 0.454 and 0.476 g/g in the presence of vanillin (2 g/L) and acetic acid (4 g/L), respectively. Similar results have also been previously reported by researchers studying the inhibitory effect on the fermentation (Moreno et al., 2013; Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000).

In the presence of higher concentrations of vanillin (4 g/L) and acetic acid (6 g/L) the trend of bioethanol productivity was almost constant from the first hour till 48 h, as the yeast consumed very little glucose. The bioethanol yield was very low 0.0243 and 0.0216 g/g in the presence of vanillin (4 g/L) and acetic acid (6 g/L), respectively, while a high yield 0.455 g/g was recorded in the control sample. The optical density was constant in the presence of inhibitors not giving an exact indication of the physiological state of the yeast; however the cell count showed a decrease of cell viability as there was reduction of the number of cell from 0 to 48 h. The OD measurement must therefore be complemented by the cell count to have an indication of the yeast physiological response to inhibition during fermentation.

Conclusion

In this study the behaviour of *S. cerevisiae* in the presence of inhibitors is enlighten by the viability test, showing that in the process of adaptation the cell biomass is reduced, but the yeast continues to grow and produce ethanol. Vanillin is found to be more toxic to the fermenting organism *S. cerevisiae*. The potency of vanillin has also been reported by Chandel et al. (2011). It was observed that at the minimum inhibitory concentrations, the inhibitors could reduce the bioethanol productivity only in the first 12 h of fermentation, which may therefore not be a serious problem if the fermentation process takes longer than 24 h. However, relatively higher concentrations have been found totally inhibitory of the yeast activity, preventing the use of glucose and reducing the bioethanol yield by approximately 95%. For such concentrations of inhibitors the inhibition may be overcome by the use of detoxification methods to avoid a significant drop of the ethanol yield.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Assessment of the safety of aqueous extract of *Aloe vera* on haematology of Wistar rats

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***Aloe vera* is used both traditionally and packaged commercially in many regions of the world for several medicinal and or cosmetic purposes. It is claimed to have rejuvenating, moisturizing, healing or soothing properties on the skin and gastrointestinal tract. This study focused on assessment of the safety of *A. vera* on blood parameters: packed cell volume (PCV), red blood cell count (RBC), haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count (WBC), its differentials neutrophils, lymphocytes and platelet counts. Thirty Wistar rats were equally and randomly divided into 3 groups and *A. vera* extract solution was administered to 2 groups for 12 or 24 h respectively, for 7 days consecutively. The third group served as control for the experiment. Blood samples were collected on day 8 to determine changes in the haemogram as a basis for toxicity. Rats administered with *A. vera* extract, particularly for 24 h showed increased levels of PCV (47.42±4.32%), RBC (9.26±0.60 X10^6/µL), WBC (12.61±0.45 X10^3/µL) and its differentials. Platelet count was also significantly increased (150.25±4.77 X10^9/L). The results from this study showed that *A. vera* stimulated increased production of all blood cell types. In conclusion, protracted consumption of the extract of *A. vera* cause stimulation of haematopoiesis which may induce or encourage the progression of haemoproliferative disorders.**

Key words: *Aloe vera*, haematology, Wistar rat.

INTRODUCTION

Aloe vera is a naturally occurring plant with succulent leaves, originating from Northern Africa (Akinyele and Odiyi, 2007). The whole leaves or juice from the leaves has been used in several cultures of the world dating back to the first century A.D. as herbal remedy for various skin conditions (Boudreau and Beland, 2006; Akinyele and Odiyi, 2007). It is being packaged and marketed alone or in combination with other substances in commercially available lotions, creams, yogurt, beverages and as desert. It is claimed to have rejuvenating, moisturezing, healing or soothing properties on the skin and gastrointestinal tract (Davies et al., 1989; Heggers et al., 1997; Vogler and Ernst, 1999; Boudreau and Beland, 2006). Preliminary reports have also been documented on its blood glucose and lipid lowering effects, suggesting possibility of its use as an anti- diabetic agent (Nassiff et

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al., 1993; Boudreau and Beland, 2006; Choudhary et al., 2014; Alinejad-Mofrad et al., 2015). Reduction of symptoms and inflammation in patients with ulcerative colitis has also been suggested amongst other medicinal uses (Langmead et al., 2004; Bottenberg et al., 2007).

Aloe vera is a stem-less or very short-stemmed succulent plant growing to 60–100 cm (24–39 in) in height and spreading by offsets. It has fleshy, thick leaves which are usually green to grey-green in colour, with some varieties showing white flecks on the upper and lower stem surfaces (Gao and Xiao, 1997; Wang et al., 2004). The margin of the leaf is serrated and has small white teeth. It has pendulous flowers which are produced in summer season and these may reach up to 90 cm (35 in) tall. The flowers have a yellow tubular corolla, 2–3 cm (0.8–1.2 in) in length. Like other *Aloe* species, *A. vera* forms arbuscular mycorrhiza, a symbiosis that allows the plant better access to mineral nutrients in soil (Gong et al., 2002).

Most evidences of the activities of *A. vera* cannot be substantiated as little scientific evidence exist on its effectiveness or safety for the medicinal or cosmetic purpose for which it is used (Cosmetic Ingredient Review Panel, 2007). The few scientific reports however, showed conflicting evidences (Vogler and Ernst, 1999; Ernst, 2000; Marshall, 2000; Boudreau and Beland, 2006). Some conflicting reports on its wound healing ability were documented by Heggars et al., (1997) and Davis et al. (1989) who reported that *A. vera* promoted wound healing, while Schmidt and Greenspoon (1991) and Kaufman et al. (1988) reported the contrary.

There are little or no reports on the effect of the plant on the blood, the vehicle of transportation of most substances. Some information of its effect on blood cells may give some insight on safety of the plant on blood cells and related organ tissues. This study was therefore designed to determine the effect of sub-chronic administration of *A. vera* on the haemogram using assessment of changes in the pack cell volume, various red and white blood cell indices and platelet counts.

MATERIALS AND METHODS

Preparation of *Aloe vera* juice

Fresh leaves of *A. vera* were plucked daily and washed. The juice was expressed by gentle milking downwards. Daily water intake of the rats was determined during the acclimatization period to be approximately 40 ml per rat per day. The fresh juice was reconstituted to 46.20 mg/ml in fresh drinking water and served to rats unprocessed. This study was carried out in September, 2012 in South West, Nigeria.

Experimental animals

Thirty male Wistar rats (140 – 160g) were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine. The animals were housed in 12 h light: dark condition and maintained on standard rat diet. Clean water was provided *ad libitum*. The animals were stabilized for 4 weeks before commencement of the experiment. All the rats were humanely managed and the study protocols were in compliance with the Faculty of Veterinary Medicine guidelines for the use of laboratory animals.

Experimental protocol

Thirty rats were randomly and equally divided into 3 groups of one control and two treatment groups. The rats in the control group were allowed free access to clean water throughout the course of the experiment. Clean water was withdrawn from rats in treatment groups 1 and 2, and replaced with the *A. vera* solution for 12 and 24 h respectively, for 7 days consecutively. For the 12 h exposure group, half of the daily water requirement was reconstituted with *A. vera* and offered for 12 h , while fresh clean drinking water was offered for the remaining 12 h.

Sample collection

On day 8, the rats were anaesthetized using anaesthetic ether and blood samples were collected from each rat via the retro-orbital sinus. About 3 ml of blood was collected into Lithium heparinized bottles for haematological analysis by Cole's method (Cole, 1986).

Statistical analysis

All values are expressed as mean ± S.E.M. Data obtained were analyzed using one-way analysis of variance (ANOVA), followed by Tukey post-test. Differences between means were considered significantly different when values $p<0.05$ were obtained using Graph-Pad Prism software Version 5 (2007).

RESULTS

Packed cell volume (PCV)

An increase in the PCV of rats administered with the extract of *A. vera* was observed with a significant ($p<0.05$) increase in rats administered with the extract for 24 h (47.42±4.32%) when compared to the control rats (41.75±2.17%) (Table 1).

Red blood cell indices

Red blood cell count (RBC) of rats administered with the extract increased from $8.22±1.57 \times 10^6$/µL observed in the control rats to 8.97±0.16 and $9.26±0.60 \times 10^6$/µL observed in rats administered with the extract for 12 and 24 h respectively. Increases were also observed in the haemoglobin concentration of the treated rats with a significant ($p<0.05$) increase in rats treated for 24 h (16.31±0.68 g/dl) compared to the control rats (13.88±0.89 g/dl). Other red cell indices also increased accordingly (Table 1).

White blood cell indices

White blood cell count (WBC) and the differential cell

Table 1. Packed cell volume and red blood cell indices obtained from rats administered with *Aloe vera* for 12 or 24 h of seven consecutive days.

Haematological parameters	Control	12 h	24 h
PCV (%)	41.75±2.17	43.30±1.43	47.42±4.32*
RBC (X10^6/µL)	8.22±1.57	8.97±0.16	9.26±0.60
Hb (g/dl)	13.88±0.89	14.25±0.41	16.31±0.68*
MCV (*f*l)	50.79±4.05	48.27±2.00*	51.21±1.21
MCH (ρg)	16.89±0.65	16.38±0.34	17.61±0.29
MCHC (g/dl)	33.25±0.41	30.39±0.26	34.39±0.22

*Significant ($p<0.05$) difference compared to control value.

Table 2. White blood cell indices and platelet count of rats administered with *Aloe vera* for 12 or 24 h of seven consecutive days.

Haematological parameters	Control	12 h	24 h
WBC (X10^3/µL)	8.70±0.27	9.48±0.67*	12.61±0.45*
Lymphocytes (X10^3/µL)	4.77±0.49	5.53±0.31	8.35±0.12*
Neutrophils (X10^3/µL)	2.23±0.25	2.93±0.14	4.34±0.13*
Eosinophils (X10^3/µL)	0.13±0.01	0.19±0.05	0.28±0.05*
Neutrophil/ Lymphocte ratio	0.47±0.01	0.53±0.05	0.52±0.02

*Significant ($p<0.05$) difference compared to control value.

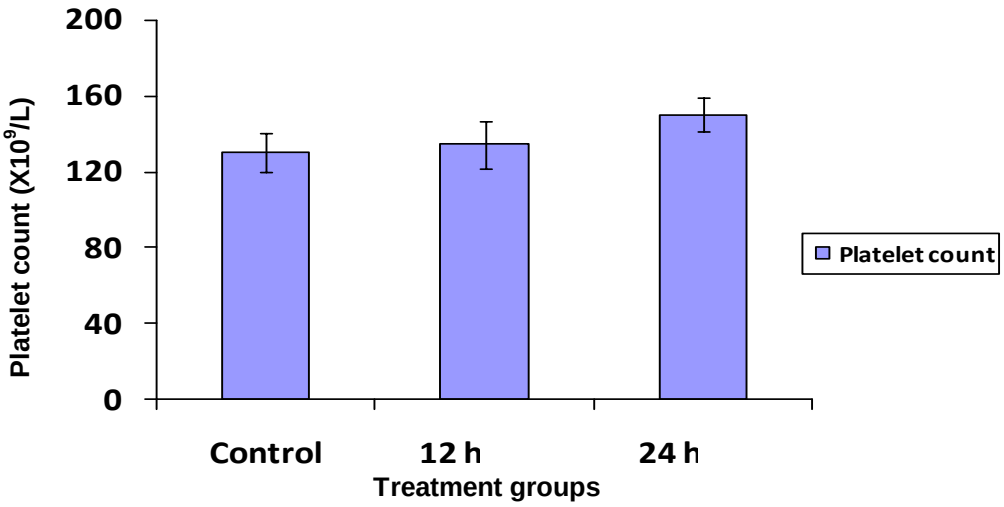

Figure 1. Mean platelet count of rats administered with aqueous extract of *Aloe vera* for a 12 or 24 h period of 7 consecutive days.

count of rats administered with *A. vera* extract increased compared to those of the control rats. Notably, WBC in rats treated for the 24 h period (12.61±0.45 ×10^3/µL) was significantly ($p<0.05$) higher than that of control rats (8.70±0.27 ×10^3/µL). The same significant ($p<0.05$) pattern was observed for the differential cell count of these rats treated for 24 h (Table 2).

Platelet count

Platelet counts were non-significantly ($p>0.05$) increased in the rats treated for 12 h (134.2±1.24 × 10^9/L) but significantly ($p<0.05$) increased in rats treated for 24 hours (150.25±4.77 × 10^9/L) compared to the control rats (130.01±2.31 × 10^9/L) (Figure 1).

DISCUSSION

In this study, rats administered with the aqueous extract of *A. vera* had increased values of the packed cell volume (PCV), red blood cell counts and other red cell indices. A significant ($p<0.05$) increase in PCV was observed in rats administered with the extract for the period of 24 h. This increase in PCV was not due to haemoconcentration because there was a generalized increase in red and white blood cells, but can be attributed to stimulation of haematopoiesis. This can further be related to the result of the red cell indices; increased MCV, MCH and MCHC, which showed that immature red cells were present in circulation, indicative of stimulation of production of immature erythrocytes, also known as reticulocytes. Morphologically, reticulocytes are characterised by increases in the size of red cells in circulation and it is usually observed as the initial response to stimulation of the haematopoietic system during active blood regeneration (Saba et al., 2009). *Telfaria occidentalis* leaves which are consumed in soups in several regions of West Africa had also been reported to have haematopoietic stimulatory ability and it is used traditional for treatment of anaemia (Alada, 2000; Dina et al., 2000).

White blood cells on the other hand showed significant ($p<0.05$) increases, particularly in rats administered with the extract for 24 h. About 2-fold increment in lymphocyte and neutrophil counts were observed in these rats. Lymphocytosis may be associated with increased immunological response to an antigenic stimulation, while the neutrophilia may be traced to increased inflammatory response in the body (Guyton and Hall, 2006a, b). Increased circulating neutrophils are usually as a result of mobilization of neutrophils into circulation in response to an antigenic stimulation. Such stimulants include trauma and bacteria endotoxins (Zekonis and Zekonis, 2004; Tang et al., 2010), of which the extract is neither. From the result obtained for this study, it can be postulated that *A. vera* may contain bioactive substances which are capable of mobilizing all blood cell types into circulation, and or stimulate haematopoiesis resulting in increased production/ release of blood cells into circulation. Our argument favours the haemopoietic theory more, considering the fact that neutrophil: lymphocyte ratios, a marker of subclinical inflammation, were approximately 0.5 in control and test groups, which were clinically and statistically non-significantly different (Sen et al., 2013; Wang, 2014).

Platelet counts were non-significantly ($p>0.05$) increased in the rats administered with the extract for 12 h, but a significant ($p<0.05$) increase was observed in rats administered the extract for the 24 h period. Thus, it can be inferred that blood clotting mechanisms may not be affected by *A. vera* extract, but this corroborates our theory in favour of indiscriminate stimulation of blood cell production.

Administration of the extract for the 12 h period showed minimal haemopoietic ability compared to rats administered with the extract for the 24 h period. A cumulative dose-dependent pattern was established from this study which discourages the continuous consumption of the extract as it is administered for certain traditional uses. The indiscriminate stimulation of blood cells may be detrimental to the body with depletion of haemopoietic stem cells in bone marrow and may eventually trigger or encourage uncontrolled stimulation of haemopoiesis which can be seen in cases of myeloproliferative disorders (Tefferi and Vainchenker, 2011; Barbui et al., 2013).

Conflict of Interest

The authors disclose that they do not have any conflict of interest.

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Full Length Research Paper

Biological, histological and ultra-structural studies of female mullet, *Mugil cephalus*, ovaries collected from different habitats during annual reproductive cycle

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This study investigates some biological characters (oocyte diameter, fecundity, histological and ultra structural features) of female *Mugil cephalus* ovaries collected from three different natural habitats: marine (MW), brackish (BW) and fresh (FW) water. Monthly gonadosomatic index (GSI) values clearly showed that the time period of reproductive activity in female *M. cephalus* from marine and brackish water habitats was from early September to late November. No peak value of GSI in females collected from freshwater was observed throughout the year. Analysis of ovum diameter for *M. cephalus* in the two habitats revealed that, there are small diameter ova (less than 0.3 mm) and large ova (larger than 0.35 mm). The percentage of small ova diameter was 5±1% in marine habitat, while 27±3% for brackish water habitat. The mean oocyte diameters in fresh water fish were less than 350 µm. The oocytes did not develop enough to be differentiated into small and large diameter ova. The total number of ripe ova in marine fish varied between 0.84 ± 0.05 to 4.14±1.01 x10^6 for a total length ranging between 35 and 52 cm, respectively; whereas, the total number of ripe ova in brackish water fish ranged from 0.57±0.14 to 3.81±0.59 x10^6 for the same length groups. There was highly significant correlation (p>0.01) between the number and length of ripe ova in 37 and 50 cm length group from the two habitats. Yolky nucleus or Balbiani's body and interstitial epithelial cells are a characteristic feature of oocytes at maturing stage, which is clearly detected in marine water fish with isolated follicular and active organelles. In brackish and fresh water fish ovary, the cytoplasm was compacted without accumulation of active organelles. Ultra structurally vacuolized oocyte wall in marine fish showed the presence of the fifth layer (cortical alveoli) while no cortical alveoli formation was observed in oocyte of brackish or fresh water females. The percentage of atretic oocytes in late vitellogenic ovary of marine water fish was about 2.5%, while in brackish water fish it was about 92±2%. In both brackish and fresh water fishes the initial stage of oocytes atresia degeneration was observed. In conclusion, the comparative study shows that ovary of marine and brackish *M. cephalus* morphologically overlaps from ripening to re-sorption stages. With the histological and fine structure characteristics, it was possible to understand the functional relationship between oocyte size and stage of fish maturation. This knowledge is of huge importance in establishing the reproductive status of the fish which is related to the functional expression of the folliculogenesis in female individuals.

Key words: Ova, *Mugil cephalus*, marine, brackish and fresh water fish.

INTRODUCTION

Mugilids are widely distributed in coastal and brackish waters of all tropical and temperate regions of the world. In many countries, mugilids are targeted by commercial fisheries (Ibanez-Gallardo-Cabello, 2004) and have also been widely cultured (Lee and Ostrowski, 2001). In Egypt, this species has been used for traditional aquaculture and culture-based fisheries since the late 1920s and is still of major importance today in other Mediterranean

countries and Taiwan Province of China (Saleh and Salem, 2005; Basurco and Lovatelli, 2003). Based on the statistics published by the Food and Agriculture Organization of the United Nations (FAO), the world total catch of mullet in 2004 was about 261,000 tones, representing only 0.3 percent of the world fish catch (FAO, 2004). Most mullet aquaculture activities rely on the use of wild seed, for example Egypt (Saleh, 1991). Commercial hatchery production of mullet seed is carried out in some countries. Induced spawning and production of fry has been achieved on an experimental and semi-commercial basis in the United States of America and Taiwan Province of China. The production of mullet fry on a limited scale for aquaculture has been reported in Italy, Israel and Egypt (Saleh, 2006). It is of vital importance to understand the reproductive biology of this species and start a national program for hatchery production of fry.

Mullets are usually grown in extensive, semi-intensive ponds and netted enclosures in shallow coastal waters. Mullet can be polycultured successfully with many other fish, including common carp, grass carp, silver carp, Nile tilapia and milkfish and can be reared in fresh, brackish and marine waters.

The reproductive biology of Mugilids was studied by many authors (McDonough et al., 2003; Ibanez and Gallardo, 2004; Kendall and Gray, 2008; Assem et al., 2008; Albieri and Araujo 2010). Teleost oocytes as in *Mugil cephalus* are surrounded by two major cell layers as an outer thecal layer and inner granulsa. In the present work, a comparative study was carried out between steroid hormone producing cells (thecal and follicular cells) and cortical alveoli layer in the ovaries of female caught in three different environments. Ovarian follicles have the ability to synthesize estrogen, androgens and corticosteroids. Matsuyama et al. (1991) indicated that the thecal cells during vitellogenesis and oocyte maturation possess organelles, characteristic of steroid-producing cells.

Therefore, this study investigates and clarifies some biological, histological and ultrastructural characters differences of ovaries of *M. cephalus* from three different habitats- marine, brackish and fresh water.

MATERIALS AND METHODS

Fish sampling biometric measurements

Specimens of *Mugil cephalus* were collected three times a month; from May 2012 to January 2013. Four hundred (400) specimens from three areas were studied. The first main natural marine habitat area was Bardawil Lagoon (El-Areesh governorate), the second brackish area was El-Deepa triangle (Port Said governorate) and the third fresh water area was Kafer El-Sheik fish farms (Bohera governorate). *M. cephalus*' total length is from 35 to 52 cm. The length is well characterized at first maturity (Assem et al., 2008), having total weight of 400 to 1380 g. For each fish, the date of capture, total length to nearest mm, total weight to nearest mg were recorded. The fish was dissected to determine the maturity stage, according to Assem et al. (2008). Ovaries at all stages of maturity were fixed in 10% formal saline solution until used for histological studies. The gonadosomatic index was calculated (ovarian percentage weight to the total weight of the fish). Paired lobes of ovaries were weighted; 0.1 g of ripe and spawning specimens was fixed in 4% neutral formalin. These samples of ovaries were counted for estimating fecundity; also, diameter of all eggs was measured. The total number of ripe ova in the ovary is known as absolute fecundity, whereas relative fecundity is the number of ripe ova per unit weight or length. The egg diameter is divided into fourteen groups; the first six groups (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mm) were small, transparent and hexagonal in shape. While, the remaining ova group ranging between 0.35 mm and 0.7 mm in diameter was yolky eggs.

Histological and fine structure examination

Fixed ovaries were washed in 70% ethyl alcohol prior to dehydration. Then they were cleaned and embedded in paraffin wax. Sections from 5-7 µm thick were stained with Eirlich Hematoxylin and eosin. Four small blocks of ovary specimens were fixed overnight in 4% buffered glutaradialdehde and then in 1% osmium tetraoxide for 1 h at room temperature; they were rinsed twice in cacodylate buffer (pH 7.2), dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in polarbed 812 (polaron) epoxyresin. Ultra-thin sections of 1 micron thick were prepared using glass and diamond knives, and stained with uranyl acetate and lead citrate. Sections were examined using transmission electron microscope.

Statistical analysis

Significant differences in GSI and fecundity were tested by Microsoft Excel 2003 one way ANOVA, followed by LSD test. For all the procedures, level of significance recorded was 0.05 and 0.01.

RESULTS

The biological studies

The gonadosomatic index (GSI)

Monthly variation of GSI values is the ovarian percentage weight to the total weight of the fish. In early September, the ovaries started to increase in weight and then GSI value increased gradually. In August and September, the GSI values of brackish and marine water female were increased to reach the peak value in October and November, and then decreased throughout December. Slight increase in GSI values in fresh water female were noticed in August, September and December, but they were still at immature stage. Highly significant correlation

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Table 1. Monthly variation in average gonadosomatic indices values (GSI) of female *Mugil cephalus* at three different habitats, natural marine, brackish and fresh water throughout the period from May 2012 to January 2013.

Month	GSI±SE		
	Marine	Brackish	Fresh
May-12	0.29±0.12	0.25±0.11	0.18±0.05
Jun	0.31±0.09	0.28±0.02	0.16±0.06
Jul**	0.86±0.06[a]	0.55±0.04[b]	0.31±0.05[c]
Aug	3.96±1.73	1.70±0.76	1.10±o.71
Sep*	6.89±1.43[a]	5.91±1.15[a]	2.34±0.38[b]
Oct**	22.55±2.62[a]	24.79±1.53[b]	0.25±0.07[b]
Nov**	18.4±11.97[a]	19.83±2.98[b]	0.43±0.07[b]
Dec**	0.72±0.20[b]	1.98±0.37[a]	0.69±0.04[b]
Jan. 2013	0.56±0.11	1.11±0.37	0.37±0.17

Values represent mean± (SE), means in same row not shearing the same superscript are ** highly significantly different (p<0.01) and * significant (P<0.05).

was detected in GSI values at p<0.01 throughout July,October, November and December. Furthermore, significant correlation at p<0.05 was noticed in GSI value throughout September as indicated in Table 1.

Fecundity

In the study of fecundity, two terms are generally used: absolute and relative fecundity.

Analysis of fecundity length relationship: Regression equation of marine females is: Fa = 211789.2 TL – 7145156.7; while for brackish water is Fa= 196203.58 TL-6634.885 (where, Fa is the absolute fecundity, and (TL) is total length (Table 2). The correlation coefficient was 0.923 marine and 0.9474 for brackish females. Highly significant correlation (p>0.01) in 37 and 50 cm length group was detected in the two habitats. The equation of relative fecundity for marine was Fr = 3742 TL – 117807.7, where, Fr is the relative fecundity hile, in brackish female was: Fr = 3515 TL – 111635.05. The correlation coefficient was found to be 0.9142 for marine and 0.9615 for brackish water.

Analysis of fecundity- weight relationship: The equation of average absolute fecundity related to each gutted weight is Fa = 3411.506 W – 619539 for marine female, while for brackish is: Fa = 3164.4 W – 677131, where, W is gutted weight in grams. Correlation coefficient recorded was 0.6370 for marine and 0.7090 for brackish female with highly significant correlation in the two habitats at p> 0.01, in all the absolute fecundity for gutted weight groups except for 800 and 1100 g.

Egg diameter determination and spawning

Marine and brackish water: At the beginning of spawning season of *M. cephalus* (early September) (Figure 1a and b), the ovaries of both habitats had large percentage of small diameter ova and small percentage of yolky eggs. In late September (Figure 2a), for marine female, there was small percentage of transparent ova compared to those of brackish water diameter groups (Figure 2b). At the peak of spawning season of *M. cephalus* (October and November), in general all the ovaries of ripe marine female were in ripe process (Figure 3a and b). While ovaries of ripe female (Figure 4a and b) have a large percentage of small diameter.

Fresh water: All ovaries of fresh water females contained small ova diameters only.

Histology and ultrastructure studies of ovaries of female *Mugil cephalus* collected from three different habitats

Pervitellogenic (immaturation) stage

At this stage, the early mother cells or persynaptic in the three habitats (marine, brackish and fresh water) are similar. Ultra structure examination of presynaptic oocyte showed that the nucleus is characterized by dense chromatin material; the presynaptic group of cells was surrounded by large number of active organelles (mitochondria and endoplasmic reticulum) in marine females, whereas in brackish and fresh water females, the mother cells had poor organelles (Figure 5a, b and c).

Early-vitellogenic (maturing) stage

Marine habitat: The maturation period is characterized by the appearance of isolated follicular epithelial cells around the oocyte. The percentage of normal oocytes ranged between 88 and 95%. The normal oocytes reached 85 µm in diameter. The cytoplasm is faintly stained and is characterized by the appearance of bright corpuscle (yolk nucleus) as shown in Figure 6, a and b. Figure 7 is an electron micrograph of two primary oocytes at maturation period, showing the interstitial follicular epithelial layer; the cytoplasm is provided with mitochondria and endoplasmic reticulum.

Brackish habitat: The wall of the ovary thickly varied in diameter between 65 and 80 µm. The percentage of normal oocytes was about 47%. All the abnormal oocytes were atretic (Figure 8a and b), then they shrank gradually till degeneration and resorption. In electron micrograph, the cytoplasm of primary oocyte was compacted without accumulation of active organelles as shown in Figure 8c.

Table 2. Average absolute fecundity, relative fecundity for each length and gutted weight groups for ripe female *Mugil cephalus* of two different habitats (marine and brackishwaters).

Measurement	Fecundity±SE			
	Absolute		Relative	
	Brackish	Marine	Brackish	Marine
Total length (cm)				
35	574584±147680	841898±56232	16416	24054
37**	777952±47880[a]	912396±24792[b]	21025	24659
40	958484±33629	971098±25305	23962	24277
42	1390366±96765	1311370±158282	33103	31223
44	1991538±351597	1730123±271943	45262	39320
46	2277693±209216	2889315±528795	49515	62811
48	2612319±495065	3077791±40864	54423	64120
50**	2049975±465093[a]	3696710±27667[b]	40999	73934
52	3816669±598870	4146903±101636	73397	79748
Gutted weight (g)				
400**	698964±24768[a]	776719±18374[b]	1747	1941
500**	794073±39247[a]	1858017±88954[b]	1588	3716
600**	1792598±158051[a]	2433067±125630[b]	2987	4055
700**	1908180±138979[a]	958104±10612[b]	2725	1368
800	1266650±130945	971331±11549	1583	1214
900**	1045338±85144[a]	1380922±80223[b]	1161	1534
1000**	2689165±178407[a]	3446815±54696[b]	2689	3446
1100	2864960±128309	2959513±110131	2604	2690
1200**	3685244±115472	4272235±102279	3071	3560
1300**	3685244±109416[a]	4272235±106011[b]	2834	3286

Values represent mean± (SE), means in same row not shearing the same superscript are **highly significantly different (p<0.01) and *significant (P<0.05).

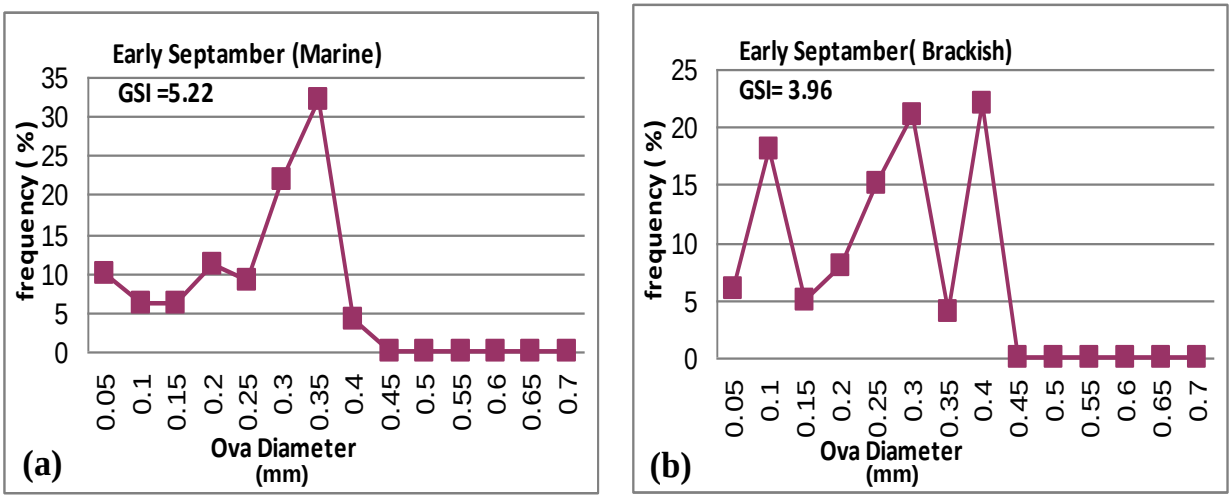

Figure 1. Ova diameter frequency distribution for female *Mugil cephalus* throughout the period from early September, 2013 caught from two different habitats (marine and brackish water).

Fresh habitat: The wall of ovary is moderate in thickness than that of marine and brackish ovaries varied in diameter between 45-50 µm. Also, the wall was characterized by rich innervations of blood capillaries.

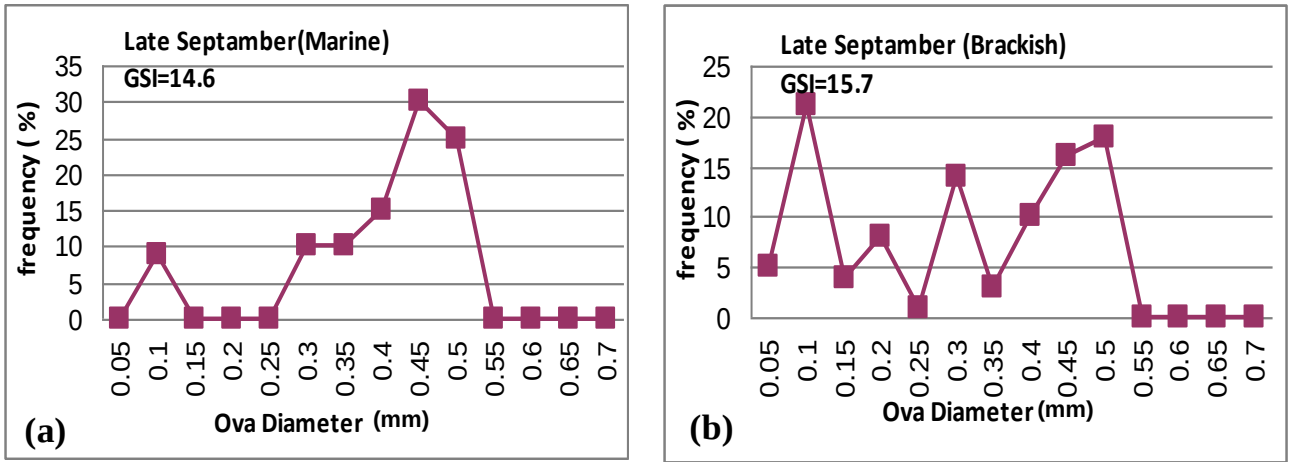

Figure 2. Ova diameter frequency distribution for female *Mugil cephalus* throughout the period from late September, 2013 caught from two different habitats (marine and brackish water).

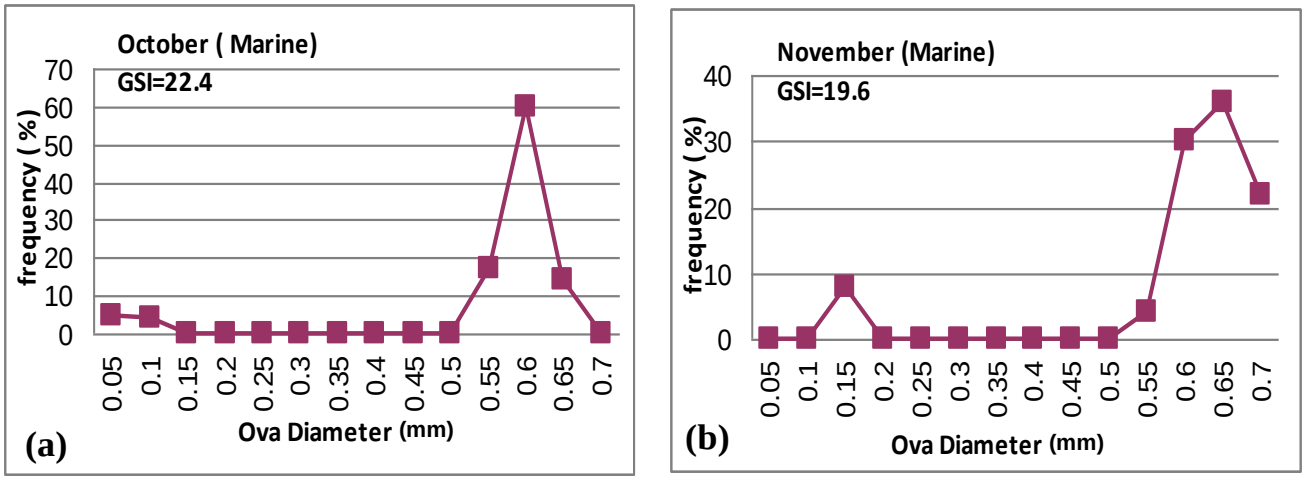

Figure 3. Ova diameter frequency distribution for female *Mugil cephalus* throughout the period from October and November, 2013 caught from marine water.

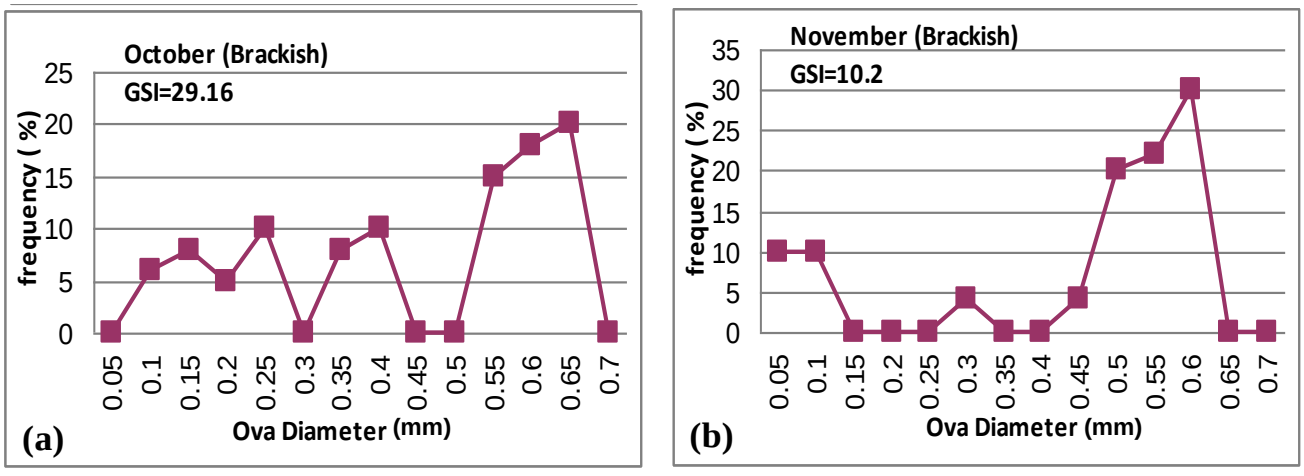

Figure 4. Ova diameter frequency distribution for female *Mugil cephalus* throughout the period from October and November, 2013 caught from brackish water.

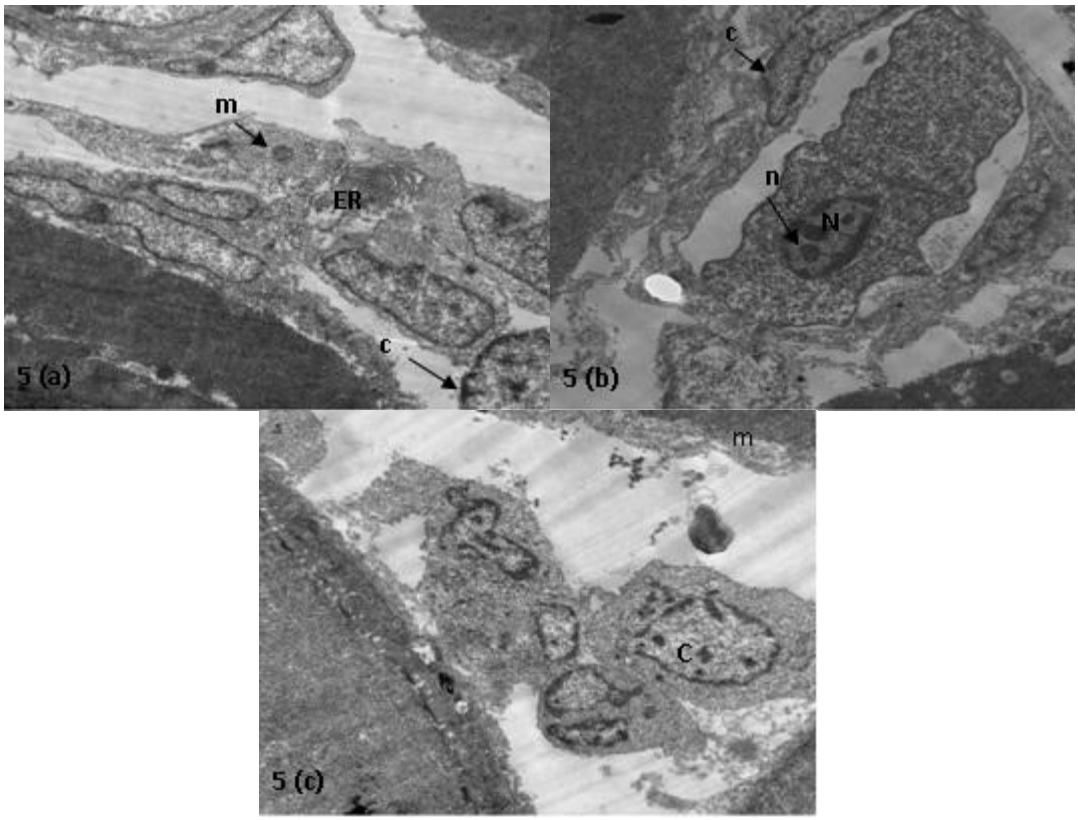

Figure 5. Photo-electron-micrography of cross section (cs) in immature ovary *of Mugil cephalus* showing **(a**) group of presynaptic mother cell in marine habitat, (ER) endoplasmic reticulum (m) mitochondria, (c) chromatin material. **(b)** Presynaptic cells in brackish habitat, (N) large nucleus with **(c)** dense chromatin and (n) nucleolus. (5c) Group of presynaptic mother cells in fresh water habitat with minute mitochondria (m) and faint chromatin (c). X 3000 stained with uranyl acetate (UA) and lead citrate (LC).

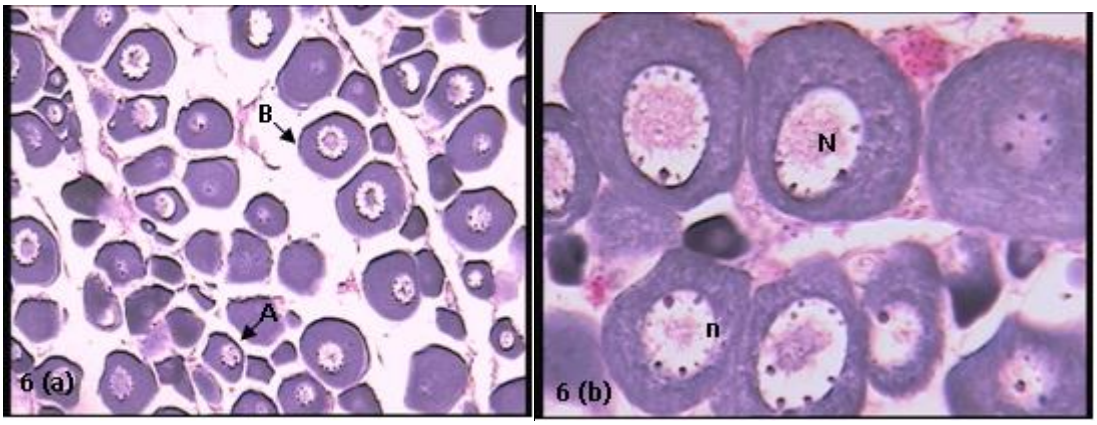

Figure 6. photo-micro and electron graph of CS in maturing ovary of marine habitat showing **(a)** primary (A) and more advanced oocyte (B) X100 Hematoxylin and Eosin (H and E). **(b)** Magnification of (a) showing follicular epithelial layer (FE), nucleus (N), nucleolus (n), yolky nucleus (Yn).

The ovary has about 19% of the total oocytes in normal shape, as shown in Figure 9a and b. The electron micrograph showed that interstitial follicular epithelial cells existed in the primary oocyte wall at maturation

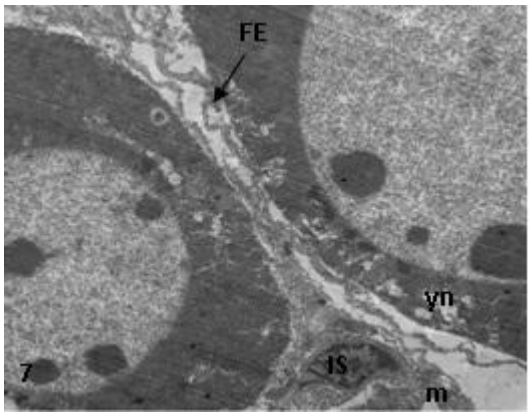

Figure 7. Two cytoplasmic growth cells with isolated follicular epithelial layer (FE) with embedded interstitial cells (IS) and yolky nucleus (Yn) rich in mitochondria (m). X2500.

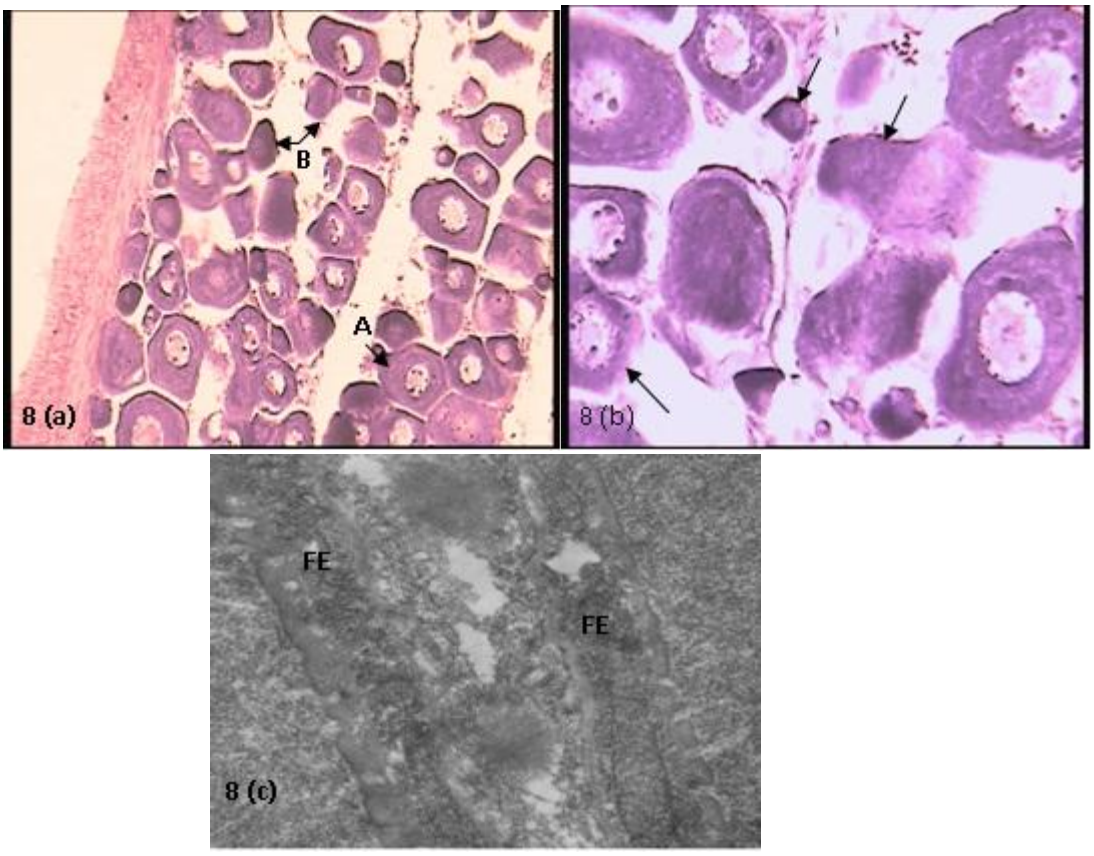

Figure 8. photo-micro and electron graph of CS in maturing brackish ovary showing, **(a)** cytoplasmic growth oocytes in normal (A) and deformed (b) shape (x 100, H and E). **(b)** Magnification of cytoplasmic growth showing shrank degeneration and resorption (arrows) of atretic cells. (X250 H and E). **(c)** Two cytoplasmic growth cells with isolated follicular epithelial layer (FE) notice no any activity in the cytoplasm (X3000).

period. However, the cytoplasm of primary oocyte was not provided by active organelles (Figure 9c).

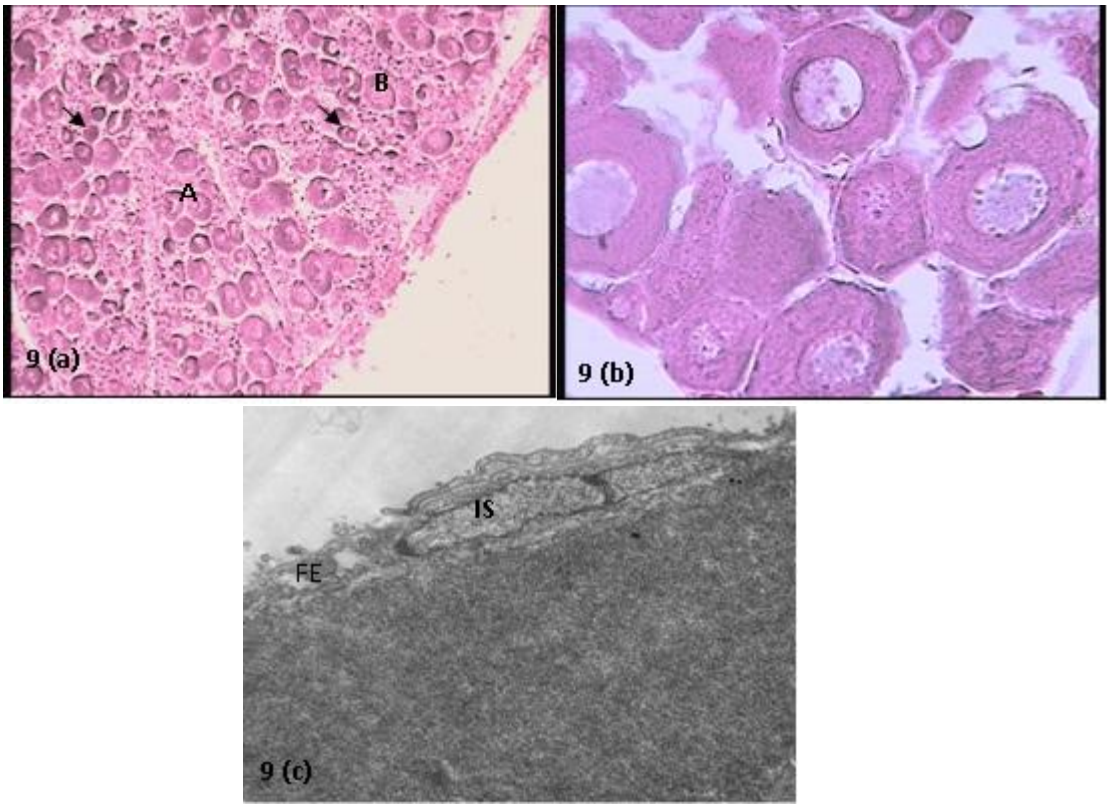

Figure 9. Photo-micro-electron graph of CS in maturing fresh water habitat ovary showing **(a and b)** cytoplasmic oocyte with magnification in normal (A) and deformed (B) shape note degeneration of atretic cells (arrows). **(c)** Cytoplasmic growth cells with interstitial cells (IS) embedded in follicular epithelial layer (FE). Note no any activity in the cytoplasm. X 5000.

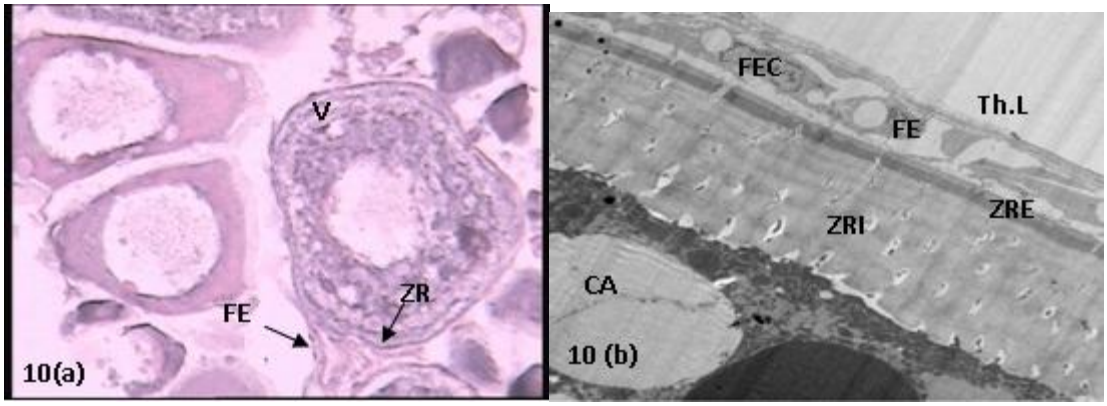

Figure 10. Photo-micro and electron graph of CS in vacuolized ovary of marine habitate showing, (X250), **(a)** one row of vacuoles (V) outer isolated follicular epithelial (FE) and inner zona radiata (ZR), **(b)** theca layer (Th.L), follicular epithelial layer (FE) with follicular epithelial cells (FEC), zona radiata externa (ZE) and zona radiata interna (ZI) and cortical alveoli (CA). X1500.

Mid-vitellogenic (nearly ripe) stage

Marine habitat: Ovaries at this stage have about 2% atretic oocytes. However, normal oocytes are characterized by the appearance of cytoplasmic vacuoles (Figure 10a). The oocyte diameter ranged from 160 to 210 µm. The oocyte wall consisted of zona radiate of about 5 µm in thickness coated with follicular epithelial layer of 6 µm

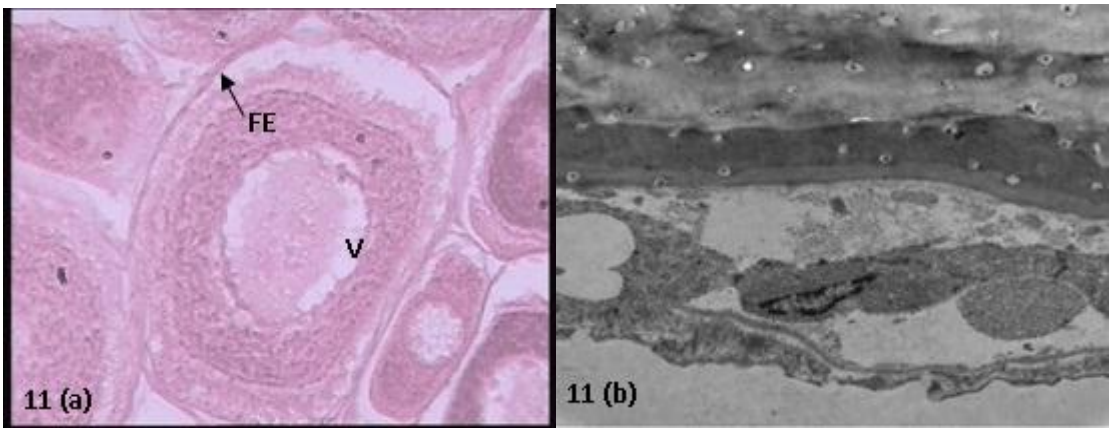

Figure 11. (a) CS in vacuolized ovary of captive brackish water (X 250) showing one row of vacuoles (V), thick follicular epithelial layer (FE) and inner zona radiata (ZR). **(b)** Absence of cortical alveoli layer. X 3000.

thick. The ultra structure of vacuolized oocyte wall showed the presence of five different layers in which the outer most layers is the theca layer. Then the follicular epithelial layer, the third and fourth layers are zona radiate externa and interna. The fifth layer is cortical alveoli (Figure 10b). Large number of mitochondria, ribosomes, endoplasmic reticulum and follicular epithelial cells were detected in the theca layer and follicular layer.

Brackish habitat: Ovaries have about 86% atretic oocytes (Figure 11a). The ultrastructure of vacuolized oocyte wall showed the presence of four layer only, the follicular epithelial layer (with few number of follicular epithelial cells) and absence of cortical alveoli as shown in Figure 11b. No organelles, mitochondria, endoplasmic reticulum and ribosomes were detected in the theca layer.

Fresh habitat: Ovaries have about 83% atretic oocytes, which ranged in diameters from 100 to 280 µm (Figure 12a). Ultrastructure of oocyte wall consists of thin zona radiata (about 3 µm) as shown in Figure 12b. No steroid producing tissue or active organelles or cortical alveoli layer was detected in the oocyte wall.

Late – Vitellogenic (Ripe) stage

Marine habitat: Ovaries of marine female have about 2-3% atretic oocyte. The wall of the normal oocytes consisted of zona radiata (about 11±2 µm in thickness), coated with follicular epithelial layer (about 5±1 µm) as shown in Figure 13a and b. The ultra structure of cell wall at ripe stage revealed the presence of five different layers (Figure 14a). Magnification of the outer most layer of the oocyte indicated the presence of elongated thecal cells and follicular epithelial cells, with dense chromatin material, mitochondria, ribosomes and endoplasmic reticulum. Zona radiata layers contained pore canals and cortical alveoli contained cortical granules (Figure 14b and c).

Brackish habitat: *M. cephalus* ovaries have about 92±2 abnormal and atretic oocytes. The atretic oocytes have disorganized nucleus and cytoplasm, distorted and hypertrophied follicle, liquified yolk globules, disintegrated and fragmented zona radiate and follicle epithelium layer, engulfed zona radiata and phagocyted yolk, as indicated in Figure 15 a and b.

The ultrastructure of the oocyte wall at this stage showed the presence of outer theca layer without special theca cells followed by basement membrane, follicular epithelial layer "without follicular epithelial cells", then zona radiata externa and zona radiata interna. No cortical alveoli layer was detected in brackish female ova as shown in Figure 15b and c.

Spawning – resorbed and spent stage

Marine habitat: The ovary displayed all the peculiarities of spawning at this period by having a large number of empty follicles due to egg production. Few atretic oocytes were also detected. At the end of spent period, ovaries were characterized by appearance of empty follicles and new generation of small oocytes as shown in Figure 16.

Brackish habitat: The ovary was characterized by presence of different stages of atretic oocytes. The wall of the oocyte consisted of thin follicular epithelial layer of about 3 µm and thick zona radiata of about 14 µm. At the end of atretic ooctye stage, the yolk granules completely resorbed, leading to the formation of lipofusin granules.

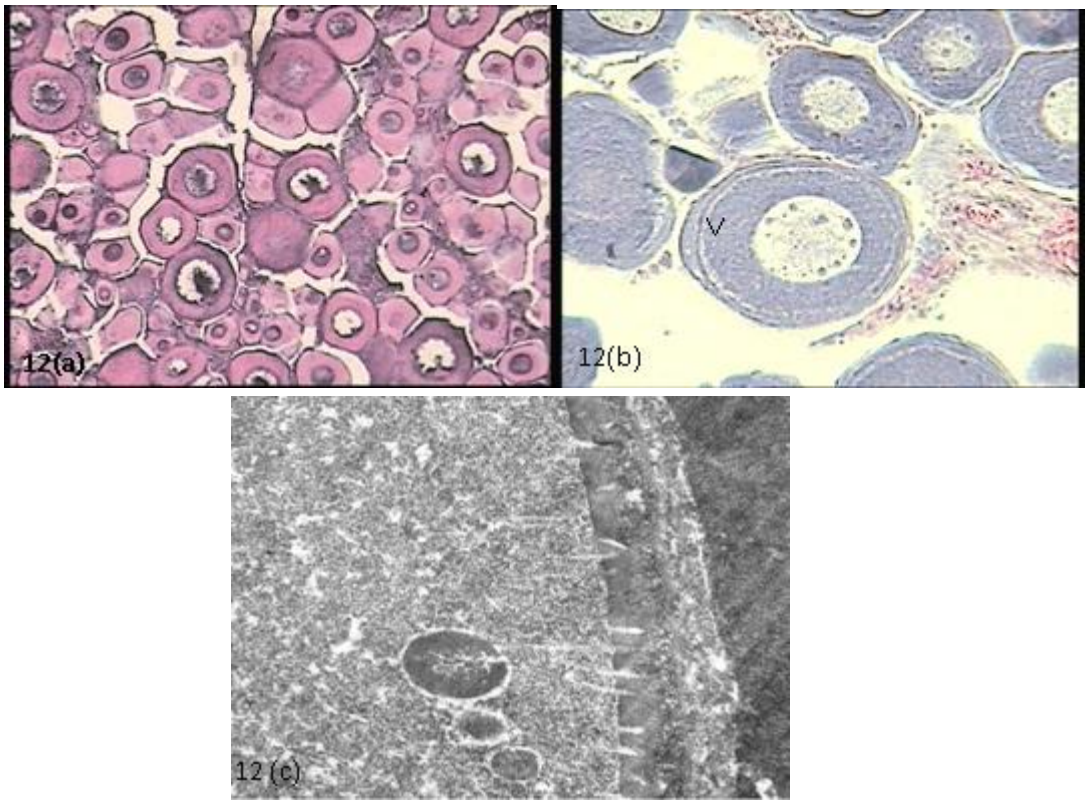

Figure 12. Vacuolized ovary of fresh water habitat ovary showing **(a & b)** X100 and X250 different diameters of cytoplasmic growth. **(c)** Fine structure of fresh water habitat wall of oocyte (X5000), note absence of cortical alveoli, follicular epithelial cells and theca cells.

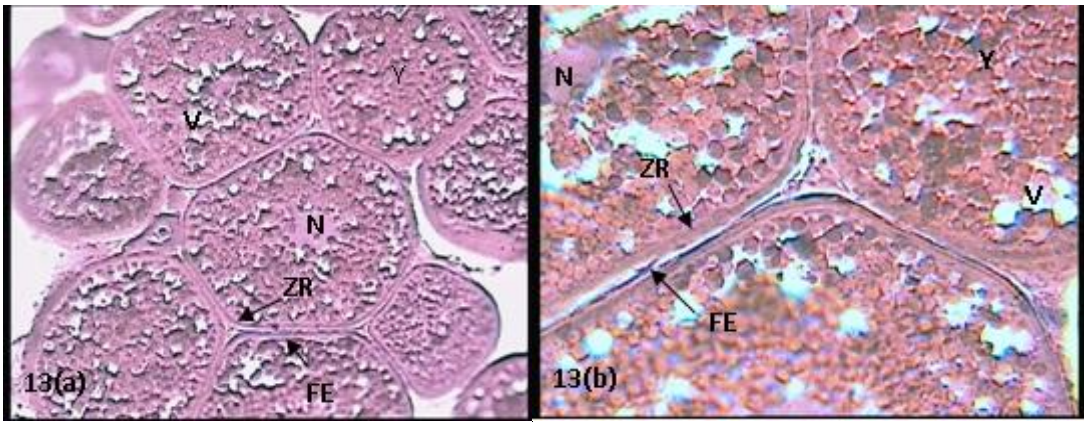

Figure 13. CS in ripe ovary of marine habitat showing ripe ova with nucleus (N), follicular epithelial layer (FE), zona radiata (ZR), yolk granules (Y) and vacuoles (V). (a- X 100 & b- X250).

Convoluted and fragmented basal membrane persisted and then the granulocytes appeared close to the atretic follicles (Figure 17).

DISCUSSION

The biological feature of *M. cephalus* has been well documented, but much less information is available on the biological aspects of reproduction in the wild (Render et al., 1995; McDonough et al., 2003). Oocytes of *M. cephalus* investigated developed on a group as synchronous pattern (McDonough et al., 2003). This is typical for many other mugilids, including Klun Zinger's mullet (*Liza Klun Zingeri)* (Day, 1888; Abou Seedo and Dadzie, 2004), large scale mullet (*Liza macrolepis)*

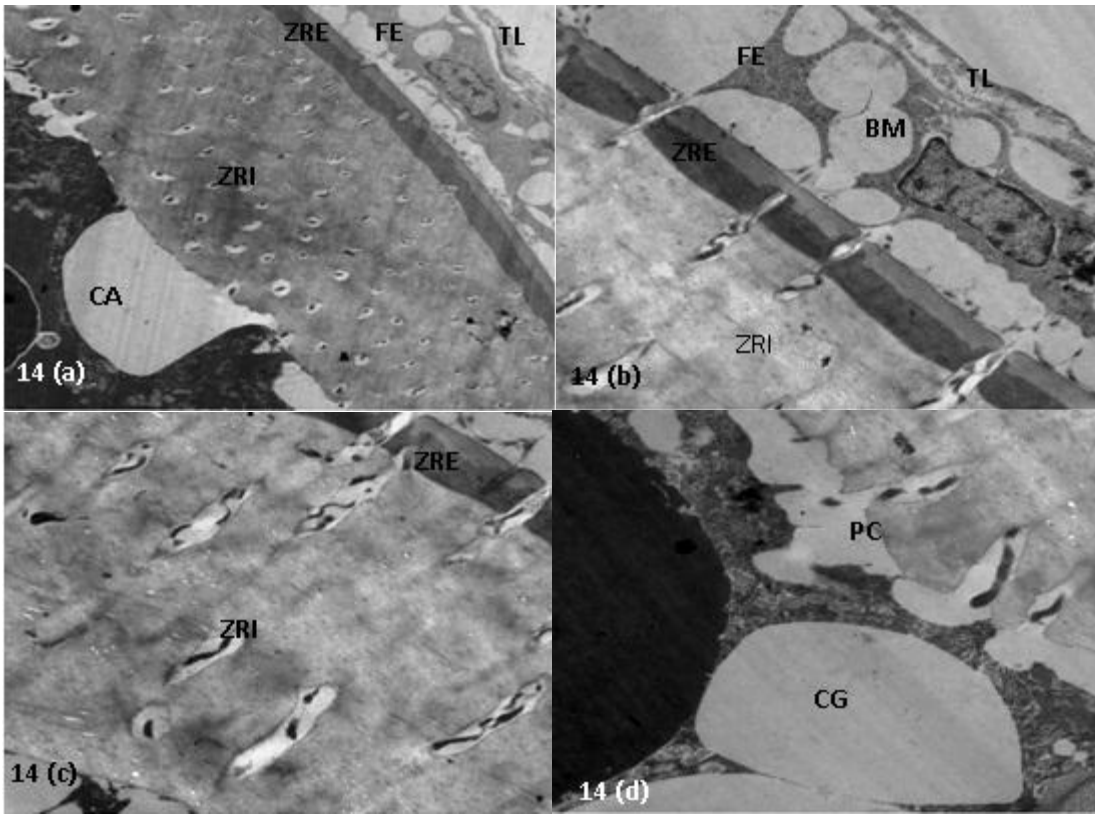

Figure 14a, b, c and d. Photo-electron graph of wall of marine oocyte, which consisted of outermost theca layer (TL) basement membrane (bm), follicular epithelial layer (FE) with follicular epithelial cells (FC), zona radiata externa (ZRE), zona radiata interna (ZRI) with pore canal (PC), then the fifth layer the cortical alveoli (CA) with cortical granules (CG).

(Smith, 1846; Chen et al., 1999), green back mullet (*Liza subviridis*) (Valenciennes, 1836; Chan and Chua, 1980) and striped mullet (*Mugil platanus)* (Gunther, 1880; Romagosa et al., 1988).

Due to the world wide distribution of *M. cephalus,* some comparison with other areas is possible. The GSI data for *M. cephalus* from Mediterranean Sea varied between 0.39 minimally and 27.22 maximally (Mourad, 2009). In estuaries in South Carolina (USA), the GSI for fecund fish ranged from 7.7 to 27.7 (McDonough et al., 2003). In another study on Egyptian gray mullet in Lake Temsah female, GSI observed was 12.4 (El-Halfawy et al., 2007). Significantly higher GSI values were also reported for Turkish waters. Ergene (2000) reported a value of 16.67 for grey mullet females. The highest GSI value, close to 40, was obtained for *M. cephalus* in the waters of North-west Gulf of Mexico (Ibanez and Gallardo Cabello, 2004). In contrast, in the waters of Korea the highest female GSI of only 5.32 was found at the peak of the reproductive season (Kim et al., 2004). These spatial differences in GSI among the same species as already discussed for *M. cephalus* could be caused by differences in food resources, living temperature or the evolutionary adaptation of different population to the specific ecological properties of specific ecosystems.

In present study, the analysis of ova diameter for *M. cephalus* marine, brackish and freshwater habitat revealed that, there are small diameter ova (less than 0.3 mm) and large ova over 0.35. All oocytes in the ovary of female in marine habitat were in vitellogenic growth phase, the oocyte size coincides with the highest GSI values and the females clearly migrate to open waters for spawning. In parallel with the growth of oocytes that will be released, the ongoing recruitment of new oocytes is apparent in the gonads. This new batch of oocytes grows during the next season as indicated by Bartulović et al. (2011). In the present study, the oocyte diameter did not change with fish size, fecundity or GSI as reported by Albieri et al. (2010) who found that oocyte diameter did not change with fish size. Also in the present study, all ovaries of fresh water female used throughout the year were not used for fecundity or oocyte diameter count. All samples had less-developed ovaries; with low GSI value and mean oocyte diameters less than 350 µm. the oocytes did not develop enough to be separable from the smaller oocytes.

The absolute and relative fecundity in marine habitat is larger than those of brackish habitat. There is a

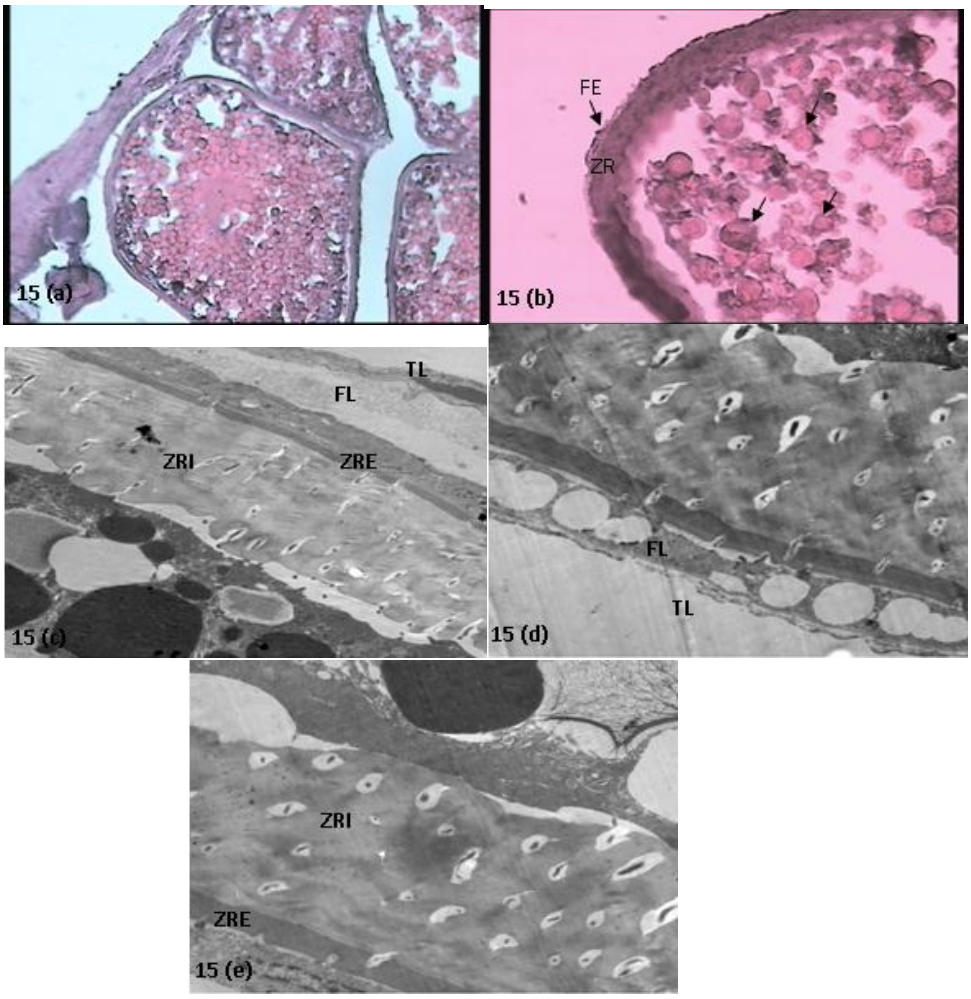

Figure 15. CS in ripe oocyte of brackish habitat **(a)** X100 normal oocyte **(b)** atretic oocyte characterized by hypertrophy of follicle layer and zona radiata (ZR) with liquification of the yolky globules (arrows). **c, d and e.** Electron- graph of the wall of brackish habitat oocyte; (c) hypertrophy of theca layer (TL) and follicular layer (FL) without theca cells or follicle cells, then zona radiata externa (ZRE) and interna (ZRI). X1500. (d & e) Magnification of outer theca (TL) and follicle layer (FL) X2500.

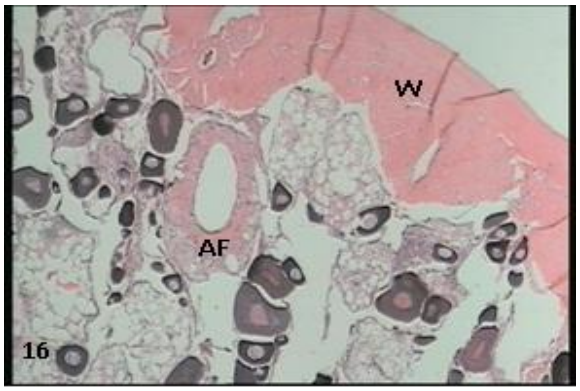

Figure 16. Light micro graph of CS in early spent ovary at marine habitat showing theca wall (W) of ovary new generation of cytoplasmic growth (arrows), atretic follicle (AF). X100.

relationship between the number of oocytes and their diameters. McDonough et al. (2003) demonstrate that there is an inverse relationship between oocyte density and oocyte diameter. Fecundity in *M. cephalus* highly correlated with length and weight. Fecundity may vary due to different adaptations to environmental habitats (Witthames et al., 1995). In female gametogenesis, the young meiotic oocyte originates from the oogonia, primary oocyte growth giving rise to the previtellogenic oocyte, secondary growth or vitellogenesis, and the formation of the cortical alveoli and yolk granules (Selman and Wallace, 1989). In *M. cephalus*, oogonia or presynaptic cells are the smallest germ cells found in the gonads, as in other Teleost species, showing a common morphologic pattern (Grier, 2000). In the present study, the fine structure of presynaptic group of cell in marine habitat was surrounded by large number of active

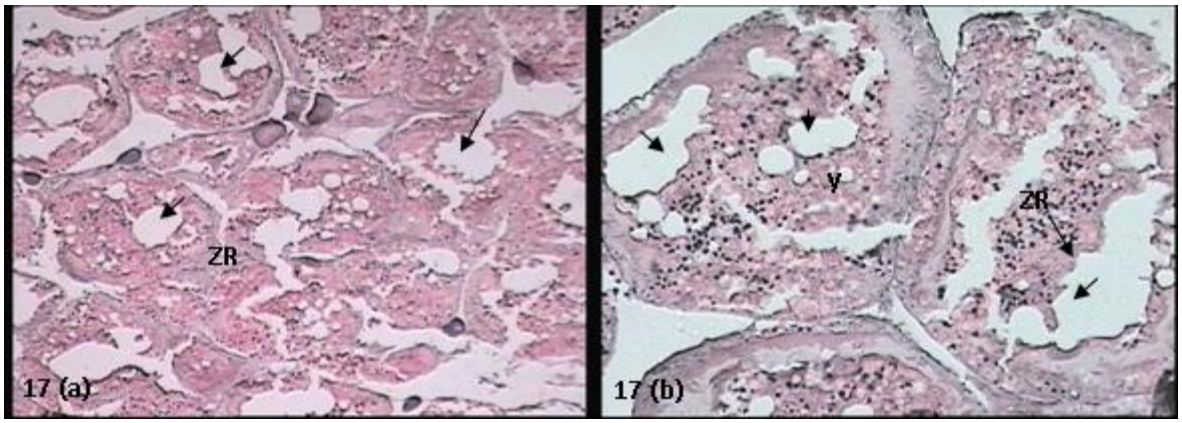

Figure 17. CS in brackish habitat resorbed ovary showing advanced atresia, liquification of yolk (Y), convoluted zona radiata (ZR), vacuolated area in the ooplasm (arrows) during the phagocytosis process. (a - X100 and b- X250).

organelles (mitochondria and endoplasmic reticulum), whereas in brackish and fresh water habitat, the presynaptic cells were poor in organelles. The presence of these cells in nests with the ovigerous lamellae also occurs in other species (Guimaraes and Quagi Gorassiotto, 2001; Spadella et al., 2005).

Oogonia can be found all through life and continue mitotic division maintaining permanent oogonia production (Grier, 2000). In present study, yolky nucleus or Balbiani's body and interstitial epithelial cells are characteristic of oocytes at maturing stage, which was clearly detected in marine habitat with isolated follicular and active organelles. In brackish and fresh habitat ovary, the cytoplasm was compacted without accumulation of active organelles.

The fine structure of atresia occurred at early vitellogenic maturing stage of female from marine habitat. Miranda et al. (1999) support the present result that revealed the changes in the ooplasm including the disintegration of the cytoplasm organelles. The annulated lamellae lost their organization, thus showing a disintegration process in the atretic oocytes of *L. reinhardti.* Selman and Wallace (1989) suggested that the Balbiani's body is involved in the intense organelle production which is frequent during this development stage of the oocyte. Other authors say that this structure is of great importance in vitellogenesis (Dettlaf and Vassetzky, 1988). All abnormal oocyte was detected in follicular atresia. Atresia is more frequent in vitellogenic oocytes, although in previtellogenic oocytes as observed in brackish ovaries in present study as well as by Rizzo and Bazzoli (1995). The first signs of atresia observed in *M. cephalus* were the disintegration in the oocyte nucleus, followed by the fragmentation and hypertrophy of the follicle cells, as also described by Mylonas et al. (1997). Miranda et al. (1999) described two types of oocyte nuclear disintegration. In the first type, in the intracellular nuclear disintegrations, the nuclear content disappears rapidly in the ooplasm following lysis of the nuclear envelope. In the second type, the oocyte nucleus together with part of the ooplasm is eliminated through an opening in the follicle wall into the ovarian lumen, where it breaks up into various fragments. These two types of atresia were observed in brackish and fresh water ovary.

The present results reveal that there was no cortical alveoli formation in oocyte of captive brackish or fresh water females. Grant (1990) characterized the vacuolization stage by the cortical alveoli layer formation. Spadella et al. (2005) indicated that: alveoli formation starts with secondary growth and at the end of this stage these vesicles are in their highest concentration in the same region. According to Patino and Sullivan (2002) and Arocha (2002), cortical alveoli are necessary during fertilization because it releases its contents into the perivitelline space, to be structurally and functionally transformed to vitelline envelope, which blocks polyspermia. In agreement with the present results, Assem 2003, indicating that the follicle cell layer generally consists of inner sub-layers which are separated by a basement membrane. In present study, the percentage of atretic oocytes in late vitellogenic ovary of marine water was about 2.5%, while in brackish water it was about 92± 2%.

The process that controls atresia in fish ovary is not well known. It is very difficult to establish the time necessary for oocytes absorption under natural conditions, but under captivity, it can be estimated (Romagosa et al., 1988). In the initial stage of atresia in *M. cephalus* degeneration in the present studies was observed; it was characterized by necrosis such as dissolution and disappearance of the nucleus and changes in the organelles, suggesting that the death of the oocyte could be involved in this process. Some studies have show apoptosis, a type of physiological cell death. Miranda et al. (1999) indicate occurrence of apoptotic cell death during regression of the atretic follicles in two teleost

species. The resorbed ovary in brackish habitat of *M. cephalus* was characterized by large number of blood cells; Ferreira (1993) mentioned the presence of blood cells derived from the theca in atretic fish oocytes which are involved in their resorption. According to Palmer et al. (1995), the cell derived from the ovarian stroma and /or the theca may act together with the follicle cells in the resorption of the atretic follicles.

Ultrastructure of vitellogenic oocyte in marine habitat showed the presence of five layers: the outer most theca layer which included special theca cell followed by basement membrane, follicular epithelial layer including follicular epithelial cells then third and fourth layer zona radita externa and internal, the cortical alveoli. Ultrastructure of vitellogenic oocyte in brackish habitat was characterized by absence of special theca cells, follicular epithelial cells and cortical alveoli layer. Dettlaf and Vassetzky, 1988 indicated that follicle and thecal cells could be involved in r RNA synthesis for the oocyte and may participate in vitellogenesis and hormone secretion. Unal et al. (2005) revealed that special theca cells possess specific organelles, which are characteristic of steroid-producing cells and suggest that during vitellogenesis the special theca cells are the sites of steroid synthesis in *Calcalburnus trichi* ovary.

In conclusion, the morphological comparative study between ovary of marine and brackish water *M. cephalus* showed an overlap between ripening and resorption stages; it was possible to understand the functional relationship between oocyte size, oocyte frequency and stage of maturity due to histological and fine structure characteristics. This knowledge is of highly importance in understanding the reproductive status of the fish which is related to the oogenesis in the Mugillidae.

Conflict of interests

The author(s) did not declare any conflict of interest.

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